Synergistic effect of Smac-mimetic and poly (I:C) on apoptosis of cholangiocarcinoma cells

Mr. Thanpisit Lomphithak

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science in Clinical Biochemistry and Molecular Medicine
Department of Clinical Chemistry
Faculty of Allied Health Sciences
Chulalongkorn University
Academic Year 2018

ผลการเสริมฤทธิ์ของสะแมกไมมิติกและโพลีไอซีต่อการเหนี่ยวนำการตายแบบอะพอพโทซิสของเซลล์มะเร็งท่อน้ำดี

นายธัญพิสิษฐ์ ล้อมพิทักษ์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาชีวเคมีคลินิกและอณูทางการแพทย์ ภาควิชาเคมีคลินิก
คณะสหเวชศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2561

Thesis Title	Synergistic effect of Smac-mimetic and poly (I:C) on apoptosis of cholangiocarcinoma cells
By	Mr. Thanpisit Lomphithak
Field of Study	Clinical Biochemistry and Molecular Medicine
Thesis Advisor	SIRIPORN JITKAEW, Ph.D.

Accepted by the Faculty of Allied Health Sciences, Chulalongkorn University in Partial Fulfillment of the Requirement for the Master of Science

.....
Dean of the Faculty of Allied Health Sciences
(Assistant Professor PALANEE AMMARANOND, Ph.D.)

THESIS COMMITTEE

.....
Chairman
(Assistant Professor TEWIN TENCOMNAO, Ph.D.)

.....
Thesis Advisor
(SIRIPORN JITKAEW, Ph.D.)

.....
Examiner
(Assistant Professor VIROJ BOONYARATANAKORNKIT, Ph.D.)

.....
External Examiner
(Patompon Wongtrakoongate, Ph.D.)

ธัญพิสิษฐ์ ล้อมพิทักษ์ : ผลการเสริมฤทธิ์ของสะแมกไมมิติกและโพลีไอซีต่อการเหนี่ยวนำการตายแบบอะพอพโทซิสของเซลล์มะเร็งท่อน้ำดี. (Synergistic effect of Smac-mimetic and poly (I:C) on apoptosis of cholangiocarcinoma cells) อ.ที่ปรึกษาหลัก : อ. ดร.ศิริพร จิตแก้ว

มะเร็งท่อน้ำดีเป็นมะเร็งที่เกิดจากการเปลี่ยนแปลงของเซลล์เยื่อบุผนังของท่อทางเดินน้ำดี ผู้ป่วยมะเร็งท่อน้ำดีมีอัตราการเสียชีวิตที่สูง อัตราการกลับเป็นโรคซ้ำสูง และมีการพยากรณ์ของโรคที่ไม่ดี โดยพบอุบัติการณ์สูงในประเทศแถบทวีปเอเชีย และพบอุบัติการณ์สูงที่สุดในประเทศไทย เนื่องจากผู้ป่วยมักได้รับการวินิจฉัยในระยะลุกลาม และยังไม่มีวิธีการรักษาที่มีประสิทธิภาพ ดังนั้นการค้นหาเป้าหมายใหม่เพื่อใช้ในการพัฒนาวิธีการรักษาให้มีประสิทธิภาพสูงขึ้นจึงเป็นสิ่งจำเป็นยิ่ง โดยจะนำไปสู่การเพิ่มอัตราการรอดชีวิตของผู้ป่วย มะเร็งท่อน้ำดีมีความสัมพันธ์กับภาวะการอักเสบเรื้อรัง ซึ่งอาจทำให้มีการเพิ่มการแสดงออกของโทลล์ไลค์รีเซฟเตอร์สาม หรือทีแอลอาร์สาม (Toll-like receptor 3, TLR3) โดยมีรายงานว่าโทลล์ไลค์รีเซฟเตอร์สามไลแกน หรือสารโพลีไอซี [poly(I:C)] สามารถกระตุ้นให้เซลล์มะเร็งบางชนิดตายแบบอะพอพโทซิส และยังกระตุ้นระบบภูมิคุ้มกันต่อมะเร็งอีกด้วย อย่างไรก็ตาม ในมะเร็งบางชนิดรวมถึงมะเร็งท่อน้ำดีพบความผิดปกติของสัญญาเอนเอฟแคปป้าบี โดยส่งผลให้มีการแสดงออกที่เพิ่มสูงขึ้นของโปรตีนยับยั้งการตายแบบอะพอพโทซิส คือ โปรตีนซีไอเอพีหนึ่ง (cIAP1) และสอง (cIAP2) ส่งผลให้ยับยั้งการตายแบบอะพอพโทซิสเมื่อกระตุ้นด้วยสารโพลีไอซี จึงนำไปสู่คำถามวิจัยที่ว่า การยับยั้งการทำงานของโปรตีนซีไอเอพีหนึ่งและสองโดยการสลายตัวด้วยสารสะแมกไมมิติก (Smac mimetic) ร่วมกับสารโพลีไอซีจะเสริมฤทธิ์กันและกระตุ้นให้เซลล์มะเร็งท่อน้ำดีตายแบบอะพอพโทซิสหรือไม่ การศึกษาการแสดงออกของโปรตีนทีแอลอาร์สามพบว่ามีความจำเพาะกับเซลล์มะเร็งท่อน้ำดีเพาะเลี้ยง จำนวน 6 ชนิด แต่ไม่พบการแสดงออกในเซลล์ท่อน้ำดีเพาะเลี้ยงปกติ อย่างไรก็ตามการกระตุ้นทีแอลอาร์สามด้วยสารโพลีไอซี ไม่สามารถกระตุ้นให้เซลล์มะเร็งท่อน้ำดีเพาะเลี้ยงตายได้ แต่เมื่อเหนี่ยวนำให้มีการสลายตัวของโปรตีนซีไอเอพีหนึ่งและสองด้วยสารสะแมกไมมิติก พบว่ามีการเสริมฤทธิ์กับสารโพลีไอซีในการเหนี่ยวนำให้เซลล์มะเร็งท่อน้ำดีเพาะเลี้ยง จำนวน 2 ชนิด ตายผ่านทางอะพอพโทซิสโดยกระตุ้นการทำงานของเอนไซม์แคสเปสแปด และไม่ส่งผลต่อเซลล์ท่อน้ำดีเพาะเลี้ยงปกติ การศึกษากลไกเพิ่มเติมด้วยสารยับยั้งการทำงานของโปรตีนไคเนสริฟหนึ่ง (Receptor-interacting protein kinase1, RIPK1) และโดยการยับยั้งการแสดงออกของโปรตีนไคเนสริฟหนึ่งด้วยเทคโนโลยีคริสเปอร์แคสไนน์ พบว่าการตายของเซลล์เกิดผ่านทางการควบคุมของโปรตีนไคเนสริฟหนึ่ง กล่าวโดยสรุปงานวิจัยนี้ถือเป็นการค้นพบครั้งแรกโดยแสดงให้เห็นว่า สารโพลีไอซีและสารสะแมกไมมิติกทำงานเสริมฤทธิ์กันในการเหนี่ยวนำการตายแบบอะพอพโทซิสผ่านทางการทำงานของโปรตีนไคเนสริฟหนึ่งในเซลล์มะเร็งท่อน้ำดีเพาะเลี้ยง และมีความสำคัญยิ่งในการนำไปพัฒนาต่อยอดการรักษารูปแบบใหม่ที่มีประสิทธิภาพสูงขึ้น และนำไปสู่การเพิ่มอัตราการรอดชีวิตของผู้ป่วยมะเร็งท่อน้ำดีในอนาคต

สาขาวิชา	ชีวเคมีคลินิกและอณูทางการแพทย์	ลายมือชื่อนิสิต .....
ปีการศึกษา	2561	ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

5976662237 : MAJOR CLINICAL BIOCHEMISTRY AND MOLECULAR MEDICINE
KEYWORD: Cholangiocarcinoma, Toll-like receptor 3, Smac mimetic, Receptor-interacting protein kinase 1, Apoptosis
Thanpisit Lomphithak : Synergistic effect of Smac-mimetic and poly (I:C) on apoptosis of cholangiocarcinoma cells. Advisor: SIRIPORN JITKAEW, Ph.D.

Cholangiocarcinoma (CCA), a malignancy transformed from cholangiocytes in the bile ducts is more common in Asia and has the highest incidence rate in Thailand. CCA is an aggressive malignancy which has high mortality, high recurrence rate and poor prognosis due to late diagnosis and lack of effective treatment, therefore identification of novel therapeutic targets could lead to the development of more efficient therapy. CCA is associated with chronic inflammation that could upregulate Toll-like receptor 3 (TLR3) in CCA cells. TLR3 agonist, poly(I:C) has been reported to directly induce apoptosis in selected cancers and also activates anti-tumor immunity. However, in some cancers including CCA, dysregulated NF-κB signaling which upregulates the expression of cellular inhibitor of apoptosis proteins (cIAPs) 1 and 2 has been demonstrated to confer resistance to poly(I:C)-induced apoptosis. This led us to ask the research question whether the combination treatment of TLR3 ligand, poly(I:C) and IAPs antagonist, Smac mimetic could synergistically induce apoptosis in CCA cells. Here, we showed that TLR3 expression was differentially expressed in 6 CCA cell lines but not in a non-tumor cholangiocyte cell line. However, stimulation with poly(I:C) alone had no effect on CCA cell death. We showed for the first time that targeting cIAP1 and cIAP2 degradation by Smac mimetic, SM-164 in the combination with poly(I:C) treatment synergistically and specifically induced apoptosis in two representative CCA cell lines, but not in a non-tumor cholangiocyte cell line. Mechanistically, poly(I:C) and Smac mimetic treatment activates caspase-8 and induces apoptosis through a receptor-interacting protein kinase-1 (RIPK1)-dependent manner which was confirmed by a pharmacological inhibitor of RIPK1, necrostatin-1, and a deletion of *RIPK1* gene using CRISPR/Cas9 technology. In conclusion, our findings demonstrated for the first time that TLR3 ligand, poly(I:C) and Smac mimetic synergistically induced apoptosis in CCA cells that have important implications for the development of a novel therapeutic strategy which could lead to increase survival rate of CCA patients.

Field of Study:	Clinical Biochemistry and Molecular Medicine	Student's Signature .....
Academic Year:	2018	Advisor's Signature .....

ACKNOWLEDGEMENTS

This thesis could not have been possible without these supports that I have received along the way from these amazing people.

First of all, I would like to thank my advisor Dr.Siriporn Jitkaew for believing in me that I could do this. I do appreciate her trust, guidance and support

throughout the years. It was my honor to work with you and learn from you. You do not only teach me, but you keep motivating me so that I can achieve my goals. This is just the beginning. I look forward to working with you in near future. I also would like to thank you for the opportunity I have received.

I would like to thank Prof.Hironobu Sasano for his kind collaboration, for letting me be an exchange student in his laboratory at Tohoku University, Sendai, Japan. It was a great experience for me to explore a world of research.

I would like to thank the Scholarship from the Graduate School, Chulalongkorn University to commemorate the 72nd anniversary of his Majesty King Bhumibol Aduladej (GCUGE12-2), the Chulalongkorn University 90th Anniversary Fund and Join Funding (Ratchadaphiseksomphot Endowment Fund) (GCUGR1125613083M).

I would like to thank all the collaborators and co-authors for their contribution in our research collaboration.

I would like to thank all my friends who have been through our lives as graduate students. We will still support each other.

Finally, I would like to thank my family who are very supportive to me in everything I do. I will keep doing what I am supposed to do to be your pride and I will never let you down.

Thanpisit Lomphithak

TABLE OF CONTENTS

Page

LIST OF FIGURES

Page

CHAPTER I

INTRODUCTION

1.1 BACKGROUND AND RATIONALE

Cholangiocarcinoma (CCA) is a heterogeneous malignancy that originates in the bile ducts. CCA has the highest incidence in Thailand especially in North East and the incidence rate is progressively increased worldwide (1,2). CCA is an aggressive cancer with high mortality rate, high recurrence rate and poor prognosis, therefore CCA becomes a major health problem. To date, there are no effective therapeutic strategies for CCA, although surgery is considered to be the best approach. However, CCA is usually diagnosed when the disease has progressed into an advanced stage in which only therapeutic option is chemotherapy without the possibility of curative surgery (3). However, there are no effective chemotherapeutic regimens for CCA because of high chemoresistance in this disease (3). The 5-year survival rate of CCA patients especially with advanced stages are lower than 5-10% (4). Therefore, more effective treatment strategies are urgently needed for a better CCA treatment and prognosis. Apart from surgery and chemotherapy, there are three more major therapeutic strategies for cancer treatment including radiation therapy, targeted therapy, and immunotherapy. Combination treatments of chemotherapy or targeted therapy with immunotherapy or other treatment strategies that have dual benefits to target cancer cell death and enhance anti-tumor immunity could conceivably be promising therapeutic strategies for more efficient cancer treatment, reducing cancer relapse, increasing survival rate and finally possible cancer cures (5,6).

Chronic inflammation has been generally considered to play important roles in the development of CCA following primary sclerosing cholangitis, liver fluke, hepatitis B and C infection, which suggested the immune related etiology of CCA and therefore, immune modulation therapy could possibly result in better prognosis of patients with CCA. In addition, cancer immunotherapy for CCA is being evaluated in clinical trials (1). Polyinosinic-polycytidylic acid (poly I:C), a synthetic analog of

double-stranded RNAs which activates toll-like receptor 3 (TLR3) signaling, an endosomal pattern recognition receptor (PRR), has been used as a potent vaccine adjuvant in cancer immunotherapy to enhance its anti-tumor activity (7,8). In addition, *in vivo* studies have demonstrated the connection between TLR3 treatments and anti-tumor activity (9). It has been reported that poly(I:C) as an adjuvant in cancer immunotherapy acts on both cancer and immune cells that leads to tumor regression. In immune cells, the binding of poly (I:C) to TLR3 receptor leads to the activation of nuclear factor kappa B (NF-κB), mitogen-activated protein kinase (MAPK), and interferon regulatory factor *(*IRF3) inflammatory signaling pathways, thereby resulting in inflammatory cytokine production and immune response activation such as dendritic cell maturation (10). Interestingly, poly (I:C) has been shown to directly induce apoptosis both *in vitro* and *in vivo* in various types of cancers such as breast cancer, melanoma, renal cell carcinoma, prostate cancer, nasopharyngeal carcinoma, multiple myeloma, head and neck squamous cell carcinoma (HNSCC), hepatocellular carcinoma, neuroblastoma and non-small cell lung cancer (8,9,18–23,10–17).

Poly(I:C)-induced apoptosis has been proposed to be mediated through the formation of TIR-domain-containing adapter-inducing interferon-β (TRIF), Fas-associated protein with death domain (FADD) and receptor interacting protein kinase 1 (RIPK1), thereby leading to the activation of caspase-8, an extrinsic apoptosis pathway (24). However, negative regulators of poly(I:C)-induced apoptosis has been reported in a subset of cancers. Cellular inhibitor of apoptosis protein 1 and 2 (cIAP1 and cIAP2), two of nuclear factor κB (NF-κB) target genes, have been shown to be the negative regulators of TLR3 signaling-induced cancer cell apoptosis (25). Overexpression of cIAP1 and cIAP2 has been reported in various cancers that facilitates cancer cell survival by inhibiting apoptosis and therefore contributes to anti-tumor therapy failure (26). Therefore, targeting cIAP1 and cIAP2 proteins are considered as attractive targets for therapeutic intervention in cancers both as a single treatment or the combination treatment with other anti-cancer drugs (27). Several small molecules targeting IAP proteins have been developed called Smac mimetics. Smac mimetics function as endogenous IAP antagonists which target cIAPs for proteasomal degradation (28,29). The combination treatment of TLR3 ligand and

Smac mimetic has been studied as an alternative anti-tumor treatment in other cancers, however there is no study in CCA.

Damage-associated molecular patterns (DAMPs) that are released from damaged or dying cells in tumor microenvironment and pathogen-associated molecular patterns (PAMPs) are two of factors that induce chronic inflammation in tumor microenvironment (30,31). Both DAMPs and PAMPs have been shown to activate and induce the expression of pattern recognition receptors (PRRs) including TLR3, suggesting that TLR3 ligand could be used for treatment of CCA which is associated with chronic inflammation through the induction of cancer cell apoptosis. In addition, dysregulated expression and activation of NF-κB proteins have been observed in CCA cells that might limit TLR3 ligands-induced apoptosis (32,33). We therefore asked if cIAP1 and cIAP2 were overexpressed in CCA cells and contributed to the resistance to TLR3 ligands-induced apoptosis. As discussed above, we therefore hypothesized that cIAP1 and cIAP2 were overexpressed in CCA cells and targeting cIAP1 and cIAP2 for proteasomal degradation could sensitize CCA cells to TLR3 agonist-induced apoptosis. This led us to ask the research question if the combination treatment by targeting cIAP1 and cIAP2 for proteasomal degradation using Smac mimetic and targeting TLR3 by TLR3 ligand or poly(I:C) treatment synergistically induced apoptosis in CCA cells.

In this study, we investigated the expression of cIAP1, cIAP2, and TLR3 proteins in CCA cell lines. The single treatment of poly(I:C) or Smac mimetic and the combination treatment of poly(I:C) and Smac mimetic to induce apoptosis in CCA cells were investigated. Finally, the role of RIPK1, a proposed key mediator of Smac mimetic/TLR3-induced apoptosis was investigated through two different approaches including a pharmacological inhibitor and CRISPR/cas9-mediated deletion of *RIPK1* gene. Because TLR3 ligand, poly(I:C) is in clinical use and is entered clinical trials for cancer immunotherapy and Smac mimetics are currently in clinical trials as anti-cancer agents for treatment of some cancers, the data obtained from our *in vitro* studies using the combination treatment of poly(I:C) and Smac mimetic-induced CCA apoptosis could be translated into the promising treatment strategy for CCA patients. In addition, since poly(I:C) is an adjuvant used in cancer vaccine immunotherapy and Smac mimetic has been shown to enhance anti-tumor immunity, a synergistic

combination of Smac mimetic and poly (I:C)-induced apoptosis could be considered as an immunogenic cell death (ICD) which can also activate anti-tumor immunity (34,35). This combination treatment strategy could be more effective therapy for CCA and will lead to increase survival rate of CCA patients.

1.2 RESEARCH QUESTION

Does Smac mimetic and poly(I:C) have a synergistic effect on apoptosis of cholangiocarcinoma cells?

1.3 HYPOTHESIS

cIAP1 and cIAP2 were overexpressed in CCA cells and contributed to the resistance to TLR3 ligand-induced apoptosis, therefore targeting cIAP1 and cIAP2 for proteasomal degradation using Smac mimetic could synergize poly (I:C)-induced CCA cell apoptosis.

1.4 OBJECTIVES (AIMS)

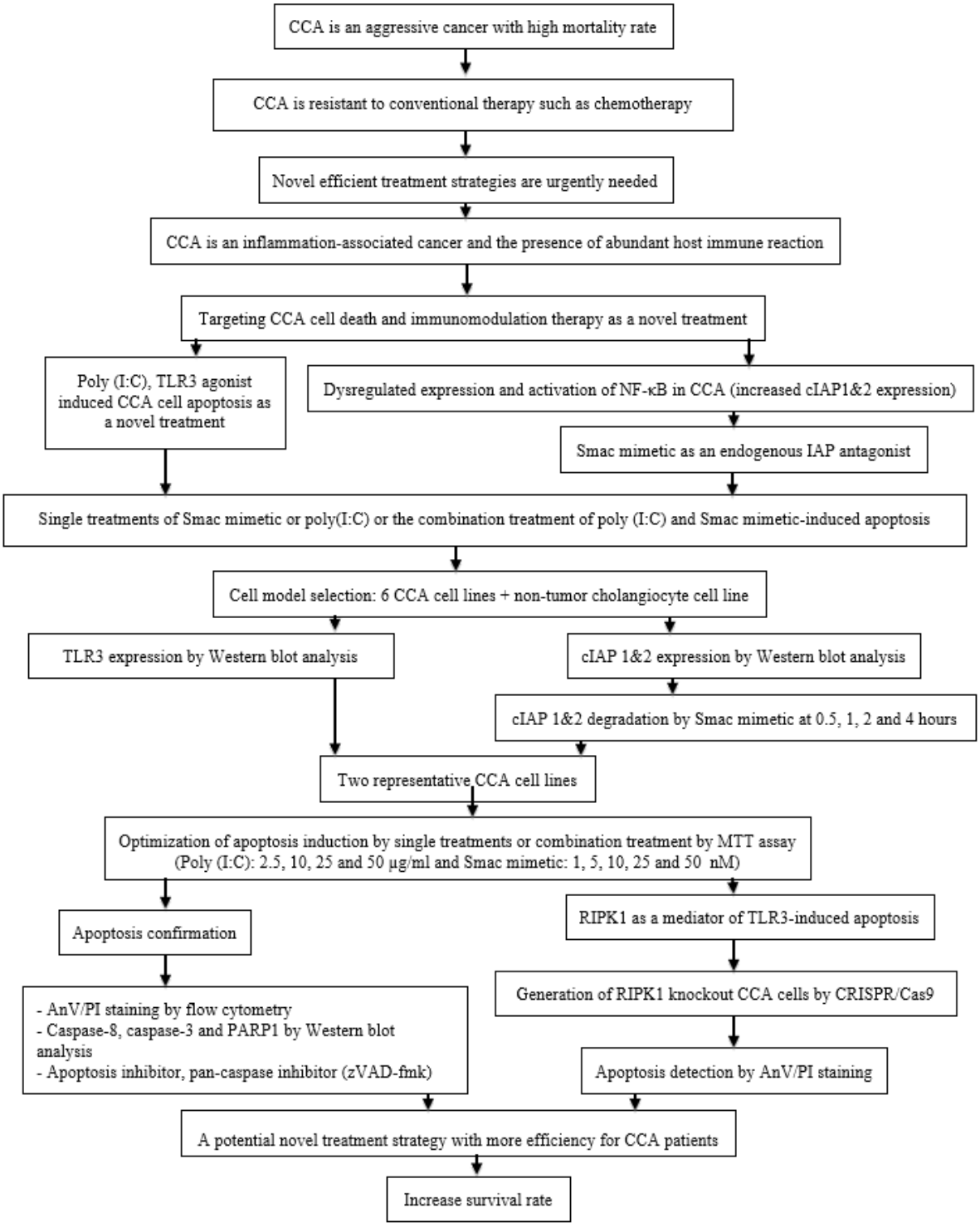
1.4.1 To examine the expression of cIAP1, cIAP2 and TLR3 proteins in CCA cell lines

1.4.2 To induce the degradation of cIAP1 and cIAP2 by Smac mimetic

1.4.3 To examine apoptosis induction by Smac mimetic or poly(I:C) single treatments or the combination of Smac mimetic and poly(I:C) treatment

1.4.4 To determine the role of RIPK1 as a mediator of Smac mimetic and poly (I:C)-induced CCA cell apoptosis

1.5 CONCEPTUAL FRAMEWORK



1.6 SCOPE OF RESEARCH

In this research, *in vitro* cell model was used to investigate the synergistic effect of Smac mimetic and poly(I:C)-induced apoptosis. We examined the expression of cIAP1, cIAP2 and TLR3 in 6 different CCA cell lines and a non-tumor cholangiocyte. A bivalent Smac mimetic called SM-164 was used to induce the degradation of cIAP1 and cIAP2 in representative CCA cell lines and a non-tumor cholangiocyte. Apoptosis induction in CCA was triggered by single treatments of Smac mimetic (SM-164) or a synthetic TLR3 ligand, poly(I:C) or the combination treatment of Smac mimetic (SM-164) and poly (I:C). Cell death was screened by MTT assay. Apoptosis was further determined by more specific markers including AnV/PI staining followed by flow cytometer analysis, caspase-8, caspase-3 activation and PARP-1 cleavage by Western blot analysis. In addition, inhibitor of apoptosis, a pan-caspases inhibitor (zVAD-fmk) was used to determine if the combination treatment induced apoptosis. Finally, the role of RIPK1 as a proposed mediator of TLR3 ligand and Smac mimetic was explored using a pharmacological inhibitor and a genetic deletion of *RIPK1* gene by CRISPR/cas9-mediated gene deletion.

1.7 BENEFIT AND APPLICATION

Lack of effective therapy is major contribution of low 5-year survival rate and high mortality rate of CCA patients. This is the first study demonstrating the combination treatment of poly(I:C) and Smac mimetic and their synergistic effect to induce apoptosis of CCA cells. Since Poly(I:C) is being used in clinics as well as a vaccine adjuvant for immunotherapy nowadays and small molecules targeting cIAP1 and cIAP2 for degradation (i.e. Smac mimetics) are being evaluated in clinical trials for treatment of some cancers, the *in vitro* studies from the current study could be translated into the promising treatment strategy for the development of a novel anti-cancer therapy for CCA patients. Moreover, both poly(I:C) and Smac mimetic have been shown to enhance anti-tumor immunity. The combination treatment strategy of poly(I:C) and Smac mimetic-induced apoptosis could be considered as an immunogenic cell death which can also activate anti-tumor immunity. This combination treatment strategy could be more effective therapy for CCA patients and will lead to increase survival rate of CCA patients.

