รายงานวิจัยฉบับสมบูรณ์

โครงการ: การยับยั้งการทำงานของกระบวนการต้านอนุมูลอิสระเพิ่มฤทธิ์การต้านมะเร็งของไปเปอร์ลองกูมินในเซลล์มะเร็งท่อน้ำดี

โดย ผู้ช่วยศาสตราจารย์.ดร.ชุติมา ตลับนิล

มิถุนายน 2562

สัญญาเลขที่ MRG6080055

รายงานวิจัยฉบับสมบูรณ์

โครงการ: การยับยั้งการทำงานของกระบวนการต้านอนุมูลอิสระเพิ่มฤทธิ์การต้านมะเร็งของไปเปอร์ลองกูมินในเซลล์มะเร็งท่อน้ำดี

ผู้ช่วยศาสตราจารย์.ดร.ชุติมา ตลับนิล
สำนักวิชาวิทยาศาสตร์ มหาวิทยาลัยเทคโนโลยีสุรนารี

สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัยและมหาวิทยาลัยเทคโนโลยีสุรนารี (ต้นสังกัด)

กิตติกรรมประกาศ

ขอขอบพระคุณ ศาสตราจารย์ ดร.โสพิศ วงศ์คำ ที่กรุณาให้ข้อเสนอแนะ และช่วยแก้ไขปัญหาต่างๆที่เกิดขึ้นในระหว่างการศึกษาวิจัย

การวิจัยครั้งนี้ได้รับการสนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย

ชุติมา ตลับนิล

มิถุนายน 2562

Abstract

Project Code: MRG6080055

Project Title: Suppression of antioxidant activity enhances antitumor effect of piperlongumine on Cholangiocarcinoma cells

Investigator: Chutima Talabnin (Ph.D.), School of Chemistry, Institute of Science, Suranaree University of Technology

Email Address: chutima.sub@sut.ac.th

Project Period: 2 Years

Chemoresistance is the main aspect in the cholangiocarcinoma (CCA) treatment. Therefore, a novel treatment strategy is an urgent need to develop effective chemotherapeutic protocols for treating CCA. Recently, redox-modulating strategies using piperlongumine (PL) become one of potential treatment for CCA patients. Piperlongumine (PL) is a known ROS inducer, and killed CCA cells by inducing G2-M phase arrest via ROS-JNK-ERK pathway. However, differential response to PL was observed in among all CCA cells which underlying mechanism is still unclear. The objective of this study was to investigate the molecular mechanisms of PL resistance to increase PL susceptibility on CCA cells. We first determined the basal mRNA expression levels of phase II detoxification enzymes and antioxidant proteins in CCA cells. High expressions of phase II detoxification enzymes (such as NADPH quinone oxidoreductase-1 (NQO-1), heme oxygenase-1 (HO-1), superoxide dismutases (SOD), and aldo-keto reductase 1 subunits C-1 and 3 (AKR1C1 and AKR1C3) were detected in KKU-100 which was least sensitive to PL in among all CCA cells (KKU-213 and KKU-214). Up-regulation of HO-1 expression via AKT activation was clearly detected in PL-treated CCA cells in a dose-dependent manner. Inhibition of HO-1 by chemical inhibitor, ZnPP, or specific siRNA eliminated their antioxidant defense machinery and lead to increased anti-tumor activity of PL in CCA cell lines via the increment of intracellular ROS level and upregulation of apoptotic proteins. These observations suggest that inhibition of Nrf2/HO-1 axis could enhance chemosensitizing effect of PL in CCA.

Keywords: Cholangiocarcinoma, Piperlongumine, Heme oxygenase -1, anti-oxidant capacity

บทคัดย่อ

รหัสโครงการ: MRG6080055

ชื่อโครงการ: การยับยั้งการทำงานของกระบวนการต้านอนุมูลอิสระ เพิ่มฤทธิ์การต้านมะเร็งของไปเปอร์ลองกูมินในเซลล์มะเร็งท่อน้ำดี

ชื่อนักวิจัย: ชุติมา ตลับนิล สาขาวิชาชีวเคมี สำนักวิชาวิทยาศาสตร์ มหาวิทยาลัยเทคโนโลยีสุรนารี

Email Address: chutima.sub@sut.ac.th

ระยะเวลาโครงการ: 2 ปี

การดื้อยาเคมีบำบัดถือเป็นปัจจัยสำคัญในการรักษามะเร็งท่อน้ำดี กลยุทธ์การรักษาแนวใหม่เป็นสิ่งที่จำเป็นในการพัฒนาแนวทางการรักษาด้วยเคมีบัดให้มีประสิทธิภาพเพื่อรักษาโรคมะเร็งท่อน้ำดี ณ ปัจจุบัน กลยุทธ์การควบคุมปฏิกิริยาการรับส่งอิเล็กตรอนหรือปฏิกิริยารีดอกซ์ ด้วยการใช้สารไปเปอร์ลองกูลมิน เป็นกลยุทธ์ที่น่าจะมีศักยภาพในการรักษาผู้ป่วยมะเร็งท่อน้ำดี เนื่องจากไปเปอร์ลองกูลมินมีคุณลักษณะเป็นตัวกระตุ้นการสร้างสารอนุมูลอิสระที่เรียกว่า อนุพันธ์ออกซิเจนที่ว่องไว ซึ่งสามารถทำลายเซลล์มะเร็งท่อน้ำดีได้ด้วยการชักนำการหยุดชะงักของกระบวนการแบ่งเซลล์ผ่านการกระตุ้นด้วยวิถี ROS-JNK-ERK แต่อย่างไรก็ตามการตอบสนองต่อไปเปอร์ลองกูลมินของเซลล์มะเร็งท่อน้ำแต่ละเซลล์นั้นมีความแตกต่างกัน และยังไม่เคยมีรายงานมาก่อนว่าเกี่ยวข้องกับกลไกใด ดังนั้นในการศึกษาครั้งนี้มีวัตถุประสงค์ในการศึกษากลไกที่เกี่ยวข้องกับการดื้อต่อไปเปอร์ลองกูลมิน เพื่อนำไปสู่แนวทางการพัฒนาการรักษาโรคมะเร็งท่อน้ำดีให้มีประสิทธิภาพ โดยผู้วิจัยเริ่มจากการตรวจวิเคราะห์การแสดงออกของยีนที่ใช้การสร้างเอนไซม์หรือโปรตีนที่มีคุณสมบัติในการกำจัดอนุมูลอิสระ ผลการศึกษาพบเซลล์มะเร็งท่อน้ำดี KKU-100 ที่มีความไวน้อยต่อไปเปอร์ลองกูลมินมีการแสดงออกของยีนในกลุ่มเอนไซม์ NADPH quinone oxidoreductase-1 heme oxygenase-1 superoxide dismutases และ aldo-keto reductase 1 subunits C-1-3 ค่อนข้างสูงเมื่อเปรียบเทียบกับเซลล์มะเร็งท่อน้ำดี KKU-213 และ KKU-214 ที่มีความไวมากต่อไปเปอร์ลองกูลมิน และผู้วิจัยพบว่าการเพิ่มขึ้นของการแสดงออกของยีน heme oxygenase-1 ในเซลล์มะเร็งท่อน้ำดีโดยผ่านกระบวนการ AKT activation นั้นจะเกิดเมื่อมีการทดสอบด้วยไปเปอร์ลองกูลมินในลักษณะที่การแสดงออกของยีนเพิ่มขึ้นตามปริมาณความเข้มของสารที่เพิ่มขึ้น และเมื่อทดสอบด้วยการยับยั้งการทำงานของ heme oxygenase-1 ด้วยตัวยับยั้งที่เรียกว่า ZnPP หรือ การทดสอบร่วมกับ HO-1 siRNA เพื่อกำจัดกระบวนการต้านอนุมูลอิสระ พบว่าประสิทธิภาพการทำลายเซลล์มะเร็งท่อน้ำดีด้วยไปเปอร์ลองกูลมินเพิ่มขึ้นเนื่องจากมีการชักนำการสร้างเพิ่มขึ้นของอนุพันธ์ออกซิเจนที่ว่องไว และโปรตีนที่เกี่ยวข้องกับกระบวนการตาย ดังนั้นจากการศึกษาครั้งนี้แสดงให้เห็นว่า การยับยั้งการทำงานของ Nrf2/HO-1 ในมะเร็งท่อน้ำดีน่าจะเป้าหมายที่สำคัญในการเพิ่มประสิทธิภาพและความไวของไปเปอร์ลองกูลมิน

