EFFECT OF PIPERLONGUMINE ON UBIQUITIN-PROTEASOME SYSTEM IN CHOLANGIOCARCINOMA

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ผลของไปเปอร์ลองกูลมินต่อระบบยูบิควิตินโปรติเอโซม ในมะเร็งท่อน้ำดี

นายนราธิป นราดุลย์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for a Master's Degree.

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มะเร็งท่อน้ำดีเป็นมะเร็งที่ร้ายแรงต่อชีวิต มะเร็งท่อน้ำดีมีจุดเริ่มต้นจากเซลล์เยื่อบุของท่อทางเดินน้ำดี การรักษาโดยการผ่าตัดถือเป็นวิธีที่มีประสิทธิภาพสูงสุด แต่ด้วยผู้ป่วยมะเร็งท่อน้ำดีส่วนใหญ่ไม่สามารถได้รับการรักษาด้วยการผ่าตัดได้ เนื่องจากได้รับการวินิจฉัยว่าเป็นมะเร็งท่อน้ำดีในระยะท้ายของโรค ดังนั้นปัจจุบันการรักษาด้วยเคมีบำบัดหรือการรักษาแบบมุ่งเป้าจึงถูกแนะนำมาใช้ในการรักษาผู้ป่วยมะเร็งท่อน้ำดีกลุ่มดังกล่าว แต่ปัญหาหลักสำคัญคือการตอบสนองต่อการรักษาดังกล่าว กระบวนการยูบิควิตินโปรติเอโซม คือวิถีหลักในการกำจัดโปรตีนเพื่อรักษาสมดุลของปริมาณโปรตีนและการทำงานภายในเซลล์ โดยเฉพาะกลุ่มโปรตีนที่เกี่ยวข้องกับกระบวนการเจริญเติบโตและการอยู่รอด การรักษาแบบมุ่งเป้าไปที่กระบวนการยูบิควิตินโปรติเอโซมของมะเร็งชนิดก้อน ได้มีการนำมาใช้ เนื่องจากความผิดปกติของโครโมโซมและการกลายพันธุ์ของยีน ทำให้มีการสร้างโปรตีนผิดปกติจำนวนมากในเซลล์มะเร็ง ไปเปอร์ลองกูลมินเป็นสารกลุ่มแอลคาลอยด์ที่มีฤทธิ์ในการเลือกฆ่าเฉพาะเซลล์มะเร็ง ในมะเร็งหลาย ๆ ชนิดรวมถึงมะเร็งท่อน้ำดีผ่านการชักนำให้มีการสร้างอนุมูลอิสระจำนวนมากภายในเซลล์ บทบาทของไปเปอร์ลองกูลมินยังมีผลต่อการยับยั้งกระบวนการยูบิควิตินโปรติเอโซมอีกด้วย ในการศึกษานี้ ผู้วิจัยมุ่งเน้นในกลไกของไปเปอร์ลองกูลมินในการชักนำให้เซลล์มะเร็งท่อน้ำดีตายผ่านกระบวนการยูบิควิตินโปรติเอโซม และความเป็นไปได้ของแผนการรักษาแบบร่วมของไปเปอร์ลองกูลมินกับบอร์ทีโซมิฟสำหรับมะเร็งท่อน้ำดี ผลการศึกษาแสดงให้เห็นว่าไปเปอร์ลองกูลมินกับบอร์ทีโซมิฟยับยั้งการเจริญเติบโตของเซลล์มะเร็งท่อน้ำดี การทดสอบร่วมกันของไปเปอร์ลองกูลมินกับบอร์ทีโซมิฟแสดงถึงการเสริมฤทธิ์ซึ่งกันและกันในมะเร็งท่อน้ำดี ซึ่งแสดงได้ด้วยค่าดัชนีผลการทดสอบร่วมที่มีค่าน้อยกว่า 1 การเสริมฤทธิ์ซึ่งกันและกันของไปเปอร์ลองกูลมินกับบอร์ทีโซมิฟถูกยืนยันด้วยการแสดงออกที่ลดลงของยีนที่เกี่ยวข้องกับการเจริญเติบโต ไปเปอร์ลองกูลมินสามารถชักนำให้เกิดการเพิ่มการสะสมของโปรตีนที่ถูกจับด้วยยูบิควิติน แต่ไปเปอร์ลองกูลมินไม่มีผลต่อแอคทิวิตี้ของโปรติเอโซม การทดสอบร่วมกันของไปเปอร์ลองกูลมินกับบอร์ทีโซมิฟสามารถชักนำให้เกิดการสะสมของโปรตีนที่ถูกจับด้วยยูบิควิตินเพิ่มมากขึ้น นำมาสู่การเกิดภาวะเครียดในเอนโดพลาสมิกเรติคูรั่มด้วยการพบการชักนำให้เกิดชิ้นส่วนของยีน XBP1s มากไปกว่านั้นการทดสอบร่วมกันของไปเปอร์ลองกูลมินกับบอร์ทีโซมิฟส่งเสริมให้เกิดการกระตุ้นกระบวนการตายแบบ unfolded protein

response ผ่านวิถี ATF4-CHOP จากผลการศึกษาทั้งหมดแสดงให้เห็นว่าไปเปอร์ลองกูลมิน ชักนำให้เซลล์มะเร็งท่อน้ำดีตายด้วยการเพิ่มการสะสมของโปรตีนที่ถูกจับด้วยยูบิควิติน ส่งเสริมการต้านมะเร็งของบอร์ทีโซมิฟด้วยการชักนำเกิดภาวะเครียดในเอนโดพลาสมิกเรติคูรั่มซึ่งส่งผลให้เกิดการชักนำการตาย ดังนั้นการรักษาร่วมของไปเปอร์ลองกูลมินและบอร์ทีโซมิฟน่าจะเป็นการรักษาทางเลือกให้กับมะเร็งท่อน้ำดีได้

สาขาวิชาเคมี
ปีการศึกษา 2564

ลายมือชื่อนักศึกษา นราธิป นาคูลย์
ลายมือชื่ออาจารย์ที่ปรึกษา

NARATHIP NARADUN : EFFECT OF PIPERLONGUMINE ON UBIQUITIN-PROTEASOME SYSTEM IN CHOLANGIOCARCINOMA. THESIS ADVISOR : ASST. PROF. CHUTIMA TALABNIN, Ph.D. 48 PP.

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Cholangiocarcinoma (CCA) is a deadly malignancy originating from the cholangiocytes lining the biliary tree. Surgical resection is a gold standard treatment, but most of CCA patients are inoperable due to diagnosed at advanced state. Currently, systemic chemotherapy and targeted therapy are recommended for the inoperable patients or metastasis but the poor response to these therapies is a major obstacle. The ubiquitin-proteasome system (UPS) is a major protein degradation pathway to maintain the proteostasis and cellular function especially those proteins involve in cell growth and survival. Targeting UPS in the treatment of solid malignancies has been established due to chromosome abnormalities and gene mutation, leading to high production of mutated proteins in cancer cells. Piperlongumine (PL) is a biologically active alkaloid that selectively kill various cancer cells including CCA through the induction of reactive oxygen species (ROS). The role of PL has also been shown via inhibiting ubiquitin-proteasome system. In current study, we focused on the mechanism of PL-induced CCA cell death via ubiquitin-proteasome system and the therapeutic potential of combined PL and proteasome inhibitor bortezomib for CCA. The result of present study demonstrated that single treatment (PL or BTZ) inhibits CCA cell growth. The combined treatment with PL with Bortezomib (BTZ) showed the synergistic interaction in CCA cell lines demonstrating via the combination index less than 1. The synergistic interaction between PL and BTZ on CCA growth was confirmed by down-regulation of cell cycle markers (*CCND1*, and *CCNB1*, *c-Myc* and *BIRC5*). PL induced the accumulation of poly-ubiquitinated proteins in CCA cells but no effect on proteasome activity. The combined treatment with PL and BTZ amplified the accumulation of poly-ubiquitinated proteins in CCA cells, leading to endoplasmic reticulum stress response stress by the induction of XBP1splicing to spliced XBP1 (*XBP1s*) mRNA. Moreover, PL-combined BTZ promotes the activation of proapoptotic

unfolded protein response via ATF-CHOP axis. Taken together, our findings showed that PL induced CCA cell death via the increase of the accumulation of the poly-ubiquitinated proteins. PL enhances anti-cancer activity of BTZ via ER stress-induced CCA cell death. Thus, combined treatment with PL and BTZ could be the therapeutic option for CCA.

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Student's signature _____
Advisor's signature _____

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Narathip Naradun

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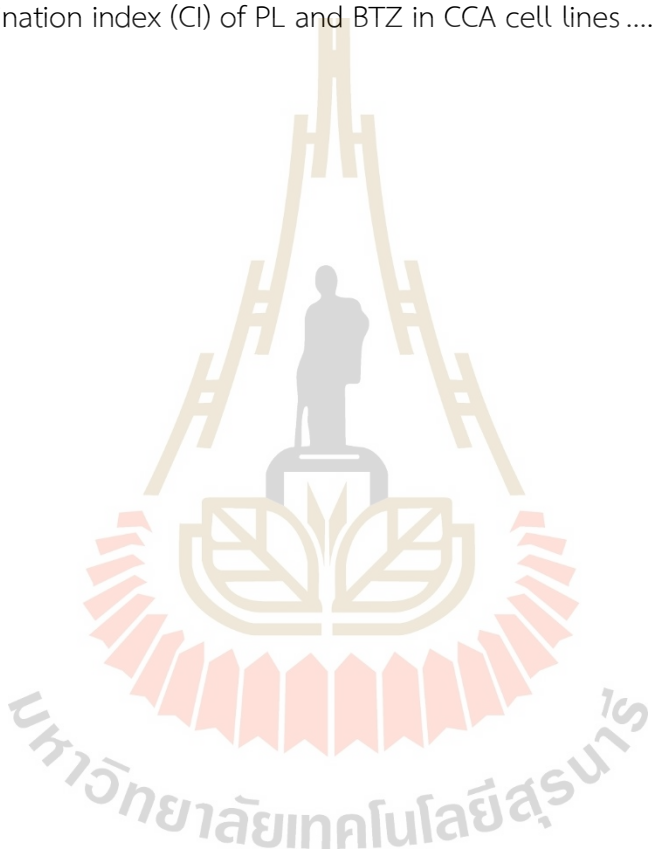

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LIST OF ABBREVIATIONS

ATF4	Activating transcription factor 4
ATF6	Activating transcription factor 6
BTZ	Bortezomib
CCA	Cholangiocarcinoma
CHOP	C/EBP homologous protein
DMEM	Duldecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
ECCA	Extrahepatic CCA
EGFR	Epidermal growth factor receptor
ER	Endoplasmic reticulum
ERAD	ER-associated protein degradation
FBS	Fetal bovine serum
FGFR	Fibroblast growth factor receptor
HRP	Horseradish peroxidase
ICCA	Intrahepatic CCA
IDH1/IDH2	Isocitrate dehydrogenase 1 and 2
PL	Piperlongumine
ROS	Reactive oxygen species
RPM	Revolution per minute
TCA	Tricarboxylic acid
UPR	Unfolded protein response
UPS	Ubiquitin proteasome system
VEGFR	Vascular endothelial growth factor
receptor	

LIST OF ABBREVIATIONS (Continued)

XBP1s	spliced X-box binding protein 1
(m, n, μ)M	(milli, nano, micro) Molar
v/v	Volume per volume
w/v	Weight per volume

CHAPTER I
INTRODUCTION

1.1 Rational for the study

Cholangiocarcinoma (CCA) is a biliary tract malignancy which represents the second most common type of primary liver cancer. CCA has very poor prognosis and is extremely aggressive with symptoms unobservable until there is a blockage of the bile duct by the tumor. For the CCA treatments, surgical resection are the potentially curative therapeutic choices. Because of the location of the tumor can affect the surgical treatments (Ghouri et al., 2015), complete surgical resection is the only therapy to afford a possibility of CCA treatment. Unfortunately, many patients were presented with unresectable disease including distance metastasis or recurrence. Additional treatment by chemotherapy needs to be included in that CCA patients.

The ubiquitin-proteasome system (UPS) is necessary for proper cell cycle progression, stress response, DNA repair, and differentiation. Because cancer cells proliferate indefinitely, they rely more heavily on proteasomal function to degrade suppressive proteins that would otherwise stop their growth and division. (Shen et al., 2013). Thus, the precise mechanism of proteasome inhibitors is the promising therapeutics in cancer.

