scientific reports

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CDK4/6 inhibitors upregulate cIAP1/2, and Smac mimetic LCL161 enhances their antitumor effects in cholangiocarcinoma cells

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Cholangiocarcinoma (CCA) is a highly aggressive bile duct cancer with a poor prognosis and high mortality rates, primarily due to the lack of early diagnosis and effective treatments. We have shown that cyclin D and CDK4/6, key regulators of cell cycle progression, are highly expressed in CCA patients. Moreover, high levels of cyclin D, CDK4, and CDK6 are associated with shorter survival in CCA patients, suggesting that cyclin D and CDK4/6 might be potential targets for CCA therapy. However, we have demonstrated that CDK4/6 inhibitor palbociclib monotherapy is less effective in CCA cells. We have identified Cellular Inhibitor of Apoptosis Proteins 1 and 2 (cIAP1/2), NF-κB target genes that their expression is associated with shorter survival in CCA patients, as potential key regulators of the CDK4/6 inhibitor response. We showed that palbociclib, a CDK4/6 inhibitor, increases phosphorylated p65 and its nuclear translocation, resulting in cIAP1/2 upregulation in CCA cells. Therefore, we hypothesized that the combination of a cIAP1/2 antagonist and a CDK4/6 inhibitor might enhance the CDK4/6 inhibitor response. Interestingly, combined treatment with the Smac mimetic LCL161, a cIAP1/2 antagonist, and palbociclib synergistically inhibits cell proliferation and induces cell death in both 2D monolayer and 3D spheroid CCA cultures. We further showed that this combination treatment has less effect on non-tumor cholangiocytes and human peripheral blood mononuclear cells (PBMCs). Our findings demonstrate for the first time that the combined treatment of Smac mimetics and CDK4/6 inhibitors is a promising novel targeted therapy for CCA patients.

Keywords Cholangiocarcinoma, Targeted therapy, CDK4/6 inhibitors, Smac mimetics, NF-κB signaling

Abbreviations

CCA	Cholangiocarcinoma
cIAP1/2	Cellular inhibitor of apoptosis proteins 1 and 2
PBMCs	Peripheral blood mononuclear cells
CDK	Cyclin dependent kinase
SMAC	Second mitochondria-derived activator of caspases
RIPK3	Receptor-interacting protein kinase 3
MLKL	Mixed lineage kinase domain-like
Rb	Retinoblastoma
TNF-α	Tumor necrosis factor-α
GEO	Gene expression omnibus
NODE	National omics data encyclopedia
TCGA-CHOL	The cancer genome atlas-cholangiocarcinoma
DMEM	Dulbecco's modification of eagle's medium
PBS	Phosphate buffered saline
DMSO	Dimethylsulfoxide

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PI	Propidium iodide
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
TBS-T	Tris-buffered saline, 0.5% Tween20
BSA	Bovine serum albumin
IC_{50}	Half-maximal inhibitory concentration
S.D.	Standard deviation
BIRC	Baculoviral IAP repeat containing
CI	Combination index

Cholangiocarcinoma (CCA) is a highly heterogeneous biliary malignancy that originates from the aberrant division of biliary tree epithelial cells[1]. Currently, the incidence of CCA is increasing globally, particularly in the northeastern region of Thailand[2]. CCA is the second most common type of liver cancer and is often caused by *Opisthorchis viverrini* (food-borne liver fluke) infection. Other contributing risk factors include primary sclerosing cholangitis (PSC), hepatolithiasis, and hepatitis B and C infections[3]. CCA is an aggressive cancer with a poor prognosis and a high mortality rate of more than 6 cases/100,000 habitants per year, with a 5-year survival rate of around 5–10%[4]. The majority of early-stage CCA patients are asymptomatic; thus, patients often present clinically at an advanced stage, leading to high mortality due to standard treatment failure[5,6]. Surgical resection is the best curative procedure for early-stage CCA patients, but most are diagnosed at advanced stages, making tumor resection unfeasible. Therapeutic options for advanced-stage CCA patients are limited, with chemotherapy being the only option, which is often ineffective and leads to chemoresistance[7]. The standard chemotherapy for CCA, including gemcitabine or gemcitabine combined with cisplatin, benefits only about 20% of cases. Therefore, the development of more effective therapeutic strategies, particularly targeted therapeutic approaches, is urgently needed to improve the survival of CCA patients.

Dysregulation of cell division is crucial aspects of cancer, driving sustained proliferation, carcinogenesis, and cancer progression[8].Therefore, targeting these unique features of cancer can more effectively inhibit proliferation and induce cell death[9]. The key regulators of the cell cycle are cyclin/cyclin-dependent kinase (CDK) complexes. At the G1/S checkpoint, cyclin D works with CDK4/6 to phosphorylate retinoblastoma (Rb) protein, resulting in its dissociation from the E2F transcription factors, which then transcribe the required genes for entering the S phase[10]. Previous studies have shown that CCA patients overexpress cyclin D and CDK4/6, which are key regulatory proteins of the cell cycle[11]. Patients with high expression of cyclin D and CDK4/6 have shorter overall survival and disease-free survival[12,13]. Currently, selective CDK4/6 inhibitors for the treatment of cancers with overexpressed cyclin D and CDK4/6 have entered clinical trials and been approved by the U.S. FDA[14]. Palbociclib, one of the FDA-approved specific CDK4/6 inhibitors, effectively inhibits both CDK4 and CDK6 kinase activities. Palbociclib causes G1-phase cell cycle arrest by preventing Rb phosphorylation through the inhibition of CDK4/6 activity and has no effect on Rb-deficient cancer types[15]. Palbociclib was approved for the treatment of hormone receptor-positive/HER2-negative advanced breast[16–18]. However, using palbociclib as a single treatment remains challenging because its main mechanism is to inhibit cell proliferation by G1 cell cycle arrest without inducing cell death, resulting in a low drug response. Additionally, it can cause major adverse effects including leukopenia and neutropenia[17]. Furthermore, cancer cells can develop acquired drug resistance and induce cellular senescence[19–21]. Therefore, combining CDK4/6 inhibitors with other agents that can induce cell death might enhance their efficacy.

Gene mutations and dysregulation of cell signaling involved in the maintenance of cell survival and inhibition of apoptosis, including NF-κB signaling and induction of cellular senescence, have been shown to contribute to low efficacy following CDK4/6 monotherapy[22,23]. Previous studies have shown that the expression levels of cellular inhibitors of apoptosis protein 1/2 (cIAP1/2) were increased in breast cancer cells upon treatment with CDK4/6 inhibitors[24]. Previous studies demonstrated that hyperphosphorylation of Rb by cyclin D-CDK4/6 promotes specific Rb-p65 interactions, inhibiting NF-κB target gene transcription. The loss of Rb phosphorylation at S249/T252 *via* CDK4/6 inhibitor or Rb deletion promotes tumor survival through NF-κB target gene transcription, such as PD-L1 and cIAP1/2, which are encoded by *CD274*, *BIRC2*, and *BIRC3*, respectively[25]. Our bioinformatics analysis results show that cIAP1/2 proteins are highly expressed in CCA patients and are associated with shorter overall survival and disease-free survival in CCA patients with high cIAP1/2 expression. In addition, cIAP1/2 expression is regulated by the NF-κB signaling pathway, which is dysregulated in CCA patients[26]. Therefore, cIAP1/2 might contribute to the low efficacy of CDK4/6 inhibitor monotherapy, necessitating therapeutic approaches that target both CDK4/6 and cIAP1/2 inhibition in CCA.

The small molecule second mitochondria-derived activator of caspases (SMAC) mimetic has been developed to antagonize cIAP1/2 activity. The binding of the Smac mimetics to cIAP1/2 results in conformational changes and stimulates RING domain dimerization, leading to cIAP1/2 auto-ubiquitination and proteasomal degradation[27]. Recently, Smac mimetics have progressed to clinical trial phases and are being recommended as combination treatment strategies for various types of cancer[28]. The combination of Smac mimetics with different agents has been evaluated in both preclinical studies and clinical trials for various types of cancers. Moreover, our previous studies show that Smac mimetics can sensitize CCA cells to different agents, inducing apoptosis and receptor-interacting protein kinase 3 (RIPK3)/mixed lineage kinase domain-like (MLKL)-mediated necroptosis, including low doses of gemcitabine, poly(I:C), quercetin, and kaempferol[29–31]. In addition, the triple combination of gemcitabine, cisplatin, and Smac mimetics can inhibit and prevent CCA cells from developing multidrug resistance[32]. Accordingly, targeting cIAP1/2 might be a new therapeutic strategy for CCA.