คำหลัก: มะเร็งท่อน้ำดี ไปเปอร์ลองกูลมิน heme oxygenase-1 ความสามารถในการต้านอนุมูลอิสระ

Introduction

Cholangiocarcinoma (CCA) is a malignancy of bile duct epithelial cells. CCA is the second commonest primary liver cancer that have a very poor prognosis with 5-10% for 5-year survival rate. Surgical resection is the most effective treatment for CCA. However, 5-year survival rates of 25%-30% have been documented in patients with a potentially curative surgery (Khan SA et al., 2012 and Nagino M et al., 2013). To control disease, and improve survival and quality of life in patients with irresectable, recurrent and metastastic CCA, various chemotherapeutic agents including gemcitabine cisplatin and oxlalipatin) have been used either alone or in combination. However, a response rate (0-40%) and median survival (2-12 months) remains low because resistance to chemotherapy is reported to be the main aspect in CCA treatment (Thongprasert et a., 2005, Fodale et al., 2011 and Holohan et al.,2013).

The regulation of redox homeostasis is an essential factor to maintaining normal cellular functions and ensuring cell survival. Cancer cells are characterized by increased aerobic glycolysis that causes to high levels of reactive oxygen species (ROS) generation. The high ROS levels in cancer cells are a consequence of alteration in several signaling pathways that link to tumorigenesis including stimulation of cellular proliferation, promoting of mutation and genetic instability (Pelicano H et al., 2004 and Gorrini C et al., 2013). Redox-modulating strategies become one of potential treatment for cancer patients that may enable therapeutic selectivity and to overcome drug resistance, such as cancer cells with increasing reactive oxygen species (ROS) level or decreasing antioxidant capacity are more susceptible to oxidative stress-induced cell death (Trachootham D et al., 2009). Currently, several anti-cancer agents including arsenic trioxide, anthracyclines, and cisplatin have demonstrated to act as ROS-generating agents that cause increased cellular ROS generation, and these anti-cancer agents are logical candidate for evaluating the strategy of preferentially killing cancer cell with increased ROS stress (Pelicano H et al., 2004). In this study, we focus on piperlongumine (PL) which is a phytochemicals act as anti-cancer agent that targets redox regulation by selectively killing various cancer cells including CCA with increment of intracellular ROS levels. PL-induced ROS generation is dependent on the activation of mitogen activated protein kinases (MAPKs) including c-Jun-N-terminal kinase (JNK), ERK and p38 (Liu JM et al., 2013 and Thongsom S et al., 2017). However, differential responses to PL has reported and may possibly involve in difference of genetics background of antioxidant mechanism defense in each cancer cells (Thongsom S et al., 2017).

Heme oxygenase 1 (HO-1) is, an inducible form of heme oxygenase, the first rate-limiting enzyme in the degradation of cellular heme to release free iron, carbon monoxide (CO) and biliverdin in mammalian cells. HO-1 is frequently upregulated in numerous types of tumors including prostate, renal, gastric, colon cancer and CCA (Maines MD et al., 1996, Goodman AI et al., 1997, Yin H et al., 2014 and Kongpetch S et al., 2012 and 2014). Up-regulation of HO-1 is associated with tumor progression including tumor growth, metastasis and chemoresistance. A recent study showed that a depletion of the key cytoprotective enzyme such as HO-1 in the cancer cells could enhances the chemosensitivty of several anti-cancer agents including gemcitabine, cisplatin, and bortezomib (Kongpetch S et al., 2012, Furfaro AL et al., 2014 and Lv X et al., 2016). In this research work, we demonstrated the possibility strategy for CCA treatment by the combination of anti-cancer agents such as PL with targeting HO-1. The result showed that HO-1 was induced during PL treatment in CCA cell lines and suppression of HO-1 by chemical inhibitor or specific siRNA could increase the intracellular ROS level and chemosensitivity to PL in the CCA cell lines

Materials and Methods

Chemicals and Reagents

Cell culture reagents were purchased from Gibco (Grand Island, NY, USA). Piperlongumine (PL), 2´,7´-dichlorodihydrofluorescein diacetate (DCFH-DA), trichloroacetic acid (TCA), Zinc-protoporphyrin IX (ZnPP; HO-1 inhibitor), and sulforhodamine B (SRB) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The HO-1 siRNA and non-target negative control siRNA were purchased from Santa Cruz (San Diego, CA, USA). DharmaFect 1 siRNA transfection reagent was purchased from Dharmacon (Lafayette, CO, USA). Primary antibodies were obtained from Cell Signaling Technology (Danvers, MA, USA) —including total Akt *(*60 kDa*)*, Ser 473 phosphorylated Akt (p-Akt) (60 kDa), poly (ADP-ribose) polymerase (PARP) (116/89 kDa), and Bcl-2 (28 kDa). Antibody against HO-1(32 KDa), Nrf2 (110 KDa) and β-Actin (42 kDa) were purchased from Santa Cruz, San Diego CA, USA. Mouse anti-rabbit IgG-HRP secondary antibody and Donkey anti-rabbit IgG-HRP secondary antibody were obtained from GE health care (Piscataway, NJ, USA). Luminata TM Forte Western HRP substrate detection reagents were purchased from Millipore (Millipore, Billerica, MA, USA). Superscript VILO^{TM} cDNA synthesis kit was purchased from Invitrogen (Carlsbad, CA, USA). LightCycler® 480 SYBR Green I Master was supplied from Roche (Mannheim Germany).

Cell culture and Transfections

Three human CCA cell lines (KKU-100, KKU-213, and KKU-214) were established from CCA patients at Srinagarind Hospital, Khon Kaen University, by Professor Banchob Sripa. Certificates of analysis were obtained from the Japanese Collection of Research Bioresources Cell Bank, Osaka, Japan. Cell were cultured in HAM-F12 containing 1% penicillin-streptomycin and 10% fetal bovine serum (FBS) Cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2. Cells with 70-80 % confluence were trypsinized with 0.25% trypsin-EDTA and subcultured in the same media.

Suppression of HO-1function was performed by HO-1 siRNA in two CCA cell lines including KKU-100 and KKU-213. Cells were seeded on to six-well plate at 3 - 4 x 10^5 cells/well and incubated for overnight. Cells were transfected with 10 μM of specific siRNA to HO-1 (siHO-1) or non-target negative control siRNA (siCon) using DharnaFect 1 siRNA transfection reagent for 24 and 48 h. Transfection procedure was performed according to the manufacturer's instruction (Dharmacon).

Drug treatments

A stock concentration of 50 mM PL and 5mM of ZnPP were dissolved in dimethyl sulfoxide (DMSO) and stored in aliquots at -20°C until its use. Various concentrations of PL (0-100 μM) or ZnPP (0-10 μM) were diluted with cell culture media for subsequent experiments. The vehicle control was DMSO with the same final concentration used in preparation of PL or ZnPP working solution. For combination treatment of PL and ZnPP, the cells were pre-treated with ZnPP for 3 h and then removed it before treating with various concentration of PL (0-100 μM) for 24 h. For treatment of PL in presence or absent of siHO-1 or siCon, the cells were treated with a selection of concentration of PL from 0-20 μM for 24-30 h. Then, the treated cells were subsequently performed the cell viability, intracellular ROS, quantitative PCR and western blot analysis.