Piperlongumine (PL) is, a bioactive alkaloid compound, isolated from the long pepper plant *Piper longum* (Chatterjee and Dutta, 1967). PL has been shown to inhibit bacterial, angiogenic, and adiabetic activities (Bezerra et al., 2013). Current studies shown that PL can selectively kill various transformed cells including head and neck cancer (Roh et al., 2014), hepatocellular carcinoma cells (Chen et al., 2015), breast cancer (Jin et al., 2017), gastric cancer (Duan et al., 2016) and lung cancers (Zheng et al., 2016). PL can induce high levels of reactive oxygen species (ROS), which results in *in vitro* and *in vivo* cell death via activate several mechanisms, including ROS-p38/JNK pathways which induce apoptotic and autophagic death of primary acute myeloid leukemia cells (Xiong et al., 2015),

ROS-ER-MAPKs-CHOP pathways in hepatocellular carcinoma cells (Chen et al., 2015), nuclear factor kappa B (NF-kB) signaling pathways in lung cancer (Zheng et al., 2016). Taken together, it was suggested that PL is a ROS inducer with distinct anti-cancer properties. However, PL has been addressed on complex mechanisms of action via inhibiting ubiquitin proteasome system. Jarvius et al. (2013) have demonstrated that PL induces the accumulation of poly-ubiquitinated proteins in breast cancer which is similarly observed in the cells treated with USP14/UCHL5 deubiquitinase inhibitor (b-AP15).

Previous study has reported that PL significantly inhibited the growth of CCA cell lines by triggering G2/M phase arrest, activating ROS level, and causing CCA cell apoptosis through the JNK/ERK pathway. Moreover, the stabilization of particular regulating proteins in cell cycle and apoptosis such as p21 and cleavage PARP were observed in PL-induced CCA cell death as strongly as MG-132 (Thongsom et al., 2017). However, the underlying mechanism of PL-inhibited ubiquitin-proteasome system in CCA have never been reported. In present study, we evaluated anti-tumor effect of PL via inhibiting ubiquitin-proteasome system in CCA cell lines. Additionally, the underlying mechanism were investigated.

1.2 Research objectives

The objectives of this study focus on the effect of Piperlongumine on ubiquitin-proteasome system and underlying mechanism in CCA cell lines.

1.2.1 To examine the effect of Piperlongumine on ubiquitin-proteasome system in CCA cell lines.

1.2.2 To investigate the combination effect of Piperlongumine and Bortezomib on cell growth in CCA cell lines.

CHAPTER II

LITERATURE REVIEW

2.1 Cholangiocacinoma

Cholangiocarcinoma (CCA) is a biliary tract malignancy. CCA is the second most common type of primary liver cancer. It is classified as intrahepatic (ICCA) or extrahepatic (ECCA), including perihilar and distal CCA, based on its anatomical location. CCA has very poor prognosis and is extremely aggressive with symptoms unobservable until there is a blockage of the bile duct by the tumor. Major risk factor of CCA in Thailand especially in Northeast is *Opisthorchis viverrini* (Ov). Ov is a food borne trematode that infected human by eating uncooked cyprinoid fish. Ov infection induces chronic inflammation that leads to hepatobiliary abnormalities enhance to cancer. Several studies have been conducted to investigate the processes by which Ov infection may promote cholangiocarcinogenesis. Inflammation, periductal fibrosis, and proliferative responses such as epithelial hyperplasia, goblet cell metaplasia, and adenomatous hyperplasia may occur with liver fluke infection, resulting in lesions that increase DNA vulnerability to mutagens. Several intermediates produced by inflammatory cells, such as nitric oxide and other reactive oxygen species, may increase cell proliferation during Ov infection, with direct cytotoxic and mutagenic effects. It has been reported that there is an increase of nitric oxide-mediated DNA damage in the epithelial biliary in rats infected with Ov (Kawanishi et al., 2006), and together with the liver fluke infections could enhance DNA damage in biliary tree (Pinlaor et al., 2006). With less apoptosis of the infected biliary epithelial cells (Sripa et al., 2007), genetic alterations may be repaired after several sequences, resulting to malignant transformation as shown in Figure 2.1 (Brindley and Loukas, 2017).

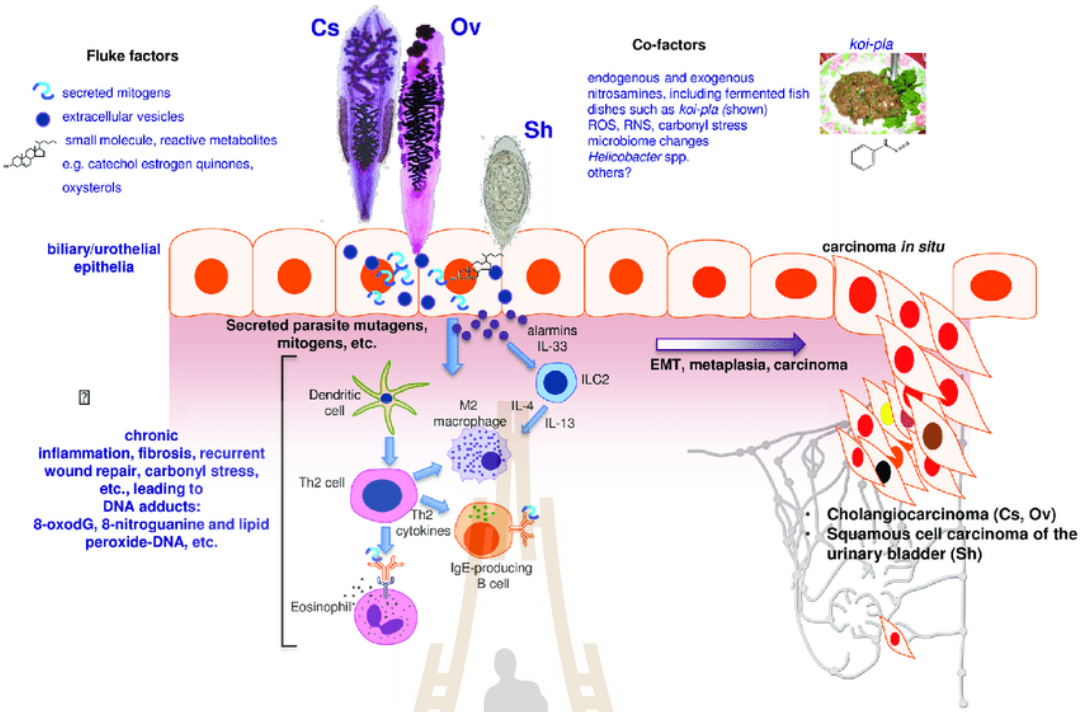

Figure 2.1 Schematic representation of CCA with liver flukes infection process. Carcinogenesis of the biliary tract and urinary bladder during severe liver fluke infection with *Opisthorchis viverrini* (Ov), *Clonorchis sinensis* (Cs), and the urinary blood fluke *Schistosoma haematobium* (Sh) have been reported (Brindley and Loukas, 2017).

2.2 Therapeutics in cholangiocarcinoma

The clinical presentation of CCA patients is unspecific. Because of CCA is usually asymptomatic during early stages. Patients with ICCA may experience nausea and vomiting, weariness, excessive sweating, losing weight, and a strong desire to eat. Patients with ECCA tent to present with obstructive jaundice. surgical resection are the potentially helpful therapeutic solutions for CCA treatment. Because of the location of the tumor can affect the surgical treatments (Ghouri et al., 2015). However, several preclinical studies have shown that targeting molecular pathways in CCA is the potential treatment for CCA.

2.2.1 Surgical resection and Orthotopic liver transplantation

Surgical resection is the common standard option for CCA treatment which may be the only potential cure. Resection becomes more difficult when the increase of dispersion within biliary trees area, amount of liver parenchyma involvement,

vascular development, lobar atrophy, and development of metastatic disease are considered (Yachimski and Pratt, 2008). Furthermore, negative margins resection enhances long-term survival; however, the tumor's anatomical location at the moment of resection, such as perineural extension and pre-vascular formation, frequently makes curative surgical resection more difficult (Hemandez et al., 2008). Recently, more extensive surgical procedures with longer survival and lower recurrence rates, such as major hepatectomy, pancreatoduodenectomy, extended lymphoadenectomy, and vascular resection have been develop to achieve negative margins (R0 resection) (Kosuge et al., 1999). Furthermore, R0 resection with sufficient margins is an crucial factor influencing survival (Jarnagin et al., 2001). CCA patients are often left with positive margins (R1 or R2 resections) despite aggressive resection because estimating the proximal extent of the microscale disease by pre-operative imaging is difficult. Only surgical resection is insufficient for CCA treatment, particularly in the advance stages. Therefore, adjuvant therapeutic modalities such as systemic-chemotherapy or radiotherapy are required to achieve long term survival. Recently, valuable right hepatic artery resection and restructuring in ECCA patients was reported, with postsurgical mortality rates of less than 5% and 5-year survival rates of 30%. The patients with portal vein invasion or local invasion, pancreaticoduodenectomy with portal vein resection or hepatopancreaticodenectomy had tumor-free margins resection rates of 80% and rates of 5-years survival ranging from 10% to 40% (Blechacz, 2017). However, the surgical resection is limited by high morbidity and mortality rates.

Orthotopic liver transplantation (OLT) is not suggested to use as a single therapy for CCA because chemoradiation followed by OLT has been shown as a successive option for ECCA treatments. Recurrence rates after the transplant procedure are 20% with 5-year recurrence-free survival of 68%. However, the patient-selection criteria are restrictive, and 30% of patients develop disease progression while waiting for the liver transplantation, resulting in rejection from the treatment procedure. There are studies have evaluated the effectiveness of liver transplant with or without adjuvant treatment for ICCA. However, recurrence rates were as high as 30% to 70% and 5-year survival only 35% to 50%. As a result, OLT is not generally approved as a treatment for ICCA (Blechacz, 2017).

Extensive research and insight gained from new-generation molecular genetic analyses have recently enabled progress in the development of targeted therapies for CCA. Several mutated genes including EGFR, VEGFR, FGFR2 and NTRK fusion, and IDH1/2 have been reported and implications for CCA therapy. Earlier efforts in this strategy concentrated on inhibiting known targets implicated in CCA carcinogenesis, such as EGFR and VEGFR. However, targeted ERGR or VEGFR did not demonstrate any benefit on survival either single agents or combined with chemotherapy in phase II studies. In recent year, the advent of next-generation sequencing technology has discovered the novel mutated genes that is frequency found in CCA including IDH1/IDH2 and FGFR2 fusion in CCA. IDH1 mutation are more common than IDH2 mutation. AG-120 (Ivosidenib) is a potent oral inhibitor of mutant IDH1 that was studied in a phase I study in solid tumor containting mutant IDH1. Phase III, randomized, double-blind study of AG-120 vs placebo in advanced CCA patients with an IDH1 mutation demonstrated a favorable trend in overall survival with AG-120. Median overall survival was 10.8 months for AG-120 and 9.7 months for placebo. Although, the outcome given by this approaches have not satisfied enough, but the precision medicine-based target therapy remains promising and is potential treatment for CCA in the future.

2.3 The ubiquitin-proteasome system (UPS)

Proteasome-mediated degradation pathway is a regular process that cells regenarate their intracellular proteins and maintain protein balance. The abundance of protein within a cell is controlled by to the cellular needs. Protein control dysregulation can result in a variety of cell disorders. There are two major pathways degrading proteins. The major one is proteasome-mediated proteolysis, has of two main steps. Protein labeling is the first step, followed by protein degradation by the 26S proteasome. Moreover, this process is divided into three minor steps: activation of the ubiquitin molecules (Ub) by the Ub-conjugating enzyme (E1), transportation of Ub by Ub-conjugating enzyme (E2) and then the bindind of Ub to the target protein by the Ub-ligase enzyme (E3) as shown in Figure 2.2 (Liu et al., 2015). There are 7 estential lysine residues in the ubiquitin molecule (Lysine 6, 11, 27, 29,K33, 48, and 63) and

Met1 that can be further ubiquitinated to allow the different types of ubiquitin chains formation (Komander, 2009). K48-linked ubiquitination is a degradation signal that targets substrate for proteasomal degradation, whereas polyubiquitination at K63 is involved in cellular signaling pathways. (Chau et al., 1989).

The 26S proteasome complex is presented in both the cytosol and nucleus, it recognizes polyubiquitinated proteins that have been designed for detaching by the ubiquitinating enzymes E1, E2, and E3. When polyubiquitinated proteins are recognized by the 19S regulatory subunit, particularly the four non-ATPase subunits (Rpn 1, 2, 10, and 13), the ubiquitin chain is detached by proteasome-associated deubiquitinating enzymes (DUB). The de-ubiquitinated target protein is then moved into the tubular core of the 20S proteasome component. Protein degradation process is aided by catalytic β-subunits with nucleophilic N-terminal threonine (Thr1) residues. Although eukaryotic 20S proteasomes have seven distinct catalytic β-subunits in their two-fold symmetrical (α1- α7)(β1- β7)(β1- β7)(α1- α7) stacked form, three of seven are the active β-subunits. β1 *(PSMB6)* exhibited caspase-like, β2 *(PSMB7)* exhibited trypsin-like, and β5*(PSMB5)* exhibited chymotrypsin-like, are proteolytically active subunits. These three catalytic β-subunits are primary targets for small molecule proteasome inhibitors. The use of particular inhibitors against one or more catalytic β-subunits to block or inhibit the 26S proteasome complex, which regulates protein degradation, can lead to a significant rise in the level of cytotoxic proteins in cells as shown in Figure 2.3 (Kubiczkova et al., 2014).