In our study, we aimed to investigate the efficacy of CDK4/6 inhibitor monotherapy and identify a key regulator of CDK4/6 inhibitor response, focusing on NF-κB signaling. Additionally, we combined the CDK4/6 inhibitor with a Smac mimetic to enhance its efficacy. Given that palbociclib, a CDK4/6 inhibitor, is approved by the U.S. FDA for the treatment of breast cancer and that cIAP1/2 inhibitors (Smac mimetics) are currently

under evaluation in several phase I and II clinical trials for certain cancers, our findings will provide valuable preclinical data. This is the first study that aims to develop novel combination strategies using CDK4/6 inhibitors and Smac mimetics, demonstrating potential for clinical translation and enhancing the efficacy of CDK4/6 monotherapy in CCA patients.

Materials and methods

Gene expression and overall survival in CCA patients

The GSE107943 cohort was downloaded from the Gene Expression Omnibus (GEO) database (https://www.ncbi.nlm.nih.gov/geo/, accessed on 10th March 2024). A comparative analysis of *CCND1* (cyclin D1), *CDK4*, *CDK6*, *BIRC2* (cIAP1), and *BIRC3* (cIAP2) gene expression was performed on 31 tumor (intrahepatic cholangiocarcinoma) samples and 30 adjacent normal liver tissue samples as controls. The data from the GSE107943 cohort were analyzed *via* an independent sample *t*-test and presented in a box plot. The OEP00115 cohort was downloaded from the National Omics Data Encyclopedia (NODE) database (https://www.biosino.org/node/). The Cancer Genome Atlas-Cholangiocarcinoma (TCGA-CHOL) dataset was downloaded from the University of California at Santa Cruz (UCSC) Xena platform (https://xena.ucsc.edu/). A Kaplan-Meier and log-rank test was used to compare overall survival between patients with high and low expression of cyclin D, CDK4, CDK6, cIAP1, and cIAP2.

Reagents and antibodies

Palbociclib and Smac mimetic LCL161 were purchased from APExBIO Technology LLC (Houston, TX, USA). Gemcitabine was purchased from Sigma (St. Louis, MO, USA). Tumor necrosis factor-α (TNF-α) was purchased from R&D systems (Minneapolis, MN, USA). Antibodies for Western blot were obtained from commercial sources including anti-cyclin D1 (2978), anti-phospho Rb (Ser780) (9307), anti-cIAP1 (7065), anti-cIAP2 (3130), anti-phospho p65 (3033), anti-PARP-1 (9542), anti-caspase-3 (9662S) and anti-β actin (4970) were from Cell Signaling Technology (Danvers, MA, USA).

CCA cell lines and culture

CCA cell lines (KKU-213A, KKU-213B, HuCCT-1) and non-tumor cholangiocyte MMNK-1 were provided from the Japanese Collection of Research Bioresources (JCRB) Cell Bank, Osaka, Japan. The MCF-7 breast cancer cell line, which is sensitive to CDK4/6 inhibitors, was provided by ATCC (Manassas, VA, USA). All CCA cell lines and MMNK-1 were grown in HAM's F-12 medium (HyClone Laboratories, Logan, Utah, USA), while MCF-7 was grown in Dulbecco's modification of Eagle's medium (DMEM; Hyclone Laboratories, Logan, Utah, USA) supplement with 10% fetal bovine serum (Sigma, St Louis, MO, USA) and 1% penicillin streptomycin (HyClone Laboratories, Logan, Utah, USA) under the standard protocol at 37˚C in 5% CO_2 humidified atmosphere. All cultures were tested for mycoplasma contamination and were mycoplasma-free.

Isolation of peripheral blood mononuclear cells (PBMC)

The PBMCs were obtained from the buffy coat of anonymous healthy blood donors. The PBMCs were isolated using Ficoll-Paque PLUS (Cytiva Life Sciences, Marlborough, MA, USA), followed by centrifugation. The buffy coat was diluted with 1X phosphate buffered saline (PBS; HyClone Laboratories, Logan, Utah, USA), layered on Ficoll-Paque, and centrifuged at 400 g for 45 min. The PBMCs were collected from the white interface layer, washed with 1X PBS, and centrifuged twice at 400 g for 15 min. The pellets were then washed with RPMI 1640 media and centrifuged twice at 200 g for 15 min. The PBMCs were counted and seeded in a 12-well plate. The cells were treated with palbociclib, LCL161, or a combination for 72 h, and cell death was determined *via* Annexin V/PI staining and flow cytometry.

Ethical approval for the use of buffy coat from anonymous healthy blood donors

Peripheral blood mononuclear cells (PBMCs) were obtained from the buffy coat of anonymous healthy blood donors, in accordance with the guidelines of the Thai Red Cross Society Institutional Review Board. Approval for this study was granted by the Thai Red Cross Society Institutional Review Board (Certificate of Approval No. NBC 7/2023, granted on March 27, 2023). The Thai Red Cross Society Institutional Review Board waived the requirement for informed consent, as the study utilized anonymized buffy coats derived from healthy blood donors. These buffy coats were classified as leftover materials and considered biological waste.

Drug treatment and cell viability assay

For primary screening of drug responses in CCA cell lines, the cells cultured in 96-well plates were treated with various concentrations of palbociclib (0.1, 1, 5, 7.5, and 10 μM) and LCL161 (25 μM), both individually and in combination (with LCL161 pre-treatment for at least 30 min) in complete HAM's F-12 media, with Dimethylsulfoxide (DMSO) as a vehicle control. We also evaluated the cell viability of Gemcitabine, the standard chemotherapeutic drug for CCA, at concentrations of 0.01, 0.025, 0.05, and 0.1 μM. After 72 h of incubation, cell viability was measured using the MTT assay. Briefly, 10 μL of 5 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution was added to each well and incubated at 37 °C ina 5% CO_2 atmosphere for 2 h. The medium was then removed, and 100 μL of DMSO was added to dissolve the formazan crystals. Absorbance was measured at 540 nm using a microplate reader, and the percentage of cell viability and IC_{50} were calculated.

Cell death analysis by Annexin V/PI staining

CCA cell lines and non-tumor cholangiocyte MMNK-1 were seeded at 15,000 cells per well with 1 mL of complete HAM's F-12 medium in a 12-well plate and incubated for 18–24 h. The cells were treated with palbociclib (0.1,

1, and 5 μM), LCL161 (25 μM), or a combination treatment in complete HAM's F-12 media for 72 h at 37 °C in a 5% CO_2 humidified atmosphere. Cell death was assessed by Annexin V/Propidium Iodide (PI) double staining followed by flow cytometry analysis. Briefly, the cells were collected, washed with 1X PBS, and resuspended in Annexin V-binding buffer. The cells were stained with Annexin V-FITC (ImmunoTools, Friesoythe, Germany) and propidium iodide (PI) (Invitrogen, Carlsbad, CA, USA) for 15 min at room temperature in the dark. The stained cells were analyzed using flow cytometry (Navios, Beckman Coulter, Indianapolis, IN, USA, and BD Accuri C6, Franklin Lakes, NJ, USA). The Combination Index (CI) was calculated based on the Chou-Talalay method, where CI = 1 indicates an additive effect, CI < 1 indicates synergism, and CI > 1 indicates antagonism[33].

Colony formation

CCA cell lines and non-tumor cholangiocyte MMNK-1 were seeded at 100 cells per well with 1.5 mL of complete HAM's F-12 medium in a 6-well plate and incubated for 18–24 h. The cells were treated with palbociclib (0.1 and 1 μM), LCL161 (25 μM), or a combination treatment in complete HAM's F-12 media for 72 h at 37 °C in a 5% CO_2 humidified atmosphere. The drug was then removed, and the cells were washed twice with 1X PBS and maintained in HAM's F-12 medium. The cells were cultured for 1–3 weeks until colonies formed (a colony is defined as consisting of at least 50 cells). The experiment was stopped when the control group reached 50–100 colonies or when the colonies no longer overlapped with each other. The colonies were then stained with 1% crystal violet and counted under a microscope, comparing the control and treatment groups.