Cell viability

Cell viability was measured using a sulforhodamine B (SRB) assay capable of determining cell density based on the measurement of cellular protein content; performed per Voigt with slight modifications. The % cell viability was calculated as (A564 in treatment wells) ÷ (A546 in control wells) × 100. Each experiment was performed three times independently. The half maximal inhibitory concentration (IC_{50}) values were calculated using CompuSyn software [16] (ComboSyn Inc.; Paramus, NJ, USA)

Measurement of intracellular accumulation of ROS

The production of intracellular ROS was detected using DCFH-DA fluorescence assay as previous described (Thongsom S et al., 2017). Briefly, transfected CCA cell lines (1.5 - 2 × 10^5 cells) were seeded in 6-well plates for overnight after 30 h of transfection. Then cells were treated with PL at 0, 10, and 20 μM for 12 h. After treatment, the intracellular ROS was detected with 20 μM of DCFH-DA in a humidified 5% CO_2 incubator at 37°C for 30 min. After washing with 1XPBS, the cells were trypsinized and re-suspended in 1XPBS. Then, the fluorescent intensity of the DCFH-DA was determined using Tali image-based cytometer (Invitrogen,Carlsbad, CA, USA).

Real-time reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted using TRIzol reagent as recommended by the manufacturer (Invitrogen). First-stand cDNA was synthesis using Superscript VILO^{TM} cDNA synthesis kit with 2 µg of total RNA according to the manufacturer's instruction (Invitrogen). The sequences of primers were listed in Table 1. Real-time PCR was performed using the LightCycler® 480 Real-Time PCR System and LightCycler® 480 SYBR Green I mastermix (Roche Diagnostics, Mannheim, Germany). The amplification condition was 95°C for 5 min; 95°C for 10 s, annealing at 60°C for 10 s, and extension at 72°C for 10 s through 45 cycles. The specificity of each of the PCR products was confirmed by melting curve analysis. Relative mRNA expression was obtained after normalization with endogenous human β-actin and calculated by $2^{-\Delta\Delta Ct}$.

Table 1 Oligonucleotide for quantitative RT-PCR

Gene	5' –Forward 3'	5'-Reverse-3'
Nrf2	5' –TACTCCCAGGTTGCCCACA- 3'	5' –CATCTACAAACGGGAATGTCTGC- 3'
GCLM	5'-GACAAAACACAGTTGGAACAGC-3'	5' –CAGTCAAATCTGGTGGCATC- 3'
GCLC	5' –ATGCCATGGGATTTGGAAT- 3'	5' –AGATATACTGCAGGCTTGGAATG- 3'
AKR1C1	5' –CATGCCTGTCCTGGGATTT- 3'	5' –AGAATCAATATGGCGGAAGC- 3'
AKR1C3	5'-CATTGGGGTGTCAAACTTCA-3'	5'-CCGGTTGAAATACGGATGAC-3
HMOX1	5´-CAACATCCAGCTCTTTGAGGA-3´	5´-GGGCAGAATCTTGCACTTTG-3´
NQO1	5´-GATATTCCAGTTCCCCCTGC-3´	5´-TTCTTACTCCGGAAGGGTCC-3´
TXN	5´-GAGAGCAAGACTGCTTTTCA-3´	5´-CAGAGAGGGAATGAAAGAAAG-3´
GSTP1	5´-TACACCAACTATGAGGCGGG-3´	5´-AGCGAAGGAGATCTGGTCTC-3´
SOD2	5´-GTTGGCCAAGGGAGATGTTAC-3´	5´-AGCAACTCCCCTTTGGGTTC-3
PARK7	5´-CGAGCTGGGATTAAGGTCA-3´	5´-CATATGGTCCCTCTTTTTTTGC-3´
B-actin	5'-GATCAGCAAGCAGGAGTATGACG-3'	5'-AAGGGTGTAACGCAACTAAGTCATAG-3'

Protein Preparation

CCA cell lines were cultured in six-well plate with 3 - 4 x 10^5 cells/well for 24 h. Then the cells were treated with siRNA and/or various concentrations of PL (0, 5, 10 and 20 µM) for another 0, 3, 6, 12 and 24 h. After incubation, whole cell lysate was harvested as described in previous study (Thongsom S et al., 2017). For nuclear and cytoplasmic fractions were extracted using nuclear extraction kit (Chemicon®, Merck, Darmstadt, Germany) according to manufacturer's instruction. Then, whole cell lysate, nuclear and cytoplasmic fraction were determined the protein concentration using Pierce® BCA protein assay kit (Thermo Scientific, Rockford, USA).

SDS-PAGE and Western blot analysis

The protein samples from whole cell lysate or nuclear or cytoplasmic fraction with 20 ug were electrophoretically separated on 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were transferred to a nitrocellulose membrane. The nitrocellulose membranes were blocked for 1h with 5% (w/v) skimmed milk in 1XPBS containing 0.05% Tween 20 (PBST). Then the membranes were incubated overnight at 4 °C with primary antibodies at a dilution of 1:500 for HO-1 and Nrf2 proteins or 1:1000 for PARP, Bcl-2, pAkt (Ser473) total Akt and lamin B1 proteins in PBST. The membrane were then incubated with 1:2,000 dilution of respective HRP-conjugated secondary antibody for 1 h at room temperature. Luminata™ Forte Western HRP substrate was applied for detecting the proteins and the bands were captured using ImageQuant LAS 4000 (GE Healthcare, Piscataway, NJ, USA).

Statistical analysis

All experiments were performed at least 2-3 times, and all results expressed as a mean ± the standard error. Statistical analyses were performed using GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA, USA). The unpaired t-test was used for a between group statistical analyses. $P<0.05$ was considered statistically significant.

Results

Expression levels of phase II detoxification enzymes and antioxidant proteins in CCA cell lines.

To eliminate the harmful reactive oxygen species (ROS) or carcinogens, Nrf2- mediated cytoprotective genes especially phase II detoxification enzymes and antioxidant proteins are thought to be a major antioxidant defense mechanism in mammalian cells. In this study, basal mRNA expression of 11 antioxidant related genes were investigated in three CCA cell lines that have been previously showed differential responses to PL including KKU-100 (CCA-less sensitive to PL), KKU-213 and KKU-214 (CCA-high sensitive to PL). We found that mRNA expression levels of phase II detoxification enzymes including NADPH quinone oxidoreductase-1 (*NQO-1*), heme oxygenase-1 (*HO-1*), superoxide dismutases (*SOD*) and aldo-keto reductase 1 subunits C-1 and 3 (*AKR1C1* and *AKR1C3*) were visibly high in KKU-100 comparing with other CCA cell lines. However, basal expression levels of *Nrf2* and other *Nrf2*-mediated cytoprotective genes especially an enzymes in glutathione synthesis including γ-glutamylcysteine synthetase catalytic subunit (*GCLC*), γ-glutamylcysteine synthetase modifier subunit (*GCLM*), Glutathione S-transferase P1 (*GSTP1*) were comparable in among all cell lines (Fig 1). This result suggests that the mechanism of action of antioxidant defense might depend on the genetic background of each CCA cell lines.