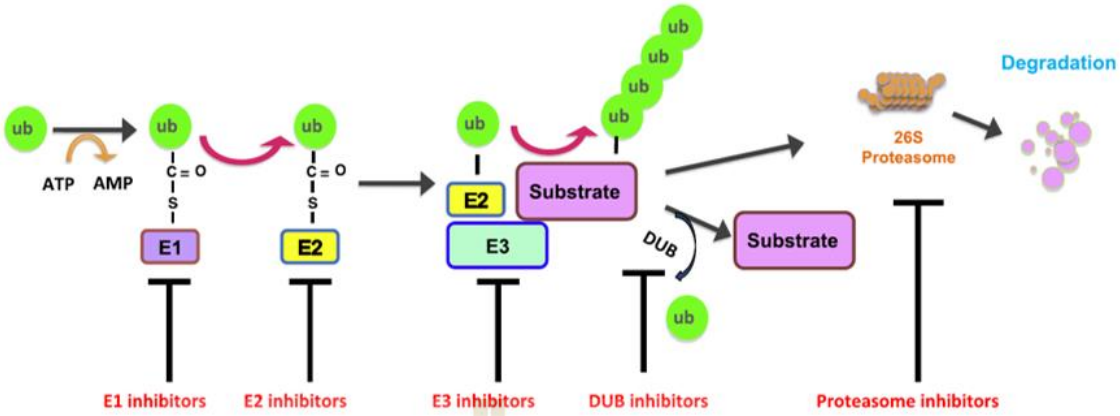

Figure 2.2 Proteasome-mediated proteolysis pathway is achieved by a series of enzymatic steps (Liu et al., 2015).

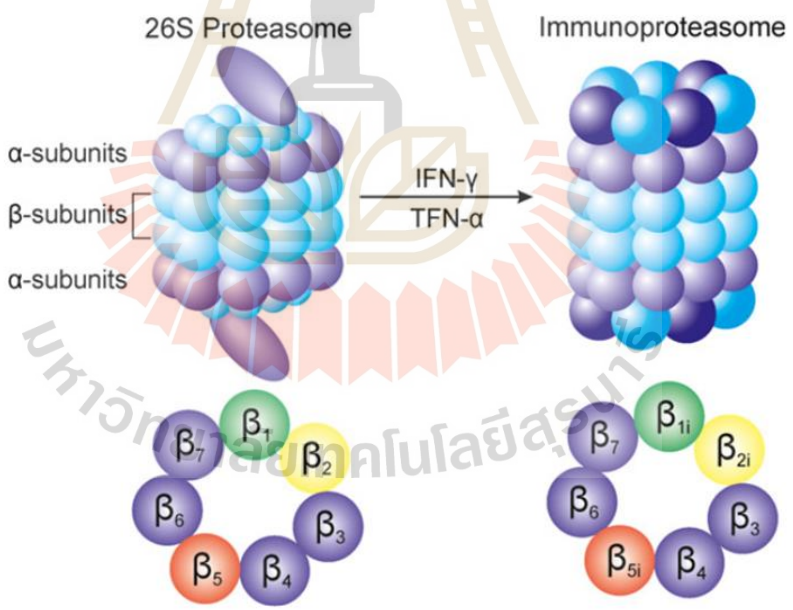

Figure 2.3 The 26S proteasome complex and immunoproteasome and its catalytic subunits (Kubiczkova et al., 2014).

2.4 The ubiquitin-proteasome system (UPS) in cancer

The ubiquitin proteasome system is required for efficient of cell cycle progression, stress response, DNA repair, and cell differentiation. It is well known that the UPS is known as plays an important role in regulating the turnover of several key proteins including cyclins, p21, p27, p53 and NF-KB that involved in cell-cycle progression and survival. Cancer cells are highly reliant on proteasomal function for the excessive breakdown of those inhibitory proteins that would otherwise stop their growth and division due to their potential for ongoing proliferative activity. (Shen et al., 2013). Elevated proteasomal activity has been detected in many types of cancers including melanoma (Ren et al., 2000), breast (Chen and Madura, 2005), colon cancers (Arlt et al., 2009; Rho et al., 2008). Although new results suggest that the increase of proteasomal subunit proteins may be involved, the exact processes causing the higher proteasome activity in malignancies remain largely unclear. For example, breast cancer tissues exhibit higher levels of proteasome activity and elevated expression of some proteasomal subunits compared to normal tissue. (Chen and Madura, 2005). In a recent study, PSMB7, a β2 protein was found to be highly expressed in colon cancer tissue (Rho et al., 2008). High expression of the proteasomal subunit, S10, has been reported in melanoma, resulting from gene amplified by a chromosomal translocation and accounting for enhanced proteasome activity (Ren et al., 2000). As a result, proteasome inhibition has emerged as a useful and potent strategy in the treatment against cancer. (Almond and Cohen, 2002; Adams, 2004). Several pre-clinical studies have shown that malignant cells are more vulnerable than normal cells to the cytotoxic effects of proteasome inhibition. The mechanisms underlying malignant cell sensitivity are remain unclear. However, it is believed that increased proteasomal activity, which preserves specific survival signal-transduction pathways like the IKB/NFKB pathway, protects the cancer cell against apoptosis (Wang et al., 2000; Karin et al., 2002; Marciniak et al., 2004).

However, the different efficacies effect of proteasome inhibition is depended on tumor types and drug resistance (Kale and Moore, 2012). Pro-survival pathway activation, proteasome expression changes, and introduction of mutations in

β-subunit genes are a few examples of resistance mechanisms. In multiple myeloma, resistance to proteasome inhibitors was observed in the patient with mutations in genes encoding catalytic beta subunits of proteasomes (Barrio et al., 2019). Moreover, patients with breast cancer who had low PSMB5 expression had poor prognosis. These data indicate that the usage of proteasome inhibitors in cancer treatment is restricted for particular cancer types. In CCA, there is a few evidence on the alteration of proteasome system, but it seems to be possible to used proteasome inhibitors for CCA treatment. Yu et al., have demonstrated that ubiquitin receptor ADRM1 was found to be overexpressed in CCA. Targeting ADRM1 with particular inhibitors, RA190, had a successive activity in CCA through inducing G2-M phase arrest and apoptosis in *in vitro* and *in vivo* (Yu et al., 2018). Moreover, Bortezomib found to perturb the proteasome activity demonstrated ER stress-induced apoptotic cell death in CCA (Vaeteewoottacharn et al., 2013). Taken together, inhibition of the ubiquitin-proteasome system may be a viable approach for CCA treatment.

2.5 Proteasome as a drug target

Since the UPS plays an important role in regulating the turnover of several key proteins that involve in cell-cycle progression and survival, inhibition of the UPS may be a viable approach cancer treatment. Malignant cells are more vulnerable than normal cells to the cytotoxic effects of proteasome inhibition, according to pre-clinical investigations. The mechanisms behind the higher sensitivity of malignant cells are unclear, however, they probably use the proteasome to control the pathways that lead to proliferative and anti-apoptotic responses. Thus, inhibition of NF-ΚB activity can alter cell cycle related proteins degradation, alter proapoptotic and anti-apoptotic protein stability, ER stress and inhibition of angiogenic development and DNA repairing process have all been reported to responds toward the apoptotic effect by using proteasome inhibitors in tumor cells (Figure 2.4) (Crawford et al., 2011).

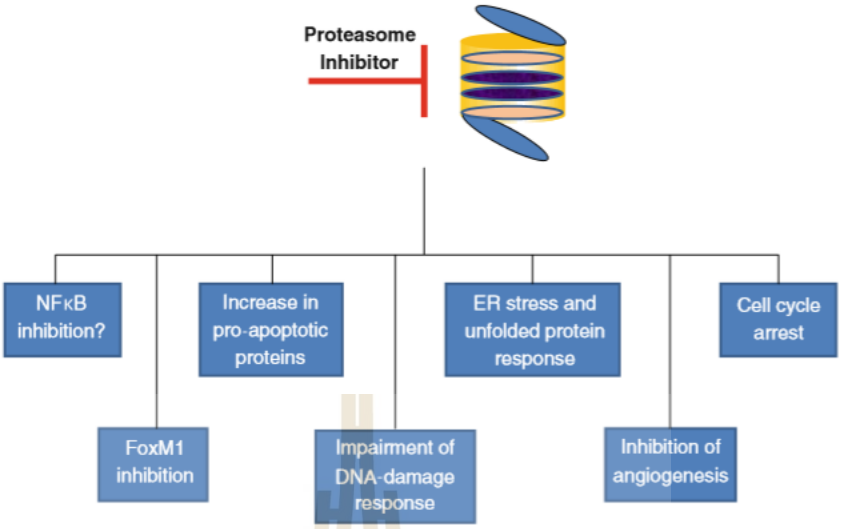

Figure 2.4 Critical targets for proteasome inhibitors in malignant cells (Crawford et al., 2011).

NF-KB has been related to tumorigenesis and resistance to chemotherapeutic drugs. It is capable of activating in a significant fraction of advanced malignancies (Luqman and Pezzuto, 2010). In the cytoplasm, NF-KB is linked to its inhibitor I-B and is only activated when IKB is broken down by proteasomes. Proteasome activity inhibition thus prevents IKB degradation and the further down activation and translocation of NF-KB to the nucleus to initiate back-line mechanisms. Pre-clinical and clinical studies with Bortezomib demonstrated the inhibition of NF-KB transcription. However, endometrial cancer cells and multiple myeloma revealed that bortezomib enhances non-proteasomal degradation of IKB and induces NF-KB DNA binding, the exact opposite impact of bortezomib on NF-KB activation. (Hideshima et al., 2009). In sum, there is ongoing debate concerning the function of NF-B in mediating the impact of proteasome inhibition.

Altered degradation of cell cycle regulatory proteins especially p27 have been report as a target of proteasome inhibitor. p27, a CDK inhibitor, prevents the cell cycle progression through the G1/S phase by regulating both cyclin D and E (Sherr and Roberts, 1999). It has been showed that proteasome inhibitor MG-132 promotes downregulation of ubiquitin ligase S-phase kinase protein 2 (Skp-2) targets p27 and

lead to accumulation of p27 resulting in cell cycle arrest (Hussain et al., 2009). In addition, Apoptosis caused by DNA damage and transforming oncogenes is crucially regulated by p53. Inhibiting proteasome with MG-132 in colon cancer causes p53 to accumulate and has been found to trigger p53 target genes such as p21, Fas ligand, PUMA and Bax (Ding et al., 2007). However, treatment with bortezomib in B-lymphoma and renal cell carcinoma showed to act independently of p53.

The unfolded protein response (UPR), which helps to prevent the buildup of unfolded proteins and restore ER function, is predominantly a pro-survival response. Usually, unfolded proteins are directed to the proteasome for degradation. Thus, if these proteins accumulation is persistent by inhibiting proteasome system, it would turn the signaling from pro-survival to pro-apoptotic. Numerous studies have shown that bortezomib causes pro-apoptotic ER stress and the accumulation of unfolded protein in a variety of cancers including multiple myeloma (Obeng et al., 2006), pancreatic (Nawrocki et al., 2005), head and neck cancer (Fribley et al., 2004), non-small cell lung carcinoma (Morgillo et al., 2011) and cholangiocarcinoma (Vaeteewoottacharn et al., 2013).

Taken all together, there are a number of pathways that alter during proteasome inhibition. Thus, the precise mechanism of action of each proteasome inhibitors is needed for future anti-cancer therapies.

2.6 Proteasome inhibitors

Traditionally, Inhibitors of transcription or translation have been utilized in the expression of a protein or other cellular adaptations to elucidate the underlying mechanisms. The accessibility of proteasome inhibitors now enables a comparable, quick study of the potential contributions of protein degradation in intact cells. Such inhibitors are very suggestive of proteasome-mediated degradation if they prevent a protein level decline or raise a protein level. Direct measurements of the protein's half-life changes and proof that the protein is functionalized following the testing with proteasome inhibitor can provide stronger evidence. Previously, various proteasome inhibitors have been identified that can easily penetrate cells and suppress this degradative pathway specifically. The peptide aldehydes, such as Cbz-leu-leu-leucinal

(MG132), Cbz-leu-leu-norvalinal (MG115) and acetyl-leu-leu-noeleucinal (ALLN) are the most commonly used. These agents predominantly block the proteasome's chymotrypsin-like activity as substrate analogues and powerful transition-state inhibitors. The peptide aldehyde inhibitor Cbz-ile-glu(O-i-Bu)-ala-leucunal (PSI) is another helpful inhibitor. The 26S proteasome's proteolytic activity is inhibited by this inhibitor, but its ATPase and isopeptidase activities are unaffected. A more recent class of proteasome inhibitors has selectivity provided by an inhibitory boronate group linked to peptide or peptidomimetic sequences. These drugs have a substantially higher potency, reversible binding to the proteasome's active sites, and they do not inhibit other cellular proteases. Recently, Andre S. Bachmann et al. (2016) revealed that the syrbactin structural analog TIR-199 inhibits proteasome activity and causes cancer cell death. The TIR-199 act as a proteasome inhibitor which inhibits catalytic subunit activities β1, β2 and β5 in multiple myeloma and neuroblastoma cells (Bachmann et al., 2016).

