Enhancement of piperlongumine chemosensitivity by silencing heme oxygenase-1 expression in cholangiocarcinoma cell lines

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Abstract. Piperlongumine (PL) produces reactive oxygen species (ROS) and induces G2/M-phase arrest in cholangiocarcinoma (CCA) cells via the JNK/ERK pathway. A differential response to PL was observed among all CCA cell lines However, the underlying mechanisms have remained to be fully elucidated. The aim of the present study was to investigate the molecular mechanisms of PL-induced heme oxygenase-1 (HO-1) expression in CCA cell lines. The anti-proliferative action of PL in the CCA cell lines KKU-100 and KKU-213A was analyzed using sulforhodamine B assays. Reverse transcription-quantitative PCR and western blot analyses were used to examine mRNA and protein expression. HO-1 inhibition was achieved using the chemical inhibitor zinc protophoryn or specific small interfering RNA to HO-1. Intracellular ROS was detected using a 2',7'-dichlorodihydrofluorescein diacetate fluorescence assay. High expression of phase-II detoxification enzymes, including NADPH quinone oxidoreductase-1, heme oxygenase-1, superoxide dismutases and aldo-keto reductase 1 subunits C-1 and 3, were detected in the KKU-100 cell line. Of the CCA cell lines tested, KKU-100 was the least sensitive to PL. Dose-dependent upregulation of HO-1 expression via PI3K/Akt activation was detected in PL-treated CCA cells. Inhibition of HO-1 eliminated the anti-oxidant defense mechanisms, leading to increased anti-cancer activity of PL in the CCA cell lines via an increase in intracellular ROS levels and apoptotic protein expression. These observations indicated that HO-1 inhibition had a chemosensitizing effect on CCA to PL.

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Introduction

Cholangiocarcinoma (CCA) is a malignancy of bile duct epithelial cells. By 2002, CCA is the second most common type of primary liver cancer in most parts of the world (1). CCA has a poor prognosis, with a 5-year survival rate of 5-10% worldwide between 2012 and 2013 (2,3). Surgical resection is the most effective treatment for CCA (1,4). When resection is performed at an early stage, the 5-year survival rate increases to 25-30% between 1997 and 2010 in Japan (4,5). In non-resectable, recurrent and metastatic CCA, various chemotherapeutic agents, including gemcitabine, cisplatin and oxaliplatin, have been used either alone or in combination (5). Resistance to chemotherapy, however, constrains the response rate to 0-40% and median survival to 2-12 months between 1994 and 2002, worldwide (6-8).

The regulation of redox homeostasis is an essential factor in maintaining normal cellular functions and ensuring cell survival (9). High reactive oxygen species (ROS) levels in cancer cells are a consequence of alteration of several signaling pathways linked to tumorigenesis, including stimulation of cellular proliferation, as well as promotion of mutation and genetic instability (9,10). Redox-modulating strategies are a potential treatment for patients with breast, ovarian, lung and pancreatic cancer that may enable therapeutic selectivity and help in overcoming drug resistance (11,12). For instance, cancer cells with an increased level of ROS or decreased antioxidant capacity are more susceptible to oxidative stress-induced cell death (13). Certain anti-cancer agents, including arsenic trioxide, anthracyclines and cisplatin, have been demonstrated to act as ROS-generating agents that cause increased cellular ROS generation (10). These anti-cancer agents are candidates for evaluating the preferential targeting of cancer cells with increased ROS-induced stress (9,10). The present study focused on piperlongumine (PL), a phytochemical that acts as an anti-cancer agent (14-16). PL induces redox dysregulation, selectively killing cancer cells (including CCA) with incremental increases in intracellular ROS (14). The increased sensitivity of cells to PL is associated with the degree of cell transformation (16). In addition, this was demonstrated in previous studies (15) on immortalized cholangiocytes and spontaneously immortalized fibroblasts (15,16). PL-induced ROS generation is dependent on the activation of MAPKs,

including JNK, ERK and p38 (14,15). Various responses to PL have been reported, which may be due to differences in the underlying genetics of the antioxidant defense mechanism in each type of cancer cell (15).

Heme oxygenase-1 (HO-1), an inducible form of HO, was the first rate-limiting enzyme discovered. In mammalian cells, HO-1 degrades cellular heme to release free iron, CO and biliverdin (17). HO-1 is frequently upregulated in numerous types of tumor, including prostate, renal, gastric, colon cancer and CCA (17-21). Upregulation of HO-1 is associated with tumor progression, including tumor growth, metastasis and chemoresistance (20,21). Previous studies have demonstrated that the depletion of critical cytoprotective enzymes in cancer cells (particularly HO-1) enhanced the chemosensitivity of several anti-cancer agents, including gemcitabine, cisplatin and bortezomib (20,22,23). The present study aimed to demonstrate that increased chemosensitivity of CCA may be achieved by a combination of anti-cancer agents, specifically PL targeting HO-1. The hypothesis was that HO-1 may be induced during PL treatment in CCA cell lines and that the suppression of HO-1 by a chemical inhibitor or specific small interfering (si)RNA may increase the level of intracellular ROS and chemosensitivity to PL.

Materials and methods

Materials. Cell culture reagents were from Gibco (Thermo Fisher Scientific, Inc.). PL, 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA; cat. no. D6883), trichloroacetic acid (cat. no. T0699), zinc-protoporphyrin IX (ZnPP; HO-1 inhibitor; cat. no. 691550) and sulforhodamine B (SRB; cat. no. 51402) were obtained from Sigma-Aldrich (Merck KGaA). Specific siRNA to HO-1 (siHO-1; cat. no. sc-35554) and non-targeted negative control siRNA (siCon; cat. no. sc-37007) were purchased from Santa Cruz Biotechnology, Inc. DharmaFect 1 siRNA transfection reagent (cat. no. T-2001-20) was purchased from GE Healthcare Dharmacon, Inc. Primary antibodies were obtained from Cell Signaling Technology, Inc., including total Akt (60 kDa) (cat. no. 4685S; 1:1,000), serine 473-phosphorylated Akt (pAkt; 60 kDa) (cat. no. 4060S; 1:1,000), poly(ADP-ribose) polymerase (PARP; 116/89 kDa) (cat. no. 9542; 1:1,000), lamin B1 (68 kDa) (cat. no. 13435; 1:1,000) and Bcl-2 (28 kDa) (cat. no. 4223; 1:1,000). Antibodies to HO-1 (32 kDa) (cat. no. sc-136960; 1:500), nuclear factor erythroid 2-related factor 2 (Nrf2; 110 kDa) (cat. no. sc-365949; 1:500), Bax (23 kDa) (cat. no. sc-526; 1:500), and β-actin (42 kDa) (cat. no. sc-47778; 1:2,000) were purchased from Santa Cruz Biotechnology, Inc. Mouse anti-rabbit IgG-horseradish peroxidase (HRP) (cat. no. NXA931; 1:2,000) and donkey anti-rabbit IgG-HRP (cat. no. NA934V; 1:2,000) secondary antibodies were obtained from Cytiva. The Luminata™ Forte Western HRP substrate detection reagents (cat. no. WBLUF0100) were purchased from Merck KGaA. The Superscript VILO™ cDNA synthesis kit (cat. no. 11754-050) was purchased from Invitrogen (Thermo Fisher Scientific, Inc.). The LightCycler® 480 RT-PCR System and the LightCycler® 480 SYBR Green I master mix (cat. no. 04707516001) were from Roche Diagnostics GmbH. Wortmannin (cat. no. 9951S) was purchased from Cell Signaling Technology, Inc.