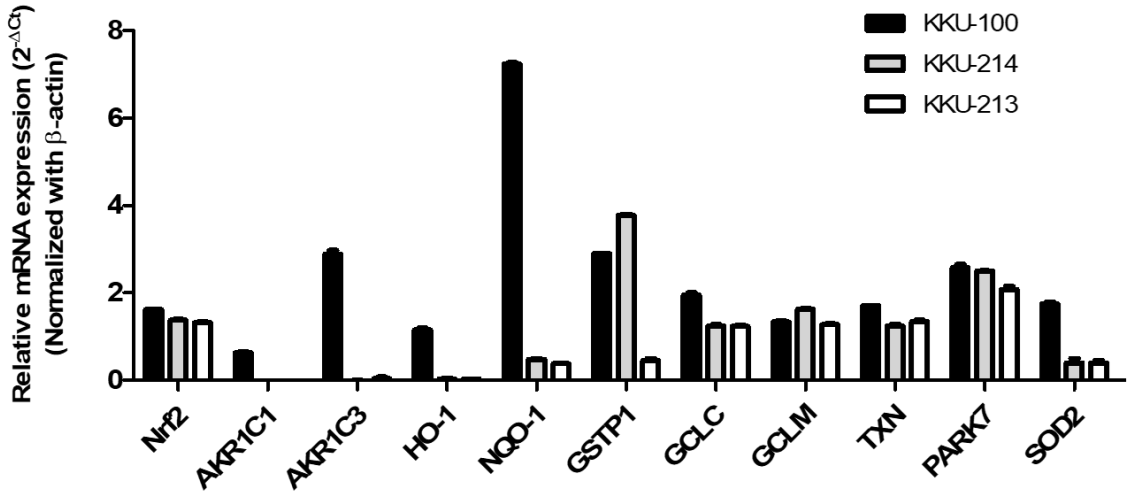
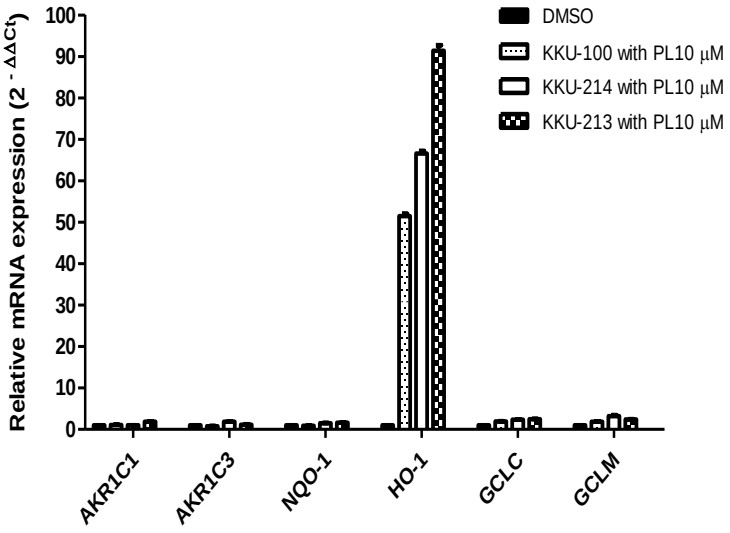


Fig 1. Antioxidants expression profiles in 3 CCA cell lines. The mRNA expressions of all antioxidant related genes in 3 CCA cell lines were analyzed by qPCR and normalized using β-actin as reference gene. The relative mRNA expression of all genes was calculated using $2^{-\Delta Ct}$ The data are presented as the mean ± SEM of two independent experiments

Induction of HO-1 expression upon piperlongumine treatment

To evaluate the role of Nrf2-mediated cytoprotective genes in the responses to PL treatment. CCA cell lines were treated with PL at 10 μM for 6 h and then the mRNA expression of Nrf2-mediated cytoprotective genes including *AKR1C1*, *AKR1C3, NQO-1*, *HO-1*, *GCLC*, and *GCLM* were determined by RT-PCR. The result showed that the expression of *HO-1*, *GCLC*, and *GCLM* were increased in all CCA cell lines after PL treatment; however, the increment of HO-1 expression was clearly observed in all CCA cell lines compare to the other genes expression (Fig 2A). Next, we examined the HO-1 expression in CCA cell lines in the presence of PL with various concentrations (0-20 μM) for 24 h or 10 μM of PL at various times (0, 3, 6 and 24 h). Expression analysis demonstrated that the expression of HO-1 in both mRNA and protein levels in both KKU-100 and KKU-213 were observed in a dose-dependent manner, but the induction of HO-1 expression indeed increased in response to the PL treatment especially at 6 h and the expression was declined after 24 h of PL treatment (Fig 2B and 2C). The results of the present study suggest that PL triggers preferentially HO-1 inducible expression in CCA cell lines and activation of HO-1 expression could be early antioxidant defense in the responses to PL treatment.

A



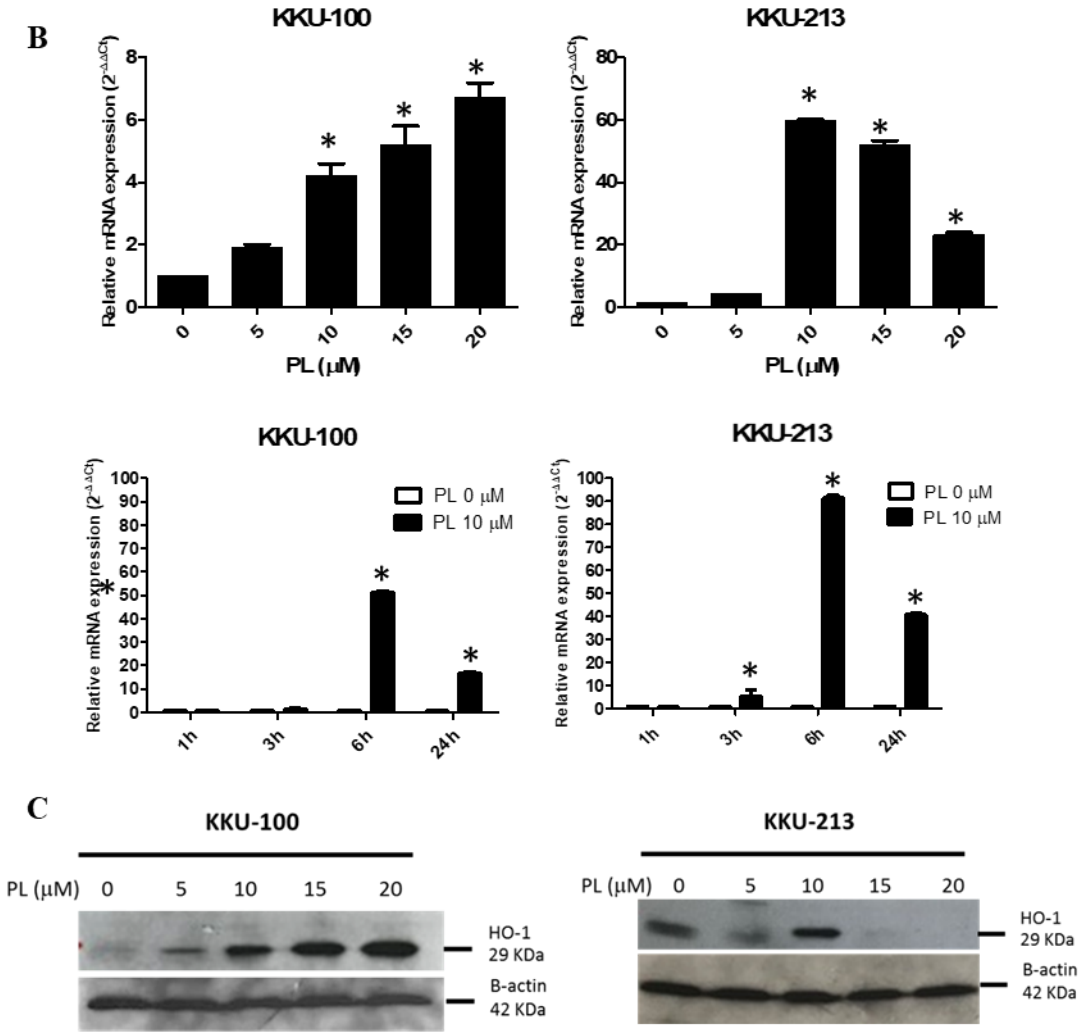


Fig 2 Piperlongumine induces HO-1 expression in CCA cell lines.