Western blot analysis

CCA cells were washed twice with cold 1X PBS and lysed in RIPA buffer (Merck Millipore, Darmstadt, Germany) containing a proteinase inhibitor cocktail (Roche, Mannheim, Germany). The protein lysate was vortexed, centrifuged at 12,000 rpm for 5 min, and the supernatant was collected. Protein concentrations were determined by Bradford assay (Bio-Rad, Hercules, CA, USA). Samples of 25 μg total protein were separated by 10% SDS-PAGE and transferred onto PVDF membranes. Membranes were blocked with 5% blotting-grade Blocker (Bio-Rad, Hercules, CA, USA) at room temperature for 1 h, then incubated with primary antibodies overnight at 4 °C. After that the membranes were washed three times with TBS-T (Tris-buffered saline, 0.5% Tween20) buffer and incubated with horseradish peroxidase-conjugated secondary antibodies (Cell Signaling Technology, Danvers, MA, USA) at room temperature for 1 h. The proteins were visualized by enhanced chemiluminescence (Bio-Rad, Hercules, CA, USA) using Amersham ImageQuantTM 800 (Cytiva, Marlborough, MA, USA). All Western blots shown were representative of at least three independent experiments.

Immunofluorescence staining

CCA cells were seeded on a poly-D-lysine coated coverslip in a 35 mm culture dish and incubated for 18–24 h to allow the cells to attach to the coverslip. The cells were then treated with 5 μM palbociclib for 24, 48, and 72 h, with treatment using 10 ng/mL TNF-α for 15 min as a positive control. The cells were fixed in 4% paraformaldehyde for 10 min, washed twice with 1X PBS, and incubated with 0.1% Triton X-100 for 10 min. After washing with 1X PBS three times, the cells were blocked with 2% bovine serum albumin (BSA)/PBS for 1 h at room temperature and probed with anti-p65 primary antibody (dilution 1:1000 in 2% BSA) overnight at 4 °C. The cells were washed twice with 1X PBS and incubated with anti-rabbit secondary antibodies conjugated with Alexa555 in 2% BSA for 1 h at room temperature. The secondary antibodies were then removed, and the nuclei were counterstained with 0.5 μg/mL Hoechst 33342 for 10 min at room temperature. Fluorescent images were acquired using a confocal microscope (Leica Microsystems, Wetzlar, Germany). The positive cells, indicated by p65 translocation into the nucleus, were analyzed and counted using Gen5 software (Agilent, Santa Clara, CA, USA).

3D tumor spheroid formation

Tumor spheroids were generated using the liquid overlay technique (LOT). The representative CCA cell line, KKU-213A, was seeded at 5,000 cells per well into a 96-well ultra-low attachment round-bottom plate (Corning, Gilbert, AZ, USA) and centrifuged at 1,000 rpm for 5 min to facilitate cell aggregation. The cells were cultured at 37 °C in a 5% CO_2 humidified atmosphere for 3 days for spheroid initiation. The end of the initiation phase was marked as day 0, and palbociclib (5 and 10 μM), LCL161 (25 μM), or a combination of both drugs were added at various concentrations. Spheroidal morphology and size were observed and measured every 2 days (day 0, 3, 6, 9, 12, 15, 18). The old medium was removed and replaced with fresh medium every two days. To visualize live and dead cells in the tumor spheroid, calcein AM and PI fluorescence dyes were used, and images were captured by BioTek Cytation7 live cell imaging (Agilent, Santa Clara, CA, USA), then analyzed for fluorescence intensity *via* Gen5 software (Agilent, Santa Clara, CA, USA). The mean fluorescent intensity of stained spheroids, either green or red, was averaged and represented in a bar graph.

Statistical analysis

All statistical analyses were conducted using the SPSS software (version 28.0.0.0, IBM Corp; Armonk, NY, USA). Results were expressed as the mean ± standard deviation (S.D.) of at least three independent experiments. Comparisons between two groups were determined by Student's t-test. Difference between two groups were considered statistically significant at p-value < 0.05 (*), p-value < 0.01 (**), and p-value < 0.001 (***), and p-value < 0.0001 (****).

Result

Differential expression of key G1/S phase cell cycle regulatory proteins in CCA patients

Sustaining proliferative signaling is one of the distinguishing characteristics of cancer. Therefore, to evaluate the targeted therapeutic potential for CCA, we analyzed the mRNA expression of key regulatory proteins in cell cycle progression, including CDK4, CDK6 and cyclin D, in CCA patients using the GSE107943 cohort from the Gene Expression Omnibus (GEO) database. The box plot shows that cyclin D1 (*CCND1* gene, Fig. 1A), *CDK4* gene (Fig. 1B), and *CDK6* gene (Fig. 1C) were significantly higher expressed in CCA primary tissues compared to their expression levels in adjacent non-tumor tissues. We then examined the relationship between high expression of these three genes and overall survival in CCA patients using the OEP00115 cohort from the national omics data encyclopedia (NODE) database. The Kaplan-Meier graph (Fig. 1F) shows that patients with high CDK6 expression were significantly associated with shorter overall survival ($p = 0.005$, HR = 1.77). In contrast, high expression levels of CDK4 and cyclin D1 were not significantly associated with overall survival (Fig. 1D,E). In addition, the results are similar when analyzed from the TCGA dataset, which shows that these three genes are highly expressed in CCA patients (Supplementary Fig. 1A,D,G). Cyclin D is associated with shorter overall survival ($p = 0.037$, HR = 2.73) (Supplementary Fig. 1B), while CDK4 ($p = 0.051$, HR = 2.88) shows a trend towards association with poor overall survival (Supplementary Fig. 1E). Furthermore, analysis of the TCGA dataset reveals that CDK4 is associated with shorter disease-free survival ($p = 0.003$, HR = 11.81) (Supplementary Fig. 1F), while cyclin D ($p = 0.053$, HR = 3.25) and CDK6 ($p = 0.090$, HR = 2.87) show a trend towards association with poor disease-free survival (Supplementary Fig. 1C,I). These results suggest that the overexpression of these cell cycle regulatory proteins, particularly the high expression of cyclin D and CDK6, is significantly associated with poor survival outcomes, and CDK6 is associated with shorter disease-free survival in CCA patients. Therefore, the cyclin D/CDK4/6 complex could be a potential therapeutic target for CCA. However, several studies have shown that overexpression of the cyclin D/CDK4/6 complex in various cancers does not always correlate with a favorable response to CDK4/6 inhibitors[34–36]. Consequently, we further investigated the sensitivity of CCA cells to CDK4/6 inhibitor.

CDK4/6 inhibitor Palbociclib monotherapy reduces cell proliferation but has minimal effect on cell death in CCA cells

To examine the effect of the CDK4/6 inhibitor palbociclib on cell proliferation and cell death, *in vitro* CCA cell models, including HuCCT-1, KKU-213A, and KKU-213B, were analyzed for cyclin D1 and phosphorylated Rb (Ser780), a specific CDK4/6 target protein, by Western blot analysis[10]. As shown in Fig. 2A, all three CCA cell lines expressed both cyclin D1 and phosphorylated Rb proteins. To evaluate the efficacy of a CDK4/6 inhibitor on cell proliferation inhibition, we initially determined the response of the CCA cell lines to the single treatment of palbociclib using an MTT assay, then calculated the IC_{50} (half-maximal inhibitory concentration). The MMNK-1 cell line was used as a non-tumor cholangiocyte control, while MCF-7, a breast cancer cell line, was used as a CDK4/6 inhibitor-sensitive cell line. Gemcitabine, a standard chemotherapy for CCA, was included for comparison. Each cell line was treated with palbociclib (0.1, 1, 5, 7.5, 10 μM) and gemcitabine (0.01, 0.025, 0.05, 0.1 μM), with DMSO as a vehicle control for 72 h. The results show that the IC_{50} values of HuCCT-1, KKU-213A, and KKU-213B were 7.18, 5.83, and 7.77 μM, respectively, while the IC_{50} values of MMNK-1 and MCF-7 cell lines were 9.90 and 2.57 μM (Supplementary Fig. 2A and 2 C). When compared to the response of CCA cells to gemcitabine, the IC_{50} value of CCA cell lines is higher than that of MMNK-1 (Supplementary Fig. 2B, C). These results indicate that gemcitabine has higher toxicity to non-tumor cholangiocytes than CCA cells when compared to palbociclib. Additionally, palbociclib treatment is less effective when compared to MCF-7 (Supplementary Fig. 2A). After that a clonogenic assay was performed to confirm that palbociclib can inhibit long-term cell proliferation and colony formation in CCA cells. The results show that palbociclib at 0.1 and 1 μM significantly inhibited colony formation in HuCCT-1, KKU-213A, and KKU-213B compared to their respective vehicle controls (Fig. 2B, C). In contrast, no significant inhibition of colony formation was observed in MMNK-1 cells compared to vehicle controls. Moreover, we found a significant decrease in phosphorylated Rb after palbociclib treatment in all CCA cell models (Fig. 2D), suggesting that targeting CDK4/6 is a potential strategy to suppress cell proliferation in CCA cells. To determine whether palbociclib induces cell death, CCA cell lines and MMNK-1 were treated with palbociclib at different concentrations for 72 h, and cell death was determined by Annexin V/PI staining and flow cytometry. Palbociclib at 0.1 μM did not induce cell death, while palbociclib at 1 and 5 μM significantly induced cell death in HuCCT-1 and KKU-213A, and to a lesser extent in KKU-213B (Fig. 2E). Taken together, although palbociclib single treatment can significantly induce cell death in CCA cells compared to MMNK-1, the cell death induction is relatively ineffective.