Cell culture and transfections. A total of 2 human CCA cell lines (KKU-100 and KKU-213A) had been established from tumor of patients with CCA with liver-fluke infection admitted to Srinagarind Hospital, Khon Kaen University (Khonkaen, Thailand), as described previously by Sripa *et al* (24,25). Certificates of analyses were obtained from the Japanese Collection of Research Bioresources Cell Bank. Cells were cultured in Ham's F12 medium (cat. no. 21700-075; Gibco; Thermo Fisher Scientific, Inc.) supplemented with 1% penicillin-streptomycin (cat. no. 15140-122; Gibco; Thermo Fisher Scientific, Inc.) and 10% FBS (cat. no. 10270-098; Gibco; Thermo Fisher Scientific, Inc.). Cells were incubated at 37˚C in a humidified atmosphere containing 5% CO_2. Cells with 70-80% confluence at 24 h were trypsinized with 0.25% trypsin-EDTA and subcultured in the same media. Mycoplasma testing with MycoAlert mycoplasma detection kit (cat. no. LT07-418; Lonza Rockland, Inc.) was conducted for the cell lines used.

Inhibition of HO-1 was performed by transfecting HO-1 siRNA into the cell lines KKU-100 and KKU-213A. Cells were seeded into 6-well plates at a seeding density of 3-4x10^5 cells/well and incubated overnight. Cells were transfected with 10 μM of siHO-1 or siCon using DharnaFect 1 siRNA transfection reagent for 24 or 48 h. The transfection procedure was performed according to the manufacturer's protocol.

Drug treatments. A stock concentration of 50 mM PL, 5 mM ZnPP and 2 mM wortmannin was prepared in DMSO and stored in aliquots at -20˚C until use. Various concentrations of PL (0.01, 0.1, 1, 10, 25, 50 and 100 μM) or ZnPP (0, 1, 5 and 10 μM) were diluted with cell culture media for subsequent experiments. The vehicle control was DMSO with 0.001% concentration used in the preparation of the PL, ZnPP or wortmannin working solutions. For the combination treatment of PL and ZnPP or PL and wortmannin, cells were pre-treated with ZnPP (0, 1, 2.5, 5.0, 10, 25, 50 and 100 μM) for 3 h or wortmannin (1, 2 and 5 μM) for 2 h. Then, ZnPP or wortmannin were removed and cultured for 24 h or were removed prior to being treated with various concentrations of PL (0, 0.01, 0.1, 1, 10, 25, 50 and 100 μM; or 0, 10 and 20 μM, respectively) for 24 h. For the PL treatment after transfection with siHO-1 or siCon, transfected cells were seeded at 5x10^3 cells per well into a 96 well plate. At 24 h after seeding, cells were treated with a range of concentrations of PL from 0, 5, 10 and 20 μM for 24- h. Treated cells were subsequently tested for cell viability at 48 h, intracellular ROS and assessed by reverse transcription-quantitative PCR (RT-qPCR) and western blot analysis.

Cell viability. KKU-100 and KKU-213A cells were seed at 5x10^3 cells per well into a 96-well plate. At 24 h after seeding, the CCA cell lines were treated with drug as aforementioned and incubated for 24 h. Cell viability was measured using an SRB assay capable of determining cell density based on the measurement of cellular protein content, performed according to Voigt with slight modifications (26). In brief, cells were fixed with 10% trichloroacetic acid for overnight, washed 5 times with distilled water and stained with 0.4% SRB in 1% acetic acid for 30 min. Plates were washed 5 times with 1% acetic acid, air-dried and then solubilized the protein-bound dye with 100 μl of unbuffered 10 mM Tris solution (pH=10). The absorbance was measured at 564 nm using a microplate

Table I. Sequences of the primers used for reverse transcription-quantitative PCR.

Gene	Forward (5'-3')	Reverse (5'-3')
Nrf2	TACTCCCAGGTTGCCCACA	CATCTACAAACGGGAATGTCTGC
GCLM	GACAAAACACAGTTGGAACAGC	CAGTCAAATCTGGTGGCATC
GCLC	ATGCCATGGGATTTGGAAT	AGATATACTGCAGGCTTGGAATG
AKR1C1	CATGCCTGTCCTGGGATTT	AGAATCAATATGGCGGAAGC
AKR1C3	CATTGGGGTGTCAAACTTCA	CCGGTTGAAATACGGATGAC
HO-1	CAACATCCAGCTCTTTGAGGA	GGGCAGAATCTTGCACTTTG
NQO1	GATATTCCAGTTCCCCCTGC	TTCTTACTCCGGAAGGGTCC
TXN	GAGAGCAAGACTGCTTTTCA	CAGAGAGGGAATGAAAGAAAG
GSTP1	TACACCAACTATGAGGCGGG	AGCGAAGGAGATCTGGTCTC
SOD2	GTTGGCCAAGGGAGATGTTAC	AGCAACTCCCCTTTGGGTTC
PARK7	CGAGCTGGGATTAAGGTCA	CATATGGTCCCTCTTTTTTTGC
β-actin	GATCAGCAAGCAGGAGTATGACG	AAGGGTGTAACGCAACTAAGTCATAG

Nrf2, nuclear factor erythroid 2-related factor 2; GCLM, γ-glutamylcysteine synthetase modifier subunit; GCLC, γ-glutamylcysteine synthetase catalytic subunit; AKR1C1 and 3, aldo-keto reductase 1 subunits C-1 and 3; HO-1, heme oxygenase-1; NQO1, NADPH quinone oxidoreductase-1; TXN, thioredoxin; GSTP1, glutathione S-transferase P1; SOD2, superoxide dismutase 2; PARK7, Parkinson disease protein 7 precursor.

reader (Bio-Rad Laboratories, Inc.). The cell viability was calculated as follows: Cell viability (%) = [optical density at 564 nm (OD564) in treatment wells]/(OD546 in control wells) x 100. Each experiment was performed independently in triplicate. The half-maximal inhibitory concentration (IC_{50}) values were calculated using GraphPad Prism software (version 5.0; GraphPad Software, Inc.).