(A) KKU-100, KKU-213 and KKU-M214 were treated with PL at 10 μM or DMSO (control) for 6 h and then were determined the mRNA expressions of AKR1C1, AKR1C3,*NQO1, HO-1*, *GGCL* and *GCLM* by qRT-PCR and normalized using *β-actin* as reference gene. The relative mRNA expression of these six genes between with or without PL treatment was calculated using $2^{-\Delta\Delta Ct}$. (B and C) KKU-100 and KKU-213 were treated with PL (at 0, 5, 10, 15 and 20 μM) for 24 h and were treated with PL at 10 uM for 1 ,3, 6 and 24 h. The relative mRNA expression of HO-1 and HO-1 protein were determined by qPCR and western blot analysis respectively. The expression data are presented as the mean ± SEM of three independent experiments.

HO-1 silencing promotes PL-induced CCA cell death by increasing ROS accumulation

We subsequently investigated whether HO-1 act as a key antioxidant defense for protecting PL-mediated ROS generation in CCA cells. First, we used ZnPP, a chemical HO-1 inhibitor, to inhibited HO-1 activity in CCA cell line (KKU-100 and KKU-213) and then checked the effect of HO-1 inhibition in relation to PL-induced cytotoxicity. Cytotoxicity test demonstrated that ZnPP at less than 10 μM could suppressed CCA cell growth with less than 50 % of the control. Therefore, ZnPP concentrations from 0-10 μM were used in combination with PL treatment (0-100 μM). The respective IC50 values of PL were reduced in correlation to the combination with ZnPP at 5 and 10 μM in both KKU-100 and KKU-213 (Fig 3A and 3B). Next, we performed knockdown of HO-1 to elucidate the role of HO-1 in the sensitivity of CCA cell lines to PL. The mRNA expression levels of HO-1 in both KKU-100 and KKU-213 were dramatically decreased at 24 and 48 h after transfection of HO-1 siRNA (Fig 4A), however, suppression of HO-1 have not influenced to CCA cell growth (Fig 4B). Then, knock down of HO-1 markedly enhanced anti-tumor activity of PL in a dose-dependent manner for both KKU-100 and KKU-213 (Fig 4C). In addition, the combination of HO-1 silencing with PL treatment at 20 μM showed significant increase of intracellular ROS accumulation (Fig 4D). Moreover, Western blot analyses also showed that up-regulation of apoptotic proteins (cleavage PARP) and down-regulation of anti-apoptotic protein (Bcl-2) were detected in response to these combination treatments at 12 h (Fig 4E). Taken together, inhibition of HO-1 promotes PL-mediated ROS generation and lead to PL-induced CCA apoptosis.

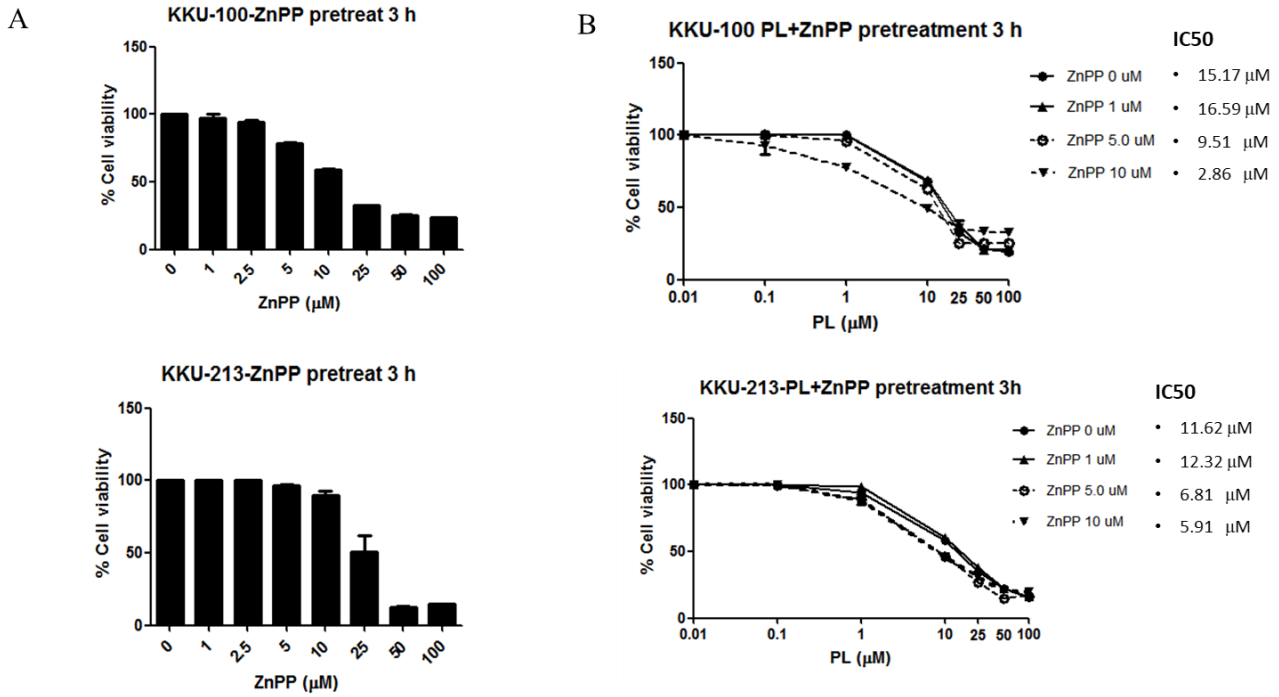


Fig 3 Inhibition of HO-1 by ZnPP increases anti-tumor activity of PL in CCA cell lines. (A) KKU-100 and KKU-213 were pretreated with ZnPP at various concentrations for 3 h and continue culture without ZnPP for 24 h. (B) KKU-100 and KKU-213 were pretreated with ZnPP at 0, 1, 5 and 10 μM for 3 h. and then ZnPP was removed and continue to treated with PL at 10 μM 24 h. Cell viability was measured by SRB assays. The data are presented as the mean ± SEM of three independent experiments.