The natural compounds lactacystin and clasto-lactacystin -lactone, which vary structurally from peptide aldehydes, are far more effective proteasome inhibitors. Lactacystin was first isolated from actinomycetes. This agent demonstrated the capacity to prevent cell division and encourage neurite outgrowth from cultured neurons. Studies of its mechanism of action revealed that it inhibits proteasome activity as a pseudo substrate by forming a covalent bond with hydroxyl groups on the active site threonine of the catalytic β-subunits. The chymotrypsin and trypsin-like activities are thus deactivated. High proteasome specificity is demonstrated by lactocystin. Lactacystin is converted into its β-lactone derivative form in aqueous solution. Because it reaches some cells far more easily than lactacystin, this derivative is a highly helpful inhibitor. It has also been discovered that a number of natural compounds contain proteasome inhibitory properties. Tea polyphenols with ester bonds founded in the serum of green tea drinkers can inhibit proteasome function and induce G1-phase arrest in tumor cells. AM114, a boronic chalcone derivative, was also discovered to be a potent proteasome inhibitors in cancel cells, inducing G2/M arrest and inhibiting cellular proliferation (Modzelewska et al., 2006).

Among these compounds, Bortezomib (BTZ), a peptide boronate inhibitor, was the first Food and Drug Administration (FDA) approved drug to treat multiple myeloma (MM) and the mantle cell lymphoma (Richardson et al., 2003). BTZ is a inhibitor that mainly reversibly inhibits the CT-L activity of the proteasome. BTZ was further investigated in *in vitro* and *in vivo* in various tumor types and showed early indications of activity many tumor types such as non-small cell lung cancer (Morgillo et al., 2011), prostate cancer (Manna et al., 2013), multiple myeloma (Richardson et al., 2003), follicular non-Hodgkin's lymphoma (Bose et al., 2014) and cholangiocarcinoma (Vaeteewoottacharn et al., 2013). Dawei Wang et al. (2019) discovered that inhibiting the proteasome increases autophagic degradation of ubiquitinated-AGR2 and improves the antitumor efficacy of bevacizumab when combined with BTZ and MG132 (Wang et al., 2019). Despite its anti-tumor activity, BTZ has been shown in preclinical studies to be toxic to non-cancer cells due to its inhibitory activity on total protein degradation. Not only to have activity against multiple myeloma (MM) cells, but also to inhibit protective interaction with bone marrow stroma cell and to inhibit angiogenesis development (Xiong et al., 2015). As a result, a novel class of structurally related proteasome inhibitors has been emerged. In addition, there are potent five proteasome inhibitors in clinical improvement, representing three distinct structural classes peptide boronated, peptide epoxyketones and β-lactones as illustrated in Figure 2.5. These inhibitors interact to catalytic sites within the proteasome either reversibly or irreversibly as summarized in Table 2.1.

Carfizomib (CFZ), another proteasome inhibitor that differs from BTZ regarding its structure and proteasome inhibition mechanisms, has been used to treat patients with multiple myeloma. CFZ binds to the β5 subunit of the 20S constitutively expressed proteasome. This binding occur via an irreversible covalent bond, which results in inhibition of CT-L activity, subsequent protein substrate accumulation within the cell, and triggering of apoptosis cell death. CFZ inhibits T-L and peptidyl-glutamyl peptide hydrolyzing-like (PGPH-L) activities at higher concentrations by inhibiting all three catalytic β-subunits (Jayaweera et al., 2021).

CFZ was approved by the US Food and Drug Administration (FDA) in July 2012 under the increased acceptance mechanism for the treatment of patients with

multiple myeloma who had received at least two prior therapies, including BTZ and an IMID, and who had tumor progression on or within 2 months of the last treatment. CFZ was shown in preclinical studies to be active against BTZ-resistant multiple myeloma cell lines. However, both BTZ and CFZ have been discovered to be ineffective when given orally, limiting their clinical application (Fostier et al., 2012).

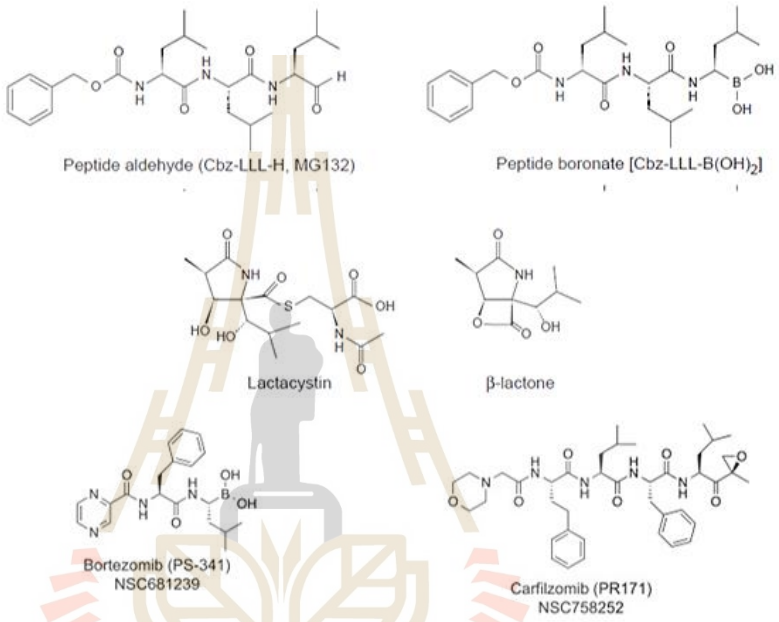

Figure 2.5 Chemical structures of proteasome inhibitors in clinical trial (Crawford et al., 2011).

Table 2.1 Properties of proteasome inhibitor in clinical trial (Merin and Kelly, 2014).

Proteasome inhibitor	Type	Target	Binding	Route of administration	Clinical development
Bortezomib	peptide boronic acid	CT-L	reversible	IV	Approved for MM and MCL
Carfilzomib	peptide epoxyketone	CT-L	irreversible	IV	Phase III in MM Phase I in AML, ALL, CLL and solid tumours
NPI-0052	β-lactone	CT-L, T-L, C-L	irreversible	Orally bioavailable	Phase I in MM, solid tumours and lymphoma
MLN9708	peptide boronic acid	CT-L	reversible	IV and orally bioavailable	Phase I/II in MM Phase I in lymphoma and solid tumours
CEP-18770	peptide boronic acid	CT-L	reversible	IV and orally bioavailable	Phase I/II in MM
ONX0912	peptide epoxyketone	CT-L	irreversible	Orally bioavailable	Phase I in solid tumours

CT-L chymotrypsin-like; *T-L* trypsin-like; *C-L* caspase-like; *IV* intravenous; *MM* multiple myeloma; *MCL* mantle cell lymphoma; *AML* acute myeloid leukaemia; *ALL* acute lymphocytic leukaemia; *CLL* chronic lymphocytic leukaemia

2.7 The involvement of ER stress and the UPR upon proteasome inhibition

Endoplasmic reticulum (ER) is an important organelle that participates in protein synthesis, post-translational modification, protein folding and secreting, and homeostasis. The inability of protein folding, or accumulation of misfolded proteins leads to ER stress condition. The ER has control mechanisms to achieve this condition, including the unfolded protein response (UPR) and ER-associated degradation (ERAD), which coordinately interacts with the ubiquitin-proteasome system (UPS) (Mao et al., 2019).

There are three major UPR pathways in mammalian cells, all of which are activated by protein sensors domain located in the ER membrane, including activating transcription factor-6 (ATF6) domain, inositol-requiring 1α (IREα) domain, and protein kinase RNA-like ER kinase (PERK) domain. Misfolded protein overabundance in the ER causes BiP dissociation from sensors (Figure 2.6). Proteases cleave ATF6 as it travels to the Golgi. The cleaved fragment (ATF6n) translocates to the nucleus and triggers transcription of ER chaperone genes, which encourage protein folding, maturation, secretion, ERAD component, and other functions. Second, IREα cleaves X-box binding protein-1 (XBP1) mRNA and change to strong transcriptional activator. Third, PERK activates the eukaryotic translation initiation factor-2α (eIF2α) phosphorylation (Alshareef et al., 2021). The three UPR pathways are activated simultaneously, but others can be selectively activated or suppressed. In general, ubiquitinated proteins are degraded by the proteasome, but in the presence of large amounts of misfolded proteins, aggregated proteins, or an impaired proteasome, the UPS function is reduced and the UPR is induced. In contrast, specific inhibition of UPS may potentially be harmful to cells as (Cybulsky, 2013).

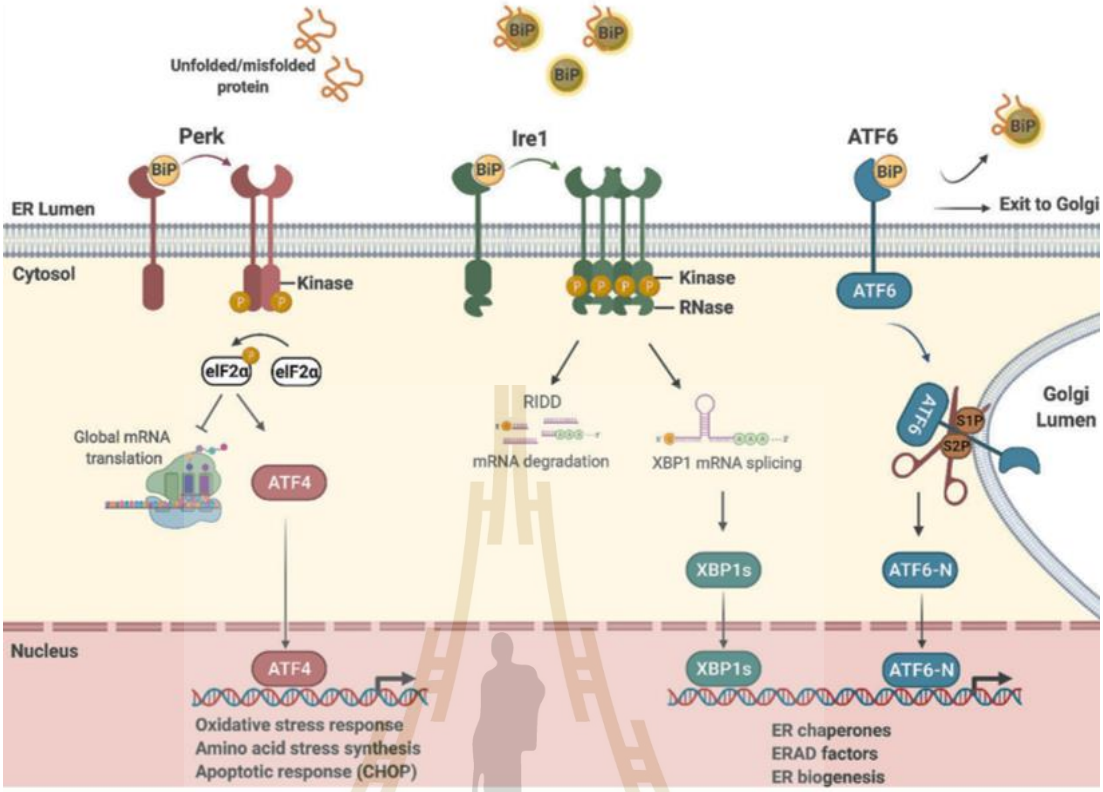

Figure 2.6 Endoplasmic reticulum (ER) stress pathways and responses (Alshareef et al., 2021).

2.8 Piperlongumine

Piperlongumine (PL) is a bioloactive alkaloid isolated from the long pepper, *Piper longum*, plant (Figure 2.7) (Chatterjee and Dutta, 1967). PL has been reported to have anti-bacterial, anti-angiogenic, and anti-diabetic properties (Bezerra et al., 2013). Current studies shown that PL can selectively kill various transformed cells including head and neck cancer (Roh et al., 2014), hepatocellular carcinoma cells (Chen et al., 2015), breast cancer (Jin et al., 2017), gastric cancer (Duan et al., 2016) and lung cancers (Zheng et al., 2016). PL has anti-tumor properties by enhancing high levels of reactive oxygen species (ROS), that results in *in vitro* and *in vivo* cell death via a variety of mechanisms, including ROS-p38/JNK pathways, which induce apoptotic and autophagic death of primary myeloid leukemia cells (Xiong et al., 2015), ROS-ER-MAPKs-CHOP pathways in hepatocellular carcinoma cells (Chen et al., 2015), nuclear factor kappa B

(NF-kB) signaling pathways in lung cancer (Zheng et al., 2016). Taken together, it was proposed that PL is a ROS inducer with diverse anti-cancer characteristics. However, PL has been addressed on complex mechanisms of action via inhibiting ubiquitin proteasome system. Jarvius et al. (2013) have demonstrated that PL causes the accumulation of high molecular weights of poly-ubiquitinated proteins which is also seen in the cells treated with a USP14/UCHL5 deubiquitinase inhibitor (b-AP15). However, the change of proteasome activity did not observe in PL-treated cells. Based on this current study, they suggested that anti-cancer activity of PL involves in the inhibition of ubiquitin-proteasome system at pre-proteasome step, prior to de-ubiquitination of misfolded protein substrates at the proteasome (Jarvius et al., 2013). Whereas HALASI et al.(2013) showed that PL has proteasome inhibitory activity through suppressing FOXM1 transcriptional activity and stabilizing the protein levels of Mcl-1 and p21 expression as efficiently as well know proteasome inhibitor including thiostrepton, bortezomib and MG-132 (Halasi et al., 2013). Gather all evidence above indicates that ROS induction by PL is a secondary effect of PL as proteasome inhibitor.