The CDK4/6 inhibitor Palbociclib upregulates cIAP1/2 expression in CCA cells

To improve the effectiveness of CDK4/6 inhibitor treatment, we aimed to explore the key target genes and signaling pathways that modulate the efficiency of CDK4/6 inhibitor treatment. Previous studies have shown that the expression levels of cellular inhibitor of apoptosis proteins 1 and 2 (cIAP1/2) were increased in breast cancer cells upon treatment with CDK4/6 inhibitors[24]. Bioinformatic analysis using the GSE107943 CCA cohort shows that the mRNA levels of cIAP1 and cIAP2, which are encoded by the *BIRC2* and *BIRC3* genes, respectively, are higher in CCA primary tissues compared to adjacent normal tissues (Fig. 3A, B). Furthermore, the overall survival of the CCA patients with high cIAP1 and cIAP2 expression was associated with a poor overall survival rate (Fig. 3C). Therefore, cIAP1/2 are promising targets that can modulate the efficiency of CDK4/6 inhibitor treatment. We determined the expression levels of cIAP1 and cIAP2 proteins in selected CCA cell models using Western blot analysis. As shown in Fig. 3D, all CCA cell lines tested expressed the cIAP1 and cIAP2 proteins (Fig. 3D)[28]. To investigate whether palbociclib can upregulate cIAP1/2 proteins in CCA cells, we initially treated HuCCT-1, KKU-213A, and KKU-213B with palbociclib at 0.1, 1, and 5 μM for 72 h, then

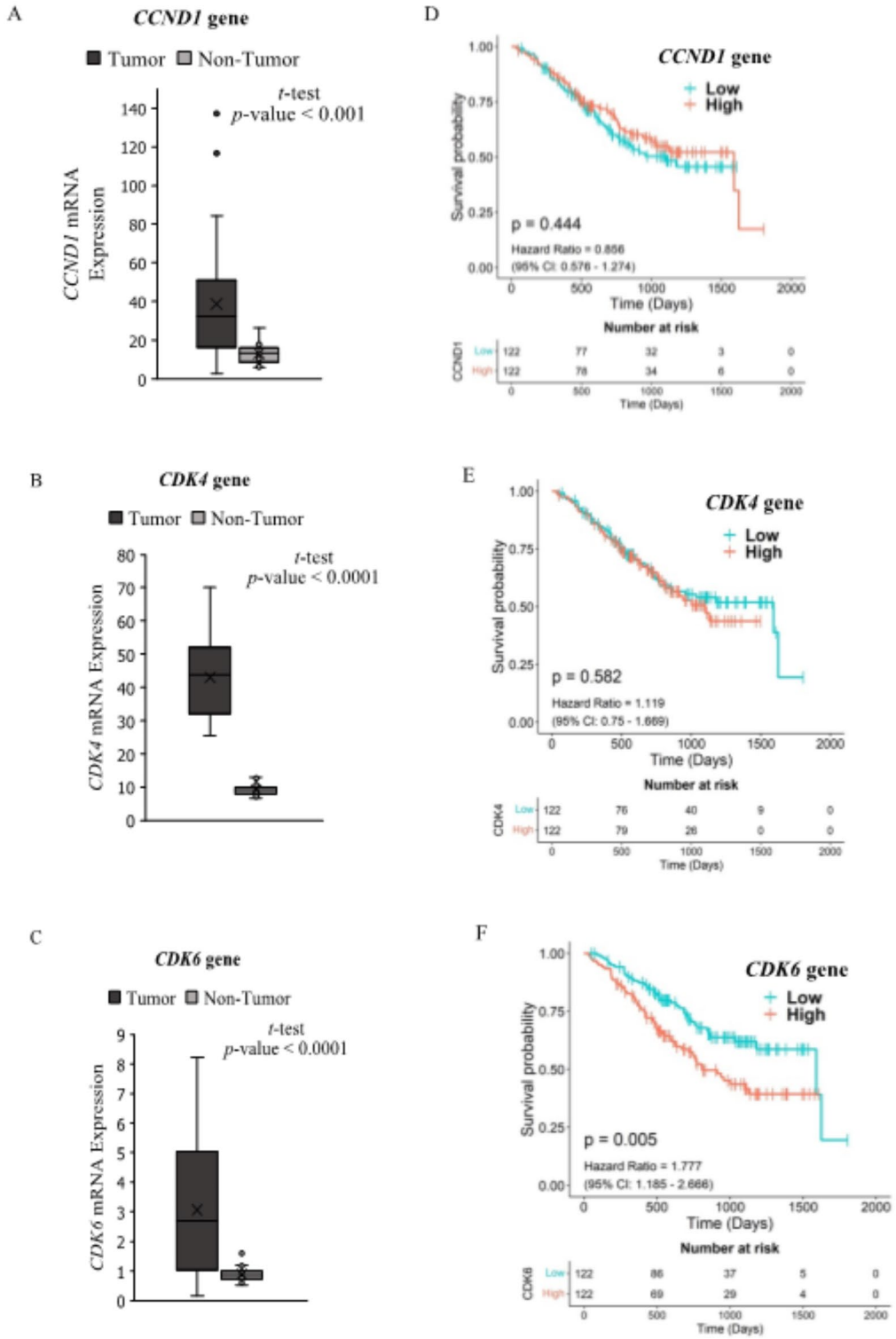


Fig. 1. Differential expression of key G1/S phase cell cycle regulatory proteins. The mRNA expression levels of cyclin D1 (*CCND1*) (**A**), *CDK4* (**B**), and *CDK6* (**C**) were obtained from the GSE107943 CCA cohort. The mRNA expression of 31 CCA tissues obtained from CCA patients, and 30 adjacent normal liver tissues were used as a control. A Kaplan-Meier curve shows the overall survival of CCA patients with high and low expression levels of the cyclin D1 (**D**), CDK4 (**E**) and CDK6 (**F**). The mRNA expression levels of cyclin D1 (*CCND1*), *CDK4*, and *CDK6* were obtained from the OEP00115 CCA cohort.