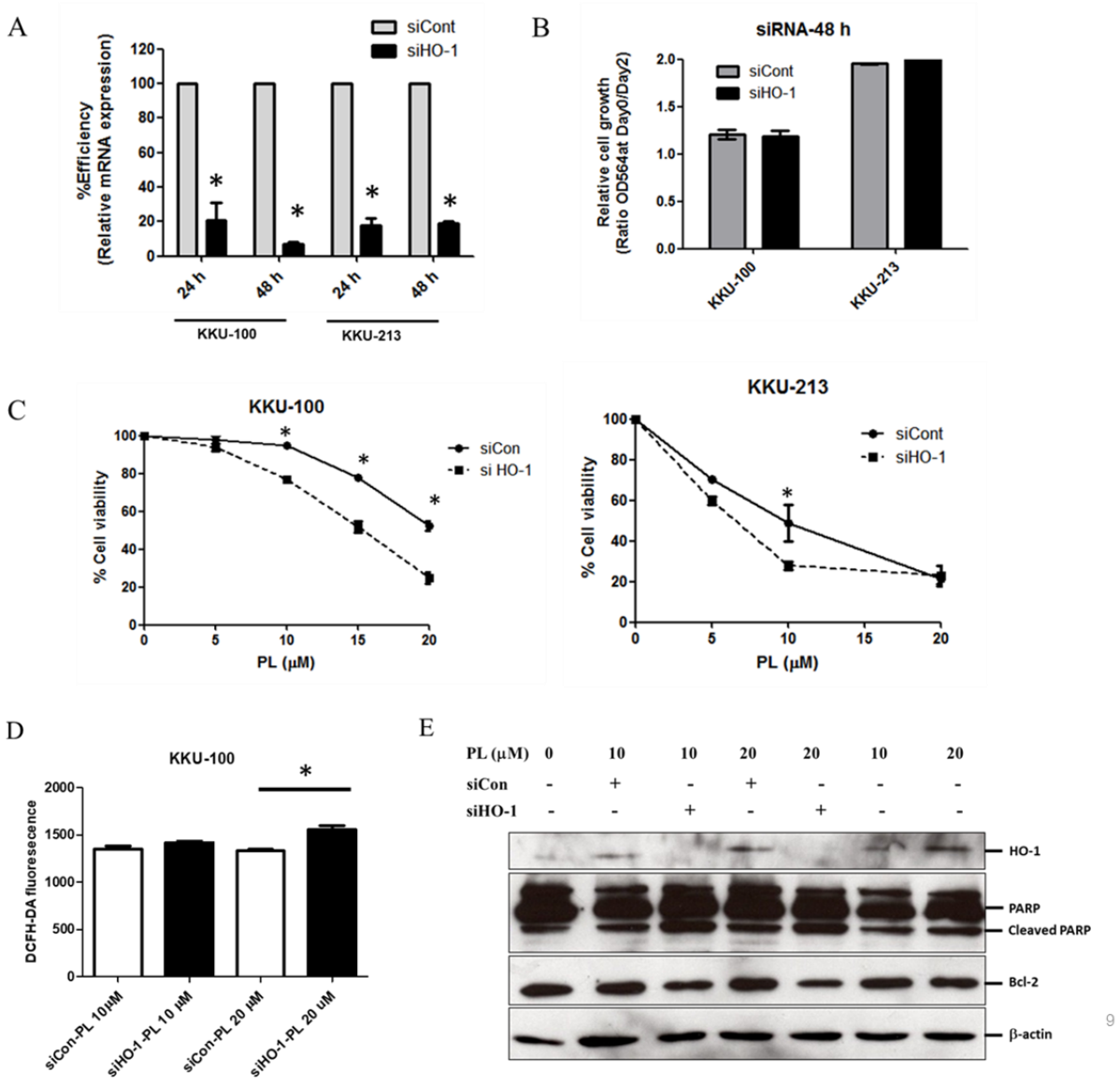


Fig 4. Knockdown of HO-1 by siRNA sensitized CCA cell lines to Piperlongumine. KKU-100 and M213 were transfected with HO-1 siRNA (si HO-1) or control transfection (si Cont) for 24-48 h. (A) Efficiency (%) of knockdown at 24 and 48 h relative to siCont was determined using real-time PCR. Cell viability at 48 h (B) and the cytotoxicity effect of PL at various concentrations (C), ROS accumulation (D) and apoptotic and anti-apoptotic proteins (E) between HO-1 knocked down cells and control were measured at 12 h of PL treatment. The data are presented as the mean ± SEM of three independent experiments.

Piperlongumine activates Nrf2-mediated HO-1 expression via activation of the AKT signal

It has been reported that PI3K/AKT pathway act as a survival signal against multiple apoptotic insults and is thought to be a main upstream signaling event to induction of Nrf2-mediated HO-1 expression (Martin D et al., 2004, Chen HH et al., 2012), so the activation/phosphorylation of AKT in PL-treated CCA cell lines was determined to elucidate the role of PI3K/AKT in PL-induced Nrf2 activation and HO-1 expression. As shown in Fig 5A, HO-1 and AKT phosphorylation indeed increased in response to PL treatment. In addition, nuclear Nrf2 and HO-1 expression were also observed in a dose-dependent manner (5,10 and 20 μM). This finding suggests that PL-induced HO-1 expression is stimulated via Nrf2/AKT activation.

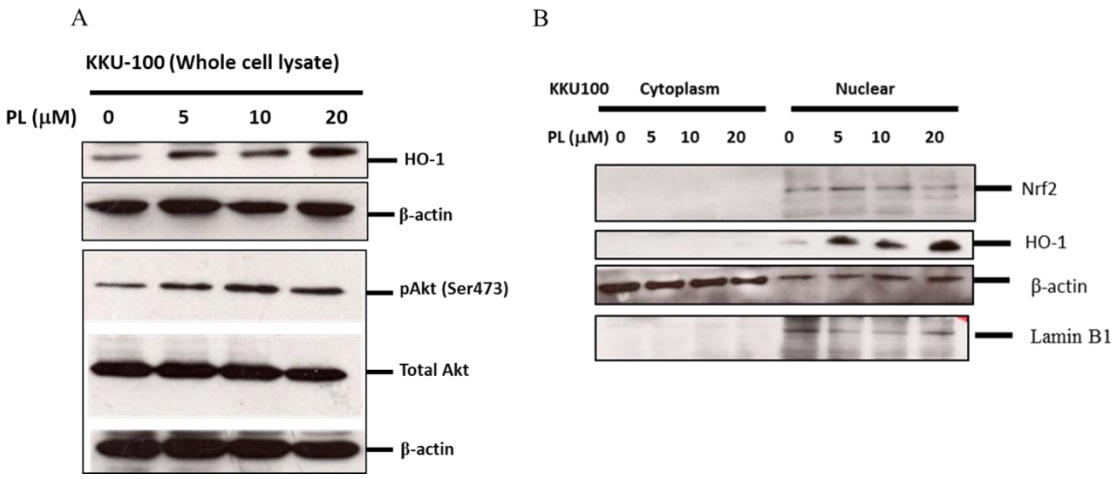


Fig 5 Piperlongumine induces HO-1 expression via Akt activation. KKU-100 were treated w ith various concentration of PL (0, 5, 10, or 20 μM) for 3 h. The whole cell lysates(A) and nucle ar extracts (B) were subjected to Western blotting and probed with an antibody specific to HO-1 , tAkt, pAkt (Ser473), Nrf2, and β-actin.

Discussion

PL exerts anti-tumor effect in variety of cancers including CCA (Thongsom S et al., 2017). PL inhibits tumor growth through the induction of ROS accumulation and activation of mitogen activated protein kinases (MAPKs) including c-Jun-N-terminal kinase (JNK), ERK and p38 (Liu JM et al., 2013 and Thongsom S et al., 2017) or inhibition of PI3K/AKT pathway (Wang Fet al., 2015, and Zhou L et al., 2019). However, sensitivity to PL varies slightly in different cancer cell lines including CCA. In this study, we aimed to investigate the underlying mechanisms of PL resistance by targeting HO-1 activity. Because, several evidences have demonstrated that HO-1 has a powerful cytoprotective effect against various apoptotic insults in both normal and cancers cells. Basal HO-1 expression levels of the cancer cells also convey the sensitivity to chemotherapeutic agents (Kongpetch S et al., 2012 and 2016). In our current study, we found that basal HO-1 expression in among CCA cell lines was associated with sensitivity to PL. In addition, HO-1 inducible expression was prominently presented after 3 and 6 h of PL treatment in all CCA cells. This result suggests that HO-1 inducible expression plays an important role in early protection against PL-induced oxidative stress reaction. Our findings are consistent with studies on well-known chemotherapeutic agents such as gemcitabine and cisplatin in which it can mediate oxidative stress and lead to induced HO-1 expression in CCA and laryngeal squamous cell cancer respectively (Lv X et al., 2016 and Kongpetch et al., 2012).