Measurement of intracellular accumulation of ROS. The production of intracellular ROS was detected using the DCFH-DA fluorescence assay, as previously described (10). In brief, KKU-100 cells were used for detecting ROS production. Thus, transfected KKU-100 cells ($2x10^5$ cells) were seeded into 6-well plates and stored overnight for 30 h post-transfection. Cells were then treated with PL at 0, 10 and 20 μM for 12 h. Following treatment, the intracellular ROS assay was performed by incubation with 20 μM of DCFH-DA in a humidified atmosphere with 5% CO_2 at 37˚C for 30 min. After washing with 1X PBS, cells were trypsinized and re-suspended in 1X PBS. The fluorescence intensity of the DCFH-DA was determined with a flow cytometer.

RT-qPCR. KKU-100 and KKU-213A cells that underwent drug treatments or transfection were harvested using TRIzol reagent for RNA preparation. In brief, total RNA was extracted using TRIzol reagent (cat. no. 15596; Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. First-stand complementary (c)DNA was synthesized using a Superscript VILO™ cDNA synthesis kit (Invitrogen; Thermo Fisher Scientific, Inc.) with 2 μg total RNA according to the manufacturer's protocol. The basal mRNA expression of 11 antioxidant-associated genes including nuclear factor erythroid 2-related factor 2 (Nrf2), NADPH quinone oxidoreductase-1 (NQO-1), heme oxygenase-1 (HO-1), superoxide dismutase 2 (SOD2), glutathione S-transferase P1 (GSTP1), aldo-keto reductase 1 subunits C-1 and 3 (AKR1C1 and AKR1C3), γ-glutamylcysteine synthetase catalytic subunit (GCLC) and γ-glutamylcysteine synthetase modifier subunit (GCLM), Parkinson disease protein 7 precursor (PARK7), thioredoxin (TXN) was investigated in the two CCA cell lines, which have differential responses to PL; KKU-100 (CCA less sensitive to PL) and KKU-213A (CCA highly sensitive to PL). The sequences of the primers used are listed in Table I. Real-time PCR was performed using the LightCycler® 480 RT-PCR System and the LightCycler® 480 SYBR-Green I master mix (Roche Diagnostics GmbH). The thermocycling conditions were as follows, according to the LightCycler 480 manufacturer's instructions: 95˚C for 5 min; 95˚C for 10 sec, annealing at 60˚C for 10 sec and extension at 72˚C for 10 sec, for 45 cycles. The specificity of each of the PCR products was confirmed via melting curve analysis. Relative mRNA expression was obtained following normalization to endogenous human β-actin and quantified using the $2^{-\Delta\Delta Cq}$ methods (27).

Protein preparation. The cell lines KKU-100 and KKU-213A were cultured in 6-well plates at $3\text{-}4x10^5$ cells/well for 24 h at 37˚C in a humidified atmosphere containing 5% CO_2. Cells were then treated with siRNA and/or various concentrations of PL (0, 5, 10 or 20 μM) for 0, 1, 3, 6 or 24 h. Following incubation, the whole-cell lysate was harvested as previously described by Thongsom *et al* (15). Nuclear and cytoplasmic fractions were extracted using a nuclear extraction kit (cat. no. 90498; Chemicon; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The whole-cell lysate and nuclear and cytoplasmic fractions were then tested for their respective protein concentrations using a Pierce® BCA protein assay kit (cat. no. 23225; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol.

SDS-PAGE and western blot analysis. Protein samples from the whole-cell lysate, nuclear or cytoplasmic fraction with a mass of 20 μg were separated using 10% SDS-PAGE. The proteins were transferred to a nitrocellulose membrane and