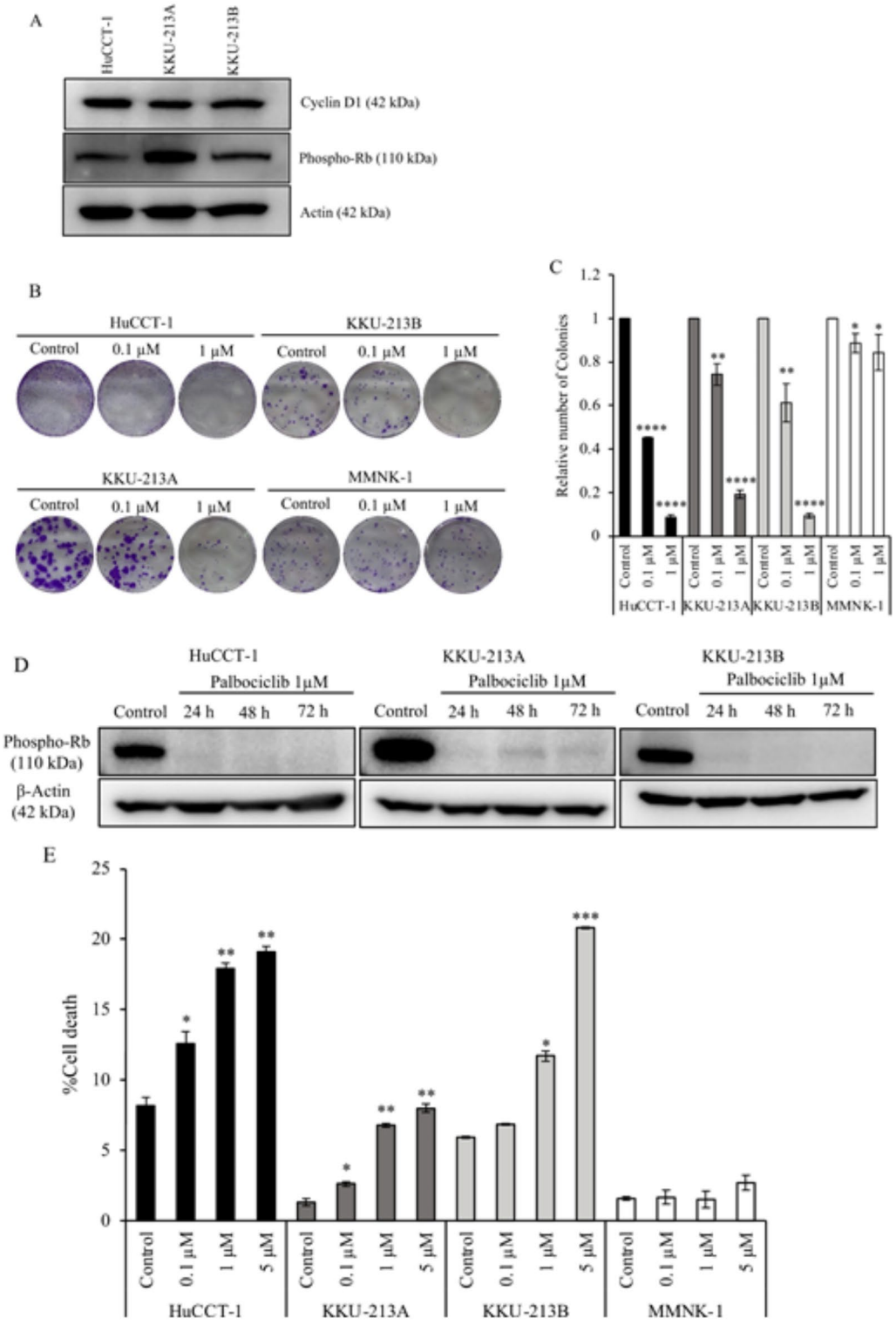


Fig. 2. Effects of palbociclib monotherapy on cell proliferation and cell death. (**A**) Expression of cyclin D1 and phospho-Rb protein in CCA cell lines was determined by Western blot analysis. (**B**) The representative image of the colony formation assay shows cell growth inhibition in CCA cell lines. The cells were treated with various doses of palbociclib, as indicated. The number of colonies, each consisting of more than 50 cells, was counted and normalized to the vehicle control. (**C**) Quantification of colony count. The data represented as mean ± S.D. (**D**) The phosphorylated Rb protein level in CCA cell lines after treatment with 1 μM palbociclib was determined by Western blot analysis. (**E**) CCA cell lines (HuCCT-1, KKU-213A, KKU-213B) and MMNK-1 were treated with palbociclib in various doses for 72 h. Cell death was determined with Annexin V and PI staining and flow cytometry. Data presented as mean ± S.D. of three independent experiments are shown. p-value < 0.05 (*), p-value < 0.01 (**), p-value < 0.001 (***), and p-value < 0.0001 (****).

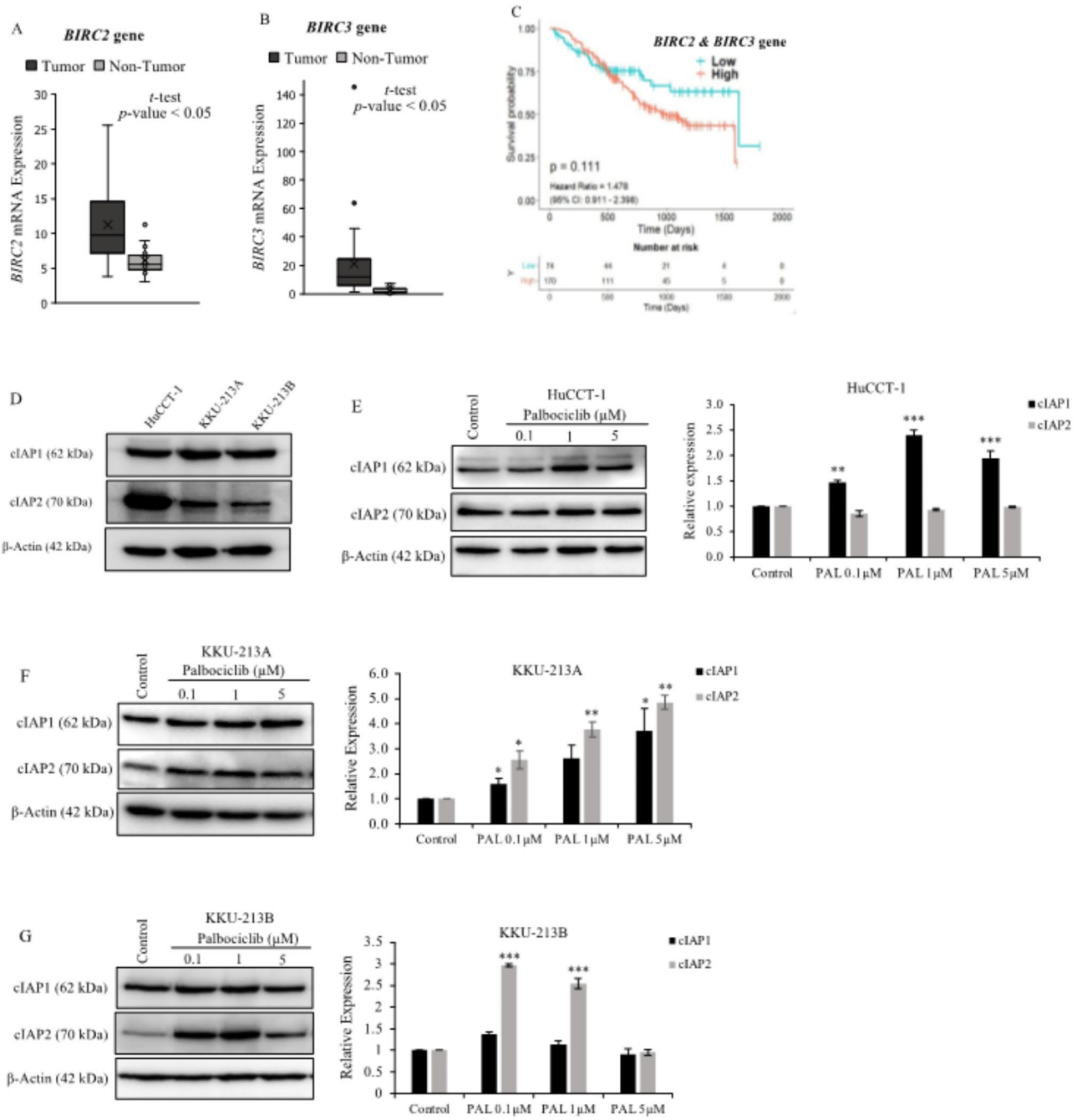


Fig. 3. Upregulation of cIAP1/2 in CCA patients and induction of cIAP1/2 expression by palbociclib in CCA cells. The mRNA expression of cIAP1 (*BIRC2*) (**A**) and cIAP2 (*BIRC3*) (**B**) was obtained from the GSE107943 CCA cohort. The mRNA expression of 31 CCA tissues obtained from CCA patients, and 30 adjacent normal liver tissues was used as a control. (**C**) A Kaplan-Meier curve shows the overall survival of CCA patients between high and low expression levels of cIAP1 and cIAP2. The mRNA expression levels of cIAP1 (*BIRC2*) and cIAP2 (*BIRC3*) were obtained from OEP00115 CCA cohort. (**D**) The protein expression of cIAP1 and cIAP2 in CCA cells was determined by Western blot analysis. The upregulation of cIAP1 and cIAP2 following treatment with palbociclib. CCA cell lines HuCCT-1 (**E**), KKU-213A (**F**), and KKU-213B (**G**) were exposed to palbociclib at 0.1, 1, and 5 μM for 72 h. The protein expression of cIAP1 and cIAP2 was detected by Western blot analysis. The quantitative results of cIAP1 and cIAP2 expression for each CCA cell line were determined by densitometry and normalized to the vehicle control. Data presented as mean ± S.D. of three independent experiments are shown. p-value < 0.01 (**) and p-value < 0.001 (***).