The PI3K/AKT pathway is involved in cellular survival, metastasis, and drug resistance in variety of cancer types (Yokoi K et al., 2017, Yoon et al., 2011, and Yothaisong S et al., 2013). The development of acquired resistance by radiation, chemotherapy and/or targeted therapy have been revealed to associate with the induction of AKT activity (Arlt A et al., 2003 and Huang WC et al.,2009). In addition, inhibition of this pathway has been proposed to increase the chemosensitivity in CCA (Leelawat K et al.,2009 and Yoon H et al., 2011). Here we found that PL treatment resulted in AKT phosphorylation and Nrf2 activation, showing PL indeed increased HO-1 inducible expression via AKT/Nrf2 activation. In contrast to PL-induced antioxidant defense in CCA, PL stimulated oxidative stress via ROS generation and induced CCA apoptosis through the activation of ROS/JNK/ERK pathway (Thongsom S et al., 2017). This finding suggests that under oxidative stress environment caused by PL, CCA cells upregulated antioxidant defense by inducing AKT-mediated HO-1 expression and enhanced anti-apoptotic capacity to resistant to PL-induced ROS generation. This result illuminates the possible cause of PL resistance in CCA.

HO-1 is a powerful antioxidant enzyme in Nrf2-mediated cytoprotective responses. HO-1 plays important role in cytoprotection and malignant transformation of cancer cells. HO-1

overexpression is found in CCA. High levels of HO-1 is associated with CCA progression and therapeutic resistance (Kongpetch S et al., 2012 and 2016). Suppression of HO-1 activity enhanced the chemosensitivity of cancer cells to various chemotherapeutic agents (Kongpetch S et. al., 2012, Lv X et al., 2016, Berberat PO et al., 2005). In present study, we found that accompanied by the inhibition of HO-1 activity using chemical inhibitor or specific siRNA to HO-1, anti-tumor activity of PL was increased in CCA cell lines through the increment of intracellular ROS accumulation and increased CCA cell death via upregulation of apoptotic proteins. Taken together, our findings provided evidence that HO-1 could serve as a major antioxidant defense in PL treatment and the levels of basal HO-1 expression indicate the efficiency of PL for treating CCA.

In conclusion, under oxidative stress environment caused by PL, AKT-mediated HO-1 activation plays an importance role in antioxidant defense to protect PL-induced CCA apoptosis. In addition, we have proved that suppression of HO-1 resulted in increased intracellular ROS generation and CCA apoptosis induced by PL. Taken all together, our results demonstrated the mechanism of PL resistance in CCA and Inhibition of HO-1 could be a feasible strategy for increasing the chemosensitivity of CCA to PL.

References

1. Khan SA, Davidson BR, Goldin RD, Heaton N, Karani J, Pereira SP, et al. Guidelines for the diagnosis and treatment of cholangiocarcinoma: an update. Gut. 2012 Dec;61(12):1657-69.
2. Nagino M, Ebata T, Yokoyama Y, Igami T, Sugawara G, Takahashi Y, et al. Evolution of surgical treatment for perihilar cholangiocarcinoma: a single-center 34-year review of 574 consecutive resections. Ann Surg. 2013 Jul;258(1):129-40.
3. Thongprasert S. The role of chemotherapy in cholangiocarcinoma. Ann Oncol. 2005;16 Suppl 2:ii93-6.
4. Fodale V, Pierobon M, Liotta L, Petricoin E. Mechanism of cell adaptation: when and how do cancer cells develop chemoresistance? Cancer J. 2011 Mar-Apr;17(2):89-95.
5. Holohan C, Van Schaeybroeck S, Longley DB, Johnston PG. Cancer drug resistance: an evolving paradigm. Nat Rev Cancer. 2013 Oct;13(10):714-26.
6. Pelicano H, Carney D, Huang P. ROS stress in cancer cells and therapeutic implications. Drug Resist Updat. 2004 Apr;7(2):97-110.
7. Gorrini C, Harris IS, Mak TW. Modulation of oxidative stress as an anticancer strategy. Nat Rev Drug Discov. 2013 Dec;12(12):931-47.
8. Trachootham D, Alexandre J, Huang P. Targeting cancer cells by ROS-mediated mechanisms: a radical therapeutic approach? Nat Rev Drug Discov. 2009 Jul;8(7):579-91.
9. Liu JM, Pan F, Li L, Liu QR, Chen Y, Xiong XX, et al. Piperlongumine selectively kills glioblastoma multiforme cells via reactive oxygen species accumulation dependent JNK and p38 activation. Biochem Biophys Res Commun. 2013 Jul 19;437(1):87-93.
10. Thongsom S, Suginta W, Lee KJ, Choe H, Talabnin C. Piperlongumine induces G2/M phase arrest and apoptosis in cholangiocarcinoma cells through the ROS-JNK-ERK signaling pathway. Apoptosis. 2017 Nov;22(11):1473-84.
11. Maines MD, Abrahamsson PA. Expression of heme oxygenase-1 (HSP32) in human prostate: normal, hyperplastic, and tumor tissue distribution. Urology. 1996 May;47(5):727-33.

12. Goodman AI, Choudhury M, da Silva JL, Schwartzman ML, Abraham NG. Overexpression of the heme oxygenase gene in renal cell carcinoma. Proc Soc Exp Biol Med. 1997 Jan;214(1):54-61.
13. Yin H, Fang J, Liao L, Maeda H, Su Q. Upregulation of heme oxygenase-1 in colorectal cancer patients with increased circulation carbon monoxide levels, potentially affects chemotherapeutic sensitivity. BMC Cancer. 2014 Jun 14;14:436.
14. Kongpetch S, Kukongviriyapan V, Prawan A, Senggunprai L, Kukongviriyapan U, Buranrat B. Crucial role of heme oxygenase-1 on the sensitivity of cholangiocarcinoma cells to chemotherapeutic agents. PLoS One. 2012;7(4):e34994.
15. Kongpetch S, Puapairoj A, Ong CK, Senggunprai L, Prawan A, Kukongviriyapan U, et al. Haem oxygenase 1 expression is associated with prognosis in cholangiocarcinoma patients and with drug sensitivity in xenografted mice. Cell Prolif. 2016 Feb;49(1):90-101.
16. Furfaro AL, Piras S, Passalacqua M, Domenicotti C, Parodi A, Fenoglio D, et al. HO-1 up-regulation: a key point in high-risk neuroblastoma resistance to bortezomib. Biochim Biophys Acta. 2014 Apr;1842(4):613-22.
17. Lv X, Song DM, Niu YH, Wang BS. Inhibition of heme oxygenase-1 enhances the chemosensitivity of laryngeal squamous cell cancer Hep-2 cells to cisplatin. Apoptosis. 2016 Apr;21(4):489-501.
18. Martin D, Rojo AI, Salinas M, Diaz R, Gallardo G, Alam J, et al. Regulation of heme oxygenase-1 expression through the phosphatidylinositol 3-kinase/Akt pathway and the Nrf2 transcription factor in response to the antioxidant phytochemical carnosol. J Biol Chem. 2004 Mar 5;279(10):8919-29.
19. Chen HH, Chen YT, Huang YW, Tsai HJ, Kuo CC. 4-Ketopinoresinol, a novel naturally occurring ARE activator, induces the Nrf2/HO-1 axis and protects against oxidative stress-induced cell injury via activation of PI3K/AKT signaling. Free Radic Biol Med. 2012 Mar 15;52(6):1054-66.
20. Wang F, Mao Y, You Q, Hua D, Cai D. Piperlongumine induces apoptosis and autophagy in human lung cancer cells through inhibition of PI3K/Akt/mTOR pathway. Int J Immunopathol Pharmacol. 2015 Sep;28(3):362-73.