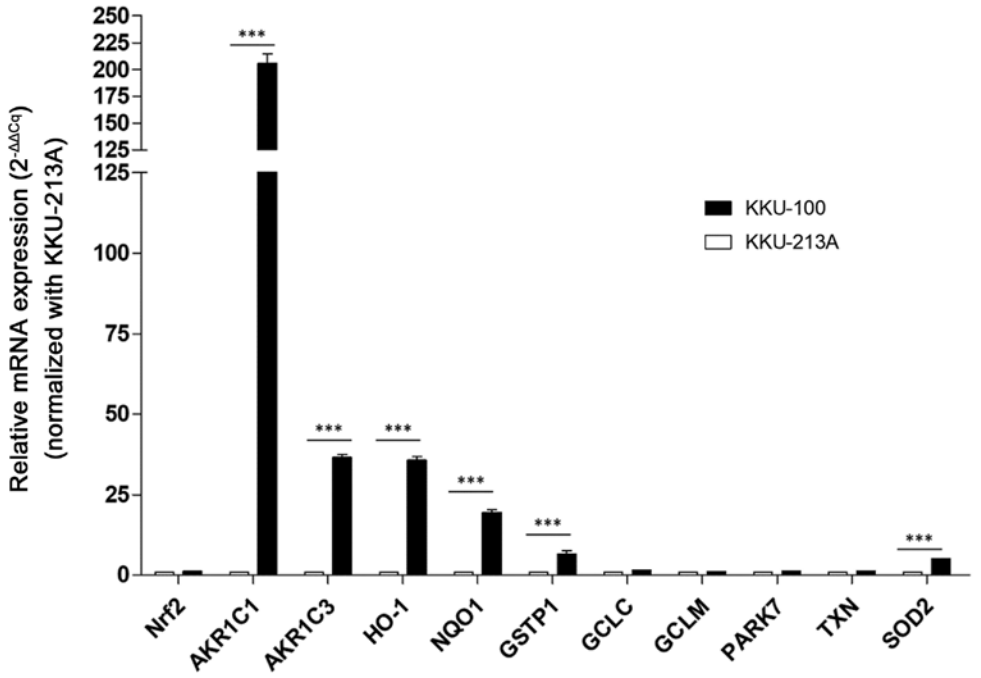

Figure 1. Antioxidant expression profiles in the cholangiocarcinoma KKU-100 and KKU-213A cell lines. mRNA expression of all antioxidant-associated genes was assessed using reverse transcription-quantitative PCR with normalization to β-actin used as the reference gene. mRNA expression of all genes was calculated using the $2^{-\Delta\Delta Cq}$ method. Values are expressed as the mean ± standard error of the mean of two independent experiments. ***P<0.001 vs. KKU-213A. Nrf2, nuclear factor erythroid 2-related factor 2; AKR1C1/3, aldo-keto reductase 1 subunits C-1/3; HO-1, heme oxygenase 1; NQO1, NADPH quinone oxidoreductase-1; GSTP1, glutathione S-transferase P1; GCLC, γ-glutamylcysteine synthetase catalytic subunit; GCLM, γ-glutamylcysteine synthetase modifier subunit; PARK7, Parkinson disease protein 7 precursor; TXN, thioredoxin; SOD2, superoxide dismutase 2.

blocked for 1 h with 5% (w/v) skimmed milk in 1X PBS supplemented with 0.05% Tween-20 (PBST). The membranes were then incubated overnight at 4˚C with primary antibodies at dilutions of 1:500 for HO-1, Bax and Nrf2 proteins; 1:1,000 for PARP, Bcl-2, pAkt (Ser473), total Akt and Lamin B1 proteins; or 1:2,000 for β-actin protein in PBST. The membranes were then incubated with HRP-conjugated secondary antibodies at a dilution of 1:2,000 for 1 h at room temperature. Luminata™ Forte Western HRP substrate (Merck KGaA) was applied for protein detection. The densities of the bands for HO-1, cleaved PARP, Bcl-2, Bax, Nrf2, Akt and pAkt were determined using Image J software version 1.52v (National Institutes of Health) and normalized to β-actin for whole cell lysate or normalized to lamin B1 for nuclear extracts. The respective ratio of each protein to β-actin, the ratio of pAkt/Akt, the ratio of cleaved PARP/Bcl-2 and ratio of Bcl-2/Bax were calculated.

Statistical analysis. All experiments were performed 2-3 times and the results are presented as the mean ± standard error of the mean. Statistical analyses were performed using GraphPad Prism software (version 5.0; GraphPad Software, Inc.). The Student's t-test was used for between-group statistical analyses. One-way and two-way analysis of variance followed by Bonferroni's correction were applied for statistical analysis of multiple groups. P<0.05 was considered to indicate a statistically significant difference.

Results

Expression levels of phase II detoxification enzymes and antioxidant proteins in CCA cell lines. Nrf2-mediated cytoprotective genes are thought to be a primary antioxidant defense mechanism in mammalian cells. The process eliminates harmful ROS or carcinogens, particularly phase II detoxification enzymes and antioxidant proteins (10). In the present study, basal mRNA expression of 11 antioxidant-associated genes was investigated in two CCA cell lines previously (15) indicated to have differential responses to PL: KKU-100 (CCA less sensitive to PL) and KKU-213A (CCA highly sensitive to PL). The results demonstrated that mRNA expression levels of phase II detoxification enzymes, including NADPH quinone oxidoreductase-1 (NQO-1), heme oxygease-1 (HO-1), superoxide dismutase 2 (SOD2), glutathione S-transferase P1 (GSTP1) and aldo-keto reductase 1 subunits C-1 and 3 (AKR1C1 and AKR1C3) were significantly higher in KKU-100 compared with KKU-213A (Fig. 1). Furthermore, the basal expression levels of Nrf2 and other Nrf2-mediated cytoprotective genes, including PARK7, thioredoxin TXN and particularly enzymes involved in glutathione synthesis, including GCLC and GCLM, were not significantly different. These results indicated that the mechanism of action for antioxidant defense may depend on the genetic background of each CCA cell line.

Induction of HO-1 expression following PL treatment. To evaluate the role of Nrf2-mediated cytoprotective genes in the responses to PL treatment, CCA cell lines were treated with 10 μM PL or DMSO (control) for 6 h. The respective mRNA expression of Nrf2-mediated cytoprotective genes (AKR1C1, AKR1C3, NQO-1, HO-1, GCLC and GCLM) was determined using RT-qPCR. The results revealed that the expression levels of HO-1, GCLC and GCLM were significantly increased in