In CCA, our previous study has reported that PL significantly inhibited CCA cell line growth by initiating G2/M phase arrest, induced ROS accumulation, and caused CCA cell apoptosis via the JNK/ERK pathway. In addition, the stabilization of particular regulating proteins in cell cycle and apoptosis such as p21 and cleavage PARP were observed in PL-induced CCA cell death as strongly as MG-132 (Thongsom et al., 2017). Based on our previous study suggest that not only ROS induction but also proteasome inhibition is the effect of PL-induce CCA cell apoptosis. However, the underlying mechanism of PL-inhibited ubiquitin-proteasome system in CCA have never been reported. In present study, we aim to explore the role of PL on ubiquitin proteasome system in CCA.

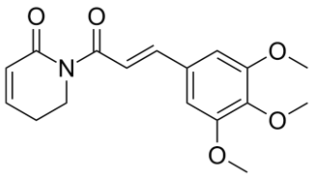

Figure 2.7 The structure of Piperlongumine (Chatterjee and Dutta, 1967).

CHAPTER III

MATERIALS AND METHODS

3.1 Materials

3.1.1 Cholangiocarcinoma (CCA) Cell lines

Human CCA cell lines (KKU-055 and KKU-213A) were established and validated (Sripa et al., 2020). The Japanese Collection of Research Bioresources Cell Bank provided certificates of analysis. All CCA cell lines were cultured in DMEM (cat. no. 12100-046; Gibco; Thermo Fisher Scientific, Inc.; USA) supplemented with 1% penicillin-streptomycin (cat. no. 15140-122; Gibco; Thermo Fisher Scientific, Inc.; USA) and 10% FBS (cat. no. 10270-098; Gibco; Thermo Fisher Scientific, Inc.; USA) and kept at 37°C in a humidified incubator with 5% CO_2. PCR detection of mycoplasma contamination was performed according (Uphoff et al., 2012)

3.1.2 Chemical and Reagents

Piperlongumine (cat. no. 528124), Bortezomib (cat no. 5.04314), MG132 (cat. no. 474791), and sulforhodamine B (SRB; cat. no. S1402) were purchased from Sigma-Aldrich (Merck, KGaA). All of chemicals were dissolved in dimethyl sulfoxide (DMSO). pZsProsensor1 was kindly provided from Professor Seiji Okada (Kumamoto University, Japan). TRIzol reagent (cat. no. 15596026) was supplied from Thermo Fisher Scien-tific, Inc. SensiFAST cDNA Synthesis Kit (cat. no. BIO-65053) was purchased from Bioline; Meridian. LightCycler® 480 SYBR green I master mix in SYBR-Green (cat. no. 04707516001) was purchased from Roche Molecular Systems, Inc. Immobilon Forte Western HRP substrate (cat. no. WBLUF0100) was obtained from Merck Millipore. Primary antibodies against mono and poly-ubiquitinated proteins (cat. no. ENZ-ABS840-0100) were purchased from EnZo Life Sciences, Inc. and α-tubulin (cat. no. sc-5286) were purchased from Santa Cruz Biotechnology, Inc. The primer sequences used were presented in Table 3.1.

Table 3.1 Primer sequences for quantitative RT-PCR.

Name	Forward primer 5'-3'	Reverse primer 5'-3'
XBP1	5'-TTA CGA GAG AAA ACT CAT GGC C-3'	5'-GGG TCC AAG TTG TCC AGA ATG C-3'
XBP1s	5'-TGC TGA GTC CGC AGC AGG T-3'	5'-GCT GGA GGC TCT GGG GAA-3'
ATF4	5'-CAT TCC TCG ATT CCA GCA AAG CAC-3'	5'-TTC TCC AAC ATC CAA TCT GTC CCG-3'
ATF6	5'-GAA CCA TTG CTT TAC ATT CCT CCA C-3'	5'-CTG CTT GAC TTG GTC CTT TCT ACT TC-3'
CHOP	5'-TGA ACG GCT CAA GCA GGA AAT CG-3'	5'-GGA TTG AGG GTC ACA TCA TTG GCA CT-3'
C-Myc	5'-CTG CTG TGG ACC CTA CTG-3'	5'-AAC TGC GTC TCT GCC AGG AC-3'
BIRC5	5'-TGA GGA GAC ACC GCC CAC-3'	5'-CAA CAT CGA TTT CTT CCT CAT CTT C-3'
CCND1	5'-CCA CTT GAG CTT GTT CAC CA-3'	5'-AAC TAC CTG GAC CGC TTC CT-3'
CCNE1	5'-GAA ATG GCC AAA ATC GAC AG -3'	5'-TCT TTG TCA GGT GTG GGG A -3'
CCNB1	5'-GAC AAC TTG AGG AAG AGC AAG C -3'	5'-ATG GTC TCC TGC AAC AAC CT -3'
ACTB	5'- GAT CAG CAA GCA GGA GTA TGA CG -3'	5'- AAG GGT GTA ACG CAA CTA AGT CAT AG -3'

XBP1, X-box binding protein 1; XBP1s, spliced X-box binding protein 1; ATF4, Activating transcription factor 4; ATF6, Activating transcription factor 6, CHOP C/EBP homologous protein; c-MYC, cellular myelocytomatosis; BIRC5, baculoviral inhibitor of apoptosis repeat-containing 5; CCND1, Cyclin D; CCNE1, Cyclin E1; CCNB1, Cyclin B1; ACTB, B-actin.

3.2 Methods

3.2.1 Determination of the anti-tumor effect of PL or BTZ on CCA cell lines

3.2.1.1 Assessment of cell proliferation

KKU-055 (5 x 10^3 cells/well) and KKU-213A (7 x 10^3 cells/well) were seeded into a 96-well plate. After seeding 24 h, the cells were treated with PL (0, 1.25, 2.5, 5. 10, 20 and 40 μM) or BTZ (0, 10, 20, 40, 80, and 160 nM) or combination treatment of PL and BTZ for 24 h. Cell viability was measured using sulforhodamine B (SRB) assay. In brief, the media was disposed and then the cells were mounted with ice-cold 10% (v/v) TCA in deionized (DI) water at 4 °C overnight. The TCA (cat. no. T0699; Merck; Sigma-Aldrich, Inc.) was removed and washed the cells five times with DI water, follow by staining with 0.4% (w/v) SRB (cat. no. 3520-42-1; Merck; Sigma-Aldrich, Inc.) dissolved in 1% (v/v) acetic acid for 30 min at room temperature. Five washes with 1% (v/v) acetic acid were used to remove unbound dye before solubilizing the cells with 10 mM unbuffered Tris base (pH 10.0). Optical density was measured at

564 nm. The half-maximum inhibitory concentration (IC_{50}) value was determined by GraphPad Prism 8 (GraphPad Software, Inc.). Then, combination index (CI) value by CompuSyn (CompuSyn Inc. software) was used to demonstrate the interactions between PL and BTZ treatment.

3.2.1.2 Colony formation assay

KKU-055 (1 x 10^3 cells/well) and KKU-213 (2 x 10^3 cells/well) were seeded into a 6-well plate. After seeding 24 h, the cells were treated with PL or BTZ or combination treatment of PL and BTZ. After 6 h, the media was disposed, and the treated cells were further cultured with DMEM supplement with 10% FBS for 14 days. SRB assay was used to visualize the colony form which was defined as consisting of at least 50 cells and the absorbance at 564 nm was used as a representative cell density. The colonies were examined under an inverted microscope with original magnification at 100X.

3.2.1.3 Protein collection and Western blot analysis

KKU-055 and KKU-213A were treated with PL or BTZ or co-treatment of PL and BTZ for 3, 6, 18, 24 h. Treated cells were lysed in lysis buffer 150 mM NaCl; 50 mM Tris-HCl pH 7.4; 1% Sodium deoxycholate; 0.1% SDS; and 1X Protease inhibitor cocktail tablets (cat. no. 05982970001, Roche Diagnostics) in order to get whole cell lysates. The Pierce BCA Protein Assay Kit (cat. no. 2322, Thermo Fischer Scientific). was used to determine the protein concentration in all samples. A similar number of proteins (30 μg/lane) were separated using 8% SDS-PAGE analysis. The separated proteins were then transferred onto a nitrocellulose membrane (Cytiva). The non-specific binding was inactivated by 5% skimmed milk at room temperature. Specific antibodies to ubiquitinylated protein (1:1000; cat. no. ENZ-ABS840-0100) and to α-tubulin (1:5000; cat. no. sc-5286, Santa Cruz Biotechnologies) were then added and incubate at 4°C, overnight. The membrane was washed with 1XPBST and then were incubated with HRP-conjugated secondary antibodies (1:2000; cat. no. NXA931 and NA934; Cytiva). The target protein signals were visualized using immunodetection utilizing chemiluminescent HRP substrate (Cytiva).

3.2.1.4 Proteasome activity assay by proteasome sensor plasmid

KKU-055 and KKU-213A were seeded at 1 x10^5 into a 4-well chamber slide. After seeding for overnight, the cells were transiently transfected with pZsProsenso-1 plasmid using TurboFect transfection reagent (ThermoFisher Scientific) in OptiMEM according to the manufacturer's protocol. After transfection 24 h, transfected cells were treated with PL or BTZ or combination treatment of PL and BTZ and incubated for 12, 18 and 24 h. The green fluorescent signals will be increased when the proteasome function is diminished and were visualized using a Nikon fluorescent microscope (Nikon Corporation) with original magnification at 100X.

3.2.2 Determination of ER stress and unfolded protein response upon PL and BTZ combination treatment

3.2.2.1 RNA extraction and quantitative PCR (qPCR)

KKU-055 and KKU-213A were treated with PL or BTZ or combination treatment of PL and BTZ for 3 and 6 h. Total RNA was extracted using the TRIzol reagent (cat. no. 15596026; Thermo Fisher Scientific, Inc.) according to the manufacturer's recommendations. cDNA was synthesized using a SensiDAST cDNA Synthesis Kit (cat. no. BIO-65053; Bioline; Meridian Bioscience). Quantitative PCR was performed to investigate the expression level using LightCycler® 480 SYBR Green I Master (Roche). The sequences of all primer are listed in Table 3.1. Gene amplification was performed by initial denaturation at 95˚C for 5 min; followed by 45 cycles of denaturation at 95˚C for 10 sec, annealing at 55˚C, or 58˚C or 60˚C for 10 sec and extension at 72˚C for 10 sec. The annealing temperature was 55˚C for *CCNB1, CCNE1, ATF4, ATF6, XBP1* and *CHOP, 58 ˚C* for *BIRC5* and *c-MYC* and 60˚C for, *CCND1* and *XBP1*. The gene expression level was normalized against the housekeeping gene β-*actin*. Relative gene expression was calculated by $2^{(-\Delta\Delta_{Cq})}$ (Livak and Schmittgen, 2001).

3.2.3 Statistical analysis

Statistical analyses in this study were performed using GraphPad Prism 8.0 (GraphPad Software, Inc.). Three independent experiments were used to calculate the results (shown as the mean SD). To compare multiple datasets, one-way and two-way ANOVAs were used, followed by Bonferroni's clarification. A statistically significant difference was indicated by a P value of 0.05.

CHAPTER IV

RESULTS

4.1 The combination effect of PL and BTZ on CCA cell growth

Both PL and BTZ have a cytotoxic effect in multiple type of cancers including CCA (Thongsom et al., 2017; Vaeteewoottacharn et al., 2013). To investigate the combination effect of PL and BTZ on CCA cell proliferation. KKU-055 and KKU-213A cells were treated with PL (0, 2.5, 5, 10, 20, and 40 μM) and/or BTZ (0, 5, 10, 20, 40, 80, and 160 nM) for 24 h. Cell viability by SRB assay demonstrated that both drugs inhibited CCA cell growth in all cell lines in a dose-dependent manner (Figure 4.1). KKU-213A cells were less sensitive to both PL and BTZ when compared with KKU-055 cells. The IC_{50} values of PL using CompuSyn software was 4.3, and 7.9 μM for KKU-055 and KKU-213A cells whereas IC_{50} values of BTZ was 17 and 281 nM for KKU-055 and KKU213A cells respectively. Additionally, the combined treatment with PL and BTZ showed significantly suppress CCA cell proliferation. CI index was calculated to determine the synergistic interaction between PL and BTZ. The CI values were less than 1 for both CCA cell lines upon combined treatment with PL and BTZ (Figure 4.1 and Table 4.1). Subsequently, we further investigated the PL enhanced anti-cancer effect of BTZ in CCA. KKU-055 was treated with PL 5 μM and BTZ 20 nM while KKU-213A was treated with PL 10 μM and BTZ 40 nM since these combinations were suppress CCA cell growth to 50% compared to control. The colony formation assay confirmed that the combined treatment with PL and BTZ in both KKU-055 and KKU-213A cells showed higher inhibitory effect when compared with single agent (PL or BTZ) as shown in Figure 4.2. Furthermore, the combination effect of PL and BTZ on CCA growth was demonstrated by down-regulation of cell cycle markers (*CCND1*, and *CCNB1*, *c-Myc* and *BIRC5*) (Figure 4.3). These findings suggest that the synergistic interaction between PL and BTZ has a potential used in CCA treatment.