CHAPTER II

LITERATURE REVIEW

2.1 CHOLANGIOCARCINOMA

Cholangiocarcinoma (CCA) is a form of cancer that originates in the bile ducts. CCAs can be classified as intrahepatic cholangiocarcinoma (iCCA), perihilar cholangiocarcinoma (pCCA) and distal cholangiocarcinoma (dCCA) due to their anatomical location. Intrahepatic cholangiocarcinoma (iCCA) originates above the second-order bile ducts, whereas perihilar cholangiocarcinoma (pCCA) and distal cholangiocarcinoma (dCCA) can be separated by the cystic duct (36). CCA has the highest incidence in Thailand especially in North East in which it has overall incidence of 85/100,000 while it has been shown that its incidence keeps rising globally (Figure 1) (2). CCA is a heterogeneous bile duct cancer that host-specific background and geographical distribution contribute as risk factors of CCA. However, the risk factors of CCA are unclear. Primary Sclerosing cholangitis (PSC) is an autoimmune disease that affects bile ducts. It leads to chronic inflammation, production of bile mutagens and proliferation of biliary epithelium that promote CCA development (37). Viral hepatitis including Hepatitis B (HBV) and Hepatitis C (HCV) has been shown to be associated with CCA. The presence of cirrhosis affected from viral hepatitis as well as chronic inflammation leads to CCA development (38,39). One of well-established risk factors is liver fluke (Opisthorchis viverrini) infection which has been classified as type I carcinogen and a strong risk factor of CCA. It is normally found in Eastern Asia, especially in Thailand, where the great majority of CCAs are caused by this parasitic infection. Chronic inflammation and cholangitis are the consequences of this parasite infection that may contribute to CCA development (40,41).

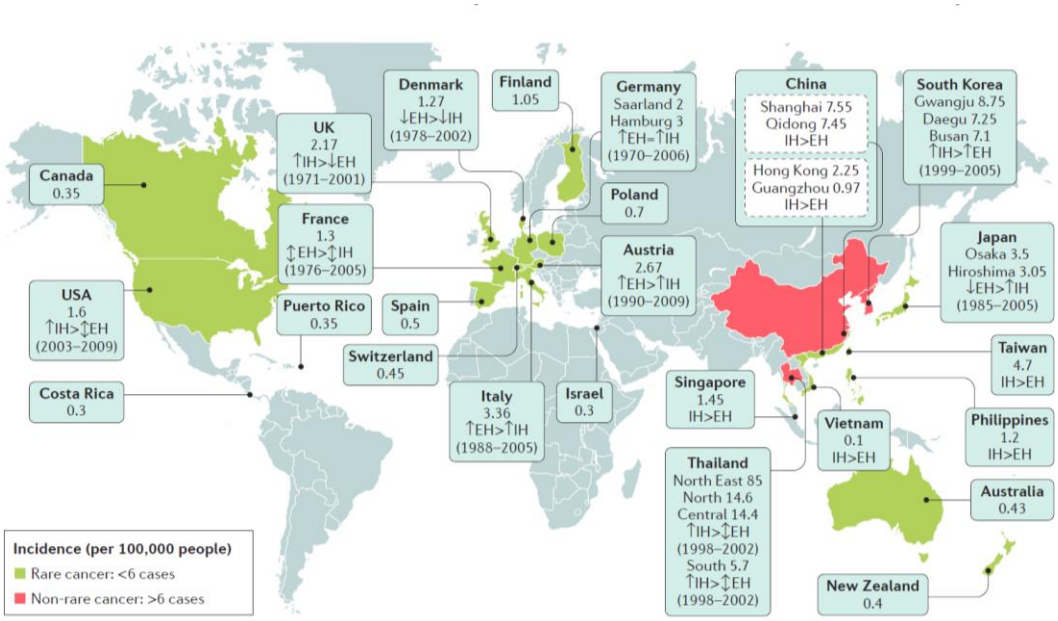


Figure 1 : Worldwide incidence of CCA

Countries with lower incidence (< 6 per 100,000 cases, rare cancer) represent in green color, whereas pink color identifies countries in which CCA is not considered a rare cancer (> 6 per 100,000 cases). North East of Thailand has highest incidence in which overall incidence reaches 85/100,000 people (case per 100,000 people)(2).

2.2 CURRENT CHOLANGIOCARCINOMA THERAPY

CCA is an aggressive cancer. In early stage, it is normally asymptomatic, and it is usually diagnosed at an advanced stage. It also has high mortality due to its aggressiveness. For CCA treatment, surgery is considered to be the treatment option for all types of CCA, but most CCA patients are presented with the advanced stage where surgical resection cannot be performed(1). The available treatments are not effective enough for CCA patients with unresectable or advanced-stage CCA. The overall median survival is less than 1 year with the current chemotherapy regimen such as gemcitabine or cisplatin (42). Therefore, more effective treatment strategies are urgently needed for a better CCA treatment. Immunotherapy is now a promising therapy for cancer treatments. It can reactivate the natural body's immune response to recognize and kill the cancer cells. Since chronic inflammation has been linked to liver fluke and primary sclerosing cholangitis associated-CCA tumorigenesis highlighting the immune etiology of CCA and thus cancer immunotherapy could be proposed as an alternative strategy for treatment of

CCA. Cancer immunotherapy alone or in combination with other treatments for CCA is being evaluated in clinical trials (1).

2.3 CANCER THERAPY AND CANCER IMMUNOTHERAPY

There are many types of cancer treatment such as surgery, radiation therapy, chemotherapy, targeted therapy and immunotherapy (43). Surgery is the best way to remove cancer from the body but most cancers with poor prognosis are usually diagnosed at an advanced stage so that surgery cannot be performed. The next common option is radiotherapy and chemotherapy, but some cancers do not respond well to chemotherapeutic drugs due to its chemoresistance property (3). There are several mechanisms of chemoresistance that help CCA cells to confer resistance to chemotherapy (44). One of major mechanisms driving chemoresistance is resisting apoptosis cell death either through the mutation of proapoptotic protein such as *p53* gene or overexpression of anti-apoptotic proteins such as Bcl-Xl, Bcl-2, Mcl-1 (45). Mcl-1 overexpression through the regulation of IL-6-induced Stat3 signaling has been observed in CCA (46). Radiotherapy and chemotherapy cannot kill all cancer cells, some population of cancer cells are still alive that lead to cancer recurrence. Besides, these treatments also have side effects on normal cells. Now, people are focusing on targeted therapy which is targeting the specific genes or molecular pathways. In order to apply targeted therapy for cancer treatment, it requires the well-known molecular mechanisms being dysregulated in cancer cells which always are heterogeneous. Another promising cancer treatment, is immunotherapy that can reactivate the immune system to recognize and fight against cancer (5). Immunotherapy has been validated in the clinical uses as an effective cancer treatment for various cancers. One of risk factors for cancers is chronic inflammation. Chronic inflammation can lead to tumor initiation. Malignant transformation frequently takes place and the immune system can also recognize and destroy the malignant transformed cells. They undergo the process of cancer immunoediting which consists of three phrases: elimination, equilibrium and escape. During these phases, there is a crosstalk among cancer cells, immune cells and inflammation. Innate and adaptive immune cells recognize and destroy cancer cells in the elimination phase. Then, cancer cells are exposed to immunoselective pressure and the immunosuppressive activity is conferred by cancer-

associated inflammation to the tumor microenvironment (TME). That leads to immune evasion of cancer cells in this equilibrium phase. In escape phase, cancer-associated inflammation is responsible for angiogenesis, invasion and also epithelial-to-mesenchymal transition (EMT). Some cancer treatments can reeducate the TME to fight cancer cells by the activation of dendritic cells. Immunogenic cell death (ICD) can be elicited in cancer cells to activate dendritic cell-mediated antitumor T cell responses (47). Targeting TME or immune cells to fight cancer could be a promising approach to sensitize cancer cells to immunotherapy. Moreover, combination of conventional therapy (radiotherapy, chemotherapy and targeted therapy) with immunotherapy is suggested to be an efficient cancer treatment which can kill cancer cells and also reactivate the immune response (5). The cancer recurrence can be prevented, and cancer treatment can be more effective.

2.4 TOLL-LIKE RECEPTOR 3 (TLR3) SIGNALING AND TLR3 AGONIST, POLY (I:C)

Toll-like receptor 3 (TLR3) is a member of an immune recognition receptor family that is essential for innate immunity. Basically, there are 10 TLRs (TLR1-10) in human which are different in their ligand specificity and their subcellular localization (48). They are class I transmembrane glycoproteins and are divided by their extracellular domains which contain different number of leucine-rich-repeat (LRR) motifs (49). Protein-protein interaction and ligands recognition of TLRs are dependent on their LRR motifs (48). TLR3 consists of a N terminal ectodomain (ECD), which is formed by 23 LRRs, and a C terminal TIR domain (50,51). Poly (I:C) (polyriboinosinic:polyribocytidylic acid), a synthetic analogs of double stranded RNAs, is now used as a potent vaccine adjuvant in cancer immunotherapy. It has been used to mimic the response to RNA virus infection recognized by TLR3 signaling which is then activated by ligand-induced dimerization of TLR3 receptors. The Toll/interleukin-1 (IL-1) receptor (TIR) domain of TLR3 engages TIR domain-containing adaptor protein inducing IFNβ (TRIF) and TRIF-related adaptor molecule (TRAM) (Figure 2). TRIF is recruited to TLR3. This step is considered to dictate downstream signaling processes. TRIF signaling activates the serine/threonine kinase TANK-binding kinase-1 (TBK-1), that phosphorylates the transcription factor

interferon (IFN) regulating factor 3 (IRF3) (52). Phosphorylated IRF3 translocates to the nucleus and activates specific proinflammatory target genes, one of the most significant gene is type I IFNs (52). In addition to activating IRF3, TLR3 signaling via TRIF also activates NF-κB (53,54). The TLR3-induced NF-κB signaling is activated by two different pathways of TRIF-dependent recruitment. First it is mediated by RIPK1 and the second is TRAF6. Both of which converge on Transforming growth factor beta-activated kinase 1 (TAK1) and the IκB kinase (IKK) complex. IKKα and IKKβ is phosphorylated by TAK1, which in turn phosphorylates IκB, the NF-κB inhibitor, resulting in IκB degradation and the nuclear translocation of NF-κB. The outcomes of TLR3 signaling are proinflammatory cytokines, chemokines production and type I IFNs response. Poly (I:C) has been used to promote an antitumor immune response. TLR3 has been reported to be expressed by various types of cells both immune and non-immune origins such as B cells, dendritic cells, hepatocytes, endothelial cells and epithelial cells from various origins. Interestingly, TLR3 expression has been observed in many cancer cells such as breast cancer and melanoma (11,55). TLR3 expressed on cancer cells might be activated by pathogen-associated molecular patterns (PAMPs) derived from microbes and damage-associated molecular patterns (DAMPs) derived from damaged and dying cells in the tumor microenvironment. Moreover, the release of nucleic acids from necrotic cancer cells and damaged normal epithelial cells in the tumor microenvironment can act as a DAMPs and activate TLR3 expression in both cancer cells (56) and immune cells (57).

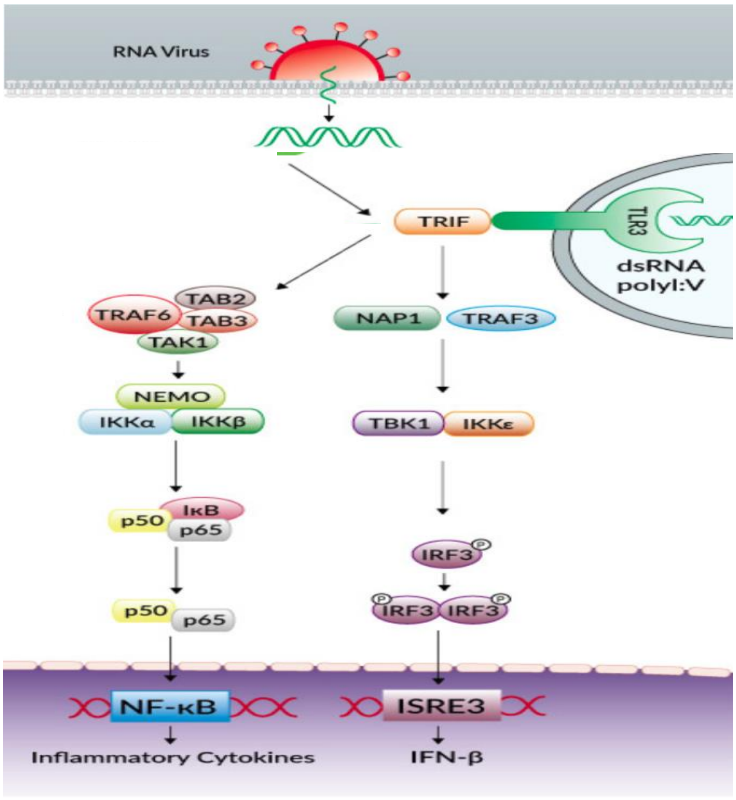


Figure 2 : Toll-like receptor 3 signaling.

Double-stranded RNA is recognized by TLR3 on endosomal membrane, which results in the recruitment of TRIF and the activation of Type I IFN and NF-κB signaling (modified from https://www.invivogen.com/review-viral-dsrna-signaling)

In addition to drive innate immune response, TLR3 activation has been shown to directly induce apoptosis in selected cancer cells. In addition, several studies have demonstrated that the combination treatment of TLR3 ligand and other agents such as chemotherapeutic drugs and Smac mimetic synergistically induce apoptosis (13,58–60). TLR3-induced apoptosis has been shown to be mediated through caspase-8 activation suggesting that TLR3 induces the extrinsic apoptotic pathway (Figure 3) (13,24). In contrast to a classical extrinsic apoptosis signaling, TLR3 induces caspase-8 activation through binding to TRIF which contains RIP Homotypic Interaction Motif (RHIM) at its C-terminus. The interaction between TLR3 and TRIF recruits RHIM-containing motif, RIPK1 that in turn recruits FADD (by interacting with dead

domain, DD, of RIPK1 and caspase-8 (by interacting with death effector domain, DED, of FADD) to form TRIF/RIPK1/FADD/caspase-8 signaling complex and mediating apoptosis (Figure 3). TLR3-induced apoptosis is negatively regulated by cIAP1/cIAP2 as well as $FLIP_L$. Therefore, poly (I:C) targeting TLR3 to induce apoptosis in cancer cells could be an efficient cancer treatment.

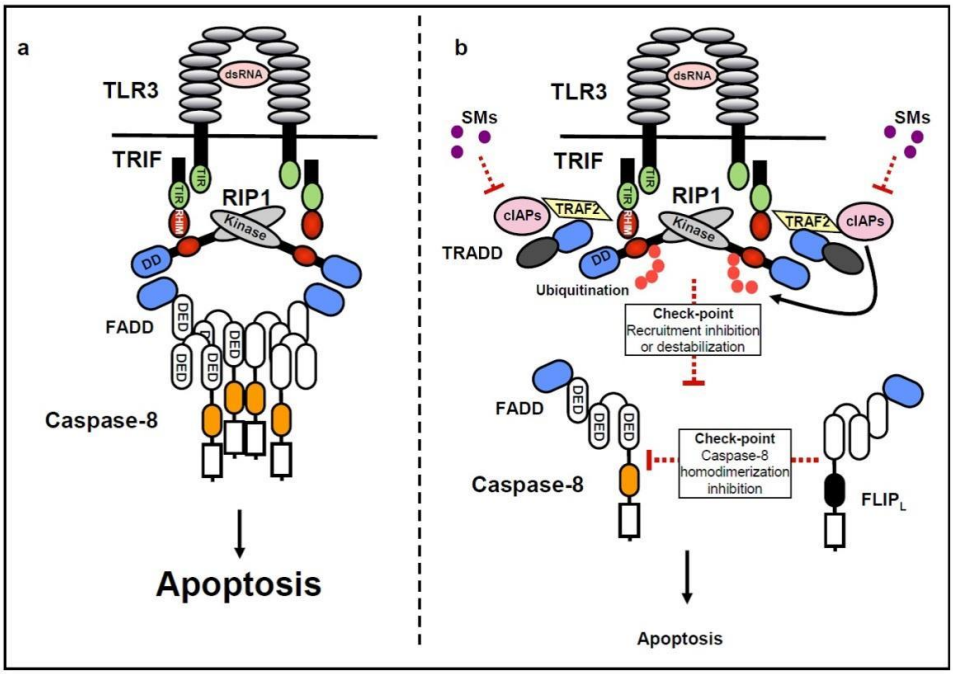


Figure 3 : Hypothetical model of TLR3-triggered apoptosis and negative regulators

(a) TLR3 activation by dsRNA induces the formation of caspase-8/FADD/RIPK1/TRIF complex. (b) Negative regulatory mechanism of caspase-8 activation and recruitment by TLR3. The adaptor protein TRIF recruits TRADD, TRAF2 and cIAP1/cIAP2 which add the ubiquitin to RIPK1. This results in the inhibition of RIPK1 recruitment to caspase-8 complex which leads to apoptosis suppression. Apoptosis resistance can be overcome by targeting cIAP1/cIAP2 for auto-ubiquitination and proteasomal degradation by small molecules such as smac-mimetics. The formation of caspase-8 signaling complex can also be regulated by FLIPL that will bind to caspase-8 resulting in the formation of caspase-8/FLIPL complex (61).

2.5 APOPTOSIS SIGNALING PATHWAYS

Apoptosis is the best studied mode of programmed cell death. Apoptotic cell death results from the disintegration of the cells by the sequential activation of cysteine proteases, called caspases that cleave numerous proteins in the cells. There are two major pathways of apoptosis: the extrinsic pathway and the intrinsic pathway (Figure 4). The extrinsic pathway also called the death receptor pathway is triggered by the ligation of members of the tumor necrosis factor (TNF) superfamily to the cell surface death receptor of TNFR superfamily including TNFRI, CD95/Fas, and TRAILR. Ligation of death receptor initiates the assembling of the multiprotein death-inducing signaling complex (DISC) containing FADD and Caspase-8. In certain cell types and conditions, DISC also contains RIPK1. The catalytic activation of caspase-8 in the DISC leads to the cleavage of an effector caspase, caspase-3. The intrinsic pathway also called the mitochondrial pathway, in contrast to the death receptor pathway; caspase-9 plays a central role in this pathway. The mitochondrial pathway is initiated by many stimuli including increased in intracellular reactive oxygen species, genotoxic stress (DNA damage), the unfolded protein response, and the deprivation of growth factors. These initiators promote the permeability of mitochondrial membrane, thereby promoting the release of cytochrome c from mitochondria into the cytosol. Once cytochrome c is in the cytosol, it binds to Apoptotic protease activating factor-1 (Apaf-1) and ATP, which then bind to procaspase-9 to form a death complex known as apoptosome. Caspase-9 is activated in the apoptosome complex which then activates an effector caspase, caspase-3. Active caspase-3 generated by the death receptor or the mitochondrial pathways will cleave numerous proteins in the cells; finally result in apoptosis (62).

In the death receptor (extrinsic) pathway observed in some cell types, ligation of death receptors such as TNF-stimulated TNF receptor 1 (TNFR1) complexes has been more complicated. Intracellular domain of TNFR1 recruits associated proteins to form a complex I. Complex I consists of TRADD, TRAF2, cIAP1/2 and RIPK1. RIPK1 is usually ubiquitinated by cIAP1/2 and ubiquitin ligases in this complex, which leads to the activation of NF-κB signaling and the upregulation of gene expressions that are related to cell survival and inflammation. When complex I is

blocked by Smac mimetic, targeting cIAP1/2 for proteasomal degradation, RIPK1 is de-ubiquitinated and leads to the formation of a new complex called complex IIa which consists of RIPK1, FADD and caspase-8. The activation of caspase-8 in complex IIa subsequently activates the executioner caspase, or caspase-3, which leads to apoptosis cell death (Figure 3) (63).

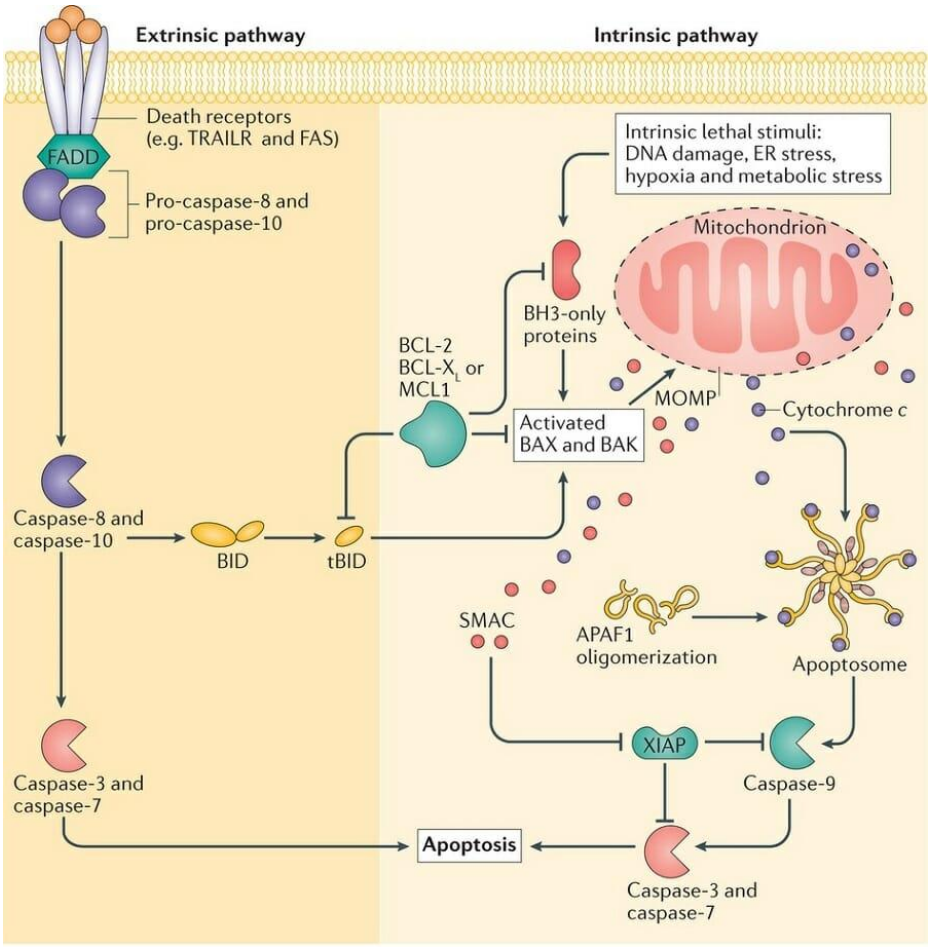


Figure 4 : Extrinsic and intrinsic apoptosis signaling pathways.

Two major types of apoptosis signaling: (1) an extrinsic pathway which is mediated through caspas-8 activation, an extrinsic initiator caspase and (2) an intrinsic pathway which is activated through cytochrome c release from mitochondria intermembrane space and then the formation of apoptosome complex which leads to the activation of caspase-9, an intrinsic initiator caspase. Both active caspase-8 and caspase-9 are then converted at executioner caspases which are caspase-3 and caspase-7. The activation of both caspase-3 and caspase-7 by initiator caspases results in the cleavage of several substrates.

(adapted from https://biologydictionary.net/apoptosis/)

2.6 CELLULAR INHIBITOR OF APOPTOSIS PROTEIN (CIAP1/2) AND SMAC MIMETIC AS AN ANTICANCER AGENT

The Inhibitor of apoptosis proteins (IAPs) consist of a family of anti-apoptotic proteins that serve as endogenous inhibitors of apoptosis by binding to second mitochondria-derived activator of caspase (SMAC, also known as DIABLO) which is released from the mitochondria during intrinsic apoptosis thereby interfering with the activation of caspases (Figure 5). There are eight IAP proteins in humans. The common feature of all IAPs is the presence of baculoviral IAP repeat (BIR) domain and some IAP proteins such as cIAP1, cIAP2, XIAP and ML-IAP also contain a carboxy-terminal RING domain (Figure 5). These RING domain-containing IAP proteins function as E3 ubiquitin ligases that play a role in survival and signaling pathways including NF-κB signaling (64). Focusing on TNF-α-induced NF-κB signaling, IAP proteins (i.e. cIAP1 and cIAP2) serve as E3 ubiquitin ligases that add ubiquitin to RIPK1 resulting in the promotion of cell survival and inflammation through the activation of NF-κB target genes and inhibition of RIPK1-mediated apoptosis (65). The expression of IAP proteins are deregulated in various human cancers due to their genetic aberrations such as an increased protein expression and the loss of endogenous inhibitors such as SMAC. Overexpression of IAPs in various cancer cells has been liked to tumor progression, poor prognosis and treatment failure (27). Therefore, IAP proteins are one of potential targets for cancer therapeutic intervention.