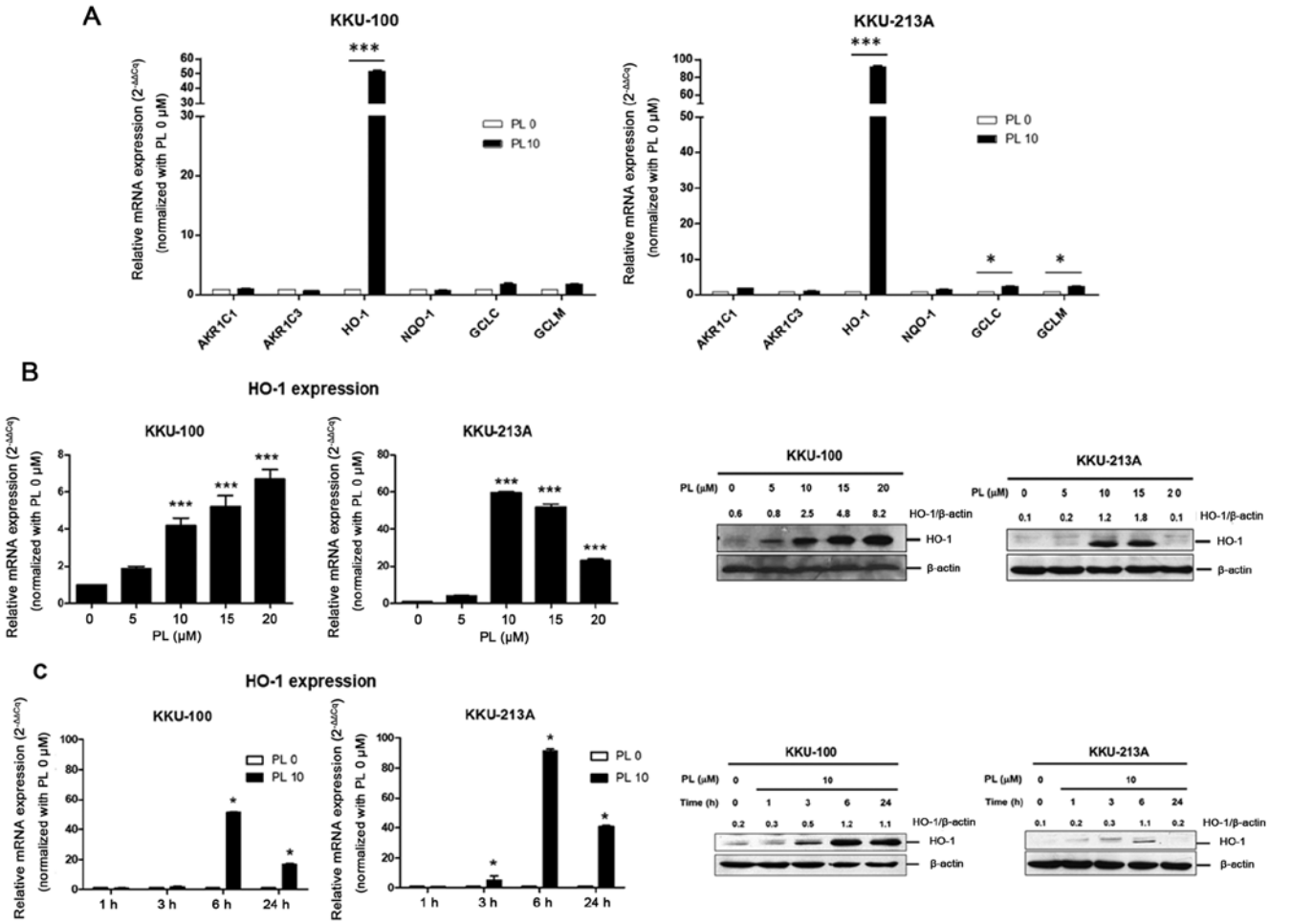

Figure 2. PL induces HO-1 expression in cholangiocarcinoma cell lines. (A) KKU-100 and KKU-213A were treated with PL at 10 μM or DMSO (control) for 6 h and the mRNA expression of AKR1C1, AKR1C3, NQO1, HO-1, GGCL and GCLM was determined using RT-qPCR. Results were normalized using β-actin as the reference gene. Relative mRNA expression was calculated using the $2^{-\Delta\Delta Cq}$ method. KKU-100 and KKU-213A treated with (B) PL (at 0, 5, 10, 15 or 20 μM) for 24 h or (C) 10 μM PL for 0,1, 3, 6 or 24 h. The relative mRNA and protein expression of HO-1 was determined using RT-qPCR ($2^{-\Delta\Delta Cq}$) and western blot analysis, respectively. Values are expressed as the mean ± standard error of the mean of 3 independent experiments. Protein expression is presented as the mean of two independent experiments. *P<0.05 and ***P<0.001 vs. PL 0 μM. PL, piperlongumine; AKR1C1 and 3, aldo-keto reductase 1 subunits C-1 and 3; NQO1, NADPH quinone oxidoreductase-1; HO-1, heme oxygenase 1; GCLC, γ-glutamylcysteine synthetase catalytic subunit; GCLM, γ-glutamylcysteine synthetase modifier subunit; RT-qPCR, reverse transcription-quantitative PCR.

KKU-213A; however, only HO-1 expression was significantly increased in KKU-100 following PL treatment. Therefore, HO-1 was the only Nrf2-mediated cytoprotective gene whose expression was increased in both CCA cell lines following PL treatment (Fig. 2A).

Subsequently, HO-1 expression in the CCA cell lines in the presence of PL at various concentrations (0, 5,10, 15 and 20 μM) for 24 h or 10 μM of PL for various lengths of time (0, 1, 3, 6 and 24 h) was examined. The expression of HO-1 at the mRNA and protein levels in KKU-100 and KKU-213A cell lines following PL treatment was altered in a dose-dependent manner; however, while HO-1 expression increased in KKU-100, expression of HO-1 in KKU-213A peaked at 10 μM and subsequently decreased (Fig. 2B). In addition, the induction of HO-1 expression increased in response to PL treatment for different durations, particularly at 6 h and then declined following 24 h of PL treatment (Fig. 2C). These results indicated that PL preferentially triggered the induction of HO-1 expression in CCA cell lines and that the activation of HO-1 expression may be an early antioxidant defense in response to PL treatment.

HO-1 silencing promotes PL-induced CCA cell death by increasing ROS accumulation. Subsequently, it was investigated whether HO-1 acts as a key antioxidant defense for protecting from PL-mediated ROS generation in CCA cells. ZnPP, a chemical HO-1 inhibitor, was utilized to inhibit HO-1 activity in KKU-100 and KKU-213A. The effect of HO-1 inhibition was then evaluated with respect to PL-induced cytotoxicity. As ZnPP acts as an enzymatic substrate of HO-1, it competes with heme for HO-1, which leads to decreasing levels of CO and bilirubin that act as an antioxidant defense (28). As previous studies have demonstrated that ZnPP is a cytotoxic agent and exhibits anti-tumor activity (29-31), the cytotoxicity of ZnPP was examined. The results demonstrated that >10 μM ZnPP suppressed CCA cell viability, with <50% viable cells remaining in the KKU-100 following 3 h of treatment, whereas <50% cell viability in the KKU-213A was observed at ZnPP >25 μM (Fig. 3A). Subsequently, cell lines were pre-treated with 0-10 μM ZnPP for 3 h in combination with 0-100 μM PL treatment to examine the effect of PL during HO-1 suppression by ZnPP. The respective IC_{50} values of PL