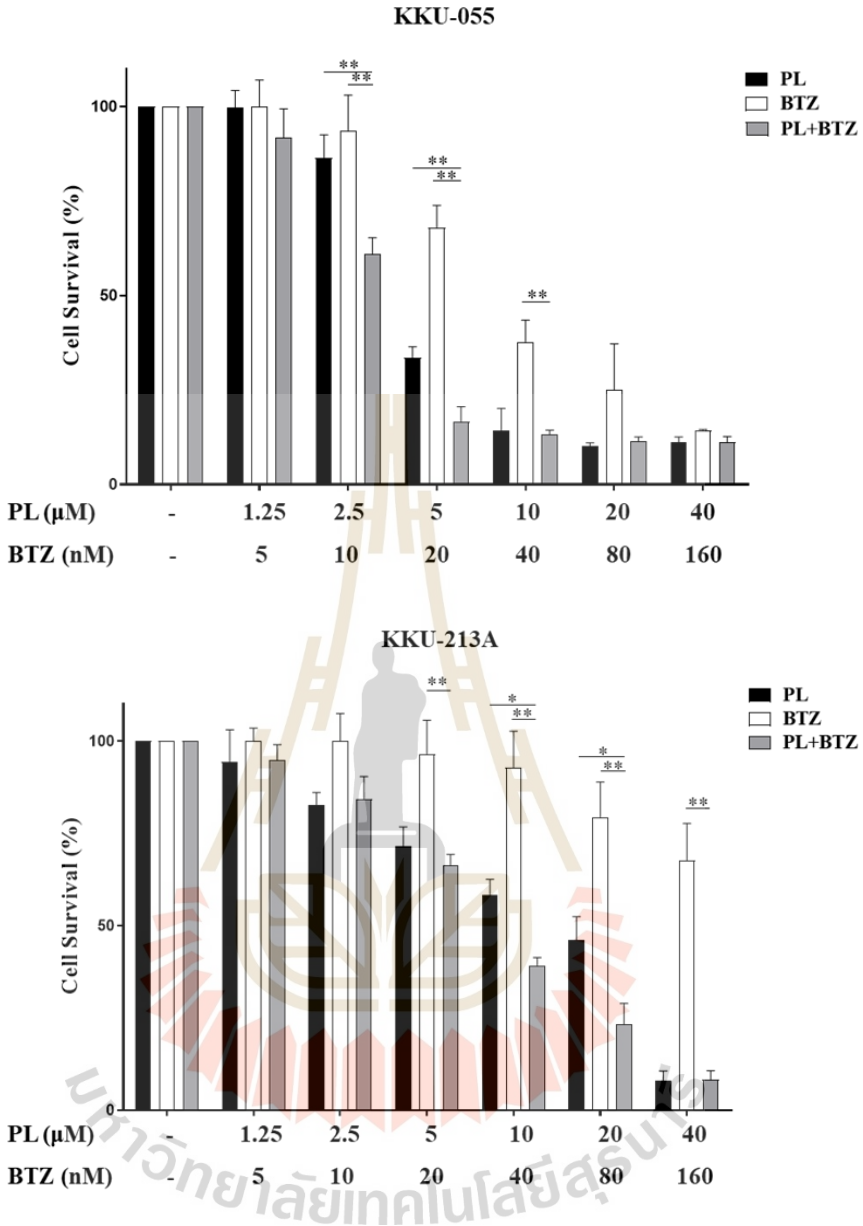

Figure 4.1 PL increases anti-proliferative effect of BTZ in CCA. KKU-055, and KKU-213A were treated with various concentrations of PL (0, 1.25, 2.5, 5, 10, 20, and 40 μM) and/or BTZ (0, 5, 10, 20, 40, 80, and 160 nM) for 24 h. Cell viability was measured by SRB assay. Values are expressed as means ± standard deviation (SD) of three independent experiments. . * P<0.05, ** P < 0.01 VS single treatment (PL or BTZ).

Table 4.1 Combination index (CI) of PL and BTZ in CCA cell lines.

Combination			KKU-055		KKU-213A	
PL (μM)	BTZ (nM)	Total dose	Fa*	CI value**	Fa*	CI value**
1.25	5	1.255	0.9051	0.9419	0.9325	1.1731
2.5	10	2.51	0.6150	0.6107	0.8262	0.9076
5	20	5.02	0.1659	0.3284	0.6783	0.8803
10	40	10.04	0.1222	0.5245	0.3772	0.5799
20	80	20.08	0.1188	1.0280	0.2738	0.7605
40	160	40.16	0.1283	2.1737	0.1001	0.5128

*, Fa : The fraction of cell death

**, the combination index (CI) of PL and BTZ in KKU-055 and KKU-213A. (CI < 1 means synergistic effect; CI > 1 means antagonistic effect; CI = 1 means additive effect.)

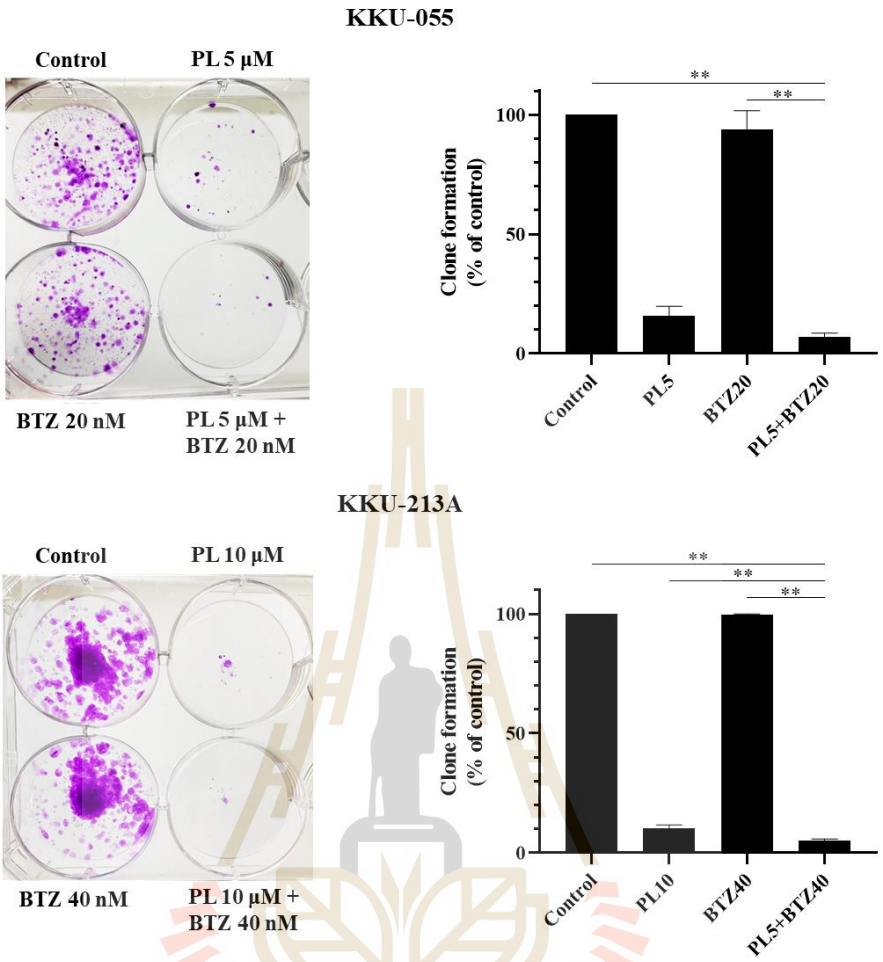

Figure 4.2 PL in combination with BTZ decrease colony formation ability in KKU-055 and KKU-213A. Left: The colony formation ability of KKU-055 after exposed with PL (5 µM) and/or BTZ (20 nM) for 6 h and KKU-213A after exposed with PL (10 µM) and/or BTZ (40 nM) for 6 h. Right: Intensity of colony formation of KKU-055 and KKU-213A were determined by SRB assay and analyzed using ImageJ software. Data were shown as the means ± SD from at least three independent experiments. * P<0.05, ** P < 0.01 VS single treatment (PL or BTZ) and control.

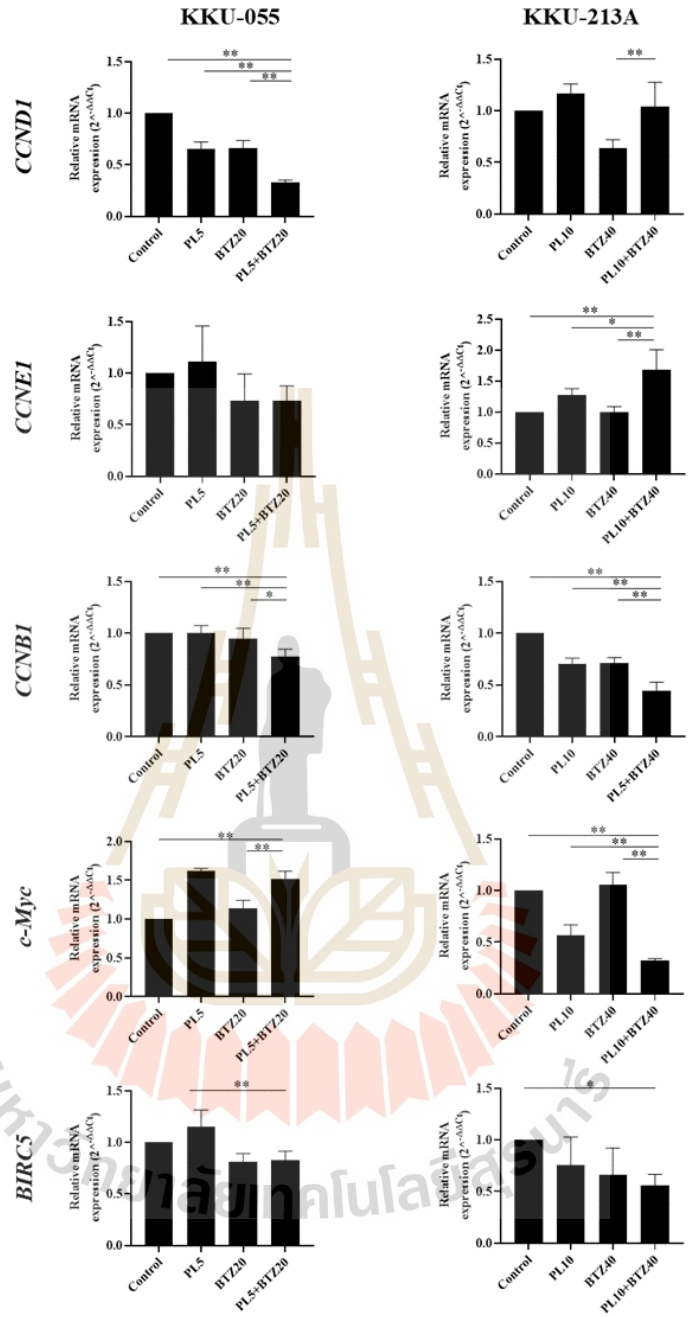

Figure 4.3 Combination effect of PL and BTZ on the expression of cell cycle-related genes. KKU-055 (left) and KKU-213A (right) were exposed to PL and/or BTZ for 6 h. mRNA expression levels of *CCND1, CCNE1, CCNB1, c-Myc* and *BIRC5* were measured by qRT-PCR relative mRNA level expression levels were determined and normalized with β-*actin*. Data were shown as the means ± SD from at least three independent experiments. * P<0.05, ** P < 0.01 VS single agent (PL or BTZ) or control.