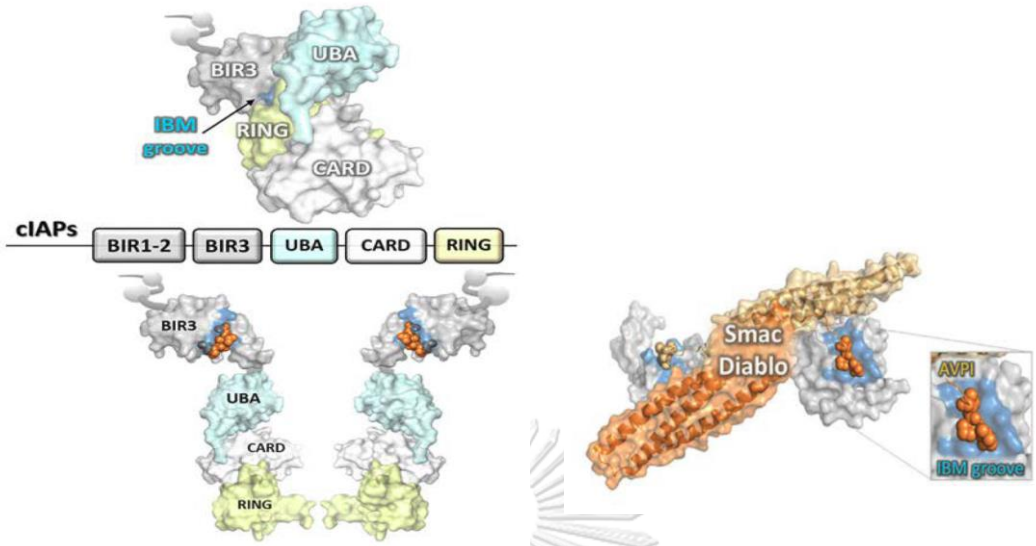


Figure 5 : Functional domains of cIAPs and binding of Smac/Diablo to dimer of cIAP protein.

cIAPs are consisting of several functional domains as indicated (in the middle) that are present in the cytosol of the cells as inactive monomers (on top). Smac/Diablo (on left) induces dimerization of cIAPs and binds to the dimer of cIAP through its N-terminal AVPI (Ala-Val-Pro-Ile) binding motif (orange color) (66).

The most widely strategy that has been used to remove IAP proteins particularly, cIAP1 and cIAP2 is based on mimicking the IAP-binding motif of SMAC, which specifically counters the anti-apoptotic activity of IAP proteins. Therefore, small molecules targeting IAP proteins have been developed called Smac mimetic, which function as an endogenous IAP antagonist (Figure 6) (27). Smac mimetic removes cIAP1 and cIAP2 through auto-ubiquitination of IAPs, thereby targeting IAPs to proteasomal degradation (28). Surprisingly, through screening a panel of 50 human non-small-cell lung cancer cell lines, it has been demonstrated that Smac mimetic induces autocrine secretion of TNF-α, therefore renders cancer cell apoptosis through the formation of RIPK1-dependent activation of caspase-8 (28,29) Smac mimetics are now in the clinical trials as an anticancer agent. Moreover, T-cell antitumor immunity can be enhanced by smac-mimetics in a cancer vaccine mouse model (67). It suggests that Smac mimetics are beneficial for cancer treatment.

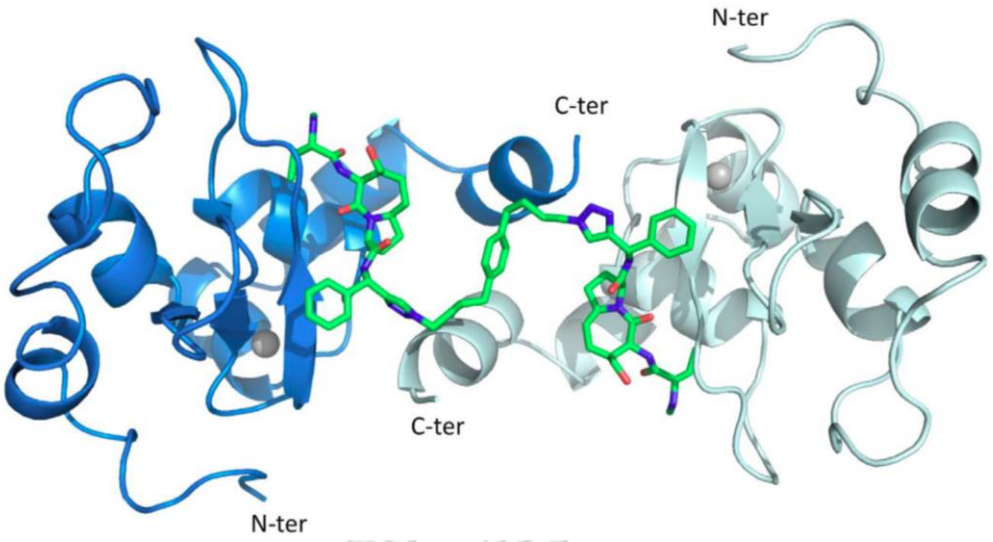


Figure 6 : Binding of Smac mimetic to dimeric assemblies of cIAP1.

X-Ray structure of dimerization of cIAP1-BIR3 dimer (cartoon in blue and pale blue) in complex with Smac mimetic (green sticks) leads to rapid auto-ubiquitination of cIAPs, thereby their degradation. (68)

2.7 RECEPTOR-INTERACTING SERINE/THREONINE-PROTEIN KINASE 1 (RIPK1)

RIPK1, a serine/threonine kinase is a multifunctional protein that regulates signaling pathways leading to opposing outcomes including inflammation and cell death both in the form of apoptosis and necroptosis (69). RIPK1 consists of a C-terminal death domain (DD) motif, a RIP homotypic interaction motif (RHIM), an intermediate domain and an N-terminal kinase domain (70). The intermediate domain of RIPK1 contains a site, K63-linked ubiquitination (K377), which is recognized by many of the ubiquitin binding proteins involved in NF-κB signaling (71). The RHIM domain of RIPK1 can interact with RHIM domain of other proteins such as TRIF and RIPK3. The death domain of RIPK1, a six helical bundle shape, can be found in FAS, TNFR1, FAS-associated death domain protein (FADD) and TNFR1-associated death domain protein (TRADD) (72). RIPK1 regulates signaling pathway through either kinase-dependent or kinase-independent manner, for example, in response to TNF-α and TLR ligands. Moreover, RIPK1 has been shown to be involved cell death

pathways. RIPK1 is well-studied for its canonical role in TNF-α signaling. Upon ligand binding to TNFR1 or TRAIL, these receptors trimerize and recruit a membrane-associated complex or complex I which contains TRADD, RIPK1 and several E3 ubiquitin ligases including cIAP1 and cIAP2 (70). The recruitment of the ubiquitin ligases to the complex resulting in RIPK1 polyubiquitination at Lys377 (70). This event leads to the pro-inflammatory response by recruiting TAK1 and activating NF-κB signaling or contributing to the IκB kinase (IKK)/mitogen-activated protein kinase (MAPK)-dependent NF-κB activation that drives inflammatory cytokine production (73,74). On the other hand, when RIPK1 is not ubiquitinated, it will form a second complex mediated by the recruitment of FADD. Then, it will lead to a formation of complex IIa by the recruitment of caspase 8 resulting in apoptosis or the recruitment of RIPK3 resulting in the formation of complex IIb and necroptosis activation. In addition to TNF-α signaling, RIPK1 has been revealed to be necessary for TLR3-mediated NF-κB activation (75) and cell death (71) as well. The physiological roles of RIPK1 and its regulation in TLR3 signaling remains unknown. Therefore, RIPK1 might play pivotal roles in TLR3-ligand-induced signaling especially in TLR3 ligand-induced apoptosis.

CHAPTER III

MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Reagents

3.1.1.1 Reagents for Annexin V/PI staining and MTT assay

- Annexin V-FITC apoptosis detection kit (ImmunoTools, Friesoythe, Germany)
- Propidium iodide (Invitrogen; Thermo Fisher Scientific, Inc., California, USA)
- 10X PBS (HyClone Laboratories, Logan, Utah, USA)
- MTT reagent (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) (AppliChem GmbH, Darmstadt, Germany)
- Dimethyl sulfoxide (DMSO), analytical grade (RCI labscan limited, Gillman SA, Australia)
- IsoFlow Sheath Fluid (1x10L) (Beckman Coulter, Inc, Atlanta, Georgia, USA)

3.1.1.2 Reagents for Western blot analysis

- Complete mini EDTA-free protease cocktail inhibitor (Roche, Mannheim, Germany)
- RIPA lysis buffer (Merck Millipore, Darmstadt, Germany)
- Bradford dye reagent (Bio-Rad, Hercules, California, USA)
- Standard bovine serum albumin (BSA) (AppliChem GmbH, Darmstadt, Germany)
- Tween-20 (AppliChem GmbH, Darmstadt, Germany)
- 30% Acrylamide/Bis Solution 29:1 (Bio-Rad, Hercules, California, USA)
- Ammonium Persulfate (APS) (AppliChem GmbH, Darmstadt, Germany)

- β-mercaptoethanol (AppliChem GmbH, Darmstadt, Germany)
- Tetramethylenediamine (TEMED) (AppliChem GmbH, Darmstadt, Germany)
- Polyvinylidene Fluoride (PVDF) membrane 0.45 µM (Amersham, GE Healthcare, Buckinghamshire, United Kingdom)
- Precision Plus Protein™ Prestained Standards Ladder (Bio-Rad, Hercules, California, USA)
- Blotting-Grade Blocker (Bio-Rad, Hercules, California, USA)
- Clarity™ Western Enhanced chemiluminescence (ECL) substrate (Bio-Rad, Hercules, California, USA)
- UltraCruz® Autoradiography Film (Santa Cruz Biotechnology, Inc., Dallas, Texas, USA)
- GBX Developer (KODAK, Rochester, New York, USA)
- GBX Fixer (KODAK, Rochester, New York, USA)

3.1.1.3 Antibodies for Western blot analysis

- Rabbit anti-human TLR3 monoclonal antibody (Cell signaling, Danvers, Massachusetts, USA) catalog#6961 clone D10F10, molecular weight 115-130 kDa, Dilution 1:1000
- Rabbit anti-human cIAP1 monoclonal antibody (Cell signaling, Danvers, Massachusetts, USA) catalog#7065 clone D5G9, molecular weight 62 kDa, Dilution 1:1000
- Rabbit anti-human cIAP2 monoclonal antibody (Cell signaling, Danvers, Massachusetts, USA) catalog#3130 clone 58C7, molecular weight 70 kDa, Dilution 1:1000
- Rabbit anti-human PARP-1 monoclonal antibody (Cell signaling, Danvers, Massachusetts, USA) catalog#9532 clone 46D11, molecular weight 116, 89 kDa, Dilution 1:5000
- Rabbit anti-human caspase-3 polyclonal antibody (Cell signaling, Danvers, Massachusetts, USA) catalog#9662, molecular weight 17, 19, 35 kDa, Dilution 1:2500

- Rabbit anti-human actin polyclonal antibody (Cell signaling, Danvers, Massachusetts, USA) catalog#4970 clone 13E5, molecular weight 42 kDa, Dilution 1:5000
- Mouse anti-human caspase-8 monoclonal antibody (Cell signaling, Danvers, Massachusetts, USA) catalog#9746 clone 1C12, molecular weight 18, 43, 57 kDa, Dilution 1:1000
- Mouse anti-human RIPK1 monoclonal antibody (BD Biosciences, San Jose, California, USA); catalog#610459 clone 38/RIP, molecular weight 74 kDa, Dilution 1:2500
- Anti-mouse IgG, Horse radish peroxidase (HRP)-linked secondary antibody; catalog#7076
- Anti-rabbit IgG, Horse radish peroxidase (HRP)-linked secondary antibody; catalog#7074

3.1.1.4 Reagents for cell culture

- Ham's Nutrient Mixture F12 medium (HyClone Laboratories, Logan, Utah, USA)
- Dulbecco's Modified Eagles Medium (DMEM) (HyClone Laboratories, Logan, Utah, USA)
- Penicillin/Streptomycin (HyClone Laboratories, Logan, Utah, USA)
- Fetal bovine serum (FBS) (Sigma, St Louis, Missouri, USA)
- Trypsin/0.25%EDTA (HyClone Laboratories, Logan, Utah, USA)
- Trypan blue (Gibco-Invitrogen; Thermo Fisher Scientific, Inc., California, USA)

3.1.1.5 Reagents for cell treatment

- Dimethyl sulfoxide (DMSO), cell culture grade (Sigma, St Louis, Missouri, USA)
- zVAD-fmk, Benzyloxycarbonyl-valine-alanine-aspartate-fluoromethyl ketone (Calbiochem, Merck Millipore, Darmstadt, Germany)
- Poly(I:C) HMW (InvivoGen, San Diego, California, USA).

- Necrostatin-1 (Sigma, St Louis, Missouri, USA)
- Smac mimetic, SM-164 (a gift from Dr. Shaomeng Wang, University of Michigan, Ann Arbor, Michigan, USA).

3.1.1.6 Lentiviral and CRISPR system and reagents

- pCMV-dr8.2-dvpr (packaging plasmid) (Addgene#8455)
- pCMV-VSV-G (envelope plasmid) (Addgene#8454)
- LentiCRISPR V2 (Addgene#52961)
- Opti-MEM I (Gibco-Invitrogen; Thermo Fisher Scientific, Inc., California, USA).
- TurboFect transfection reagent (Invitrogen; Thermo Fisher Scientific, Inc., California, USA).
- Sterile filter membrane (0.45 μM) (Merck Millipore, Darmstadt, Germany)
- Polybrene transfection reagent (Merck Millipore, Darmstadt, Germany)
- Puromycin (Merck Millipore, Darmstadt, Germany)

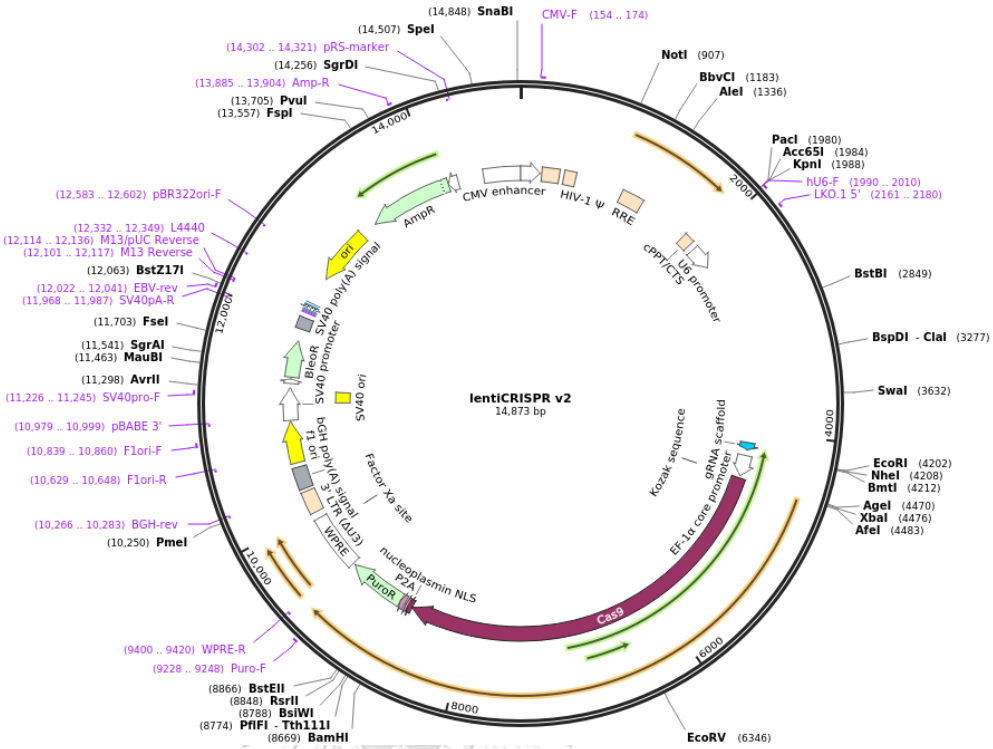


Figure 7 : LentiCRISPR V2 plasmid map
(Addgene#52961) (https://www.addgene.org/52961/)

3.1.2 Equipment and Instruments

- Steri-Cycle CO_2 Forma 371 Incubator, CO_2 incubator (Thermo Fisher Scientific, Inc., California, USA)
- Laminar flow biosafety cabinet class II (Haier Biomedical, Qingdao, China)
- Inverted microscope (Olympus Optical, Tokyo, Japan)
- Water bath (Memmert GmbH, Schwabach, Germany)
- Liquid Nitrogen Tank (Taylor-Wharton, Osaka, Japan)
- Hemacytometer (Hausser scientific, Horsham, Pennsylvania, USA)
- Flow cytometer; Navios (Beckman Coulter, Inc, Atlanta, Georgia, USA)
- BioTek Synergy Mx microplate reader (BioTek, inc., Winooski, Vermont, USA)
- Mini-Protein Tetra Vertical Electrophoresis Cell and Module (Bio-Rad, Hercules, California, USA)

- Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad, Hercules, California, USA)
- PowerPac^{TM} HC high-current power supply ((Bio-Rad, Hercules, California, USA)
- Block heater (Wealtec Corp., Way Sparks, Nevada, USA)
- Vortex Genie 2 (Scientific Industries, Bohemia, New York, USA)
- Refrigerated microcentrifuge (Hitachi, Tokyo, Japan)
- Tissue cell culture plates (96 wells/ 24 wells /12 wells/ 6 wells) and dishes (3.5 cm^2/ 6 cm^2/ 10 cm^2) (Corning, Inc., New York, USA)
- Centrifuge tubes (15 mL/50 mL) and microcentrifuge tube (1.5 mL) (Wuxi NEST Biotechnology Co., Ltd, Jiangsu, China)
- Cryovial tube 2.0 mL (Wuxi NEST Biotechnology Co., Ltd, Jiangsu, China)
- Round-bottom polystyrene tube 5 ml, 12x75 mm (BD Falcon Biosciences, San Jose, California, USA)
- Pipette tips (10 µl/200 µl/ 1000 µl) (Wuxi NEST Biotechnology Co., Ltd, Jiangsu, China)
- Serological pipettes (5 mL/10 mL/25 mL) (Wuxi NEST Biotechnology Co., Ltd, Jiangsu, China)

3.2 METHODS

3.2.1 Selection of *in vitro* cell model

Research plan: To study the effect of Smac mimetic and poly(I:C) combination treatment on apoptosis of CCA cells, representative *in vitro* CCA cell lines were selected according to three criteria. First, TLR3 receptor expression, a receptor for poly(I:C) stimulation. Second, cIAP1 and cIAP2 expression, negative regulator of poly(I:C)-induced apoptosis and targets of Smac mimetic. Last, the sensitivity to poly(I:C) and Smac mimetic treatment-induced cell death. Therefore, the expression of TLR3, cIAP1, and cIAP2 were determined by Western blot analysis in 6 CCA cell lines and an immortalized non-tumor cholangiocyte cell line. Cell death was determined by MTT assay.

3.2.1.1 Cell culture

Six CCA cell lines which are KKU100, KKU213, KKU214, RMCCA-1, KKU-M055 and HuCCT-1 were included in this research (3,33). MMNK1 cell line, an immortalized non-tumor cholangiocyte with SV-40 and hTERT transduction, was used as a comparative non-tumor cholangiocyte control (76). KKU213, KKU100, KKU214, KKU-M055, HuCCT-1, and MMNK1 were obtained from the Japanese Collection of Research Bioresources (JCRB) Cell Bank, Osaka, Japan. RMCCA-1 cells were developed from Thai patients with CCA (77). Human transformed keratinocyte HaCaT cells were used as a positive control for TLR3 expression. Human MDA-MB231, a breast cancer cell line and human HT-29, a colon cancer cell line were used as a positive control and comparative expression of cIAP1 and cIAP2. HaCaT, MDA-MB231, and HT-29 were obtained from the *American* Type Culture Collection (ATCC).

All CCA cell lines and non-tumor cholangiocytes were cultured in Ham's F12 with 10% fetal bovine serum and 1% Penicillin/Streptomycin. HaCaT, MDA-MB231, HT-29 cell lines were maintained in DMEM containing 10% FBS and 100 U/mL penicillin/100 μg/mL streptomycin. All cell lines were maintained at 37 ºC in a 5% CO_2 humidified atmosphere. All cell lines were tested for mycoplasma contamination and were mycoplasma free.

3.2.1.2 Western blot analysis

We hypothesized that cIAP1 and cIAP2 are overexpressed in CCA cells, therefore the expression of cIAP1, cIAP2 and TLR3 were detected in six CCA cell lines and non-tumor cholangiocyte cell line using Western blot analysis. First, cultured medium was discarded from cells growing in 3.5 cm^2 dish, and cells were washed with 1 mL of 1X ice-cold PBS for 3 times. Ice-cold 1X RIPA buffer containing protease inhibitor cocktails was added in the amount of 50-60 μL. Cells were scraped and collected into a new 1.5 mL microcentrifuge tube which was left on ice for 30 minutes. Then, cell lysates were centrifuged at 12,000 rpm for 10 minutes, 4°C. Supernatant was collected and protein concentration was measured by Bradford assay. Five concentrations of standard bovine serum albumin (BSA) (0.5, 0.25, 0.125, 0.06, 0.03 μg/μL) were used to generate standard curve and RIPA lysis buffer was

used as a blank. Protein samples and RIPA lysis buffer (blank) were diluted with water in a ratio of 1:15. Protein standards or protein samples were added into each well of 96-well plate in the amount of 10 µL. Bradford reagent was diluted 1:5 with water from a stock reagent and 200 µL of Bradford reagent was added each well. After that, the absorbance was measured at 595 nm with a microplate reader. Protein samples were prepared with 4X loading dye containing beta-mercaptoethanol and then denatured at 95 ºC for 10 minutes. Total 25 µg of protein was separated by SDS-PAGE. The 6% stacking gel was run at 70 volts for 30 minutes and 10% separating gel was run at 100-120 volts for 60-90 minutes. Then, proteins were transferred to Polyvinylidene Fluoride (PVDF) membrane with 100 volts for 1 hour. Membrane was activated with methanol for a minute prior using in this step. After transferring, the membrane was blocked with 5% blocking grade non-fat dry milk for 1 hour. The membrane was further incubated with primary antibodies specific for rabbit anti-TLR3 (dilution 1:1000), rabbit anti-cIAP1 (dilution 1:1000), rabbit anti-cIAP2 (dilution 1:1000) for overnight. Rabbit anti-beta actin (dilution 1:5000) was used as a loading/internal control. The membrane was washed with 0.5% TBST three times for 10 minutes each. The membrane was incubated with rabbit horseradish peroxidase-conjugated secondary antibody for 1 hour and then was washed with 0.5% TBST for three times, 10 minutes each. The membrane was developed using Enhanced Chemiluminescense system. The image was acquired using X-ray film developing. Each of experiments were performed in three independent experiments. HaCaT cells were used as a positive control for TLR3 expression and MDA-MB231 and HT-29 cells were used as positive control and comparative expression of cIAP1 and cIAP2 expression.

3.2.1.3 A pilot study: screening for cell death sensitivity and treatment conditions

Poly(I:C), a synthetic analog of *double-stranded RNA* (*dsRNA*), has been reported to directly induce apoptosis in some cancer cells and Smac mimetics are now in the clinical trials as an anticancer agent. In order to neutralize the anti-apoptotic activity of cIAP1 and cIAP2, a small molecule or Smac mimetics called SM-164 that has been developed previously by Sun *et al.* was used in this experiment

(41). SM-164 is a kind gift from Dr. Shaomeng Wang (University of Michigan, Ann Arbor, Michigan, USA). The sensitivity of CCA cells to poly (I:C) or Smac mimetic single treatments, or the combination treatment of poly (I:C) and Smac mimetic were examined. CCA cells and MMNK1 cells were seeded at 5,000 cells/100 µl culture medium into 96-well plate.

Treatment with poly(I:C) were carried out through two different approaches. First, by direct adding of poly(I:C) at 2.5, 12.5, 25, 50 µg/mL into cell culture medium. Treatment with water alone was used as a vehicle control. Second, since TLR3 receptor is mostly presented on endosomal membrane in several cells, transfection of poly(I:C) with transfection reagent, TurboFect (cationic polymer formula) at 2.5 (low concentration) and 12.5 (high concentration) µg/mL were used to deliver poly (I:C) and the sensitivity was compared to direct adding into cell culture medium. Treatment with transfection reagent alone was used as a vehicle control. The volume of TurboFect was varied based on amount in µg of poly(I:C). Poly (I:C) was diluted in Opti-MEM and TurboFect was then added and mixed well [poly (I:C) 2.5 µg/mL (0.25 µg): TurboFect 0.25 µL; poly (I:C) 12.5 µg/mL (1.25 µg): TurboFect 1.25 µL]. After that the complex was incubated at RT for 15-20 minutes. The TurboFect/poly(I:C) complexes were added (drop-wise) in each well of 96-well plate and incubated in 5% CO_2 at 37 °C, in incubator for 24 and 48 hours. Smac mimetic was used at 10 nM, the optimal dose used in most studies (78). Treatment with DMSO alone was used as a vehicle control of Smac mimetic. For the combination treatment of poly (I:C) and Smac mimetic, the cells were pretreated with Smac mimetic for 30 minutes followed by transfection with 2.5 µg/mL of poly (I:C) for 24 and 48 hours. The cell morphological changes were observed under microscope and cell death was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. In addition, treatment with 10 ng/mL of TNF-α in the combination with 10 nM Smac mimetic was included as a positive control for apoptosis.