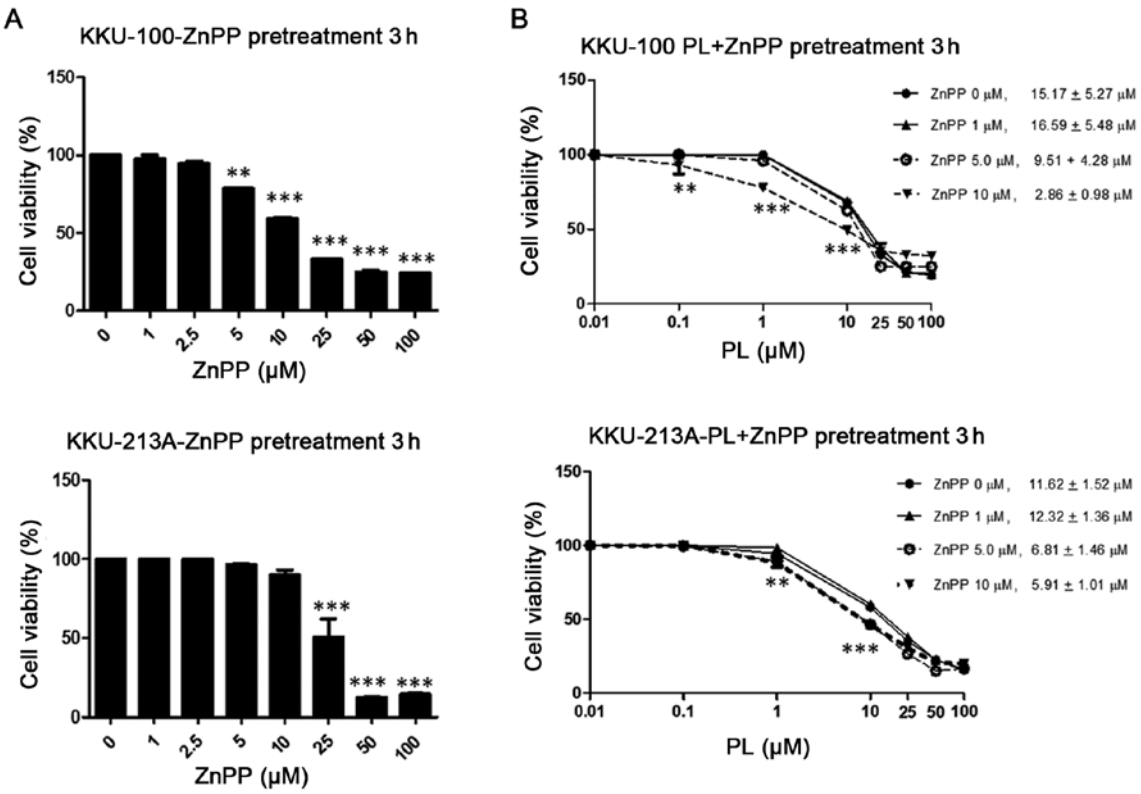

Figure 3. Inhibition of heme oxygenase 1 by ZnPP increases the anti-tumor activity of PL in cholangiocarcinoma cell lines. (A) KKU-100 and KKU-213A were pre-treated with ZnPP at various concentrations for 3 h then cultured without ZnPP for 24 h. (B) KKU-100 and KKU-213A were pre-treated with ZnPP at 0, 1, 5 and 10 μM for 3 h. ZnPP was then removed and cells were treated with 10 μM PL for 24 h. Cell viability was measured using sulforhodamine B assays. Values are expressed as the mean ± standard error of the mean of 3 independent experiments. **P<0.01 and ***P<0.001 vs. ZnPP 0 μM. ZnPP, zinc-protoporphyrin IX; PL, piperlongumine.

were reduced compared with the combination of ZnPP at 5 and 10 μM in both KKU-100 and KKU-213A cells (Fig. 3B).

Following this, HO-1 knockdown was used 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-213A were significantly decreased following transfection with HO-1 siRNA at the time-points of 24 and 48 h (Fig. 4A); however, suppression of HO-1 was not influenced by CCA cell growth (Fig. 4B). The knockdown of HO-1 significantly enhanced the anti-tumor activity of PL in a dose-dependent manner for both KKU-100 and KKU-213A (Fig. 4C). In addition, the combination of HO-1-silencing with PL treatment at 20 μM resulted in a significant increase in the accumulation of intracellular ROS. This result indicated that the effective dose of PL to induce ROS accumulation in KKU-100 was 20 μM (Fig. 4D). Response to combination treatments in KKU-100 at 12 h was detectable via western blot analysis, which confirmed the upregulation of apoptotic proteins (cleaved PARP) and downregulation of anti-apoptotic proteins (Bcl-2; Fig. 4E and F). Furthermore, the increase of PL-induced CCA apoptosis through HO-1 suppression was clearly demonstrated by a high ratio of cleaved PARP/Bcl-2 and a low ratio of Bcl-2/Bax (Fig. 4F). Therefore, inhibition of HO-1 promoted PL-mediated ROS generation, leading to PL-induced CCA cell apoptosis.

PL induces Nrf2-mediated HO-1 expression via activation of the PI3K/Akt pathway. Previous studies have reported that the PI3K/Akt pathway acts as a survival signal against multiple apoptotic insults and is hypothesized to be a major upstream signaling event prior to the induction of Nrf2-mediated HO-1 expression (32,33). Furthermore, Lee *et al* (34) demonstrated that PL directly binds to cysteine residues by a thiol modification within kelch-like ECH-associated protein (Keap1) and that this direct binding promoted nuclear translocation of Nrf2 and subsequent upregulation of HO-1 expression. The activation/phosphorylation of Akt in PL-treated CCA cell lines was determined in order to elucidate the role of PI3K/Akt in PL-induced Nrf2 activation and HO-1 expression. HO-1 and Akt phosphorylation increased in response to PL treatment (Fig. 5A). Nuclear Nrf2 was also observed in a dose-dependent manner (5, 10 and 20 μM; Fig 5A). To demonstrate the association between PI3K/Akt signaling and HO-1 expression, various concentrations (1, 2 and 5 μM) of wortmannin, a specific inhibitor of PI3K, were used to inhibit PI3K/Akt activation. The level of HO-1 expression was then determined via western blot analysis. Wortmannin significantly inhibited Akt phosphorylation and PL-induced HO-1 expression in CCA cells in a dose-dependent manner (Fig. 5B). These results indicated that PL-induced HO-1 expression is stimulated via Nrf2/PI3K/Akt activation.