4.2 PL in combination of BTZ increases the accumulation of poly-ubiquitinated proteins in CCA

The major mechanism of anti-cancer activity of PL has been addressed through the induction of reactive oxygen species (ROS) production in multiple types of cancers including CCA (Thongsom et al., 2017; Talabnin et al., 2020). However, little is known about anti-cancer effect of PL via the inhibition of ubiquitin-proteasome system (Halasi et al., 2013; Jarvius et al., 2013). In this study, we firstly determined whether PL-induced CCA cell death via inhibiting ubiquitin-proteasome system. KKU-055 and KKU-213A were treated with various concentrations of PL. Western blot analysis showed a dosage-dependent increase in poly-ubiquitinated proteins in both KKU-055 (5, 10, 20 μM) and KKU-213A (10, 20, 40 μM) after PL treatment for 3 h. Additionally, the effect of PL on the accumulation of poly-ubiquitinated protein in both CCA cell lines was clearly seen at 3 and 6 h of PL treatment and it gradually declined at 18 and 24 h (Figure 4.4 a.). Next, we further investigated the effect of PL on proteasome activity. KKU-055 and KKU-213A were transiently transfected with proteasome sensor, pZsProsensor-1, and then were treated with various concentration of PL and/or and BTZ for 24 h. The accumulation of ZsGreen signals in the cells indicates the decrease of proteasome activity. The resulted showed that there was no obvious green fluorescent signal in PL-treated KKU-055 and PL-treated KKU-213A when compared to that of BTZ-treated CCA cells (Figure 4.4 b.). These finding suggested that PL has no effect on proteasome activity but the accumulation of poly-ubiquitinated proteins could be another mechanism of action of PL-induced CCA cell death. Subsequently, to access the synergistic effect of combined treatment between PL and BTZ on ubiquitin-proteasome pathway, the Western blot analysis showed that the accumulation of poly-ubiquitinated proteins was increased upon combined treatment with PL and BTZ in both KKU-055 and KKU-213A at 3 and 6 h (Figure 4.5). However, there was no significant difference of green fluorescent signal between combined treatment with PL and BTZ and BTZ-treated cells (Figure 4.6). Altogether, the results suggested that PL enhances the proteasome inhibitory activity of BTZ in CCA through the induction of poly-ubiquitinated protein accumulation.

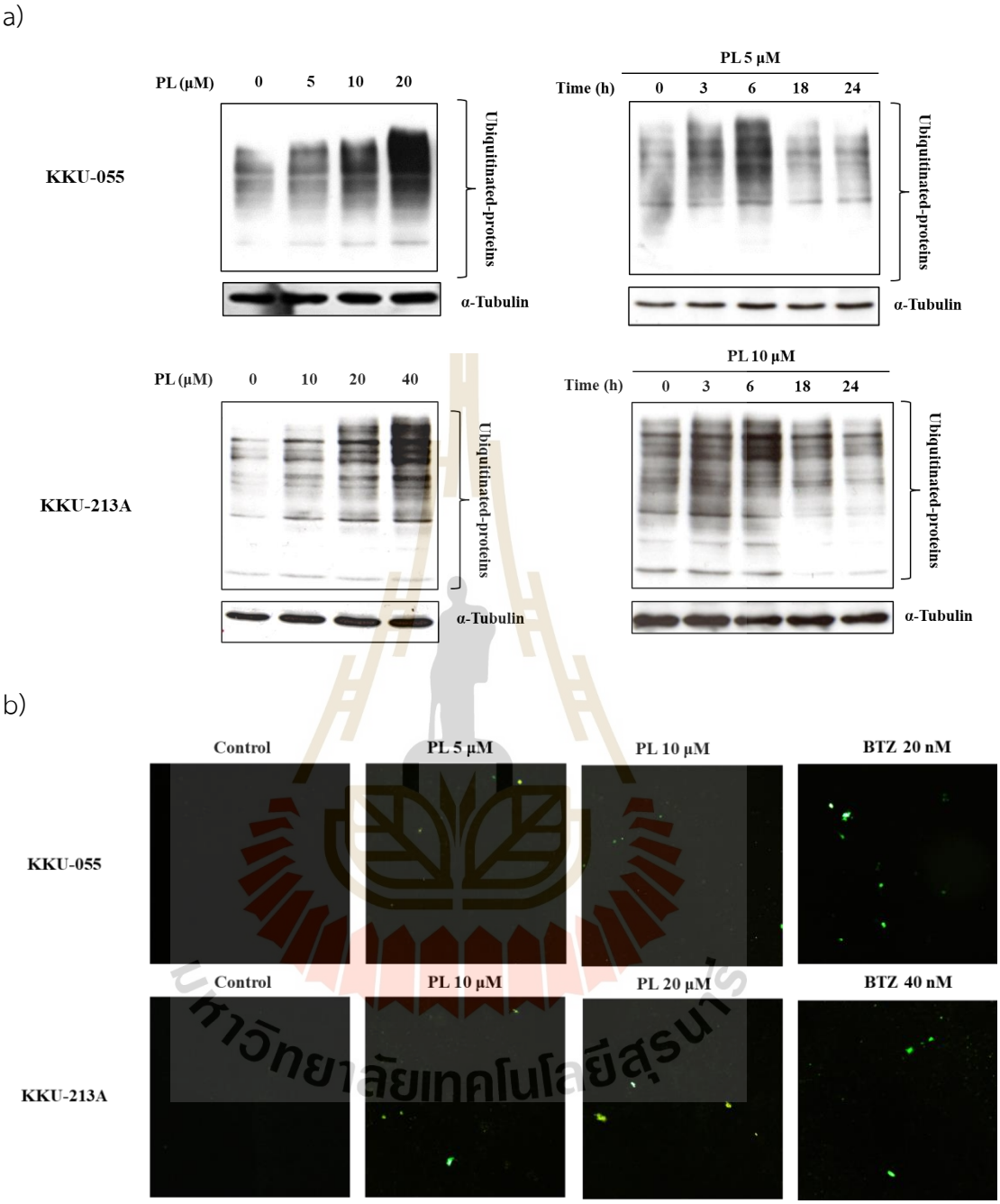

Figure 4.4 PL induces poly-ubiquitinated protein accumulation. KKU-055 and KKU-213A were treated with various concentrations of PL (0, 5, 10, 20, 40 μM) and at various time points (3, 6, 18, 24 h). The poly-ubiquitinated protein accumulation were detected by Western blot analysis. KKU-055 and KKU-213A were transiently transfected with pZsProSensor-1. Cells were treated with PL or BTZ for 24 h. A fluorescent microscope was used to observe fluorescent signals (magnification 100X).

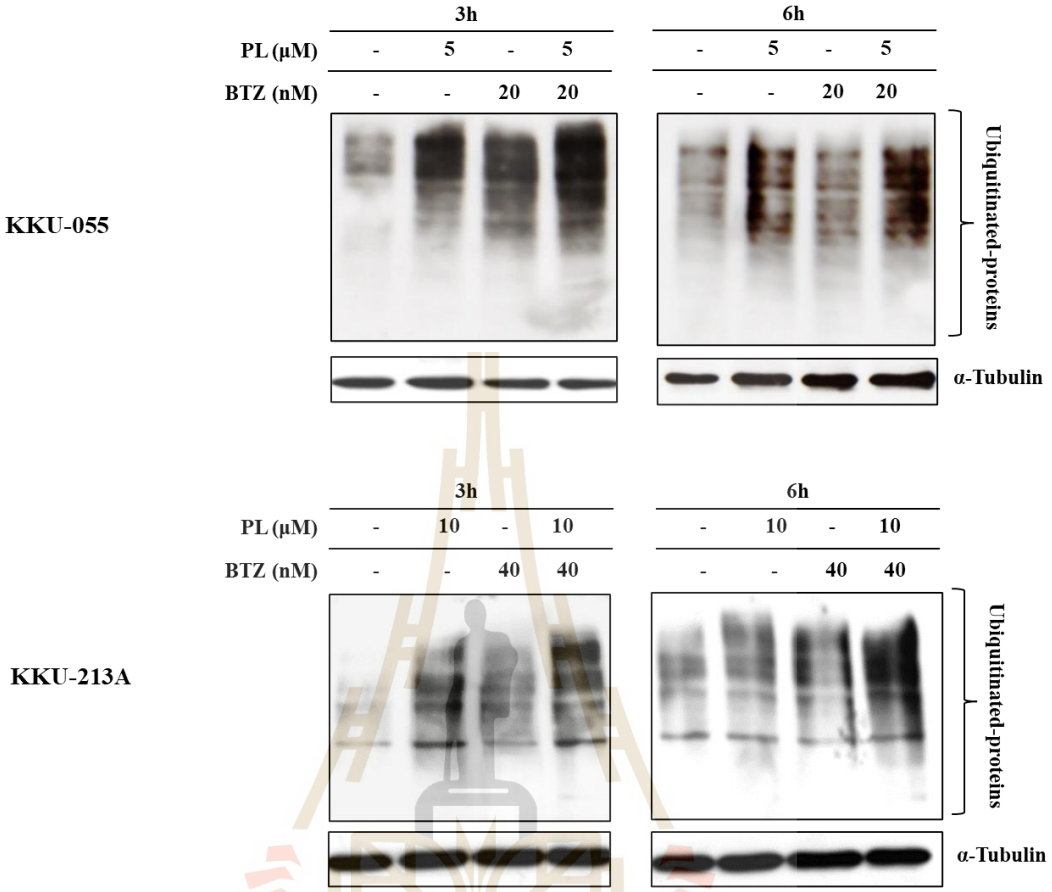

Figure 4.5 Combined treatment with PL and BTZ increase poly-ubiquitinated protein accumulation. KKU-055 and KKU-213A were treated with PL and/or BTZ for 3 and 6 h. The poly-ubiquitinated protein accumulation were detected by Western blot analysis.

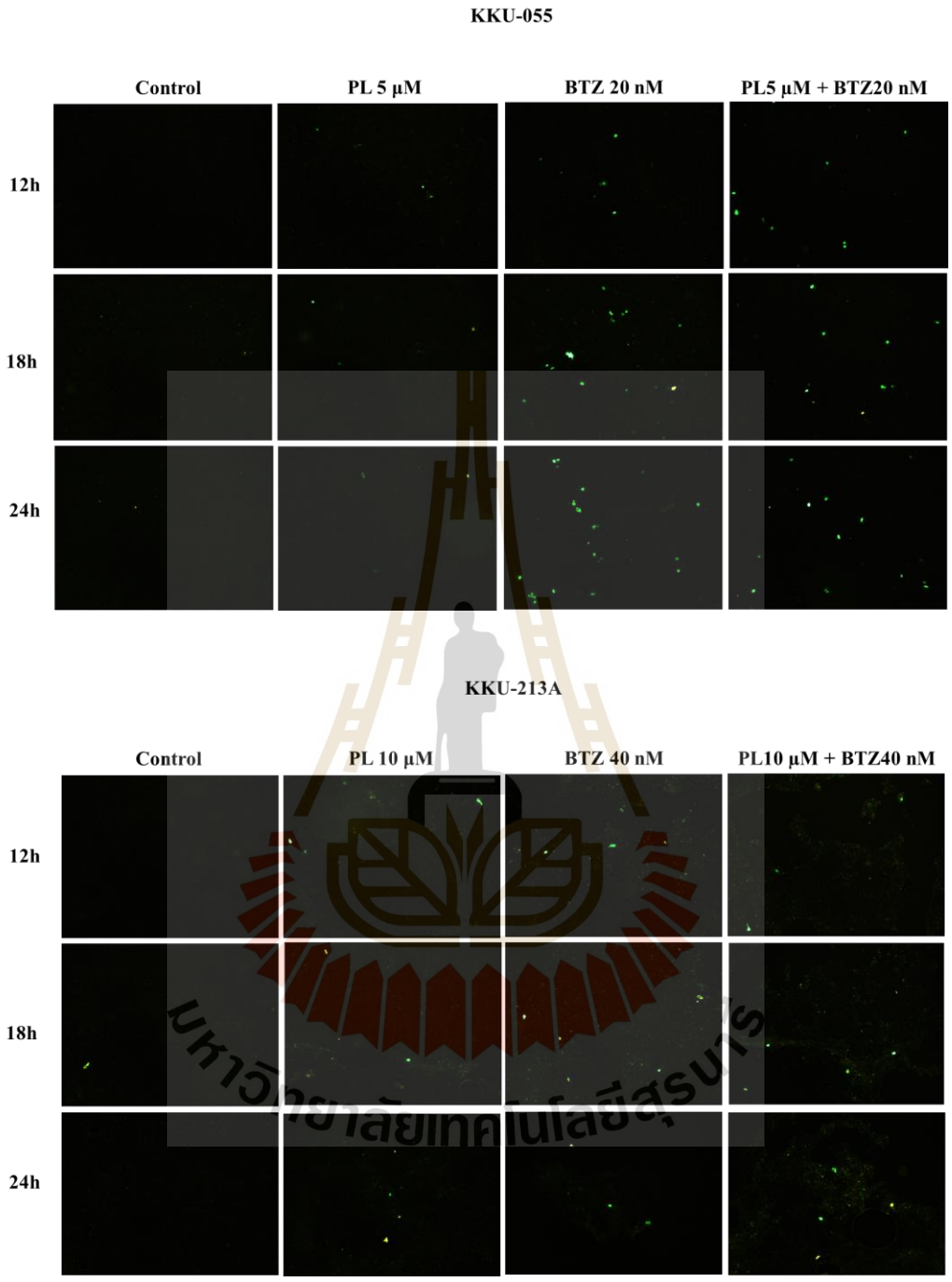

Figure 4.6 Effect of combined treatment with PL and BTZ on proteasome activity. KKU-055 and KKU-213A were transiently transfected with pZsProSensor-1. Cells were treated with PL or/and BTZ for 12, 18 and 24 h. A fluorescent microscope was used to observe fluorescent signals (magnification 100X).