21. Zhou L, Li M, Yu X, Gao F, Li W. Repression of Hexokinases II-Mediated Glycolysis Contributes to Piperlongumine-Induced Tumor Suppression in Non-Small Cell Lung Cancer Cells. Int J Biol Sci. 2019;15(4):826-37.
22. Lv X, Song DM, Niu YH, Wang BS. Inhibition of heme oxygenase-1 enhances the chemosensitivity of laryngeal squamous cell cancer Hep-2 cells to cisplatin. Apoptosis. 2016 Apr;21(4):489-501.
23. Yokoi K, Kobayashi A, Motoyama H, Kitazawa M, Shimizu A, Notake T, et al. Survival pathway of cholangiocarcinoma via AKT/mTOR signaling to escape RAF/MEK/ERK pathway inhibition by sorafenib. Oncol Rep. 2018 Feb;39(2):843 1.
24. Yoon H, Min JK, Lee JW, Kim DG, Hong HJ. Acquisition of chemoresistance in intrahepatic cholangiocarcinoma cells by activation of AKT and extracellular signal-regulated kinase (ERK)1/2. Biochem Biophys Res Commun. 2011 Feb 18;405(3):333-7.
25. Yothaisong S, Dokduang H, Techasen A, Namwat N, Yongvanit P, Bhudhisawasdi V, et al. Increased activation of PI3K/AKT signaling pathway is associated with cholangiocarcinoma metastasis and PI3K/mTOR inhibition presents a possible therapeutic strategy. Tumour Biol. 2013 Dec;34(6):3637-48.
26. Arlt A, Gehrz A, Muerkoster S, Vorndamm J, Kruse ML, Folsch UR, et al. Role of NF-kappaB and Akt/PI3K in the resistance of pancreatic carcinoma cell lines against gemcitabine-induced cell death. Oncogene. 2003 May 22;22(21):3243-51.
27. Huang WC, Hung MC. Induction of Akt activity by chemotherapy confers acquired resistance. J Formos Med Assoc. 2009 Mar;108(3):180-94.
28. Leelawat K, Narong S, Udomchaiprasertkul W, Leelawat S, Tungpradubkul S. Inhibition of PI3K increases oxaliplatin sensitivity in cholangiocarcinoma cells. Cancer Cell Int. 2009 Jan 8;9:3.
29. Berberat PO, Dambrauskas Z, Gulbinas A, Giese T, Giese N, Kunzli B, et al. Inhibition of heme oxygenase-1 increases responsiveness of pancreatic cancer cells to anticancer treatment. Clin Cancer Res. 2005 May 15;11(10):3790-8.
30.

Appendix

บทความสำหรับการเผยแพร่

มะเร็งท่อน้ำดีเป็นมะเร็งที่เกิดขึ้นบริเวณท่อทางเดินน้ำดีทั้งภายในและภายนอกตับ มะเร็งท่อน้ำดีเป็นมะเร็งที่มีอุบัติการณ์สูงเป็นลำดับที่สองในกลุ่มของมะเร็งตับ ซึ่งผู้ป่วยที่เป็นมะเร็งท่อน้ำดี มักมีพยากรณ์โรคที่แย่และมีอัตราการรอดชีวติต่ำ การรักษาด้วยการผ่าตัดถือเป็นการรักษาที่มีประสิทธิภาพแต่สามารถเห็นผลได้ชัดเจนในผู้ป่วยมะเร็งท่อน้ำดีในระยะแรกเท่านั้น ดังนั้นเพื่อควบคุมการพัฒนาของโรคหรือการทำให้ผู้ป่วยมะเร็งท่อน้ำดีมีชีวิตอยู่ได้นั้น ผู้ป่วยมะเร็งท่อน้ำดีที่ไม่สามารถผ่าตัดรักษาได้ จะได้รับการรักษาด้วยยาเคมีบำบัด แต่อย่างไรก็ตามการรักษาด้วยยาเคมีบำบัดก็ยังมีประสิทธิภาพไม่เพียงพอ เนื่องจากผู้ป่วยมะเร็งท่อน้ำดีอาจมีภาวะดื้อยาเคมีบำบัดเกิดขึ้น ดังนั้นการพัฒนาแนวทางการรักษาแนวใหม่เป็นสิ่งจำเป็นได้แก่ การรักษาด้วยสารที่มีฤทธิ์กระตุ้นกระบวนสร้างอนุมูลอิสระ ในการศึกษาก่อนหน้านี้ทางผู้วิจัยได้ทำการศึกษาสารไปเปอร์ลองกูลมินซึ่งเป็นสารสำคัญในสมุนไพรดีปลีพบว่ามีฤทธิ์ในการทำลายเซลล์มะเร็งท่อน้ำดี โดมีผลข้างเคียงต่อเซลล์ปกติค่อนข้างน้อย เนื่องจากไปเปอร์ลองกูลมินมีผลกระตุ้นอนุมูลอิสระในเซลล์มะเร็งมากกว่าเซลล์ปกติ แต่อย่างไรก็ตามการตอบสนองต่อไปเปอร์ลองกูลมินในมะเร็งท่อน้ำดีมีค่อนข้างหลากหลายคือ ตอบสนองได้น้อยบ้างมากบ้างในแต่ละรายของมะเร็งท่อน้ำดี ดังนั้นทางผู้วิจัยได้ศึกษาพบว่าเซลล์มะเร็งท่อน้ำดีแต่ละรายมีความสามารถของระบบต้านอนุมูลอิสระต่างกัน โดยที่เซลล์มะเร็งท่อน้ำดีตอบสนองน้อยต่อไปเปอร์ลองกูลมิน มักมีความสามารถของระบบต้านอนุมูลอิสระสูง จึงทำให้สามารถกำจัดอนุมูลอิสระที่เกิดจากการชักนำด้วยไปเปอร์ลองกูลมินได้ดีกว่าเซลล์มะเร็งท่อน้ำดีที่ตอบสนองต่อไปเปอร์ลองกูลมินได้มาก และเมื่อทำศึกษาโดยกดการทำงานของระบบต้านอนุมูลอิสระในเซลล์มะเร็งท่อน้ำดีที่มีความไวน้อยต่อไปเปอร์ลองกูลมินแล้ว เซลล์มะเร็งจะมีความไวต่อไปเปอร์ลองกูลมินเพิ่มขึ้น จากผลการศึกษานี้แสดงให้เห็นว่าการใช้ไปเปอร์ลองกูลมินร่วมกับการกดกระบวนการต้านอนุมูลอิสระ น่าจะเป็นแนวทางที่สามารถนำไปใช้ในการรักษาผู้ป่วยมะเร็งท่อน้ำดีที่มีภาวะดื้อยาเคมีบำบัดได้

Output จากโครงการวิจัยที่ได้รับทุนจาก สกว.

1. การเสนอผลงานในที่ประชุมวิชาการนานาชาติ European Association for Cancer Research conference : Cancer Genomics 2019 ณ เมือง แคมบริดจ์ สหราชอาณาจักร ระหว่างวันที่ 23-26 พ.ศ. 2562 นั้น โดยจะเสนอผลงานวิจัยในหัวข้อเรื่อง “Induction of heme oxygenase-1 expression promotes piperlongumine resistance via Akt activation in cholangiocarcinoma cell lines”
2. ผลงานตีพิมพ์ที่คาดหวัง ในวารสารวิชาการนานาชาติ
   Enhancement of piperlongumine’ chemosensitivity by silencing HO-1 expression in cholangiocarcinoma cell lines (on process of submission to Biomedicine & Pharmacotherapy, Impact Factor = 3.457 – Quartile 1)