Discussion

PL exerts an anti-tumor effect on various types of cancer, including glioblastoma, lung and CCA (14,15,35). PL inhibits

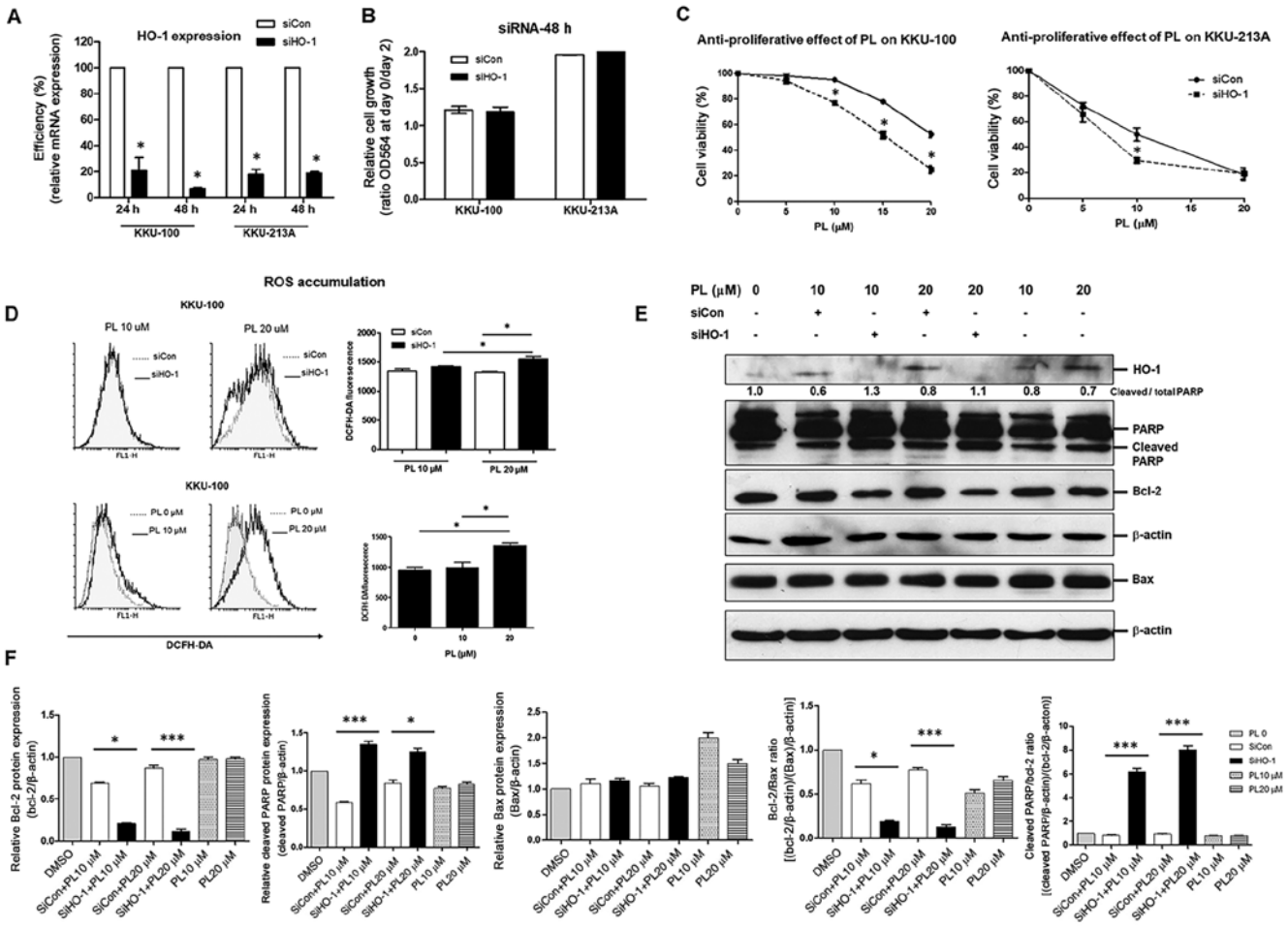

Figure 4. Knockdown of HO-1 by siRNA sensitizes cholangiocarcinoma cell lines to PL. KKU-100 and KKU-213A were transfected with siHO-1 or siCon for 24 and 48 h. (A) The efficiency of siHO-1 knockdown at 24 and 48 h relative to siCon was determined using reverse transcription-quantitative PCR. (B) Proliferative effect at 48 h and (C) the anti-proliferative effect of PL at various concentrations at 24 h were determined. (D) Reactive oxygen species accumulation at 3 (left graph) and 12 h (right graph). (E) Apoptotic and anti-apoptotic proteins at 12 h were determined and compared between HO-1-knockdown cells and controls. (F) Relative protein levels of cleaved PARP and Bcl-2, Bax and the Bcl-2/Bax ratio and cleaved PARP/Bcl-2 ratios were determined. Values are expressed as the mean ± standard error of the mean of 3 independent experiments. *P<0.05 and ***P<0.001 vs. siCon. HO-1, heme oxygenase 1; siRNA, small interfering RNA; PL, piperlongumine; siHO-1, HO-1 siRNA; siCon, siRNA control (scrambled); PARP, poly(ADP-ribose) polymerase; DCFH-DA, 2',7'-dichlorodihydrofluorescein diacetate; OD564, optical density at 564 nm.