4.3 PL in combination of BTZ induce endoplasmic reticulum (ER) stress response

The activation of ER stress-induced cell death has been proposed as a mechanism of BTZ-induced CCA cell death (Vaeteewoottacharn et al., 2013). To determine whether combined treatments with PL and BTZ enhance ER stress-induced CCA cell death. KKU-055 and KKU-213A were treated with PL and/or BTZ for 3 and 6 h, then the expression of ER-stress response genes including activating transcription factor 4 (*ATF4*), activating transcription factor 6 (*ATF6*), x-box binding protein 1 (*XBP1*), spliced XBP1 (*XBP1s*) and C/EBP homologous protein (*CHOP*) was determined. Gene expression experiment demonstrated that the expression of ER stress marker, XBP1s, was up-regulated in all treatments including PL and/or BTZ treatments. Interestingly, the up-regulation of *XBP1s* and *ATF4* was obviously seen in combined treatment with PL and BTZ at 6 h when compared with that of single agent (PL or BTZ) in both KKU-055 and KKU-213A cells (Figure 4.6). To examine combined treatments with PL and BTZ induce ER stress and proapoptotic unfolded protein response (UPR). The expression of proapoptotic UPR markers, including *ATF4* and *CHOP* was determined. The results showed that up-regulation of *ATF4* and *CHOP* expression was clearly seen at 6 h in combined treatment with PL and BTZ of both CCA cell lines (Figure 4.6). Taken together, these results demonstrate that PL enhances proteasome inhibitory activity of BTZ via ER stress-induced CCA cell death.

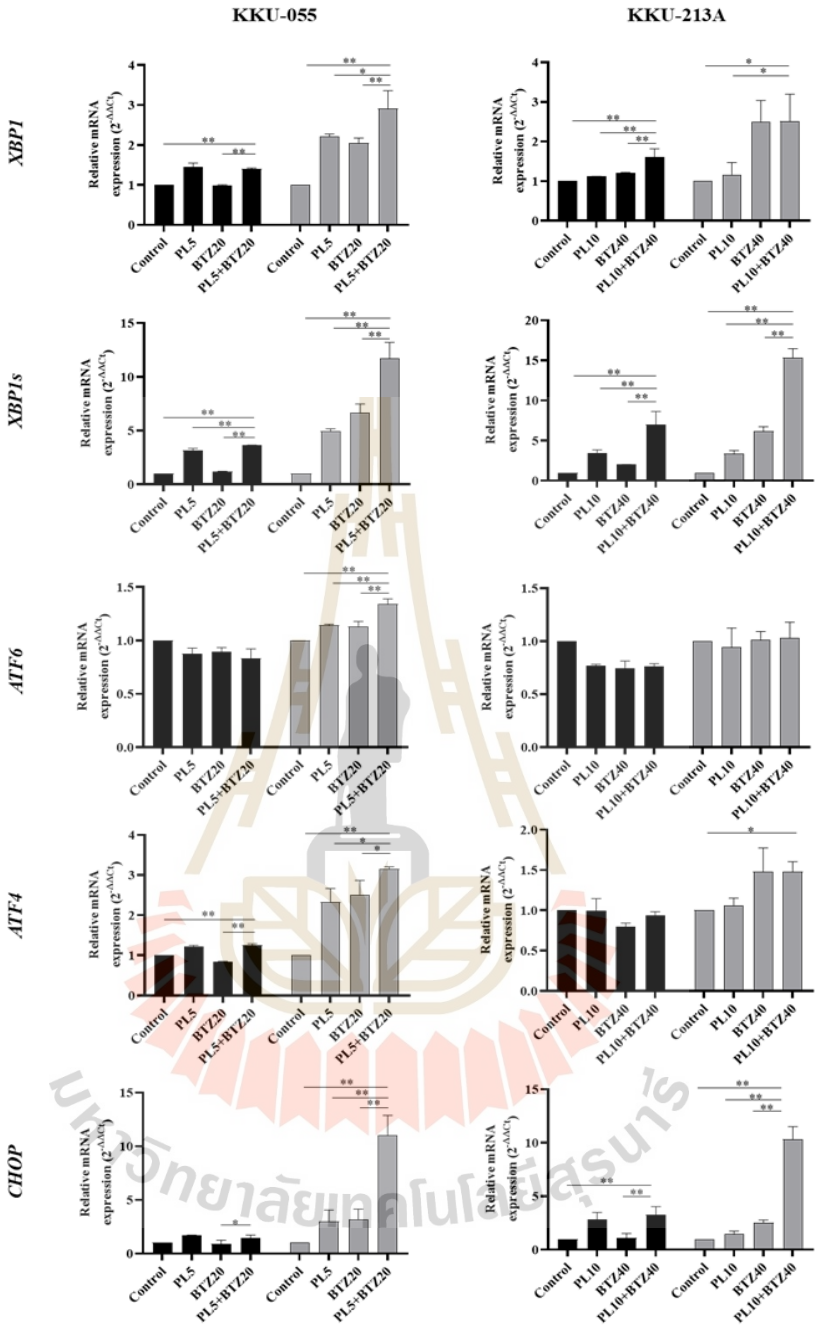

Figure 4.7 Combined treatment with PL and BTZ enhances ER stress and proapoptotic UPR. KKU-055 and KKU-213A were exposed to PL or/and BTZ for 3 h (black bar) and 6 h (grey bar). mRNA expression levels of *XBP1, XBP1s, ATF6, ATF4* and *CHOP* were measured by qRT-PCR. Relative mRNA level expression levels were calculated by normalized with β-*actin*. Data were shown as the means ± SD from three independent experiments. * P<0.05, ** P < 0.01 VS single agent (PL or BTZ) or control.

CHAPTER V
DISCUSSION AND CONCLUSION

To maintain the proliferative capacity and survival, cancer cells must need high capacity of ubiquitin-proteasome system (UPS) for regulating the turnover of several key proteins including tumor suppressive proteins and oncoproteins that involve in cell-cycle progression, apoptosis and survival (Crawford et al., 2011; Shen et al., 2013). Thus, targeting UPS might be valid and feasible in cancer treatment. Currently, many natural products show anti-cancer activity through the inhibition of UPS (Selvaraju et al., 2019). Piperlongumine (PL) is a bioactive plant alkaloid, which has been demonstrated the anti-cancer activity via several mechanisms including inhibition of UPS (Jarvius et al., 2013; Halasi et al., 2013) . PL selectively kill CCA via the activation of reactive oxygen species (ROS)-JNK-ERK pathway (Thongsom et al., 2017). In our current study, we demonstrated that PL induced the accumulation of poly-ubiquitinated protein in CCA cells. Anti-cancer effect of PL has synergistic interaction with BTZ treatment in CCA cells. Moreover, PL in combination with BTZ enhanced the poly-ubiquitinated protein accumulation, leading ER stress-induced CCA cell death.

The potential of BTZ or PL in CCA treatment has been proposed through both in vitro and in vivo models (Vaeteewoottacharn et al., 2013 and Thongsom et al., 2017). As a result, the combined treatment of PL and BTZ would be a promising choice for CCA treatment. Nevertheless, the combination effect of PL and BTZ has not demonstrated. In present study, either PL or BTZ inhibited growth of both CCA cells including KKU-055 and KKU-213A. But the cytotoxicity effect in CCA cells was obviously observed upon combined treatment with PL and BTZ. The colony formation of both CCA cells was significantly inhibited via the combined treatment with PL and BTZ when compared with that of single agent. Moreover, the combination index (CI) values were less than 1 for both CCA cells upon combined treatment which demonstrated the synergistic interaction between PL and BTZ. Additionally, inhibition of cell-cycle progression has been proposed as the mechanism of PL or BTZ (Thongsom et al., 2017;

Chun-fung et al., 2021; Ao et al., 2019). The synergistic effect of PL in combination of BTZ was clearly demonstrated through the downregulation of cell cycle markers (*CCND1*, *CCNB1*, *c-Myc*) and survival marker (*BIRC5*). However, the pattern of cyclin expressions upon drug treatments varied in between KKU-055 (*CCND1* down-regulation) and KKU-213A (*CCNB1* down-regulation). This may due to the cell-type specific responses to anti-cancer effect of PL and/or BTZ. Taken together, our findings indicate the promising efficacy of PL-combined BTZ in CCA treatment. However, the effect of combined treatment with PL and BTZ on *in vivo* experiments are required in further investigation.

The proteasome inhibitor, bortezomib, has been approved to use for treating multiple myeloma, leukemia and some solid tumors (pancreatic and gastric cancers) (Hideshima et al., 2003; Nakata et al., 2011; Nawrocki et al., 2002). BTZ is a reversible inhibitor primarily acting on the CT-L activity of the proteasome. The cytotoxic mode of action, BTZ mainly suppress IKB degradation, leading to inhibit NF-KB activation (Hideshima et al., 2003). However, the mechanism of BTZ-induced CCA death is ER stress-induced apoptotic cell death via NF-KB independent. As such, PL would be a good choice to enhance the effect of BTZ against CCA due to the inhibition effect of PL on ubiquitin-proteasome system (Jarvius et al., 2013; Halasi et al., 2013). The result of the present study demonstrated that PL induced the accumulation of poly-ubiquitinated proteins in CCA cells but no effect on proteasome activity. PL in combination of BTZ increases the accumulation of poly-ubiquitinated proteins in CCA cells. However, the synergistic effect of combined treatment between PL and BTZ was not improve the proteasome inhibitory activity of BTZ alone. These findings are consistent with the previous studies in which PL plays a role in the inhibition of ubiquitin-proteasome system at pre-proteasome (prior deubiquitination) because PL has no effect on both 20S proteolytic activity and 19S deubiquitinating activity (Jarvius et al., 2013; Halasi et al., 2013; Yao et al., 2016).

In CCA, anti-cancer effect of BTZ via UPS results in perturbation of intracellular protein homeostasis by accumulation of the poly-ubiquitinated proteins and then induction of the proapoptotic unfolded protein response (Vaeteewoottacharn et al., 2013). The results of the present study demonstrated that single agent; either PL or

BTZ induced endoplasmic reticulum (ER) stress by the induction of XBP1splicing to spliced XBP1 (*XBP1s*) mRNA. Interestingly, mRNA expression of *XBP1s* was approximately 10-fold increase in combined treatment with PL and BTZ. Moreover, induction of *ATF4-CHOP* axis under ER stress was clearly detected in the present study. These results indicate that PL in combination of BTZ enhanced ER stress and are consistent with those of studies on mechanism of BTZ-induced cancer cell death, which activate ER stress through the activation of *ATF4* and *XBP1* splicing (Fels et al., 2008; Xu et al., 2012). Under prolonged ER stress, *ATF4* induces *CHOP* expression. Following that, *CHOP* induces the proapoptotic unfolded protein response by enhanced protein synthesis and oxidation in the stressed endoplasmic reticulum (Marciniak et al., 2004). Taken together, the result of the present study suggests that PL in combination of BTZ promotes the accumulation of the poly-ubiquitinated proteins, leading to prolonged ER stress and then activation of proapoptotic unfolded protein response.

In summary, the mechanisms of PL induced CCA cell death not only via the induction of ROS production, but also the increase of the accumulation of the poly-ubiquitinated proteins, leading to ER stress. Moreover, PL in combination with BTZ demonstrated the synergistic cytotoxicity in CCA through the induction of ER stress-induce proapoptotic UPR (Figure 5.1). As a result, PL use as either monotherapy or combination could be alternative strategy for CCA treatment.

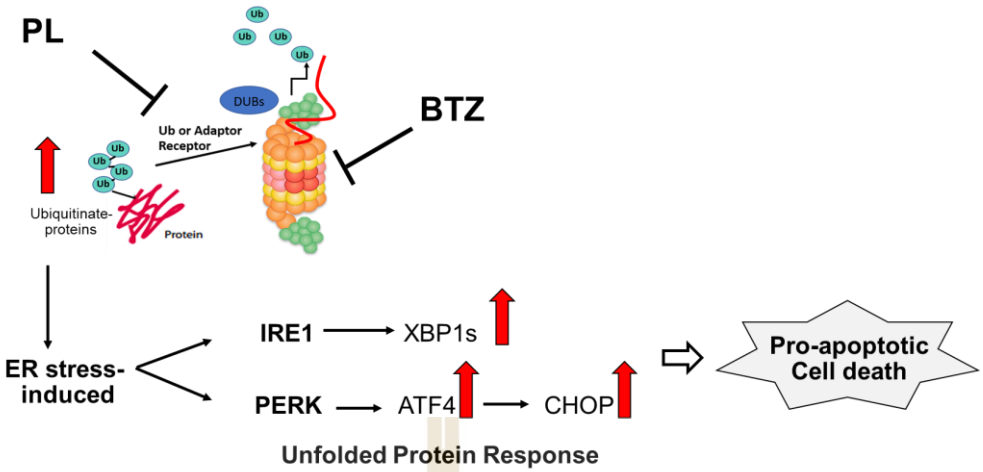

Figure 5.1 Mechanism of PL in combination with BTZ on CCA are proposed. PL in combination with BTZ synergically inhibited CCA cell growth via the accumulation of poly-ubiquitinated proteins, leading to ER stress-induced unfolded protein response (UPR). Induction of proapoptotic UPR was demonstrated by increasing *XBP1* mRNA splicing and *ATF4-CHOP* expression.

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