determined the cIAP1 and cIAP2 levels using Western blot analysis. The results show that cIAP1 was upregulated in HuCCT-1 (Fig. 3E), while both cIAP1 and cIAP2 were upregulated in KKU-213A and KKU-213B, with cIAP2 exhibiting stronger upregulation after exposure to palbociclib for 72 h (Fig. 3F, G). These results demonstrate that the CDK4/6 inhibitor palbociclib can upregulate cIAP1 and cIAP2 expression in CCA cells.

CDK4/6 inhibitor Palbociclib activates phosphorylated p65 and its nuclear translocation in CCA cells

Previous studies have demonstrated that the loss of phosphorylation of the Rb protein, whether caused by CDK4/6 inhibitors or *Rb* gene deletion, led to a loss of Rb S249/T252 interaction with the p65 transcription factor, resulting in NF-κB target gene transcription, including PD-L1 and cIAP1/2[25]. We investigated whether palbociclib can activate phosphorylation of p65 and its nuclear translocation in KKU-213A, a representative CCA cell line, using Western blot analysis and immunofluorescent staining. As shown in Fig. 4A, B, palbociclib significantly induced the phosphorylation of p65 from 24 h to 96 h, with TNF-α, a well-known inducer of NF-κB signaling, included as a positive control (Fig. 4A,B). To determine whether this phosphorylated p65 translocated to the nucleus, we analyzed the translocation by immunofluorescence staining. Interestingly, after exposure to palbociclib, p65 translocated into the nuclei starting from 24 h up to 72 h (Fig. 4C,D). Our findings demonstrate that palbociclib induces phosphorylation of p65 and its translocation into nuclei. Therefore, cIAP1/2 upregulation after palbociclib treatment might be regulated by NF-κB signaling, suggesting the potential for combination treatment with CDK4/6 inhibitor and cIAP1/2 inhibition.

The combination of the CDK4/6 inhibitor Palbociclib and the Smac mimetic LCL161 synergistically suppresses cell proliferation and induces cell death in 2D monolayer cultures of CCA cells

To investigate the potential combination of CDK4/6 inhibitor and cIAP1/2 inhibition, the Smac mimetic LCL161, a cIAP1/2 antagonist, was used to inhibit cIAP1/2 through proteasomal degradation. The validation of the Smac mimetic LCL161-induced degradation of cIAP1 and cIAP2 is shown in Fig. 5A, where the Smac mimetic LCL161 almost completely reduces the levels of both cIAP1 and cIAP2. The results revealed that the combination treatment of palbociclib and the Smac mimetic LCL161 significantly inhibited long-term colony

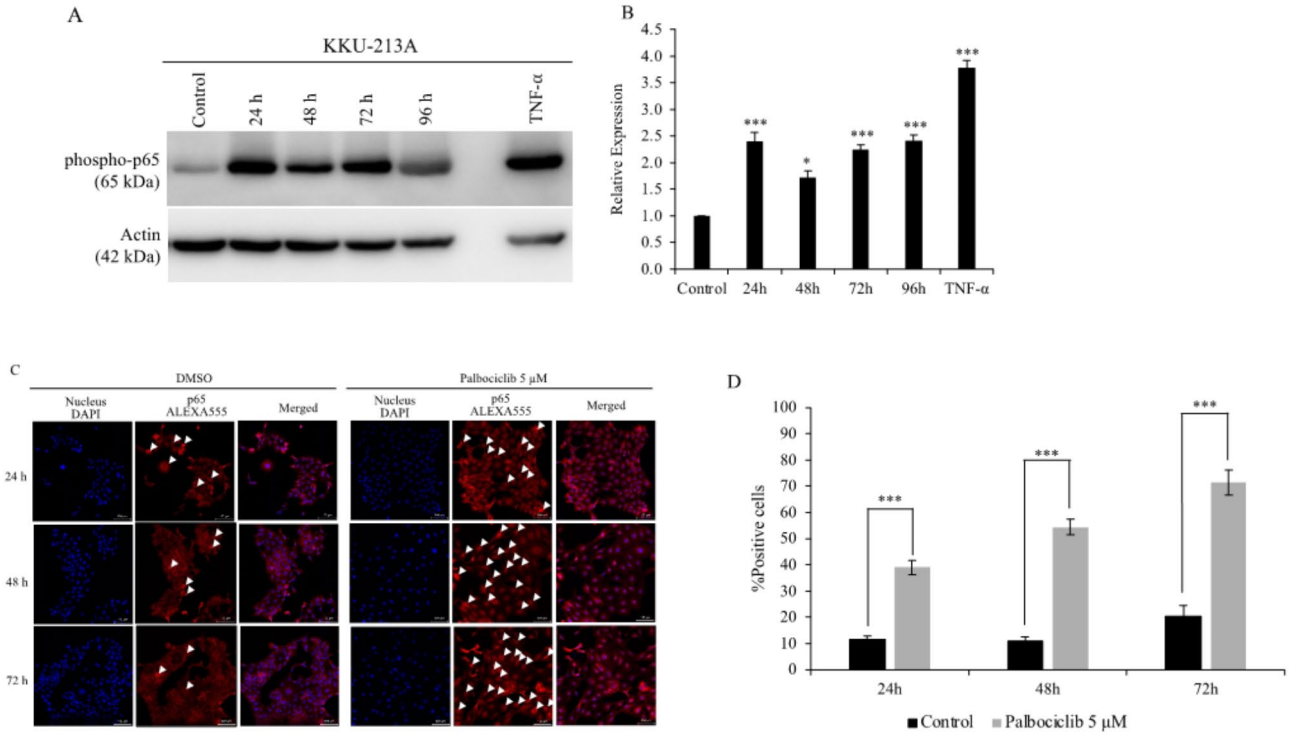
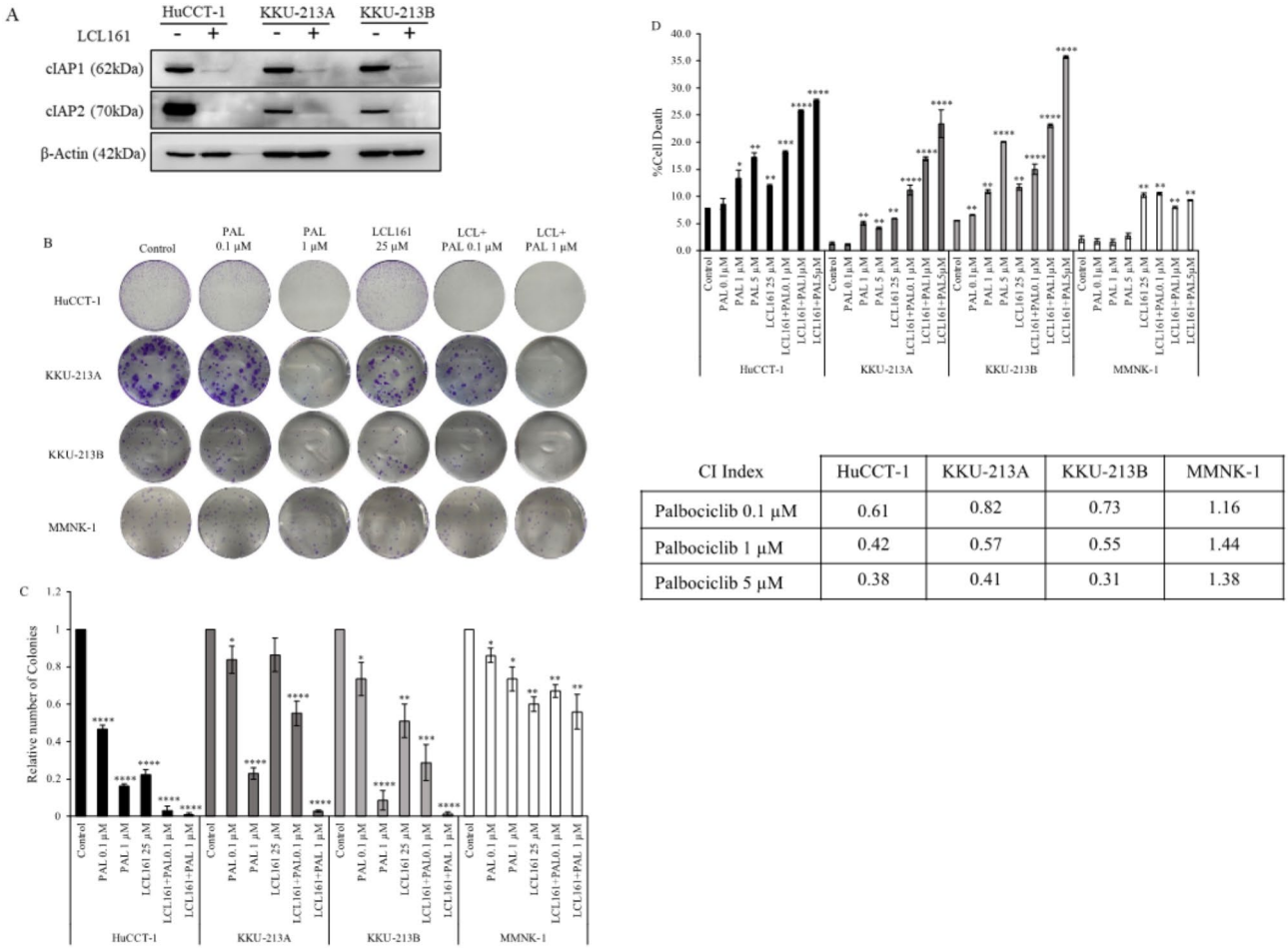