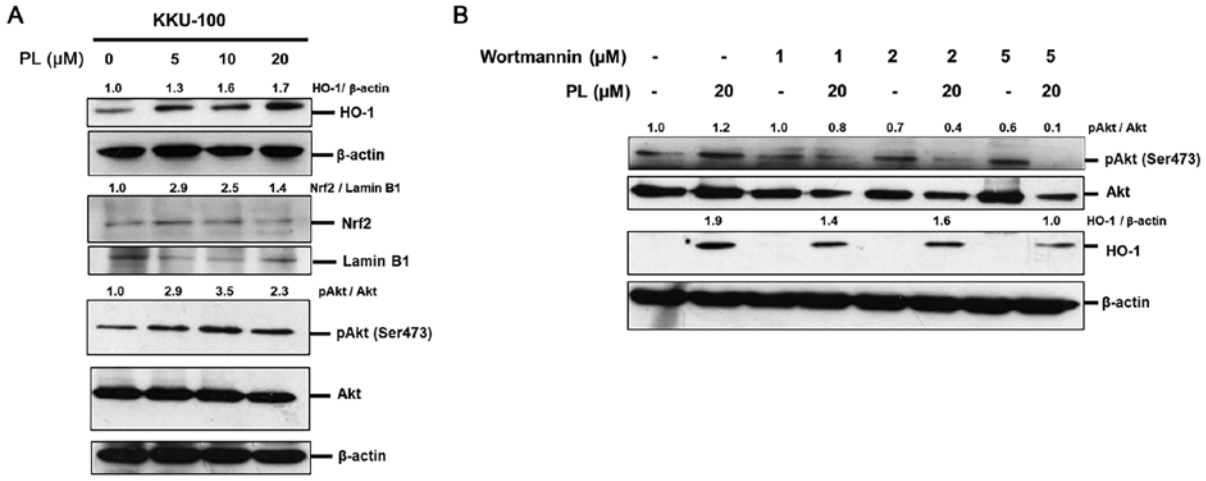

Figure 5. PL induces HO-1 expression via PI3K/Akt activation. (A) KKU-100 was treated with various concentrations of PL (0, 5, 10 or 20 μM) for 3 h. Whole-cell lysates (for HO-1, Akt and β-actin) and nuclear extracts (for Nrf2 and Lamin B1) were analyzed via western blotting and probed with antibodies specific to HO-1, Akt, pAkt (Ser473), Nrf2 and β-actin. (B) The effects of the Akt signaling inhibitor wortmannin on PL-induced HO-1 expression were determined. KKU-100 was pre-treated with various concentrations of wortmannin (1, 2 and 5 μM) for 2 h and treated with 20 μM PL for an additional 24 h. The expression of HO-1, Akt, pAkt and β-actin were determined via western blot analysis. Values are expressed as the mean of 2 independent experiments. PL, piperlongumine; pAkt, phosphorylated Akt; HO-1, heme oxygenase 1; Nrf2, nuclear factor erythroid 2-related factor 2.

tumor growth via the induction of ROS accumulation and the activation of MAPKs (including JNK, ERK and p38) (14,15) or the inhibition of the PI3K/Akt pathway (35,36). Sensitivity to PL varies among different cancer cell lines, including breast cancer and CCA cell lines (15,34). The present study aimed to investigate the underlying mechanisms of PL-induced HO-1 expression. Several previous studies have demonstrated that HO-1 has a powerful cytoprotective effect against various apoptotic insults in normal and cancer cells (19-23). The basal HO-1 expression levels of each type of cancer cell indicate its sensitivity to chemotherapeutic agents (20,21). Similar observations were reported for CCA cells treated with PL, as cells with a low basal level of HO-1 expression were more sensitive to PL compared with those with high HO-1 expression (15). In addition, in the present study, HO-1 inducible expression was evident following 3 and 6 h of PL treatment in all CCA cell lines. This result indicated that induction of HO-1 expression serves a vital role in the early protection against the PL-induced reaction to oxidative stress. The results of the present study are consistent with those of studies on well-known chemotherapeutic agents (including gemcitabine and cisplatin), which mediate oxidative stress and lead to induced HO-1 expression in CCA and laryngeal squamous cell cancer (20,23).

The PI3K/Akt pathway is involved in cellular survival, metastasis and drug resistance in various types of cancer including CCA, pancreatic and oral cancer (37-41). Development of acquired resistance to radiation, chemotherapy and/or targeted therapy has been revealed to be associated with the induction of the PI3K/Akt pathway (41,42). In addition, PI3K/Akt signaling is a key pathway for the activation of HO-1 through the Nrf2/Keap pathway. Certain studies have demonstrated that PL bears two electrophilic α,β-unsaturated carbonyl groups that cause oxidation or covalent modification of cysteine residues within Keap1. PL directly binds to cysteine residues within Keap1 by thiol modification (34). This direct binding was observed to promote nuclear translocation of Nrf2 and subsequent upregulation of HO-1 expression (34,40). Inhibition of this pathway has been proposed to increase the chemosensitivity of CCA (38,43). The present study revealed that PL treatment resulted in Akt phosphorylation and Nrf2 activation, suggesting that PL induced HO-1 expression via PI3K/Akt/Nrf2 activation. However, these results are preliminary and should be confirmed in a future study. In contrast to PL-induced antioxidant defense in CCA, PL stimulated oxidative stress via ROS generation and induced CCA apoptosis through the activation of the ROS/JNK/ERK pathway (15). These results indicated that in an oxidative stress environment, i.e. that caused by PL, CCA cells upregulate anti-oxidant defenses by inducing PI3K/Akt-mediated HO-1 expression and enhance the anti-apoptotic capacity in order to protect against PL-induced ROS generation. As Keap1 was not assessed in PL-induced Nrf2 activation and HO-1 expression in the present study, investigating the effect of PL on the Nrf2/Keap1 pathway in CCA is required for further study.

HO-1 is a powerful antioxidant enzyme in Nrf2-mediated cytoprotective responses and serves a role in the malignant transformation of cancer cells (10). High levels of HO-1 are associated with the progression of CCA and its therapeutic resistance (20,21). Concurrently, suppression of HO-1 activity has been observed to enhance the chemosensitivity of cancer cells to various chemotherapeutic agents including cisplatin and gemcitabine (20,23,44). The results of the present study demonstrated that inhibition of HO-1 activity using chemical inhibitors or specific siRNA to HO-1 increased the anti-tumor activity of PL against CCA cell lines through an increase in intracellular ROS accumulation and increased CCA cell death via upregulation of apoptotic proteins. In the present study, the increase of intracellular ROS was not significantly different between siCon KKU-100 cells treated with PL at 10 and 20 μM. This may be due to the insufficient concentration of PL, as the IC_{50} value of PL in KKU-100 is 15.9 μM at 24 h (15), or PL may have altered the activity of a redox-sensitive enzyme/transcription protein. In summary, the results of the present study suggest that induction of HO-1 expression may provide a significant antioxidant defense to PL treatment and the level of basal HO-1 expression indicates the efficiency of PL for treating CCA. It should be noted that the CCA cell lines used in the present study were established from tumors of patients with CCA with liver-fluke infections. Therefore, the results may not be generalizable to patients with non-liver fluke-associated CCAs. From a technical point, the experiments should ideally be performed three times. However, the determination of protein expression via western blot analysis was performed only twice. This limitation could influence the interpretation of the findings; thus, future studies are required to confirm.

In conclusion, in a PL-induced oxidative stress environment, PI3K/Akt-mediated HO-1 activation served an important role in antioxidant defenses, thereby protecting CCA from PL-induced apoptosis. The results demonstrated that suppression of HO-1 resulted in increased intracellular ROS generation and CCA cell apoptosis induced by PL. The present results provide strong evidence that the mechanism of PL-induced HO-1 expression in CCA and inhibition of HO-1 may be a potential strategy for increasing the chemosensitivity of CCA to PL.

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Availability of data and materials

All data generated or analyzed during the present study are included in this published article.

Authors' contributions

CT and SW conceived and designed the present study. CT performed the experiments. CT, KT and SW analyzed the data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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