3.2.1.4 MTT assay

MTT[3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay is a colorimetric assay for measuring the cell metabolic activity due to NADPH flux in the cells. Mitochondrial reductase reduces tetrazole (yellow) into formazan

crystals (blue-purple) in the living cells. The MTT was added to the culture medium to the final concentration of 0.25 mg/mL and the plate was incubated in 5% CO_2 at 37 ºC for 2-3 hours. Then, the culture medium was removed and 100 µL of DMSO was added to solubilize the formazan crystals. The absorbance of each sample was measured at 550 nm in a microplate reader and was calculated into the percentage of cell viability normalized to vehicle control.

Throughout 3 different criteria: TLR3- and cIAP1 and cIAP2-expressing CCA cells and the sensitive to the combination treatment of poly(I:C) and Smac mimetic were selected as two representatives *in vitro* CCA cell models.

3.2.2 Time optimization of Smac mimetic pretreatment and validation of cIAP1 and cIAP2 degradation

Research plan: To find an optimal time for targeting cIAP1 and cIAP2 for proteasomal degradation by Smac mimetic, time courses were varied from 0.5, 1, 2, and 4 hours and cIAP1 and cIAP2 degradation were determined by Western blot analysis

Smac mimetic has been shown to neutralize the anti-apoptotic activity of cIAP1 and cIAP2 through targeting cIAP1 and cIAP2 for proteasomal degradation. In order to find an optimal time for pretreatment and to validate the activity of Smac mimetic on the removal of cIAP1 and cIAP2 expression, CCA cell lines and a non-tumor cholangiocyte were cultured in 3.5 cm^2 dish. The next day, the cells were treated with Smac mimetic at high (50 nM) and low (5 nM) doses depending on cell types for 0.5, 1, 2 and 4 hours. The cells were washed with 1X PBS for three times and were collected with 50-60 µL of RIPA lysis buffer and subjected to Western blot analysis using specific primary antibodies to detect cIAP1 and cIAP2 expression following Smac mimetic treatment. Beta-actin was used as a loading/internal control.

3.2.3 Concentration optimization of poly(I:C) and Smac mimetic-induced cell death and calculation of synergistic combination index (CI)

Research plan: To find an optimal concentration of Smac mimetic and poly(I:C) that gives the high synergistic effect, different concentrations of both Smac

mimetic and poly(I:C) were varied. Cell death was determined by MTT assay and the synergistic effect was analyzed by combination index (CI) calculation.

3.2.3.1 Smac mimetic and poly(I:C) optimization

To study the synergistic effect of Smac mimetic and poly(I:C) on apoptosis, the concentration of both Smac mimetic and poly(I:C) were optimized. Non-tumor cholangiocyte and two CCA cell lines were seeded at 5,000 cells/100 µl culture medium into 96-well plate. The cells were transfected with 1, 2.5, 5 and 12.5 µg/mL of poly(I:C) using TurboFect alone or 1, 5 and 10 nM of Smac mimetic alone in KKU213, MMNK1 or 10, 25, 50 nM in KKU100, MMNK1 for 24 and 48 hours. For the combination of poly(I:C) and Smac mimetic, the cells were pretreated with Smac mimetic for 2 hours followed by poly (I:C) for 24 and 48 hours. Treatment with 10 ng/mL of TNF-α in the combination with 10 nM Smac mimetic was included as a positive control for apoptosis. Cell death was determined using MTT assay. Each experiment was performed in triplicate.

3.2.3.2 Calculation of synergistic combination index (CI)

Drug interactions were analyzed by the Combination Index (CI) method based on Chou-Talalay method using CalcuSyn software (Biosoft, Cambridge, UK). CI < 0.9 indicates synergism, 0.9-1.1 additivity and > 1.1 antagonism (79). The concentration of poly(I:C) and Smac mimetic with the lowest CI (CI < 0.9, synergism) indicating the highest synergistic manner was selected for further experiments.

3.2.4 Cell death detection

Research plan: To further confirm whether Smac mimetic and poly(I:C) specifically induced apoptosis of CCA cells, different approaches were used 1) pretreatment with inhibitor of apoptosis, a broad-spectrum caspases inhibitor (zVAD-fmk) 2) Annexin V/PI staining by flow cytometer analysis and 3) Caspase-8 and caspase-3 activation and PARP-1 cleavage by Western blot analysis

3.2.4.1 Inhibition of caspase activation by caspase inhibitor

Caspase activation is crucial for apoptosis induction, therefore inhibition of caspase activity using caspase inhibitor could be used to determine if the combination treatment of poly(I:C) and Smac mimetic-induced apoptosis. A broad-

spectrum caspases inhibitor (zVAD-fmk) that are able to inhibit the activity of all caspases for example caspase-8, caspase-9, and caspase-3/-7 were used in this study. Cells were seeded at 70,00 cells/1 mL in 12 well plate. The next day, cells were pretreated with 20 μM zVAD-fmk and Smac mimetic (5 nM KKU213, 50 nM KKU100) for 2 hours. After that cells were transfected with 2.5 μg/mL poly(I:C) using TurboFect for 24 hours. Cells were collected, and apoptosis was determined by annexin V/PI staining by flow cytometer analysis.

3.2.4.2 Annexin V/PI staining by flow cytometer analysis

An increase in cell death and a decrease in cell proliferation cannot be discriminated by the MTT assay. The combination treatment of poly(I:C) and Smac mimetic-induced cell death as evaluated by MTT assay was further confirmed with a more specific cell death assay by annexin V and propidium iodide (PI) staining. During cells undergo apoptosis, phosphatidylserine (PS) which normally localizes in the inner leaflet of plasma membrane is externalized to the outer leaflet of plasma membrane and that is able to be detected by recombinant protein annexin V-FITC in the presence of calcium ion. In contrast to apoptotic cell death, when cells undergo necrosis, the plasma membrane is disrupted resulting in the cell impermeable dye, propidium iodide (PI) can enter the cells and binds to DNA.

After treatment with the combination of poly(I:C) and Smac mimetic, first, supernatant was collected and put into a new 1.5 mL microcentrifuge tube and centrifuged at 1,000 rpm for 5 minutes. Supernatant was then discarded. The remaining cells in the plate were washed with cold 1X PBS and collected into the same microcentrifuge tube. Next, 200 μl of trypsin-EDTA was added into each well to detach cells. After 5 minutes, cells were collected into the same microcentrifuge tube and centrifuged at 1,000 rpm for 5 minutes and washed once with cold 1X PBS. Cell pellets were stained with 1.5 μL of FITC-conjugated annexin V and 1.5 μl of PI (50 μg/ml) in 50 μL annexin V staining buffer. The cells were collected and quantitated by flow cytometry. Viable cells were negative for both annexin V and PI. The early apoptotic cells are positive for annexin V and negative for PI, while late apoptotic/ necrotic cells are positive for both annexin V and PI staining. The viable and dead cells were collected and quantitated by flow cytometry.

3.2.4.2 Caspase-8 and caspase-3 activation and PARP-1 cleavage by Western blot analysis

To further confirm the induction of apoptosis, the more specific markers of apoptosis were used. Cleaved caspase-8, caspase-3 activation and PARP1 cleavage were investigated using Western blot analysis after stimulation with Smac mimetic or poly(I:C) alone or the combination treatment.

Since poly(I:C) and Smac mimetic have been shown to activate extrinsic apoptosis pathway, 1) cleaved caspase-8, an initiator caspase of extrinsic pathway 2) caspase-3 activation, an executioner of caspase activation, and 3) PARP1 cleavage, a substrate of caspase-3 were all explored in this experiment. Extrinsic induction of apoptosis leads to the activation of caspase-8 through the cleavage of pro-caspase-8 (55/53 kDa) to the active form of caspase-8 (cleaved caspase-8 p43/p41 and p18). Cleaved caspase-8 activates downstream of executioner caspases such as caspase-3. Caspase-3 activation then cleaves many important proteins for cell survival including Poly (ADP-Ribose) Polymerase 1 (PARP1), a DNA repair enzyme, into two smaller fragments (89 and 27 kDa) in which un-cleaved PARP-1 (116 kDa) and cleaved 89 kDa fragment can only be detected by PARP-1 antibody used in this study.

The cells were seeded at 500,000 cells/3 mL in 6 cm^2 dish. The following day, the cells were treated with 2.5 μg/mL of poly(I:C) or Smac mimetic (5 nM KKU213, 50 nM KKU100) alone or the combination of poly(I:C) and Smac mimetic for 6 and 12 hours. Treatment with TNF-α and Smac mimetic for 6 hours were used as a positive control for caspase-8 and caspase-3 activation. The cells were collected and lysed in 60-70 μL of RIPA buffer and subjected to Western blot analysis using specific antibodies for mouse anti-caspase-8 (1;1000), rabbit anti-caspase-3 (1;2500), rabbit anti-PARP1 (1:5000). Beta-actin were used as a loading control.

3.2.5 The role of RIPK1 in poly(I:C) and Smac mimetic-induced apoptosis

Research plan: To study the role of RIPK1 in the combination treatment of poly(I:C) and Smac mimetic-induced apoptosis in CCA cells, both pharmacological inhibitor of RIPK1 kinase and genetic deletion of *RIPK1* gene by CRISPR/cas9 technology were used to explore the role of RIPK1.

3.2.5.1 Pharmacological inhibitor

An inhibitor of RIPK1, necrostatin-1 (Nec-1) that inhibits kinase domain of RIPK1 was used to investigate the role of RIPK1 as a proposed key mediator of poly(I:C) and Smac mimetic-induced apoptosis (80). Cells were seeded at 70,000 cells/1 mL in 12 well plate. The following day, cells were pretreated with 60 μM of necrostatin-1 (Nec-1), Smac mimetic (5 nM KKU213, 50 nM KKU100) for 2 hours and poly (I:C) was transfected into the cells using TurboFect for 24 hours. Vehicle control for this experiment was DMSO, since both necrostatin-1 (Nec-1) and Smac mimetic was dissolved in DMSO. In addition, TNF-α and Smac mimetic treatment with or without 60 μM of necrostatin-1 (Nec-1) was served as a positive and comparative control. Cells were collected and stained with annexin V/PI followed by flow cytometer analysis.

3.2.5.2 Functional genetic deletion of RIPK1 gene by CRISPR/cas9-mediated genome editing

Concerning the stability and specificity in the cells of necrostatin-1 (Nec-1), and also the role of RIPK1 in this pathway could be through both kinase-dependent or kinase-independent (scaffold function), therefore the more permanent and efficient approach was used to explore the role of RIPK1 in this process. Our laboratory has been observed that disrupting RIPK1 at mRNA level using siRNA or shRNA resulted in low knockdown efficiency (lower than 50% knockdown efficiency). Therefore, a novel technology, CRISPR/cas9 that is used to delete gene of interest through cas9 nuclease and non-homologous end joining (NHEJ) DNA repair was used to delete *RIPK1* gene and generate *RIPK1* knockout cells.

3.2.5.2.1 RIPK1 CRISPR construction

Lentiviral CRISPR plasmid targeting human *RIPK1* gene (NM_003804) has been generated previously from the laboratory of Dr. Zheng-Gang Liu as research collaboration at National Cancer Institute, National Institutes of Health, USA (81). The CRISPR backbone was obtained from Addgene version lentiCRISPR V2 (plasmid #52961) (Figure 7) in which cas9 nuclease and trans-activating RNA scaffold are identified from *Streptococcus pyogenes.* Both cas9

nuclease and trans-activating RNA scaffold fused with guide RNA (sgRNA) are combined in the same vector backbone. The expression of cas9 nuclease are under control of EFS promoter, while guide RNA (sgRNA) and trans-activating RNA scaffold are under control of U6 promoter. Puromycin resistance in the presence of Puromycin is used to select infected cells. The construction of CRISPR plasmid is according to Zhang's protocol (82). RIPK1-CRISPR plasmid was verified by DNA sequencing.

The sequence for CRISPR-RIPK1 was 5'-CACCGGATGCACGTGCTGAAAGCCG-3'.

3.2.5.2.2 Generation of RIPK1 knockout CCA cell lines and validation of knockout cells by Western blot analysis

The lentiviral system consisting of pCMV-dr8.2-dvpr (*packaging* plasmid) and pCMV-VSV-G (envelope plasmid) was used to produce viral particles in HEK293T cells. The lentiviral system was provided by the laboratory of Dr. Zheng-Gang Liu at NIH, USA as research collaboration. HEK293T cells that were obtained from the *American* Type Culture Collection (ATCC) was cultured in DMEM with 10% fetal bovine serum and 1% Penicillin/Streptomycin and was incubated in 5% CO_2 at 37 ºC. HEK293T cells were seeded at 200,000 cells/1.5 mL in 6 well plate. HEK293T cells were co-transfected with 1 µg of pCMV-dr8.2-dvpr (*packaging* plasmid) and 0.125 µg of pCMV-VSV-G (envelope plasmid) and either 1 µg of empty vector control CRISPR (lentiCRISPRV2) or CRISPR-RIPK1 plasmids using TurboFect transfection reagent. After 24 hours of transfection, culture and transfection media were removed and replaced with 1.5 mL of fresh DMEM containing 10% FBS without Penicillin/Streptomycin and incubated for further 24 hours. Supernatants containing viral particles were collected and centrifuged at 2,000 rpm for 5 minutes to remove cells and all debris. After that, viral particles were filtered with 0.45 µm syringe-sterile filter membrane. Lentiviral preparations (1 mL) were mixed with Polybrene at a final concentration of 8 µg/mL that was used to facilitate the viral infection efficiency. After that the viral particles were used to infect CCA cells. After 24 hours of infection, infected CCA cells were selected with 2 µg/mL of puromycin both in KKU213 and KKU100 for a further 48 hours. Around 5-10 days after Puromycin selection, the expression of RIPK1 in CRISPR-RIPK1

knockout cells (pooled cells) and CRISPR-V2 control cells (pooled cells) were examined by Western blot analysis using mouse anti-RIPK1 specific antibody (1:1000). The CRISPR RIPK1 knockout cells were used for further studies.

3.2.5.2.3 Apoptosis induction in RIPK1 knockout cells

KKU213 and KKU100 RIPK1 knockout CCA cells (CRISPR-RIPK1) and CRISPR control cells (CRISPR-V2) were seeded at 70,000 cells/1 mL in 12 well plate. The next day, cells were pretreated with Smac mimetic (5 nM KKU213, 50 nM KKU100) for 2 hours following by transfetction with 2.5 µg/mL of poly(I:C) for 24 hours. Treatment with 10 ng/mL of TNF-α in the combination with 5 nM Smac mimetic was included as a positive and comparative control. Cells were collected, and apoptosis was determined by annexin V/PI staining by flow cytometer analysis.

3.3 Statistics and data analysis

All experiments were carried out in three independent experiments. In addition, MTT assay was performed in triplicate. The results were expressed as mean ± S.D. Student's t test was used to evaluate the statistical significance, and differences between mean values were considered significant when *p*-value is less than 0.05 ($p<0.05$).

CHAPTER IV

RESULTS

4.1 Selection of *in vitro* CCA cell models

Cholangiocarcinoma (CCA) patients have high mortality rate and poor prognosis with 5-year survival rate is low as of 5-10%, especially with advance stage diagnosis, mainly due to lack of effective therapy (2). Therefore, development of novel therapeutic strategies is urgently needed. It has been reported previously and also our preliminary studies in CCA patients showing that CCA tumor microenvironment is associated with chronic inflammation with the recruitment of immune cells. Damage-associated molecular patterns (DAMPs) that are released from damaged or dying cells in tumor microenvironment and pathogen-associated molecular patterns (PAMPs) are two of factors that induce chronic inflammation in tumor microenvironment (30,31). Both DAMPs and PAMPs have been shown to activate and induce the expression of pattern recognition receptors (PRRs) including TLR3, suggesting that TLR3 ligand could be used for treatment of CCA patients through the induction of cancer cell apoptosis. We therefore hypothesized that TLR3 was expressed in CCA cells and stimulation of TLR3 with TLR3 ligand, a synthetic analog of *double-stranded RNA* (*dsRNA*) could be developed as a novel potential therapeutic strategy for improvement of efficient therapy for CCA patients. In some of cancers, negative regulators of TLR3 signaling have been reported. First, cellular inhibitor of apoptosis proteins (cIAPs) including cIAP1 and cIAP2 represent a major negative regulator of TLR3-induced apoptosis (59). Later, cellular FLICE-like inhibitory protein (c-FLIP), a strong negative regulator of caspase-8-mediated

apoptosis has recently been reported as a negative regulator of TLR3-induced apoptosis (23).

4.1.1 TLR3 expression in CCA cell lines

Firstly, we examined TLR3 expression in a panel of CCA cell lines including KKU100, KKU213, KKU214, RMCCA-1, KKU-M055, HuCCT-1, and an immortalized non-tumor cholangiocytes, MMNK1, by Western blot analysis. We also included an immortalized keratinocyte cell line, HaCaT, as a positive control for TLR3 expression. We found that TLR3 was expressed in all CCA cell lines. However, TLR3 was differentially expressed in CCA cell lines in which HuCCT-1 and KKU100 exhibited higher expression when compared to other cell lines. Moreover, TLR3 expression was not detected in non-tumor cholangiocytes (Figure 8). Since, TLR3 expression has been shown to be induced by the stimulation with poly(I:C) through Type I IFN signaling (83), we therefore investigated whether stimulation of poly(I:C) upregulated the expression of TLR3. As shown in figure 9, stimulation with 2.5 μg/mL of poly(I:C) at 8 and 24 hours induced the expression of TLR3 in KKU100, KKU213, and HuCCT-1, but not in MMNK1. TLR3 expression was upregulated at 8 hours of treatment in both KKU100 and HuCCT-1, while its expression was increased later at 24 hours in KKU213, probably because of lower basal level of TLR3 in KKU213 when compared to KKU100 and HuCCT-1.

Altogether, this result demonstrated that TLR3 was differentially expressed in all CCA cell lines but not detected in non-tumor cholangiocytes. This finding from our study is the first time to reveal the existence and TLR3 expression in CCA cell lines. Therefore, targeting TLR3 signaling using poly(I:C) could probably be used as a novel potential therapeutic target for CCA patients.

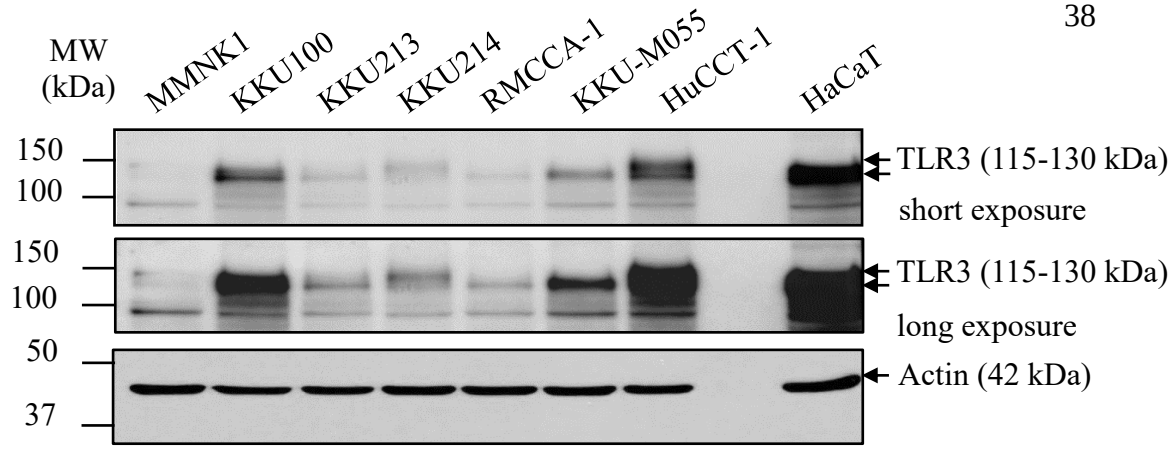


Figure 8 : Expression of TLR3 in 6 CCA cell lines (KKU100, KKU213, KKU214, RMCCA-1, KKU-M055 and HuCCT-1) and a non-tumor cholangiocyte (MMNK1).

HaCaT cells were used as a positive control for TLR3 expression. TLR3 expression was determined by Western blot analysis. β-actin was served as loading control.

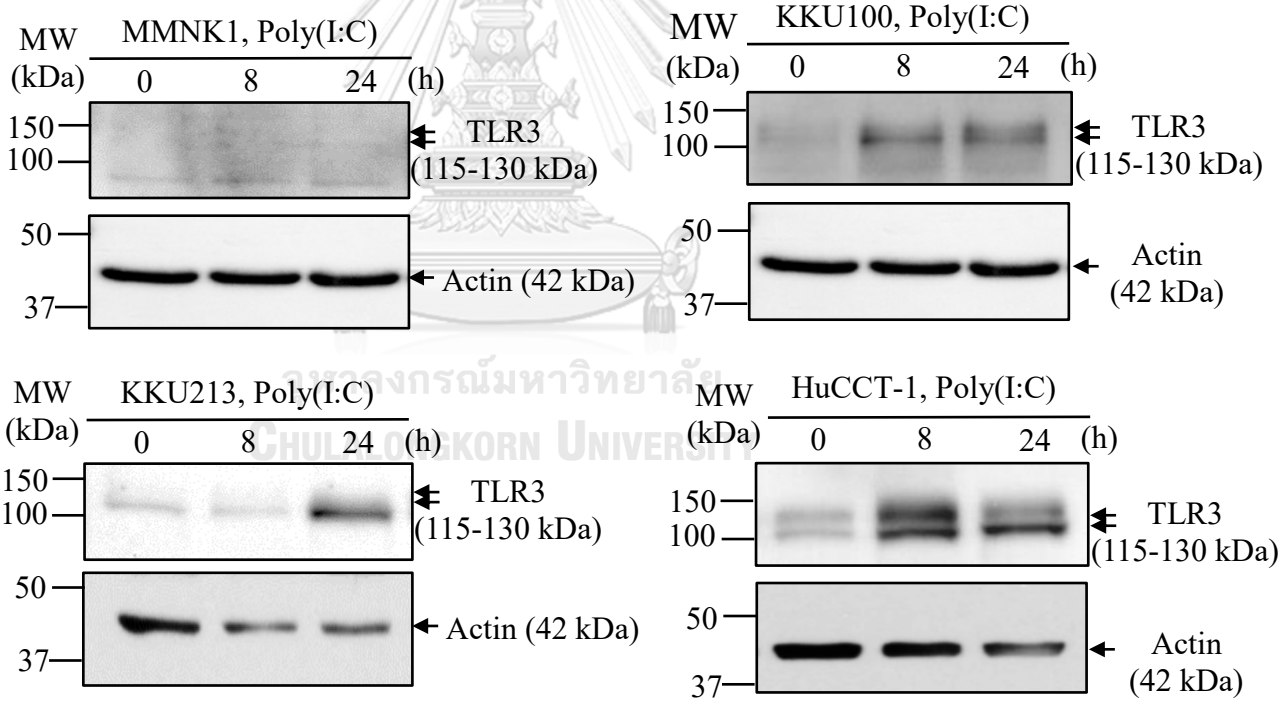


Figure 9 : Stimulation of TLR3 expression by poly(I:C) in CCA cell lines (KKU100, KKU213, HuCCT-1) and a non-tumor cholangiocytes (MMNK1).

Cells were transfected with 2.5 μg/mL Poly(I:C) at 8 and 24 hours. The expression of TLR3 was determined by Western blot analysis. β-actin was served as loading control.