Fig. 4. Effects of palbociclib on phosphorated p65 activation and its nuclear translocation. (**A**) and (**B**) The effect of palbociclib on phosphorylated p65 activation. (**A**) KKU-213A cells were exposed to 5 μM palbociclib for 24, 48, 72, and 96 h. The phosphorylation level of p65 was determined by Western blot analysis (**A**) and the quantitative results are represented as a bar graph (**B**). (**C**) and(**D**) The effect of palbociclib on p65 nuclear translocation. KKU-213A cells were exposed to 5 μM palbociclib for 24, 48, and 72 h. The localization of p65 was determined by immunofluorescence staining (**C**). TNF-α was included as a positive control for p65 nuclear translocation. The white arrowheads indicate positive cells with nuclear translocation; Scale bar: 100 μm. Quantitative data are shown as the percentage of cells displaying p65 translocation in the nucleus, calculated from at least 3 different fields (**D**). Data presented as mean ± S.D. of three independent experiments are shown. p-value < 0.05 (*) and p-value < 0.001 (***).



CI Index	HuCCT-1	KKU-213A	KKU-213B	MMNK-1
Palbociclib 0.1 μM	0.61	0.82	0.73	1.16
Palbociclib 1 μM	0.42	0.57	0.55	1.44
Palbociclib 5 μM	0.38	0.41	0.31	1.38

Fig. 5. The effect of the combination treatment of palbociclib and LCL161 on cell proliferation and cell death in 2D monolayer cultures of CCA cells. (**A**) The expression of cIAP1 and cIAP2 proteins following Smac mimetic LCL161 treatment in CCA cell lines. HuCCT-1 cells were treated with 25 μM LCL161, while KKU-213A and KKU-213B were treated with 5 μM LCL161 for 5 h. The expression of cIAP1 and cIAP2 was determined by Western analysis. β-actin was included as an internal control. (**B**) and (**C**) The effect of the combination treatment on colony formation and long-term cell survival. CCA cells and MMNK-1 cells were exposed to palbociclib, LCL161, and drug combinations for 1–3 weeks. The number of colonies, consisting of more than 50 cells, was counted and compared between the control and treatment groups. (**B**) Representative pictures from the colony formation assay showing cell growth inhibition of CCA cells and MMNK-1 cells. (**C**) Quantitative data represented as mean ± S.D. (**D**) The effect of the combination treatment on cell death. Cell death was determined by Annexin V and PI staining and flow cytometry. The combination index (CI) was calculated, where CI = 1 indicates an additive effect, CI < 1 indicates synergism, and CI > 1 indicates antagonism. Data presented as mean ± S.D. of three independent experiments are shown. p-value < 0.05 (*), p-value < 0.01 (**), p-value < 0.001 (***), and p-value < 0.0001 (****); ns = not significant.

formation in HuCCT-1, KKU-213A, and KKU-213B compared to their respective vehicle controls (Fig. 5B,C). To evaluate whether palbociclib combined with the Smac mimetic LCL161 induces cell death, we analyzed cell death using Annexin V/PI staining and calculated the combination index (CI). The combination treatment of palbociclib and LCL161 significantly induced cell death in HuCCT-1, KKU-213A, and KKU-213B cells compared to single treatment with either palbociclib or LCL161 (Fig. 5D). Additionally, apoptosis markers including cleaved caspase-3 and cleaved PARP-1, were prominently observed in the combination treatment (Supplementary Fig. 4). The CI for all combination treatments at different concentrations of palbociclib was less than 1, indicating that these combinations were synergistic in inducing CCA cell death (Fig. 5D). While all CCA cell lines exhibit similar cIAP1/2 profiles (Fig. 5A), the observed differences in cell death following combination treatment likely reflect natural variations among cell lines. Altogether these findings collectively demonstrate that the combination of CDK4/6 inhibitor and Smac mimetic synergistically induce cell death and inhibit cell growth in 2D monolayer cultures of CCA cells.

Combined CDK4/6 inhibitor Palbociclib and Smac mimetic LCL161 suppresses cell proliferation and induces cell death in 3D spheroid cultures of CCA cells

To further evaluate the effect of the combination treatment of CDK4/6 inhibitor and Smac mimetic, a 3D spheroid model that more closely mimics the tumor microenvironment was employed. The KKU-213A cell line was used as a representative cell line for this experiment. Once the spheroids were formed, they were treated with palbociclib at 5 and 10 μM and LCL161 at 25 μM. Following treatment, the morphological changes of the spheroids were observed every two days. In addition, TNF-α and Smac mimetic treatment, known apoptosis inducers, were used as a positive control. To illustrate the viability of the spheroids, they were stained with calcein AM and propidium iodide (PI) fluorescence dyes to track live and dead cells, respectively. After treatment with palbociclib and LCL161 for 18 days, PI staining significantly increased while calcein AM staining decreased, indicating that the combination treatment significantly induced cell death at the edge of the spheroid and inhibited cell growth at the core (Fig. 6A–C). These results suggest that the combination of CDK4/6 inhibitor and Smac mimetic is effective in inducing cell death and inhibiting cell growth in a tumor spheroid model.

The CDK4/6 inhibitor Palbociclib and the Smac mimetic LCL161 has minimal effects on cell death induction in PBMCs

The most common adverse symptoms of CDK4/6 inhibitors are neutropenia and leukopenia[37]. LCL161 is currently being tested in phase 1 and phase 2 clinical trials, with results indicating favorable pharmacological effects, including good tolerability and low toxicity[38]. To examine the hematological adverse events of the CDK4/6 inhibitor and Smac mimetic combination treatment, we initially analyzed the cell death of PBMCs after treating these combinations by Annexin V/PI staining and flow cytometry. The PBMCs were isolated from healthy blood donors and treated with palbociclib at 0.5, 1, and 5 μM, either as a single treatment or combined with LCL161 at 25 μM for 72 h. Interestingly, the combination treatment of palbociclib and LCL161 slightly induced cell death in PBMCs compared to CCA cell lines (Fig. 7). Taken together, these results suggest that the CDK4/6 inhibitor combined with Smac mimetic has minimal effects on cell death induction in PBMCs, further supporting the potential of combining CDK4/6 inhibitors and Smac mimetics for targeted therapy in CCA patients.