4.1.2 cIAP1 and cIAP2 expression in CCA cell lines**.**

We hypothesized that cIAP1 and cIAP2 were overexpressed in CCA cells that might suppress poly(I:C)-induced CCA apoptosis. We therefore investigated the expression of cIAP1 and cIAP2 in all CCA cell lines and a non-tumor cholangiocyte cell line. We included a breast cancer cell line, MDA-MB-231 and a colon cancer cell line, HT-29 in this experiment as positive and comparative control for cIAP1 and cIAP2 expression. These two cell lines have been reported to overexpress both cIAP1 and cIAP2 (84,85). Our Western blot analysis revealed that both cIAP1 and cIAP2 were expressed in both CCA cell lines and a non-tumor cholangiocyte (Figre 10). cIAP1 was higher expressed in all CCA cell lines except KKU100 which exhibited the lowest expression and a non-tumor cholangiocyte, MMNK1. cIAP2 was higher expressed in KKU100, KKU-M055, and HuCCT-1 than MMNK1, KKU213, KKU214, and RMCCA-1. In order to compare if CCA cells expressed high level of cIAP1 and cIAP2, MDA-MB-231 and HT-29 cell lines which have previously reported to express high level of both cIAP1 and cIAP2 and contribute to therapy resistance were used as comparative cell lines and a positive expression of both cIAP1 and cIAP2. Two representative CCA cell lines including KKU213 and RMCCA-1 which expressed similar level of cIAP1 and expressed low level of cIAP2 were selected as two representative cell lines. As seen in figure 11, the expression of cIAP1 in CCA cell lines including KKU213 and RMCCA-1 was similar to both MDA-MB-231 and HT-29. In contrast, although both KKU213 and RMCCA-1 exhibited the lowest expression of cIAP2 among CCA cell lines tested, this low level was still much higher when compared to the expression of cIAP2 in both MDA-MB-231 and HT-29 (Figure 11). These results suggested that both cIAP1 and cIAP2 were overexpressed in both a non-tumor cholangiocyte cell line and CCA cell lines that might limit the sensitivity of poly(I:C)-induced apoptosis. In addition, another negative regulator of poly(I:C)-induced apoptosis which was cFLIP was also investigated in the same cell lines. As shown in figure 12A, cFLIP with a long isoform was differentially expressed in MMNK1 and CCA cell lines. In addition, the level of cFLIP was normalized to β-actin and presented as fold increase relative to MMNK1 with its mean set to 1, as shown in figure 12B, HuCCT-1 exhibited the highest expression of cFLIP when compared to other cell lines suggesting that in

addition to high expression of both cIAP1 and cIAP2, the high expression of cFLIP might contribute to poly(I:C)-induced apoptosis in HuCCT-1.

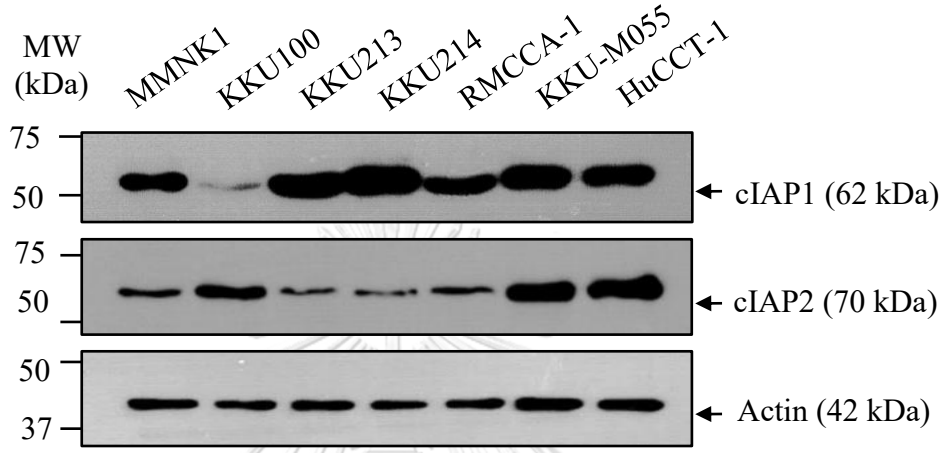


Figure 10 : Expression of cIAP1 and cIAP2 in 6 CCA cell lines (KKU100, KKU213, KKU214, RMCCA-1, HuCCT-1, KKU-M055) and a non-tumor cholangiocyte (MMNK1).

cIAP1 and cIAP2 expression was determined by Western blot analysis. β-actin was served as loading control.

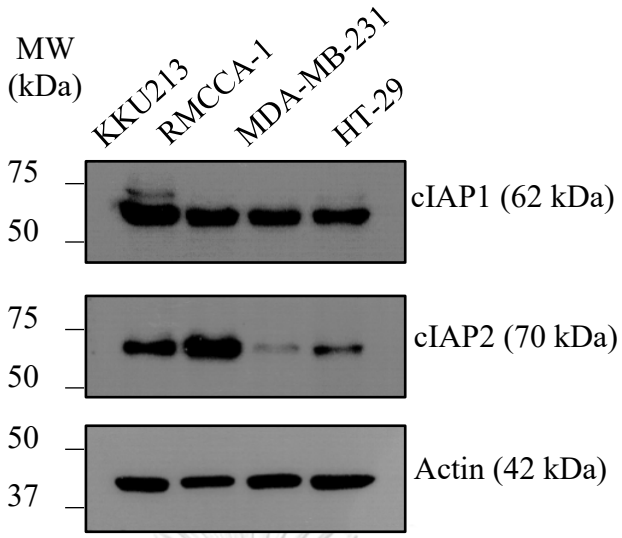
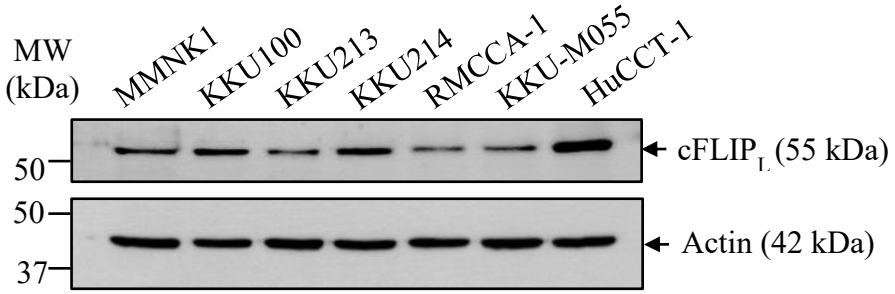


Figure 11 : cIAP1 and cIAP2 expression in CCA, breast cancer, and colon cancer cell lines.

The expression of cIAP1 and cIAP2 in KKU213 and RMCCA-1 as well as MDA-MB-231 and HT-29 cell lines were analyzed by Western blot analysis. β-actin was used as loading control.

(A)



(B)

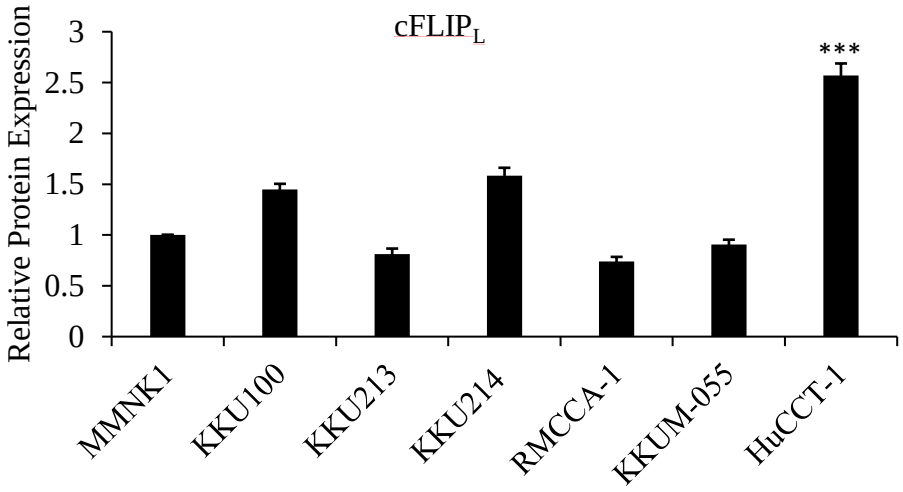


Figure 12 : cFLIP expression in 6 CCA cell lines (KKU100, KKU213, KKU214, RMCCA-1, HuCCT-1, KKU-M055) and a non-tumor cholangiocyte (MMNK1).

(A) cFLIP expression was determined by Western blot analysis. β-actin was served as loading control. (B) cFLIP was normalized to actin protein expression and presented as fold increase relative to MMNK1 with its mean set to 1.

4.1.3 Screening for the sensitivity of poly(I:C)-induced cell death in CCA cell lines

Since TLR3, cIAP1 and cIAP2 were differentially expressed in all CCA cell lines. Therefore, we conducted a pilot study to test the sensitivity of TLR3 stimulation by poly(I:C)-induced cell death as a single agent as stimulation of TLR3 by poly(I:C) has been showed to induce apoptosis in some cancers. We initially tested the sensitivity to poly(I:C) by direct adding of poly(I:C) at 2.5, 12.5, 25, 50 µg/mL into cell culture medium for 48 hours. After treatment with different concentration of poly(I:C), by observing the cell morphological changes under microscope, we observed no significant cell death up to 48 hours treatment. Although, % survival was slightly lower with high concentration of poly(I:C) at 25 and 50 µg/mL treatment in KKU213, KKU214, RMCCA-1, this effect was mainly due to decrease in cell proliferation rather than cell death (Figure 13).

Since TLR3 receptor has been previously reported to express mainly in an endosomal cell membrane inside the cells in some cancer cells, we therefore hypothesized that transfection of poly(I:C), a form of dsRNA could help to deliver poly(I:C) to its target site. We therefore did transfection of poly(I:C) at 2.5 (low concentration) and 12.5 (high concentration) µg/mL with a transfection reagent, a cationic polymer formula called TurboFect. However, as seen in figure 13, all CCA cell lines and a non-tumor cholangiocyte remained resistance to poly(I:C) treatment.

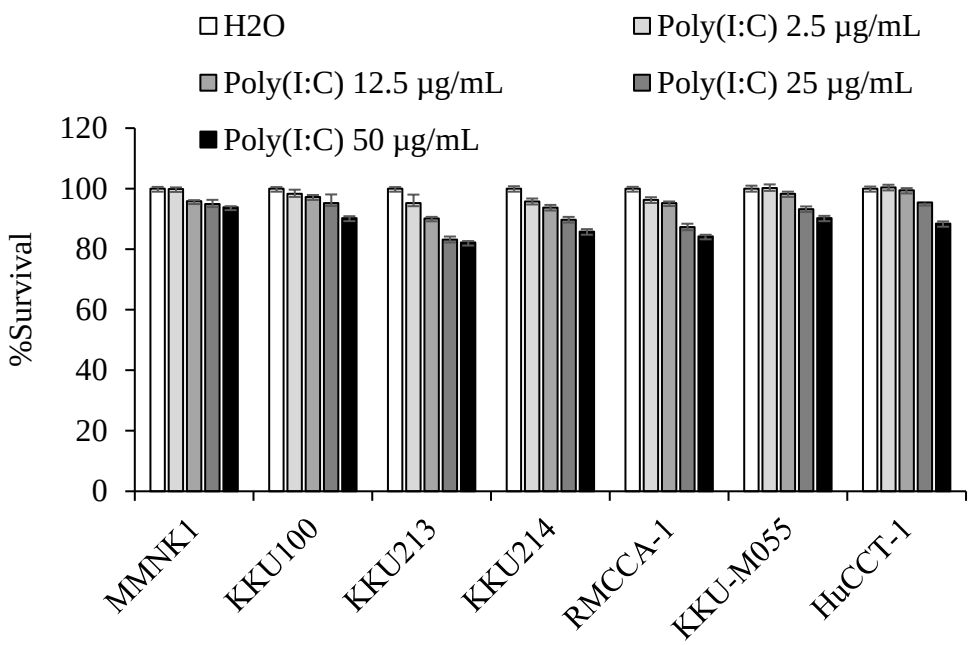


Figure 13 : Effect of poly(I:C) on cell survival by direct adding into culture medium.

Six CCA cells and a non-tumor cholangiocyte, MMNK1 were treated with different concentrations of poly(I:C) at indicated for 48 hours. Cell viability was measured by MTT assay and % survival was calculated by normalizing poly(I:C) treatment to vehicle control (H_2O). Data from two independent experiments was presented as mean $\pm$ S.D.

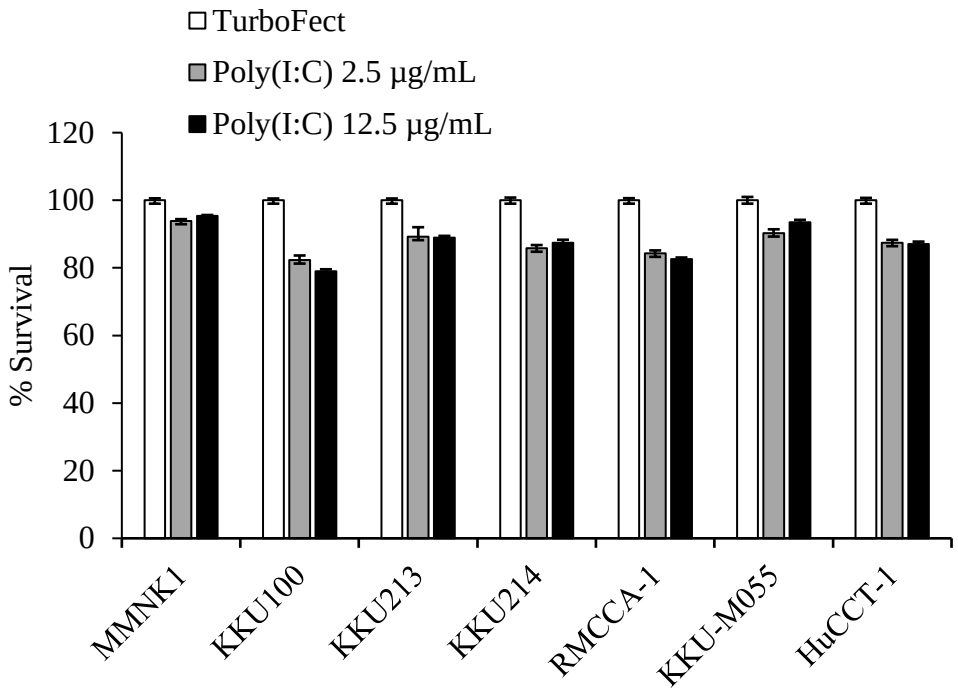


Figure 14 : Effect of poly(I:C) on cell survival by transfection.

Six CCA cells and a non-tumor cholangiocyte, MMNK1 were transfected with two different concentrations of poly(I:C) at 2.5 and 12.5 µg/mL for 48 hours. Cell viability was measured by MTT assay and % survival was calculated by normalizing poly(I:C) treatment to vehicle control (transfection reagent). Data from two independent experiments was presented as mean ± S.D.

We therefore hypothesized that the expression of cIAP1 and cIAP2 might contribute to the resistance to poly(I:C)-induced apoptosis in CCA cell lines. Since, a small molecule which functions to remove cIAP1 and cIAP2 called Smac mimetic was developed as an anti-cancer agent. We therefore tested the sensitivity of CCA cells to poly(I:C) in combination with Smac mimetic. Poly(I:C) at 2.5 µg/mL was used for transfection approach, while poly(I:C) at 25 µg/mL was used for direct adding, the most common dose used in several studies. Smac mimetic (SM-164) at 10 nM, the most common dose used in several studies was selected for a pilot study. Cells were pretreated with 10 nM Smac mimetic, after that the cells were treated with poly(I:C). TNF-α at 10 ng/mL and Smac mimetic at 10 nM (TS), a well-known inducer of apoptosis was included as a positive control Morphological changes

associated with cell death was observed under microscope and cell death was determined by MTT assay at 48 hours. Single treatment of Smac mimetic at 10 nM induced cell death at different degree in different cell lines as observed by morphological changes that were similar to apoptotic morphology (Figure 14). KKKU213, KKU214, and RMCCA-1 cells were more sensitive to Smac mimetic-induced cell death than other cell lines including MMNK-1, KKU100, KKU-M055, and HuCCT-1. Our results are consistent with other studies showing that Smac mimetics by itself can induce apoptosis through autocrine TNF-α secretion in sensitive cells (29). Interestingly, when poly(I:C) was combined with Smac mimetic, the combination treatment significantly induced cell death in KKU100 and KKU213 followed by KKU214 and RMCCA-1, while KKU-M055, HuCCT-1, and MMNK-1 were remained resistance to the combination treatment as a number of cell death in the combination treatment was similar to a single treatment with Smac mimetic (Figure 15). However, all cell lines used in this study, except HuCCT-1 were sensitive to TNF-α and Smac mimetic treatment. Different methods in the delivery of poly(I:C) into the cells resulted in different sensitivity to the combination treatment as transfection of poly(I:C) into the cells exhibited a more pronounced effect on cell death when compared to direct addition of poly(I:C) into the cells indicating that TLR3 might express in endosome in CCA cells which necessitates further investigation (Figure 15,16). To this end, we therefore delivered poly(I:C) by transfection for further experiment. All of our results including basal level of TLR3 expression, TLR3 expression in response to poly(I:C) stimulation, cIAP1 and cIAP2 expression, and the most important which is sensitivity to the combination treatment of poly(I:C) and Smac mimetic, we therefore selected KKU100 and KKU213 as two representative cell models for further experiments. In addition, MMNK1 was included as a non-tumor cholangiocyte control.

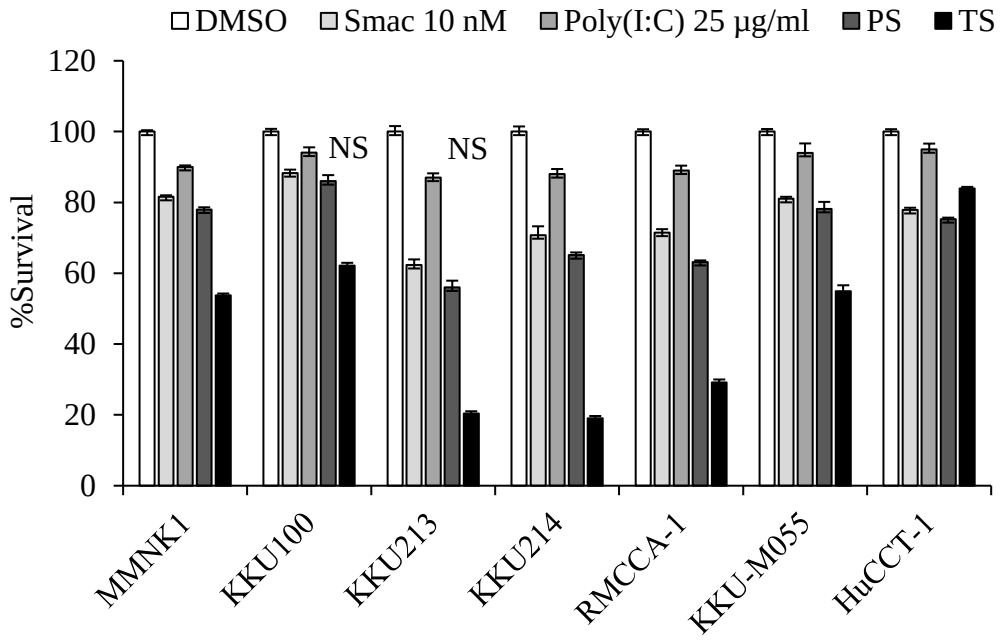


Figure 15 : The effect of Smac mimetic and direct stimulation of poly (I:C) on cell death in CCA cell lines.

Six different CCA cell lines and a non-tumor cholangiocyte (MMNK1) were pretreated with 10 nM Smac mimetic (SM-164) for 2 hours and were directly treated with 25 µg/mL poly(I:C) for 48 hours. TNF-α at 10 ng/mL and Smac mimetic at 5 nM (TS) were included as a positive control. Cell viability was determined by MTT assay.

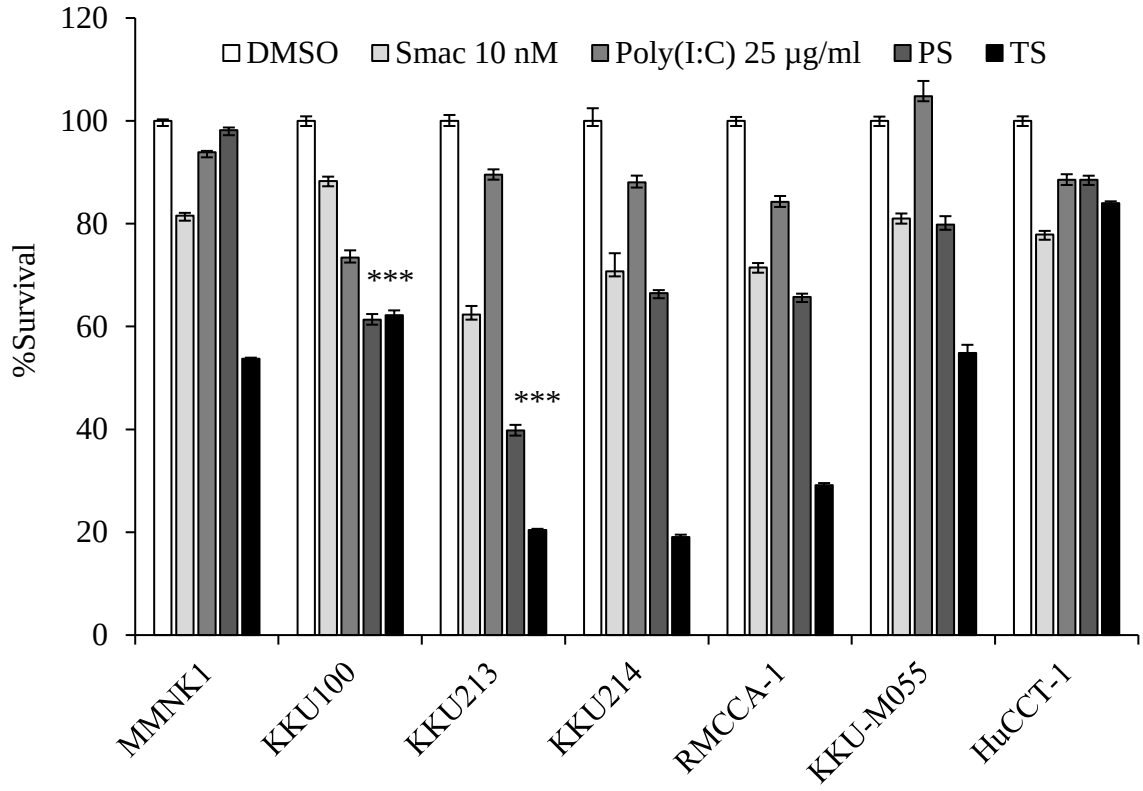


Figure 16 : The effect of Smac mimetic and transfection of poly(I:C) on cell death in CCA cell lines.

Six different CCA cell lines and a non-tumor cholangiocyte (MMNK1) were pretreated with 10 nM Smac mimetic (SM-164) for 2 hours and were transfected with 25 µg/mL of poly(I:C) for 48 hours. TNF-α at 10 ng/mL and Smac mimetic at 10 nM (TS) were included as a positive control. Cell viability was determined by MTT assay.

4.2 Validation of Smac mimetic-induced cIAP1 and cIAP2 degradation

Both cIAP1 and cIAP2 have been reported to be target of Smac mimetic that functions by binding to cIAP1 and cIAP2 and targets these proteins for proteasomal degradation. In addition, both cIAP1 and cIAP2 were highly expressed in CCA cell lines, we therefore investigated whether Smac mimetic specifically induced cIAP1 and cIAP2 degradation. To this end, we treated MMNK1 (50 nM), KKU100 (50 nM), and KKU213 (5 nM) with Smac mimetic at indicated concentration in different cells and in order to identify pretreatment time course for Smac mimetic treatment when it was combined with poly(I:C), time course analysis of Smac mimetic-induced the degradation of cIAP1 and cIAP2 was also evaluated in this study. Smac mimetic at high concentration at 50 nM rapidly induced cIAP1 and cIAP2 degradation as early as 30 minutes treatment in MMNK1 and KKU100, while in KKU213, cIAP1 and cIAP2 degradation was dramatically reduced after 2 hours treatment with low concentration of Smac mimetic at 5 nM and was degraded early after 30 minutes with high concentration fo Smac mimetic at 50 nM (Figure 17). From these results, we therefore selected 2 hours pretreatment with Smac mimetic for the combination treatment with poly(I:C) in which low dose of Smac mimetic with less effect on cell death induction was used for the combination treatment. These results indicated that Smac mimetic specifically induced both cIAP1 and cIAP2 degradation in MMNK1, KKU100, and KKU213.

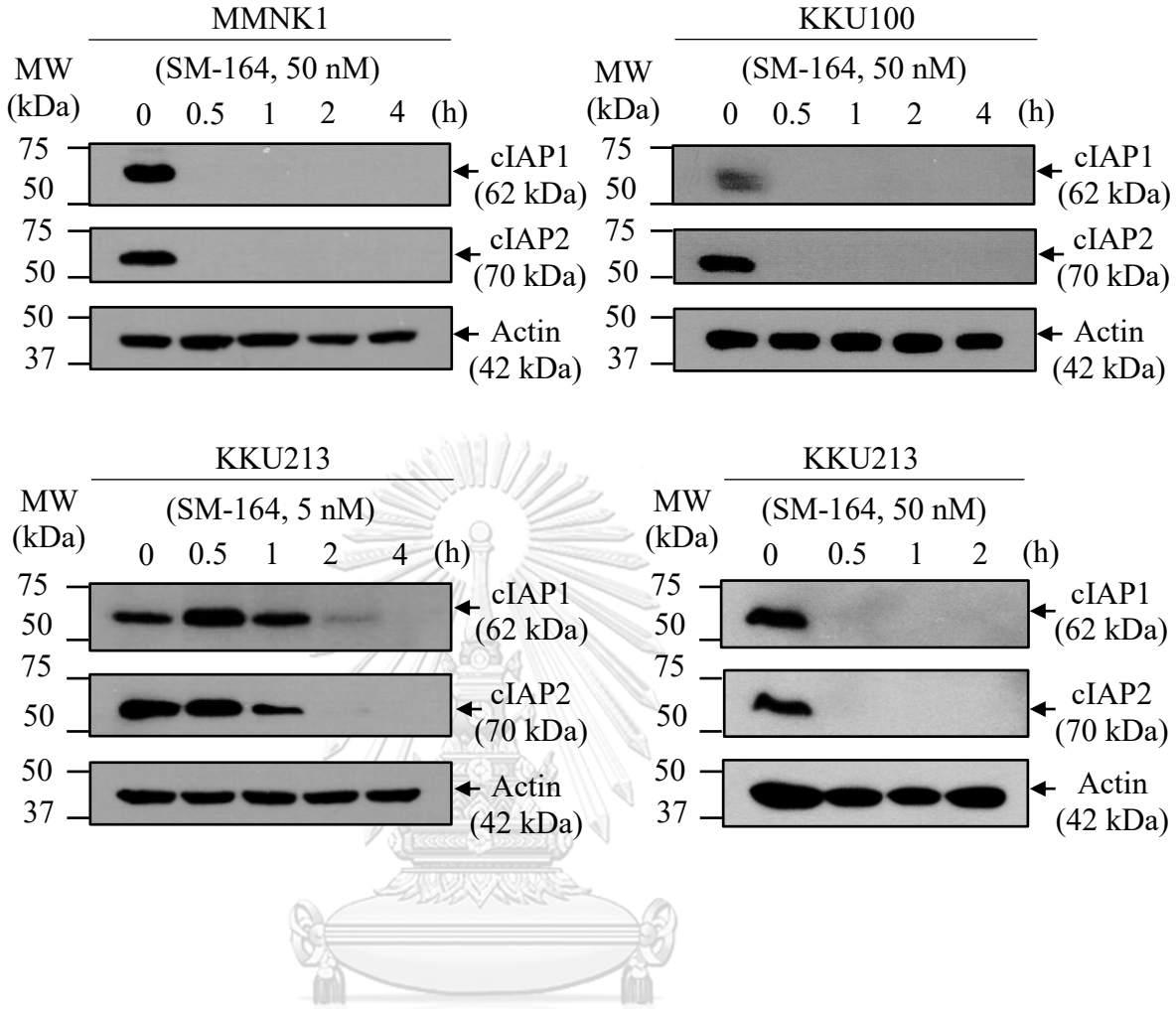
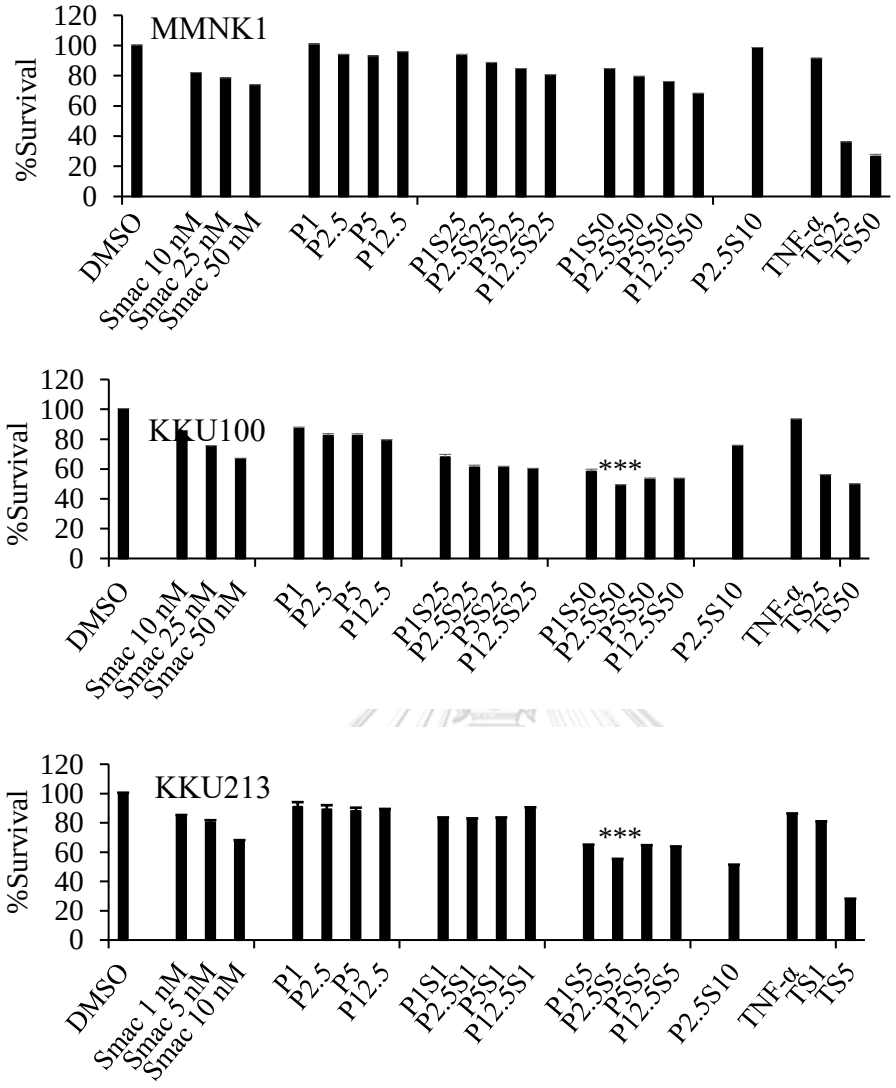


Figure 17 : Degradation of cIAP1 and cIAP2 by Smac mimetic, SM-164.

MMNK1, KKU100, and KKU213 cells were treated with Smac mimetic at indicated concentrations and time points. The expression of cIAP1 and cIAP2 was determined by Western blot analysis. β-actin was served as loading control.

4.3 Optimization of poly(I:C) and Smac mimetic-induced cell death and calculation of combination index (CI)

We next further examined for the optimal concentration of both Smac mimetic and poly(I:C) by treating KKU100, KKU213 and MMNK1 with varying concentrations of poly(I:C) (0, 1, 2,5, 5 and 12.5 µg/mL) or Smac mimetic, SM-164, (1, 5 and 10 nM for KKU213 and 10, 25 and 50 nM for KKU100 and MMNK1) or the combination of poly(I:C) and Smac mimetic and cell death was determined by MTT assay. The combination index was calculated to indicate the synergistic effect of the combination treatment and the concentration of both poly(I:C) and Smac mimetic that gave the highest synergistic effect was chosen for further experiments (79). We found that treatment with Poly(I:C) alone had a minimal effect on cell death, whereas treatment with Smac mimetic gradually induced cell death at high concentration (Figure 18). However, when poly(I:C) was combined with Smac mimetic, the combination treatment synergistically induced cell death in KKU100 (poly(I:C) at 2.5 µg/ml, Smac mimetic at 25-50 nM) (Figure 18.) and KKU213 (poly(I:C) at 2.5 µg/ml, Smac mimetic at 5 nM) (Figure 18.). In contrast, MMNK1, a non-tumor cholangiocyte was completely resistant to the combination treatment, but still sensitive to TNF-α and Smac mimetic-induced cell death (Figure 18.). Altogether, these results suggested that poly(I:C) and Smac mimetic synergistically induced cell death in CCA cells, but not in non-tumor cholangiocytes.



MNNK1		CI index		
Smac (nM)		10	25	50
Poly(I:C) (μg/ml)	1	ND	69.09	17.11
	2.5	16.51	52.14	3.13
	5	ND	9.16	1.18
	12.5	ND	3.12	0.99

KKU100		CI index		
Smac (nM)		10	25	50
Poly(I:C) (μg/ml)	1	ND	0.58	0.61
	2.5	0.53	0.38	**0.34**
	5	ND	0.39	0.45
	12.5	ND	0.41	0.47

KKU213		CI index		
Smac (nM)		1	5	10
Poly(I:C) (μg/ml)	1	0.64	0.20	ND
	2.5	0.56	**0.07**	0.49
	5	0.65	0.19	ND
	12.5	1.14	0.17	ND

Figure 18 : The optimization of poly(I:C) and Smac mimetic combination treatment and calculation of combination index (CI).

MMNK1, KKU100, and KKU213 were pretreated with indicated concentration of Smac mimetic and were transfected with different concentrations of poly(I:C) (1, 2.5, 5, and 12.5 μg/mL) for 48 hours. Cell viability was determined by MTT assay. The Chou-Talalay method was used to calculate combination index (CI) where C > 1 antagonism, CI = 0.9-1 additive, and CI < 0.9 synergism.

4.4 Poly(I:C) and Smac mimetic induced apoptosis in CCA cells

4.4.1 Inhibition of caspases (zVAD-fmk) inhibited poly(I:C) and Smac mimetic-induced apoptosis in CCA cells

To investigate whether poly(I:C) and Smac mimetic specifically triggered apoptosis, a pan-capase inhibitors (zVAD-fmk) which is a broad spectrum of caspase inhibition was used to examined whether this inhibitor inhibited poly(I:C) and Smac mimetic-induced cell death. KKU100, KKU213 and MMNK1 were pretreated with zVAD-fmk and Smac mimetic (KKU100 50 nM, KKU213 5 nM, MMNK1 50 nM) for 2 hours followed by transfection with 2.5 μg/mL of poly(I:C) for 24 hours. Annexin V/PI staining, a more specific method to evaluate cell death when compared to MTT assay which is unable to discriminate between cell death and a decrease in proliferation was used in this experiment. The representative flow cytometry analysis and cell morphology of control cells (vehicle control) and the combination treatment of poly(I:C) and Smac mimetic were shown in figure 19 and figure 20. Similar to cell viability assay as evaluated by MTT assay, transfection of poly(I:C) single treatment did not induce cell death, whereas Smac mimetic slightly induced cell death in both KKU100 and KKU213 which was determined by Annexin V/PI staining (Figure 21). Interestingly, when poly(I:C) was combined with Smac mimetic, combined Annexin V+/PI- and Annexin V+/PI+ population, the cell death was dramatically increased to 42.2 % and 48.2 % in KKU100 and KKU213, respectively (Figure 21.) In contrast, MMNK1 cells were remained completely resistant to poly(I:C) and Smac mimetic treatment, while they were sensitive to TNF-α and Smac mimetic-induced cell death (Figure 21). The pan-caspase inhibitor, zVAD-fmk completely protected cell death in KKU100 from 42.2% to 13.8% in the presence of zVAD-fmk, while partially inhibited cell death in KKU213 from 48.2% to 23.6% with zVAD-fmk (Figure 21). The effect of zVAD-fmk in the protection of TNF-α and Smac mimetic-induced cell death was similar to poly(I:C) and Smac mimetic treatment. These results suggested that poly(I:C) and Smac mimetic treatment induced capase-dependent apoptosis in CCA cells but not in non-tumor cholangiocyte cells.

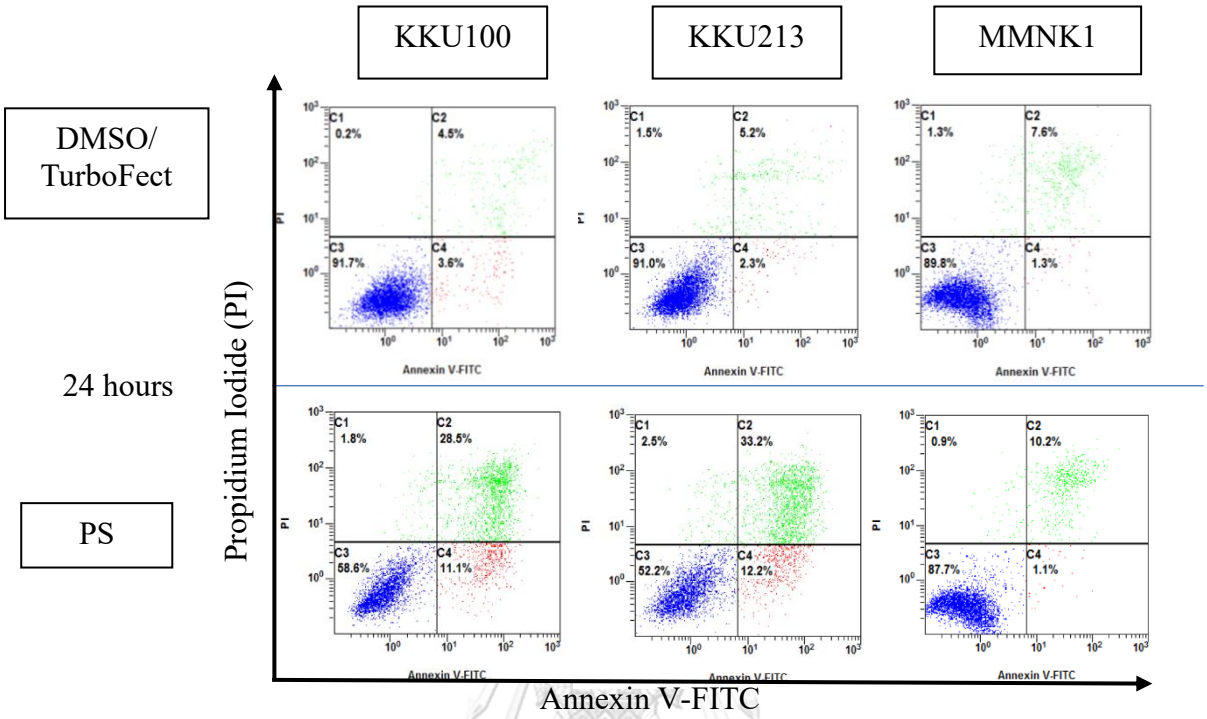


Figure 19 : Representative of flow cytometry analysis upon treatment with poly(I:C)/Smac mimetic in CCA cell lines (KKU100 and KKU213) and a non-tumor cholangiocyte, MMNK1

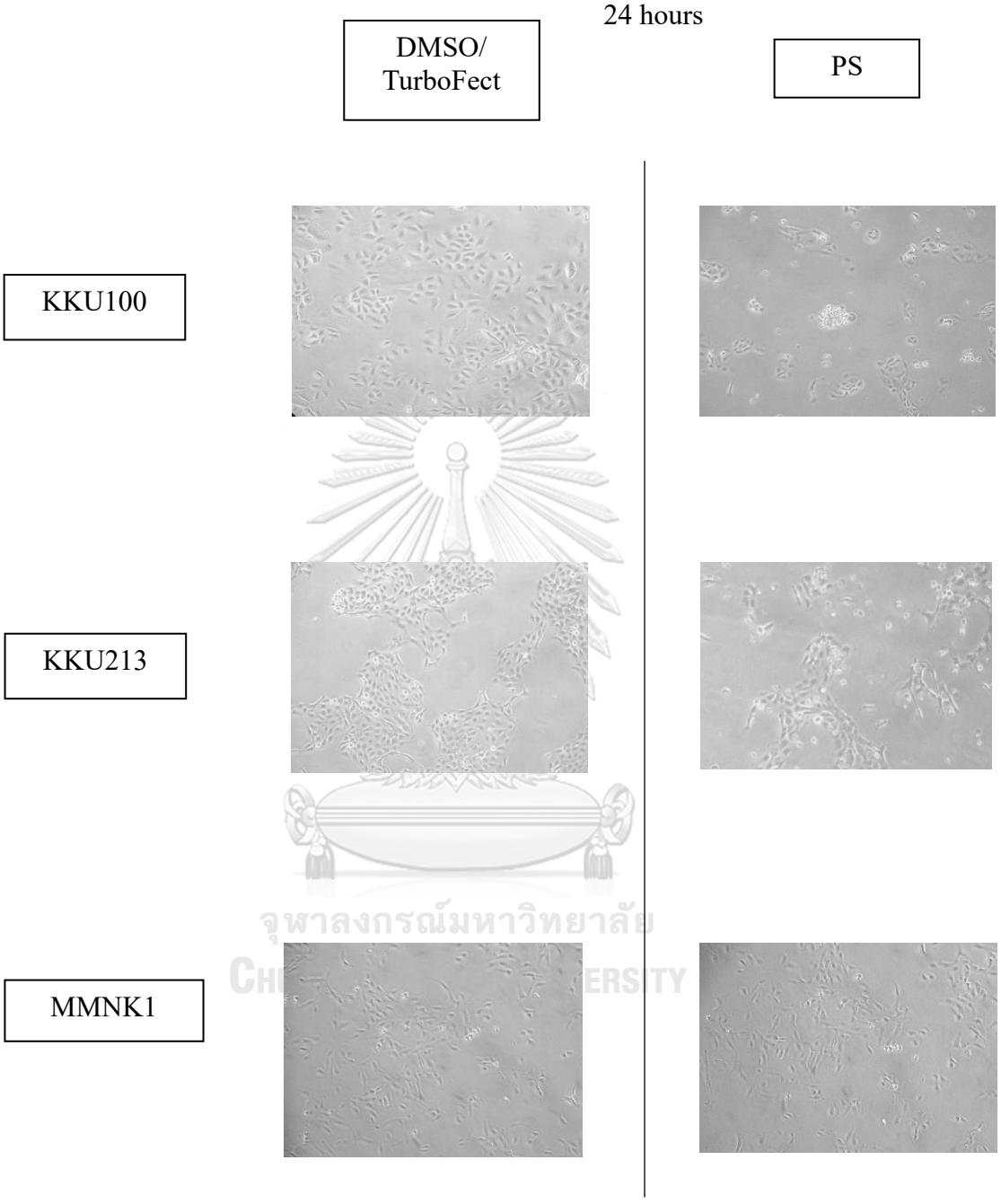


Figure 20 : Representative of cell morphology upon treatment with poly(I:C)/Smac mimetic in CCA cell lines (KKU100 and KKU213) and a non-tumor cholangiocyte, MMNK1

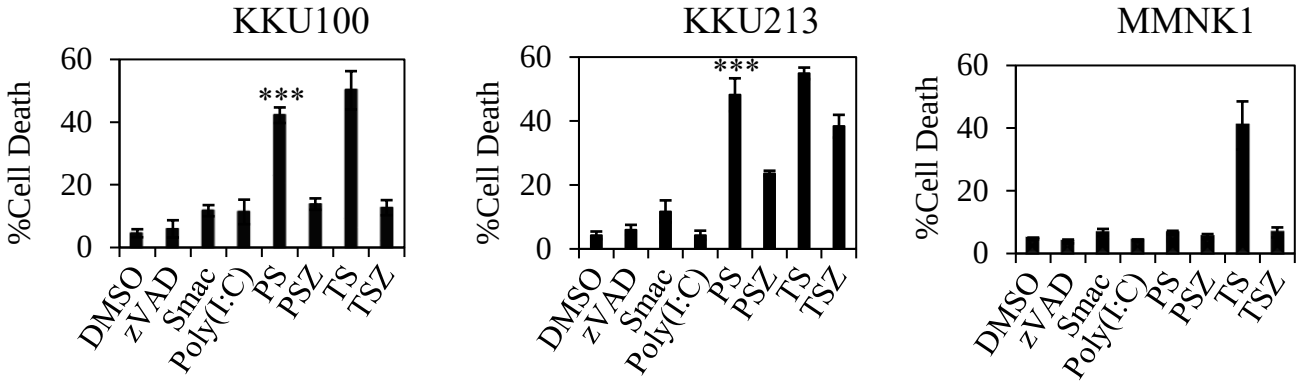


Figure 21 : Poly(I:C) and Smac mimetic induced caspase-dependent apoptosis in CCA cell lines.

MMNK1, KKU100, and KKU213 cells were pretreated with Smac mimetic (50 nM MMNK1 and KKU100, and 5 nM KKU213) (Smac) or 20 μM zVAD-fmk (zVAD) for 2 hours. After that the cells were transfected with 2.5 μg/mL poly(I:C) (PS, PSZ) for 24 hours. TNF-α at 10 ng/mL and Smac mimetic or zVAD-fmk at the same concentration as with poly(I:C) (TS, TSZ) were used as a positive control. Cell death was determined by Annexin V and propidium iodide staining followed by flow cytometry. Data from three independent experiments was presented as mean ± S.D.; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

4.4.2 Poly(I:C) and Smac mimetic induced an extrinsic caspase-8 activation in CCA cells.

Since it has been previously shown that poly(I:C) activates an extrinsic apoptosis pathway which is dependent on caspase-8 activation, an initiator of extrinsic apoptosis pathway. We therefore investigated whether poly(I:C) and Smac mimetic induced capase-8 activation. In addition, caspase-3 activation, an executioner caspase of apoptosis pathway and PARP-1 cleavage were also detected in this study which were used as specific markers of apoptosis. KKU100 and KKU213 were transfected with poly(I:C) alone, treated with Smac mimetic alone or treated with both poly(I:C) and Smac mimetic for 6 hours and 12 hours. Treatment with TNF-α and Smac mimetic was included as a positive control for apoptosis and caspase activation. The results showed that upon the combination treatment with poly(I:C) and Smac mimetic,

the smaller fragments of cleaved caspase-8 were generated which were started with the cleavage of pro-caspase-8 (p55/p53) to generate p43/p41 fragments, followed by the generation of p18 fragment in both KKU100 and KKU213 after 6 and 12 hours in which the activation of capase-8 was observed early at 6 hours when compared to KKU213 which was significantly observed after 12 hours treatment (Figure 22). Caspase-3, a downstream caspase activation was cleaved later by active caspase-8. The results revealed that pro-caspase-3 (35 kDa) was significantly decreased after 12 hours treatment in both KKU100 and KKU213. In consistent with caspase-3 activation, a substrate of caspase-3 which is PARP1 cleavage was observed after 12 hours treatment in both cell lines (Figure 22). Altogether, these results suggested that poly(I:C) and Smac mimetic activated caspase-8 in CCA cells.

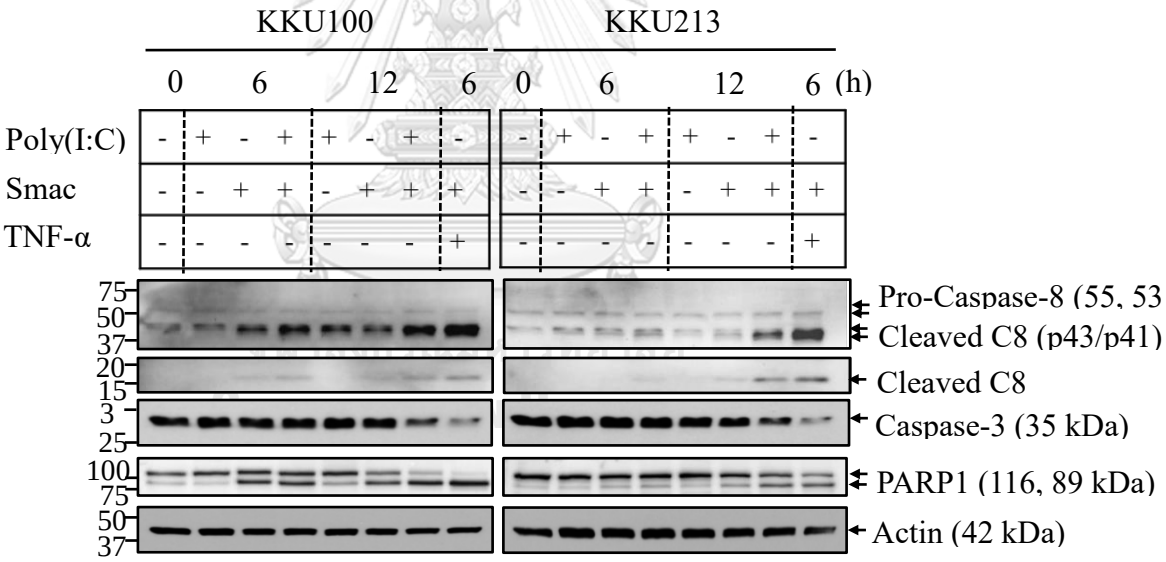


Figure 22 : Poly(I:C) and Smac mimetic induced caspase activation and apoptosis in CCA cell lines.

KKU100 and KKU213 cells were treated as in figure 21 for 6 hours and 12 hours. TNF-α and Smac mimetic was included as a positive control for apoptosis and caspase activation. Cell lysates were collected, after that activation of caspase-8 and caspase-3 and cleavage of PARP-1 were analyzed by Western blot analysis. β-actin

was served as loading control. Data shown was a representative of two independent experiments.

4.5 Poly(I:C) and Smac mimetic-induced apoptosis through RIPK1 kinase-dependent in CCA cells.

Receptor-interacting protein kinase 1 (RIPK1) has previously been proposed as a key mediator of TLR3-induced cell death (59). Mechanistically, RIPK1 recruits TLR3/TRIF to FADD/caspase-8 death complex which leads to apoptosis (59). We therefore hypothesized that RIPK1 might play an important role as a central mediator of poly(I:C) and Smac mimetic-induced apoptosis (PS) in CCA cells. Since a pharmacological inhibitor of RIPK1, necrostatin-1 (Nec-1) was developed which binds to RIPK1 and inhibits its kinase activity. To explore the involvement of RIPK1 in this pathway, we initially started with a RIPK1 inhibitor (Nec-1) to investigate whether necrostatin-1 inhibited poly(I:C) and Smac mimetic-induced apoptosis in KKU100 and KKU213. KUU100 and KKU213 cells were pretreated with 60 μM Nec-1 and Smac mimetic for 2 hours, followed by transfection with poly(I:C) for 24 hours and cell death was analyzed by Annexin V/PI staining. The results showed that Nec-1 partially reduced poly(I:C)-induced apoptosis from around 48.2% to 26.6% in KKU100 and 42.2% to 17.6% in KKU213 (Figure 23). These results suggested that poly(I:C) and Smac mimetic induced apoptosis in RIPK1 kinase-dependent manner.

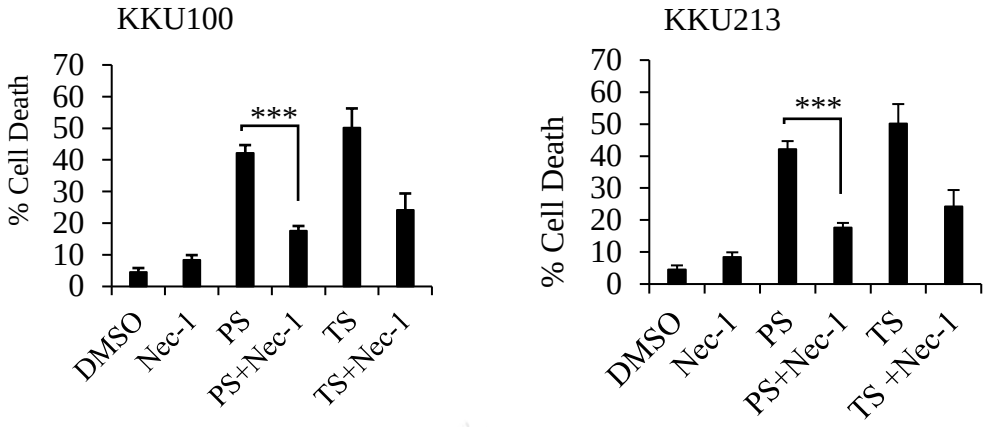


Figure 23 : The effect of a RIPK1 inhibitor, Necrostatin-1 (Nec-1) on poly(I:C)-induced apoptosis in KKU100 and KKU213 cell lines.

KKU100 and KKU213 were pretreated with 60 μM of Nec-1 and Smac mimetic (50 nM in KKU100 and 5 nM in KKU213) for 2 hours followed by transfection with 2.5 μg/mL poly(I:C) for 24 hours. TNF-α and Smac mimetic was included as a positive control. Cell death was determined by Annexin V/PI staining followed by flow cytometry. Data from three independent experiments was presented as mean $\pm$ S.D.; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Moreover, the more efficient for functional inhibition of RIPK1 was used which is a genetic disruption of *RIPK1* gene, we therefore constructed a genetic deletion of *RIPK1* in CCA cells using CRISPR/cas9 system. Western blot analysis results showed that the expression of RIPK1 was completely absent both in RIPK1 knockout KKU100 and KKU213 cells (Figure 24). Then, we investigated the protective effect of RIPK1-knockout CCA cells against poly(I:C) and Smac mimetic-induced apoptosis. The results showed that RIPK1 deletion by CRISPR/cas9 significantly reduced poly(I:C) and Smac mimetic-induced apoptosis from 42.7% to 13.3% and 42.7% to 10.5% in KKU100 and KKU213, respectively (Figure 24). Throughout two different approaches of RIPK1 inhibition, these results suggested that poly(I:C) and Smac mimetic induced CCA cells apoptosis in a RIPK1 kinase-dependent manner.

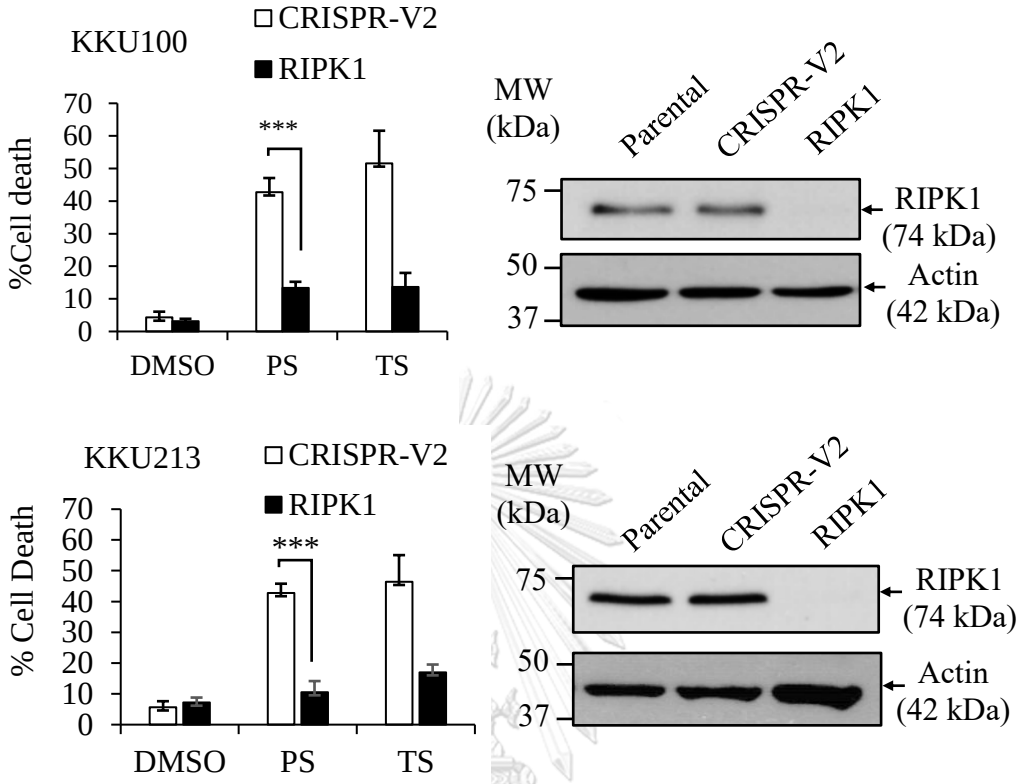


Figure 24 : The effect of RIPK1 gene deletion by CRISPR/cas9 on poly(I:C)-induced apoptosis in KKU100 and KKU213 cell lines.

CRISPR-V2 and CRISPR-RIPK1 KKU100 and KKU213 were pretreated with Smac mimetic (50 nM in KKU100 and 5 nM in KKU213) for 2 hours followed by transfection with 2.5 μg/mL poly(I:C) for 24 hours. TNF-α and Smac mimetic was included as a positive control. Cell death was determined Annexin V/PI staining followed by flow cytometry. Data from three independent experiments was presented as mean $\pm$ S.D.; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

CHAPTER V

DISCUSSION AND CONCLUSION

Cholangiocarcinoma (CCA), a heterogeneous malignancy of biliary tract which is the second most common of liver cancers. Because of late diagnosis, high aggressiveness, and heterogeneous mixed population of CCA cells, CCA patients have high mortality rate and poor prognosis with 5-year survival rate is low as of 5-10%, particularly with advanced stage diagnosis, mainly due to lack of effective therapy (2). Most of patients are diagnosed with an advanced stage, because of lack of early diagnosis tools and biomarkers. Therefore, surgery which is only a curative treatment cannot be operated. Chemotherapy and radiotherapy become two of palliative treatments for CCA patients. However, the efficacy of both treatments are limited, one of major hindrances for therapeutic success is chemoresistance and severe side effects (86). Therefore, in the present study our final goal was to development a novel treatment with more efficient in order to improve quality of life and increase survival rate of CCA patients.

Development of therapeutic treatments targeting molecules-associated with or specific-to cancer cells are a key for therapeutic success with high efficient and more specific treatments, so less side effects. As tumor microenvironment of CCA is associated with chronic inflammation which are amplified by damage-associated molecular patterns (DAMPs) releasing from damaged or dying cells in tumor microenvironment and pathogen-associated molecular patterns (PAMPs) (our unpublished data,(30)). Since both DAMPs and PAMPs have been shown to activate and induce the expression of pattern recognition receptors (PRRs) including Toll-like receptor 3 (TLR3), a novel therapeutic target for cancer therapy with immunomodulation. In this study, we therefore hypothesized that TLR3 was expressed in CCA cells and could be developed as a novel target for treatment of CCA patients. To this end, we investigated the expression of TLR3 in CCA cell lines to raise the possibility of using TLR3 ligand that activates TLR3 signaling, as a novel

therapeutic strategy for CCA patients to receive more efficient cancer treatment. We demonstrated that 6 different human CCA cell lines differentially expressed TLR3 protein but not detected in non-tumor cholangiocytes which suggested for further therapeutic development targeting TLR3 signaling.

As being an important and a novel target for cancer treatment particularly on cancer immunotherapy, several TLR3 agonists which mimic double-stranded RNA (dsRNA) have been developed in several forms such as polyinosinic acid: polycytidylic acid [poly(I:C)]. Some of them are used in clinics for immunomodulation and others are entered clinical trials for cancer immunotherapy such as Ampligene (87), Hiltonol or Oncovior (88–90), and BO-112 (91) that would make the possibility for clinical translations. In addition, stimulation with TLR3 ligand, poly(I:C) enhanced TLR3 expression in CCA cell lines, but it was restricted in non-tumor cholangiocytes. TLR3 ligand stimulation has been reported to enhance TLR3 expression (12,55) and the possible mechanism might be through type I IFNs (83). It suggested that non-tumor cholangiocytes might not be responsive to TLR3 ligand stimulation. Moreover, TLR3 expression and activation were occurred only in tumor cells so that it could emphasize the possibility and the more safety of using therapy targeting TLR3 signaling.

Although several studies have reported that a single treatment of TLR3 agonist or poly(I:C) directly induce apoptosis in some cancer cells (13,58–60), our resulted demonstrated that treatment with poly(I:C) alone by two different approaches including direct addition of poly(I:C) into cell culture medium and transfection using transfection reagent had no effect on cell death in 6 CCA lines. We therefore hypothesized that negative regulators of poly(I:C)-induced apoptosis could be one of factors that lead to resistance to poly(I:C)-induced cell death. As both cIAP1 and cIAP2 and cFLIP have been reported to be key negative regulators of poly(I:C)-induced cell death (23,59), we therefore investigated the expression of those three proteins in CCA cell lines. In comparison to known cIAP1 and cIAP2 overexpressed cell lines including MDA-MB-231, a breast cancer cell line and HT-29, a colon cancer cell line, all CCA cell lines except KKU100 expressed similar level of cIAP1 when compared to both MDA-MB-231 and HT-29 cell lines, while the expression of cIAP2 in KKU213, KKU214, and RMCCA-1 was higher than MDA-MB-231 and

HT-29 cell lines. In addition, cIAP2 expression in KKU100, KKU-M055, and HuCCT-1 was much higher than in MDA-MB-231 and HT-29 cell lines. Another negative regulator which is cFLIP was expressed highest in HuCCT-1. Altogether, these results suggested that CCA cell lines expressed high level of negative regulator particular on cIAP1 and cIAP2 that might contribute to resistance to poly(I:C)-induced apoptosis.

Since TLR3 ligand stimulation has been reported to induce cell death with or without the sensitizers in several cancers. In consistence with the high expression of cIAP1 and cIAP2 in CCA cell lines, our results did demonstrate for the first time that poly(I:C) could induce CCA cell death in the combination with Smac mimetic, an IAPs antagonist which are entered clinical trials as anti-cancer treatment (92). The over-expression of cIAP1 and cIAP2 in CCA cells plays an important role in suppression of poly(I:C)-induced CCA death as critical negative regulators. Smac mimetic specifically induced the degradation of cIAP1 and cIAP2, thereby removal key negative regulators of poly(I:C)-induced apoptosis in CCA cells. We found that a single treatment with Smac mimetic can also induce CCA apoptosis with high concentration in which KKU213, KKU214, and RMCCA-1 were more sensitive to Smac mimetic-induced apoptosis. The proposed mechanism of Smac mimetic-induced apoptosis in sensitive cell lines is an autocrine TNF-α production through a non-canonical NF-κB signaling pathway (29). In contrast, KKU100, KKU-M055, and HuCCT-1 were less sensitive which required higher dose of Smac mimetic-induced cIAP1 and cIAP2 degradation and cell death. Therefore, the correlation of CCA cell apoptosis and Smac mimetic is probably dependent on the higher relative expression of cIAP2 in which KKU100, KKU-M055, and HuCCT-1 were expressed high level of cIAP2 when compared to other cell lines. It has been reported the binding affinity of SM-164 to cIAP1 and cIAP2 using fluorescence-polarization based assays. SM-164 has higher K_i (the inhibitor constant) values to cIAP2 than cIAP1, it means that higher concentration of SM-164 is needed to inhibit cIAP2 when compared to cIAP1 (93). Therefore, it would suggest that the responsiveness of CCA cells to Smac mimetic was dependent on cIAP2 expression. There have been reported that cIAP2, but not cIAP1 expression is a contributing factor to TNF-α-induced apoptosis (94) and 5-Fluorouracil resistance and poor prognosis in squamous cell cacinoma (95). The

combination treatment of poly(I:C) and Smac mimetic significantly triggered apoptosis with high synergism. Concerning a non-tumor cholangiocyte, MMNK1, Smac mimetic significantly removed both cIAP1 and cIAP2 in this cell line, however they were not responsive to the combination treatment of poly(I:C) and Smac mimetic, yet they were highly sensitive to TNF-α and Smac mimetic treatment, a well-known caspas-8-dependent apoptosis inducer, suggesting that the unresponsiveness to poly(I:C) and Smac mimetic treatment is not due to a defective in apoptosis signaling pathway. We therefore hypothesized that TLR3 expression might contribute to apoptosis response in MMNK1 which necessitates further investigation. Among CCA cell lines investigated in this study, HuCCT-1 expressed the highest level of TLR3 and Smac mimetic with high concentration completely removed both cIAP1 and cIAP2 expression, however this particular cell line was less responsive to both poly(I:C) and TNF-α in the combination with Smac mimetic-induced apoptosis. According to our screening of known negative regulators of caspase-8-dependent apoptosis, high expression of cFLIP in this cell line might contribute to less responsiveness to apoptosis (23).

Moreover, in the presence of z-VAD-fmk, a pan-caspase inhibitor, CCA cell death could not be completely protected in KKU213 cell line, while zVAD-fmk almost completely inhibited apoptosis in KKU100 cell line. Since TLR3 agonist-mediated cell death has been reported not only through apoptosis but also a recently identified novel cell death called necroptosis in RIPK3-expressing cells (96). Our study that was not included in this thesis demonstrated that KKU213 expressed RIPK3, while KKU100 exhibited no RIPK3 expression. Therefore, it suggested that the combination treatment of poly(I:C) and Smac mimetic could switch cell death mode from apoptosis to necroptosis in KKU213, a RIPK3-expressing cell in the presence of zVAD-fmk. Of particular interested, since necroptosis is proposed to be a promising novel therapeutic treatment in cancer through dual therapeutic benefits including direct cell killing and anti-tumor immunity activation, therefore further studies in CCA cells-expressing RIPK3 could lead to more efficient therapy (97–99).

We found that treatment of CCA cells using poly(I:C) with transfection method exhibited more potent CCA cell death compared to direct adding method. Therefore, it suggested that the sensitivity of poly(I:C) on CCA cell death was

dependent on TLR3 localization. TLR3 localization can be found at cell surface and intracellular compartments mainly on an endosomal membrane depending on cell types (21,100,101). Our preliminary studies revealed that TLR3 mainly immunolocalized in cytoplasm of human CCA tissues, therefore it was not surprised that the more sensitivity of CCA cell lines to poly(I:C) treatment was towards the delivery of poly(I:C) by transfection. These results also pointed to the importance of further development of poly(I:C) delivery method into cancer cells.

RIPK1 has been reported to regulate cell fate both inflammation and cell death signaling (71). Our results revealed that RIPK1 was indispensable for poly(I:C) and Smac mimetic-induced apoptosis in a kinase-dependent manner. In addition, the deletion of RIPK1 using CRISPR/Cas9 almost completely protected apoptosis, however Nec-1, a RIPK1 kinase-domain inhibitor, only partially inhibited apoptosis while in parallel experiments, the same inhibitor almost completely inhibited TNF-α-induced necroptosis which is known that kinase domain of RIPK1 is a major mediator of necroptosis suggesting the effectiveness of this inhibitor (102). Therefore, in addition to its kinase activity, RIPK1 could also serve as a scaffold, in addition to kinase-dependent manner in poly(I:C) and Smac mimetic-induced apoptosis.

Altogether, all of these results provided the possibility to develop a novel potential therapeutic approach targeting TLR3 in combination with Smac mimetic in which apoptosis is dependent on RIPK1 expression. Although, RIPK1 was expressed in similar level in all CCA cell lines and a non-tumor cholangiocytes (data not shown), the investigation of RIPK1 expression in CCA tissue patents is of great importance for development of personalized therapy targeting TLR3 in combination with Smac mimetic which mainly depends on RIPK1 status.

TLR3 ligand-induced cancer cell death is a promising anti-cancer therapy. As mentioned above, TLR3 ligands e.g. poly(I:C) are now being evaluated in clinical trials as adjuvants for cancer immunotherapy. Interestingly, intratumoral injection of a nanoplexed poly(I:C) is undergoing phase I/II clinical trials in melanoma. It has been reported that BO-112, a conjugation of poly(I:C) to polyethylenimine (PEI) induced immunogenic cell death and exerted potent anti-tumor immunity (91). On the other hand, Smac mimetics, for example LCL-161, are also currently in clinical trials as anti-cancer agents for treatment of some cancers (103). Moreover, it has been

revealed that Smac mimetic did not induce cell death in immune cells such as T-cells and dendritic cells but not in monocytes (104). T-cell anti-tumor immunity can be enhanced by Smac mimetic in a cancer vaccine mouse model (34,35). Collectively, it suggested that the combination treatment of TLR3 ligand, poly(I:C) and Smac mimetic, while with further development could be translated into clinical uses and has significant clinical implications through dual beneficial therapeutic effects on both direct cancer cell killing and reactivating anti-tumor immunity.

In conclusion, this is the first study demonstrated that using TLR3 ligand, poly(I:C) which activates TLR3 signaling in the combination with Smac mimetic, an IAPs antagonist induced apoptosis in CCA cells but restricted to non-tumor cholangiocytes. Therefore, therapy targeting TLR3 in combination with Smac mimetic could provide a novel therapeutic concept with more effective for CCA patients. Finally, we suggested that further studies such as 1) investigation of poly(I:C) and Smac mimetic on tumor growth *in vivo*, 2) investigation of poly(I:C) and Smac mimetic in anti-tumor immunity, 3) investigation of TLR3 and RIPK1 expression in the patients and 4) development of delivery system should be examined that could benefit greatly as a targeted and personalized therapy.

REFERENCES

1. Rizvi S, Khan SA, Hallemeier CL, Kelley RK, Gores GJ. Cholangiocarcinoma-evolving concepts and therapeutic strategies. Nat Rev Clin Oncol. 2018;15(2):95–111.
2. Banales JM, Cardinale V, Carpino G, Marzioni M, Andersen JB, Invernizzi P, et al. Expert consensus document: Cholangiocarcinoma: current knowledge and future perspectives consensus statement from the European Network for the Study of Cholangiocarcinoma (ENS-CCA). Nat Rev Gastroenterol Hepatol. 2016;13(5):261–80.
3. Tepsiri N, Chaturat L, Sripa B, Namwat W, Wongkham S, Bhudhisawasdi V, et al. Drug sensitivity and drug resistance profiles of human intrahepatic cholangiocarcinoma cell lines. World J Gastroenterol. 2005 May 14;11(18):2748.
4. Waseem D, Tushar P. Intrahepatic, perihilar and distal cholangiocarcinoma: Management and outcomes. Ann Hepatol. 2017;16(1):133–9.
5. Koh S, Bertoletti A. Cancer immunotherapy: Targeting the difference. J Hepatol. 2014 Nov;61(5):1175–7.
6. Chai Y. Immunotherapy of biliary tract cancer. Tumor Biol. 2016 Mar 4;37(3):2817–21.
7. Galluzzi L, Vacchelli E, Eggermont A, Fridman WH, Galon J, Sautès-Fridman C, et al. Trial Watch. Oncoimmunology. 2012 May 27;1(3):306–15.
8. Levine AS, Levy HB. Phase I-II trials of poly IC stabilized with poly-L-lysine. Cancer Treat Rep. 1978 Nov;62(11):1907–12.
9. Salaun B, Zitvogel L, Asselin-Paturel C, Morel Y, Chemin K, Dubois C, et al. TLR3 as a Biomarker for the Therapeutic Efficacy of Double-stranded RNA in Breast Cancer. Cancer Res. 2011 Mar 1;71(5):1607–14.
10. Chin AI, Miyahira AK, Covarrubias A, Teague J, Guo B, Dempsey PW, et al. Toll-like Receptor 3–Mediated Suppression of TRAMP Prostate Cancer Shows the Critical Role of Type I Interferons in Tumor Immune Surveillance. Cancer Res. 2010 Apr 1;70(7):2595–603.
11. Salaun B, Coste I, Rissoan M-C, Lebecque SJ, Renno T. TLR3 can directly trigger apoptosis in human cancer cells. J Immunol. 2006 Apr 15;176(8):4894–901.

12. Bernardo AR, Cosgaya JM, Aranda A, Jiménez-Lara AM. Synergy between RA and TLR3 promotes type I IFN-dependent apoptosis through upregulation of TRAIL pathway in breast cancer cells. Cell Death Dis. 2013 Jan 31;4(1):e479–e479.

13. Weber A, Kirejczyk Z, Besch R, Potthoff S, Leverkus M, Häcker G. Proapoptotic signalling through Toll-like receptor-3 involves TRIF-dependent activation of caspase-8 and is under the control of inhibitor of apoptosis proteins in melanoma cells. Cell Death Differ. 2010 Jun 18;17(6):942–51.

14. Morikawa T, Sugiyama A, Kume H, Ota S, Kashima T, Tomita K, et al. Identification of Toll-Like Receptor 3 as a Potential Therapeutic Target in Clear Cell Renal Cell Carcinoma. Clin Cancer Res. 2007 Oct 1;13(19):5703–9.

15. Paone A, Starace D, Galli R, Padula F, De Cesaris P, Filippini A, et al. Toll-like receptor 3 triggers apoptosis of human prostate cancer cells through a PKC-a-dependent mechanism. Carcinogenesis. 2008;29(7):1334–42.

16. Gambara G, Desideri M, Stoppacciaro A, Padula F, De Cesaris P, Starace D, et al. TLR3 engagement induces IRF-3-dependent apoptosis in androgen-sensitive prostate cancer cells and inhibits tumour growth *in vivo*. J Cell Mol Med. 2015 Feb;19(2):327–39.

17. Vérillaud B, Gressette M, Morel Y, Paturel C, Herman P, Lo K, et al. Toll-like receptor 3 in Epstein-Barr virus-associated nasopharyngeal carcinomas: consistent expression and cytotoxic effects of its synthetic ligand poly(A:U) combined to a Smac-mimetic. Infect Agent Cancer. 2012 Dec 3;7(1):36.

18. Chiron D, Pellat-Deceunynck C, Amiot M, Bataille R, Jego G. TLR3 Ligand Induces NF- B Activation and Various Fates of Multiple Myeloma Cells Depending on IFN- Production. J Immunol. 2009 Apr 1;182(7):4471–8.

19. Nomi N, Kodama S, Suzuki M. Toll-like receptor 3 signaling induces apoptosis in human head and neck cancer via survivin associated pathway. Oncol Rep. 2010 Jul;24(1):225–31.

20. Umemura N, Zhu J, Mburu YK, Forero A, Hsieh PN, Muthuswamy R, et al. Defective NF- B Signaling in Metastatic Head and Neck Cancer Cells Leads to Enhanced Apoptosis by Double-Stranded RNA. Cancer Res. 2012 Jan 1;72(1):45–55.

21. Yuan M-M, Xu Y-Y, Chen L, Li X-Y, Qin J, Shen Y. TLR3 expression correlates with apoptosis, proliferation and angiogenesis in hepatocellular carcinoma and predicts prognosis. BMC Cancer. 2015 Dec 9;15(1):245.

22. Hsu W-M, Huang C-C, Lee H-Y, Wu P-Y, Wu M-T, Chuang H-C, et al. MDA5 complements TLR3 in suppression of neuroblastoma. Oncotarget. 2015 Sep 22;6(28):24935–46.

23. Alkurdi L, Virard F, Vanbervliet B, Weber K, Toscano F, Bonnin M, et al. Release of c-FLIP brake selectively sensitizes human cancer cells to TLR3-mediated apoptosis. Cell Death Dis. 2018 Aug 29;9(9):874.

24. Estornes Y, Toscano F, Virard F, Jacquemin G, Pierrot A, Vanbervliet B, et al. dsRNA induces apoptosis through an atypical death complex associating TLR3 to caspase-8. Cell Death Differ. 2012 Sep 16;19(9):1482–94.

25. Chen L, Chen L, Zhu Y, Zhang Y, He S, Qin J, et al. Double-stranded RNA-induced TLR3 activation inhibits angiogenesis and triggers apoptosis of human hepatocellular carcinoma cells. Oncol Rep. 2011 Nov 8;27(2):396–402.

26. Gyrd-Hansen M, Meier P. IAPs: from caspase inhibitors to modulators of NF-κB, inflammation and cancer. Nat Rev Cancer. 2010 Aug 1;10(8):561–74.

27. Fulda S, Vucic D. Targeting IAP proteins for therapeutic intervention in cancer. Nat Rev Drug Discov. 2012 Feb 1;11(2):109–24.

28. Vince JE, Wong WW-L, Khan N, Feltham R, Chau D, Ahmed AU, et al. IAP Antagonists Target cIAP1 to Induce TNFα-Dependent Apoptosis. Cell. 2007 Nov 16;131(4):682–93.

29. Petersen SL, Wang L, Yalcin-Chin A, Li L, Peyton M, Minna J, et al. Autocrine TNFα Signaling Renders Human Cancer Cells Susceptible to Smac-Mimetic-Induced Apoptosis. Cancer Cell. 2007 Nov;12(5):445–56.

30. Hernandez C, Huebener P, Schwabe RF. Damage-associated molecular patterns in cancer: a double-edged sword. Oncogene. 2016 Nov 18;35(46):5931–41.

31. Grivennikov SI, Greten FR, Karin M. Immunity, Inflammation, and Cancer. Cell. 2010 Mar;140(6):883–99.

32. Seubwai W, Vaeteewoottacharn K, Kraiklang R, Umezawa K, Okada S, Wongkham S. Inhibition of NF-κB Activity Enhances Sensitivity to Anticancer Drugs in Cholangiocarcinoma Cells. Oncol Res Featur Preclin Clin Cancer Ther. 2016 Jan 21;23(1):21–8.

33. Seubwai W, Wongkham C, Puapairoj A, Khuntikeo N, Pugkhem A, Hahnvajanawong C, et al. Aberrant Expression of NF-κB in Liver Fluke Associated Cholangiocarcinoma: Implications for Targeted Therapy. Makishima M, editor. PLoS One. 2014 Aug 29;9(8):e106056.

34. Beug ST, Beauregard CE, Healy C, Sanda T, St-Jean M, Chabot J, et al. Smac mimetics synergize with immune checkpoint inhibitors to promote tumour immunity against glioblastoma. Nat Commun. 2017 Apr 15;8(1):14278.

35. Kim D-S, Dastidar H, Zhang C, Zemp FJ, Lau K, Ernst M, et al. Smac mimetics and oncolytic viruses synergize in driving anticancer T-cell responses through complementary mechanisms. Nat Commun. 2017 Dec 24;8(1):344.

36. Rizvi S, Gores GJ. Pathogenesis, Diagnosis, and Management of Cholangiocarcinoma. Gastroenterology. 2013 Dec;145(6):1215–29.

37. Tyson GL, El-Serag HB. Risk factors for cholangiocarcinoma. Hepatology. 2011 Jul;54(1):173–84.

38. Ralphs S, Khan SA. The role of the hepatitis viruses in cholangiocarcinoma. J Viral Hepat. 2013 May 1;20(5):297–305.

39. Lee CH, Chang CJ, Lin YJ, Yeh CN, Chen MF, Hsieh SY. Viral hepatitis-associated intrahepatic cholangiocarcinoma shares common disease processes with hepatocellular carcinoma. Br J Cancer. 2009 Jun 12;100(11):1765–70.

40. Sithithaworn P, Yongvanit P, Duenngai K, Kiatsopit N, Pairojkul C. Roles of liver fluke infection as risk factor for cholangiocarcinoma. J Hepatobiliary Pancreat Sci. 2014 May;21(5):301–8.

41. Honjo S, Srivatanakul P, Sriplung H, Kikukawa H, Hanai S, Uchida K, et al. Genetic and environmental determinants of risk for cholangiocarcinoma via Opisthorchis viverrini in a densely infested area in Nakhon Phanom, northeast Thailand. Int J cancer. 2005 Dec 10;117(5):854–60.

42. Zheng W, Ying J, Zhou Y, Lu Z, Min K, Wang W, et al. The Efficacy and Safety of First-line Chemotherapies for Advanced Biliary Tract Cancer: A Network Meta-analysis. J Cancer. 2019;10(1):257–66.

43. Gotwals P, Cameron S, Cipolletta D, Cremasco V, Crystal A, Hewes B, et al. Prospects for combining targeted and conventional cancer therapy with immunotherapy. Nat Rev Cancer. 2017 May 24;17(5):286–301.

44. Marin JJG, Lozano E, Herraez E, Asensio M, Di Giacomo S, Romero MR, et al. Chemoresistance and chemosensitization in cholangiocarcinoma. Biochim Biophys Acta - Mol Basis Dis. 2018 Apr;1864(4):1444–53.

45. Hanahan D, Weinberg RA. Hallmarks of Cancer: The Next Generation. Cell. 2011 Mar;144(5):646–74.

46. Isomoto H, Mott JL, Kobayashi S, Werneburg NW, Bronk SF, Haan S, et al. Sustained IL-6/STAT-3 Signaling in Cholangiocarcinoma Cells Due to SOCS-3 Epigenetic Silencing. Gastroenterology. 2007 Jan;132(1):384–96.

47. Nakamura K, Smyth MJ. Targeting cancer-related inflammation in the era of immunotherapy. Immunol Cell Biol. 2017 Apr;95(4):325–32.

48. Chaturvedi A, Pierce SK. How Location Governs Toll-Like Receptor Signaling. Traffic. 2009 Jun;10(6):621–8.

49. O'Neill LAJ, Golenbock D, Bowie AG. The history of Toll-like receptors — redefining innate immunity. Nat Rev Immunol. 2013 Jun 17;13(6):453–60.

50. Manavalan B, Basith S, Choi S. Similar Structures but Different Roles - An Updated Perspective on TLR Structures. Front Physiol. 2011;2:41.

51. Lin A, Slack N, Ahmad A, Koltover I, George C, Samuel C, et al. Structure and Structure—Function Studies of Lipid/Plasmid DNA Complexes. J Drug Target. 2000 Jan 20;8(1):13–27.

52. Takeda K, Akira S. TLR signaling pathways. Semin Immunol. 2004 Feb;16(1):3–9.

53. Sasai M, Oshiumi H, Matsumoto M, Inoue N, Fujita F, Nakanishi M, et al. Cutting Edge: NF- B-Activating Kinase-Associated Protein 1 Participates in TLR3/Toll-IL-1 Homology Domain-Containing Adapter Molecule-1-Mediated IFN Regulatory Factor 3 Activation. J Immunol. 2005 Jan 1;174(1):27–30.

54. Jiang Z, Mak TW, Sen G, Li X. Toll-like receptor 3-mediated activation of NF- B and IRF3 diverges at Toll-IL-1 receptor domain-containing adapter inducing IFN-. Proc Natl Acad Sci. 2004 Mar 9;101(10):3533–8.

55. Salaun B, Lebecque S, Matikainen S, Rimoldi D, Romero P. Toll-like Receptor 3 Expressed by Melanoma Cells as a Target for Therapy? Clin Cancer Res. 2007 Aug 1;13(15):4565–74.

56. Goto Y, Arigami T, Kitago M, Nguyen SL, Narita N, Ferrone S, et al. Activation of toll-like receptors 2, 3, and 4 on human melanoma cells induces inflammatory factors. Mol Cancer Ther. 2008 Nov 1;7(11):3642–53.

57. Karikó K, Ni H, Capodici J, Lamphier M, Weissman D. mRNA is an endogenous ligand for Toll-like receptor 3. J Biol Chem. 2004 Mar 26;279(13):12542–50.

58. Taura M, Fukuda R, Suico MA, Eguma A, Koga T, Shuto T, et al. TLR3 induction by anticancer drugs potentiates poly I:C-induced tumor cell apoptosis. Cancer Sci. 2010;101:1610–7.

59. Feoktistova M, Geserick P, Kellert B, Dimitrova DP, Langlais C, Hupe M, et al. cIAPs Block Ripoptosome Formation, a RIP1/Caspase-8 Containing Intracellular Cell Death Complex Differentially Regulated by cFLIP Isoforms. Mol Cell. 2011 Aug 5;43(3):449–63.

60. Friboulet L, Pioche-Durieu C, Rodriguez S, Valent A, Souquère S, Ripoche H, et al. Recurrent overexpression of c-IAP2 in EBV-associated nasopharyngeal carcinomas: critical role in resistance to Toll-like receptor 3-mediated apoptosis. Neoplasia. 2008 Nov;10(11):1183–94.

61. Estornes Y, Micheau O, Renno T, Lebecque S. Dual role of TLR3 in inflammation and cancer cell apoptosis. Oncogene Cancer-from Bench to Clin Novi Sad (Croatia) INTECH. 2013;247–70.

62. Hotchkiss RS, Strasser A, McDunn JE, Swanson PE. Cell Death. N Engl J Med. 2009 Oct 15;361(16):1570–83.

63. Fuchs Y, Steller H. Live to die another way: modes of programmed cell death and the signals emanating from dying cells. Nat Rev Mol Cell Biol. 2015 Jun;16(6):329–44.

64. Sun S-C, Ley SC. New insights into NF-kappaB regulation and function. Trends Immunol. 2008 Oct;29(10):469–78.

65. Bertrand MJM, Milutinovic S, Dickson KM, Ho WC, Boudreault A, Durkin J, et al. cIAP1 and cIAP2 Facilitate Cancer Cell Survival by Functioning as E3 Ligases that Promote RIP1 Ubiquitination. Mol Cell. 2008 Jun 20;30(6):689–700.

66. Cossu F, Milani M, Mastrangelo E, Lecis D. Targeting the BIR Domains of Inhibitor of Apoptosis (IAP) Proteins in Cancer Treatment. Comput Struct Biotechnol J. 2019;17:142.

67. Dougan M, Dougan S, Slisz J, Firestone B, Vanneman M, Draganov D, et al. IAP inhibitors enhance co-stimulation to promote tumor immunity. J Exp Med. 2010 Sep 27;207(10):2195–206.

68. Cossu F, Milani M, Vachette P, Malvezzi F, Grassi S, Lecis D, et al. Structural Insight into Inhibitor of Apoptosis Proteins Recognition by a Potent Divalent Smac-Mimetic. Srinivasula SM, editor. PLoS One. 2012 Nov 15;7(11):e49527.

69. Newton K. RIPK1 and RIPK3: critical regulators of inflammation and cell death. Trends Cell Biol. 2015 Jun;25(6):347–53.

70. Vandenabeele P, Declercq W, Van Herreweghe F, Vanden Berghe T. The Role of the Kinases RIP1 and RIP3 in TNF-Induced Necrosis. Sci Signal. 2010 Mar 30;3(115):re4–re4.

71. Ofengeim D, Yuan J. Regulation of RIP1 kinase signalling at the crossroads of inflammation and cell death. Nat Rev Mol Cell Biol. 2013 Nov 16;14(11):727–36.

72. Park HH, Lo Y-C, Lin S-C, Wang L, Yang JK, Wu H. The Death Domain Superfamily in Intracellular Signaling of Apoptosis and Inflammation. Annu Rev Immunol. 2007 Apr;25(1):561–86.

73. Rickard JA, O'Donnell JA, Evans JM, Lalaoui N, Poh AR, Rogers T, et al. RIPK1 Regulates RIPK3-MLKL-Driven Systemic Inflammation and Emergency Hematopoiesis. Cell. 2014 May 22;157(5):1175–88.

74. Kelliher MA, Grimm S, Ishida Y, Kuo F, Stanger BZ, Leder P. The death domain kinase RIP mediates the TNF-induced NF-kappaB signal. Immunity. 1998 Mar;8(3):297–303.

75. Meylan E, Burns K, Hofmann K, Blancheteau V, Martinon F, Kelliher M, et al. RIP1 is an essential mediator of Toll-like receptor 3–induced NF-κB activation. Nat Immunol. 2004 May 4;5(5):503–7.

76. Maruyama M, Kobayashi N, Westerman KA, Sakaguchi M, Allain JE, Totsugawa T, et al. Establishment of a highly differentiated immortalized human cholangiocyte cell line with SV40T and hTERT. Transplantation. 2004 Feb 15;77(3):446–51.

77. Rattanasinganchan P, Leelawat K, Treepongkaruna S, Tocharoentanaphol C, Subwongcharoen S, Suthiphongchai T, et al. Establishment and characterization of a cholangiocarcinoma cell line (RMCCA-1) from a Thai patient. World J Gastroenterol. 2006;12(40):6500.

78. Lu J, McEachern D, Sun H, Bai L, Peng Y, Qiu S, et al. Therapeutic potential and molecular mechanism of a novel, potent, nonpeptide, Smac mimetic SM-164 in combination with TRAIL for cancer treatment. Mol Cancer Ther. 2011 May;10(5):902–14.

79. Chou T-C. Drug Combination Studies and Their Synergy Quantification Using the Chou-Talalay Method. Cancer Res. 2010 Jan 15;70(2):440–6.

80. Degterev A, Huang Z, Boyce M, Li Y, Jagtap P, Mizushima N, et al. Chemical inhibitor of nonapoptotic cell death with therapeutic potential for ischemic brain injury. Nat Chem Biol. 2005 Jul 29;1(2):112–9.

81. Xu Q, Jitkaew S, Choksi S, Kadigamuwa C, Qu J, Choe M, et al. The cytoplasmic nuclear receptor RARγ controls RIP1 initiated cell death when cIAP activity is inhibited. Nat Commun. 2017 Dec 4;8(1):425.

82. Ran FA, Hsu PD, Wright J, Agarwala V, Scott DA, Zhang F. Genome engineering using the CRISPR-Cas9 system. Nat Protoc. 2013 Nov 24;8(11):2281–308.

83. Tanabe M, Kurita-Taniguchi M, Takeuchi K, Takeda M, Ayata M, Ogura H, et al. Mechanism of up-regulation of human Toll-like receptor 3 secondary to infection of measles virus-attenuated strains. Biochem Biophys Res Commun. 2003 Nov 7;311(1):39–48.

84. An MJ, Cheon JH, Kim SW, Kim ES, Kim T Il, Kim WH. Guggulsterone induces apoptosis in colon cancer cells and inhibits tumor growth in murine colorectal cancer xenografts. Cancer Lett. 2009 Jun 28;279(1):93–100.

85. Bockbrader KM, Tan M, Sun Y. A small molecule Smac-mimic compound induces apoptosis and sensitizes TRAIL- and etoposide-induced apoptosis in breast cancer cells. Oncogene. 2005 Nov 25;24(49):7381–8.

86. Marin JJG, Lozano E, Briz O, Al-Abdulla R, Serrano MA, Macias RIR. Molecular Bases of Chemoresistance in Cholangiocarcinoma. Curr Drug Targets. 2017 May 4;18(8):889–900.

87. Armstrong JA, McMahon D, Huang XL, Pazin GJ, Gupta P, Rinaldo CR, et al. A phase I study of ampligen in human immunodeficiency virus-infected subjects. J Infect Dis. 1992 Oct;166(4):717–22.

88. Salem ML, Kadima AN, Cole DJ, Gillanders WE. Defining the antigen-specific T-cell response to vaccination and poly(I:C)/TLR3 signaling: evidence of enhanced primary and memory CD8 T-cell responses and antitumor immunity. J Immunother. 28(3):220–8.

89. Salem ML, El-Naggar SA, Kadima A, Gillanders WE, Cole DJ, Beeching NJ, et al. The adjuvant effects of the toll-like receptor 3 ligand polyinosinic-cytidylic acid poly (I:C) on antigen-specific CD8+ T cell responses are partially dependent on NK cells with the induction of a beneficial cytokine milieu. Vaccine. 2006 Jun 12;24(24):5119–32.

90. Okada H, Butterfield LH, Hamilton RL, Hoji A, Sakaki M, Ahn BJ, et al. Induction of robust type-I CD8+ T-cell responses in WHO grade 2 low-grade glioma patients receiving peptide-based vaccines in combination with poly-ICLC. Clin Cancer Res. 2015 Jan 15;21(2):286–94.

91. Aznar MA, Planelles L, Perez-Olivares M, Molina C, Garasa S, Etxeberría I, et al. Immunotherapeutic effects of intratumoral nanoplexed poly I:C. J Immunother Cancer. 2019 Dec 2;7(1):116.

92. Fulda S. Promises and Challenges of Smac Mimetics as Cancer Therapeutics. Clin Cancer Res. 2015 Nov 15;21(22):5030–6.

93. Lu J, Bai L, Sun H, Nikolovska-Coleska Z, McEachern D, Qiu S, et al. SM-164: A Novel, Bivalent Smac Mimetic That Induces Apoptosis and Tumor Regression by Concurrent Removal of the Blockade of cIAP-1/2 and XIAP. Cancer Res. 2008 Nov 15;68(22):9384–93.

94. Zhao X, Laver T, Hong SW, Twitty GB, Devos A, Devos M, et al. An NF-κB p65-cIAP2 link is necessary for mediating resistance to TNF-α induced cell death in gliomas. J Neurooncol. 2011 May 30;102(3):367–81.

95. Nagata M, Nakayama H, Tanaka T, Yoshida R, Yoshitake Y, Fukuma D, et al. Overexpression of cIAP2 contributes to 5-FU resistance and a poor prognosis in oral squamous cell carcinoma. Br J Cancer. 2011 Oct 25;105(9):1322–30.

96. Schmidt S V., Seibert S, Walch-Rückheim B, Vicinus B, Kamionka E-M, Pahne-Zeppenfeld J, et al. RIPK3 expression in cervical cancer cells is required for PolyIC-induced necroptosis, IL-1α release, and efficient paracrine dendritic cell activation. Oncotarget. 2015 Apr 20;6(11):8635–47.

97. Koo G-B, Morgan MJ, Lee D-G, Kim W-J, Yoon J-H, Koo JS, et al. Methylation-dependent loss of RIP3 expression in cancer represses programmed necrosis in response to chemotherapeutics. Cell Res. 2015 Jun;25(6):707–25.

98. Geserick P, Wang J, Schilling R, Horn S, Harris PA, Bertin J, et al. Absence of RIPK3 predicts necroptosis resistance in malignant melanoma. Cell Death Dis. 2015 Sep 10;6(9):e1884.

99. Gong Y, Fan Z, Luo G, Yang C, Huang Q, Fan K, et al. The role of necroptosis in cancer biology and therapy. Mol Cancer. 2019 Dec 23;18(1):100.

100. Nishiya T, Kajita E, Miwa S, Defranco AL. TLR3 and TLR7 are targeted to the same intracellular compartments by distinct regulatory elements. J Biol Chem. 2005 Nov 4;280(44):37107–17.

101. Matsumoto M, Funami K, Tanabe M, Oshiumi H, Shingai M, Seto Y, et al. Subcellular Localization of Toll-Like Receptor 3 in Human Dendritic Cells. J Immunol. 2003 Sep 15;171(6):3154–62.

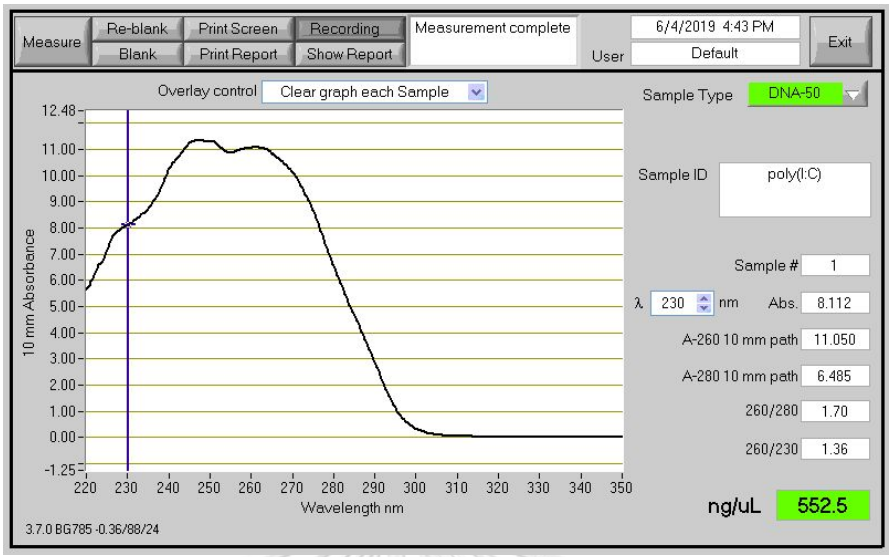
102. Vandenabeele P, Grootjans S, Callewaert N, Takahashi N. Necrostatin-1 blocks both RIPK1 and IDO: consequences for the study of cell death in experimental disease models. Cell Death Differ. 2013 Feb;20(2):185–7.

103. Runckel K, Barth MJ, Mavis C, Gu JJ, Hernandez-Ilizaliturri FJ. The SMAC mimetic LCL-161 displays antitumor activity in preclinical models of rituximab-resistant B-cell lymphoma. Blood Adv. 2018 Dec 11;2(23):3516–25.

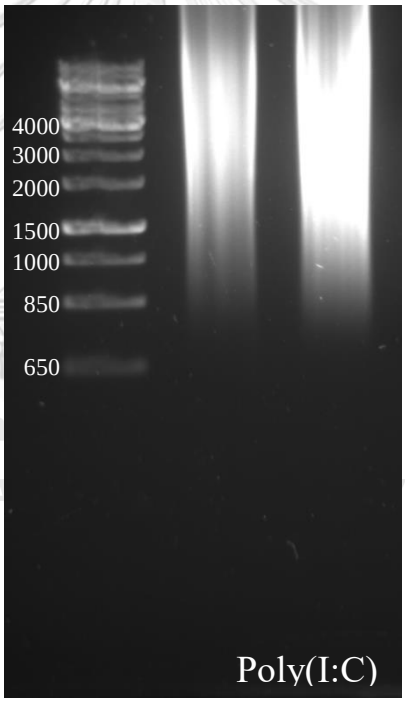
104. Mü ller-Sienerth N, Dietz L, Holtz P, Kapp M, tz Ulrich Grigoleit G, Schmuck C, et al. SMAC Mimetic BV6 Induces Cell Death in Monocytes and Maturation of Monocyte-Derived Dendritic Cells.

APPENDICES

(A)



(B)



Appendix A : Poly(I:C) Quality Control

(A) Stock solution (5 mg/mL) of poly(I:C) was diluted to 1:10 and subjected to NanoDrop Spectrophotometer for poly(I:C) quantitation. (B) Quality of poly(I:C) was determined by gel electrophoresis. High molecular weight (HMW) poly(I:C): average size of 1.5-8 kb was used in this study. Lane 1 indicated DNA marker, while lane 2 and lane 3 were loaded with 25 and 50 µg of poly(I:C), respectively.

Appendix B : Western Blot Reagents

10X inhibitor
tablet (Boetringer Mannheim Com. 1697498)

1X RIPA Lysis buffer

10x RIPA buffer	1 mL
MilliQ	9 mL

Add 1 tablet protease inhibitor cocktail

Aliquot 1 mL into each tube

5X laemmli buffer

0.5M Tris-HCL pH6.8	1.75	mL
Glcerol(Glycrin)	4.5	mL
SDS (0.25g dissolved in 1ml Tris-HCL)	2	mL
0.5g total 0.25% Bromophenol blue (25mg in 10 mL H_20)	0.5	mL
B-mercaptoethanol	1.25	mL
Total	10	mL

1M Tris-HCl pH 6.8 (MW = 121.14)
121.14 g in 1000 mL dH_20

2M Tris-HCl pH 8.8 (MW = 121.14)
242 .28 g in 1000 mL dH_20

1.5M Tris-HCl pH 8.8 (MW = 121.14)
181.71 g in 1000 mL dH_20

20% SDS
SDS 20 g
MilliQ 100 mL

10X TBS pH7.4
500 mM Tris-HCl (MW=121.4)
1.5 M NaCl (MW=58.44)

Add 60.57 g Tris
87.66 g NaCl
adjust pH to 7.4 and add dH_2O to 1000 mL

1X TBS (0.05% Tween 20)

10X TBS	100 mL	50 mM
dH_2O	900 mL	150 mM
Tween	0.5 mL	0.05%

10X Running buffer (Tris-Glycine)		*10X*	*1X*
Add	30.3 g Tris (MW=121.4)	0.25 M	2.5 mM
	144.1 g Glycine (MW=75.07)	1.9 M	190 mM
	adjust pH to 8.3 and add dH_2O to 1000 mL		

1X Running buffer (Tris-Glycine-SDS)

10X Running buffer	100 mL	
dH_2O	900 mL	
20%SDS	5 mL	(0.1%)

1X Wet Transfer buffer

100 mL 10X Running buffer
700 mL dH_2O
200 mL Methanol (20%)

VITA

NAME	Thanpisit Lomphithak
DATE OF BIRTH	24 December 1993
PLACE OF BIRTH	Bangkok
INSTITUTIONS ATTENDED	Bachelor of Science (Medical Technology), Chulalongkorn University
HOME ADDRESS	88/47 Lumpini Ville Mary-Sathon Chan 27, Chan Road, Thung Wat Don, Sathon, 10120