Discussion

Presently, therapeutic strategies for CCA remain a major challenge, leading to poor prognosis and a high mortality rate. We identified the cyclin D/CDK4/CDK6 complex and cIAP1/2 NF-κB signaling as therapeutic targets, providing a strong rational for developing a combinatorial therapeutic strategy for targeted therapy in CCA patients. First, we showed that cyclin D, CDK4, CDK6, and cIAP1/2 are highly expressed in CCA patients and are associated with poor prognosis. Second, we demonstrated the effectiveness of palbociclib in three CCA cell lines, which express phosphorylated Rb and cyclin D1 proteins. Although palbociclib single treatment can inhibit cell proliferation, it has less effect compared to a representative palbociclib-sensitive cell line and in inducing cell death. Third, palbociclib can upregulate cIAP1/2 expression in CCA cell lines, correlated with an increase in phosphorylated p65 and its nuclear translocation. Fourth, the combination treatment of the CDK4/6 inhibitor palbociclib and the cIAP1/2 antagonist Smac mimetic LCL161 synergistically inhibited cell proliferation and induced cell death in 2D monolayer CCA cultures, while having less effect on the non-tumor cholangiocyte

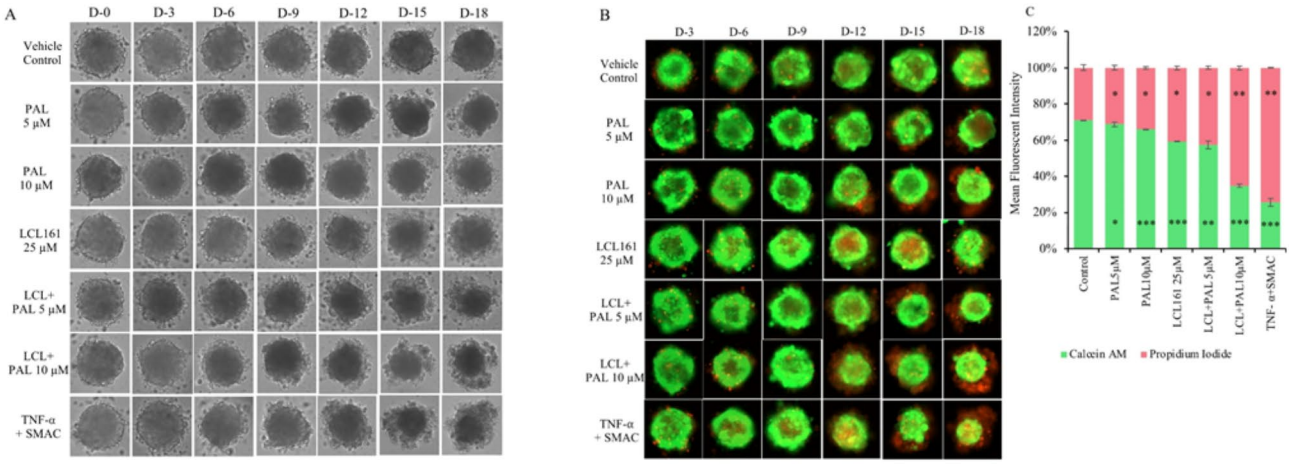


Fig. 6. The effect of the combination treatment of palbociclib and LCL161 on cell proliferation and cell death in 3D tumor spheroid. (**A**) The morphological changes of KKU-213A spheroids treated with palbociclib and LCL161 as single treatments and combination treatments under a brightfield microscope. (**B**,**C**) The viability and death of KKU-213A spheroids following drug treatment. KKU-213A spheroids were stained with calcein AM and PI fluorescent dye and imaged using live cell imaging. The images show the overlay of spheroids stained with calcein AM (green) and PI (red), indicating live cells and dead cells, respectively. (**B**) Representative image of spheroids. (**C**) The mean fluorescent intensity (MFI) of stained spheroids was averaged and calculated as a percentage of MFI. Note: p-value < 0.05 (*), p-value < 0.01 (**), and p-value < 0.001 (***).

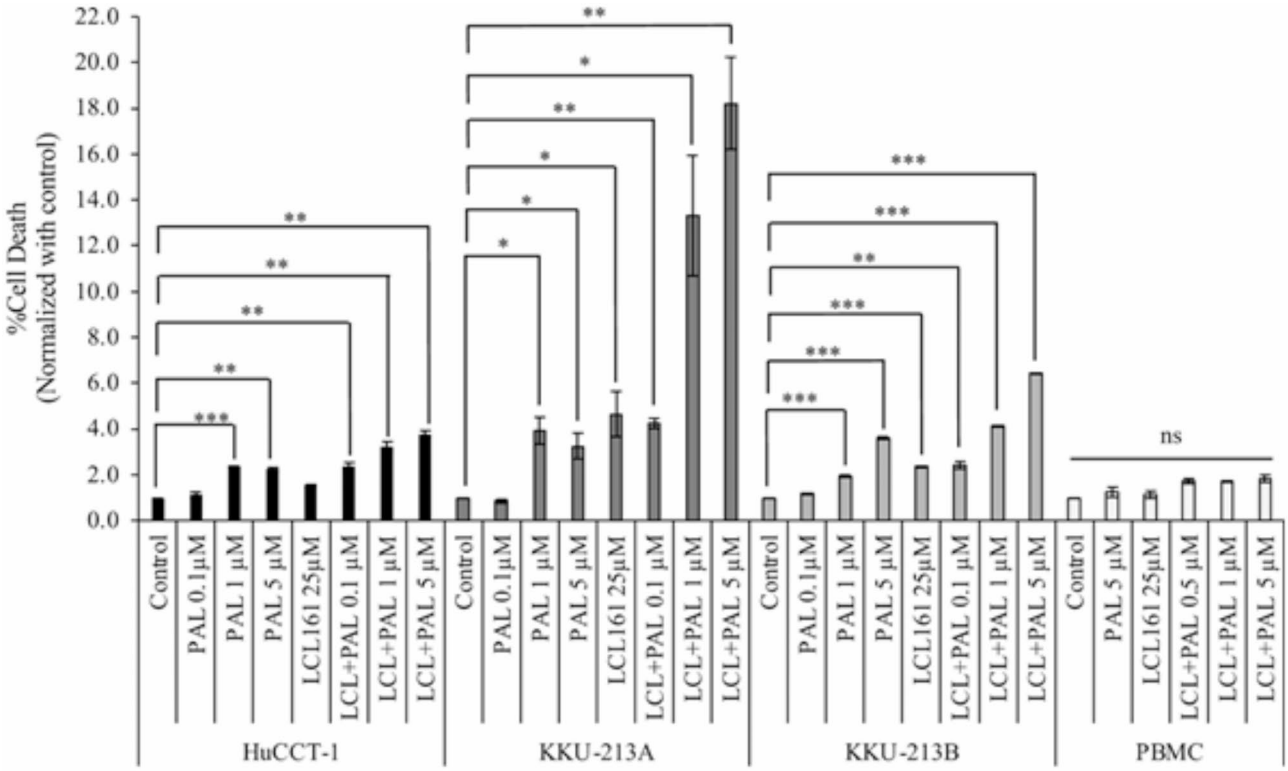


Fig. 7. The effect of the combination treatment of palbociclib and LCL161 on cell death induction in PBMCs. CCA cell lines and PBMCs were treated with palbociclib (0.1, 1, and 5 μM) and LCL161 (25 μM) as single treatments and in combination. Cell death was determined by Annexin V and PI staining and flow cytometry. The number of dead cells in the treatment groups was normalized to the control group and reported as percentage of cell death. The results are shown as mean ± S.D. of triplicate determinations from three independent experiments. Note: p-value < 0.05 (*), p-value < 0.01 (**), and p-value < 0.001 (***); ns = not significant.

MMNK-1. Fifth, these combination treatment results were recapitulated in the 3D spheroid tumor model, which better mimics the tumor microenvironment. Finally, the combination treatment exhibited low toxicity in PBMC cultures. Taken together, our findings provide a novel therapeutic combination of CDK4/6 inhibitors and Smac mimetics. This novel combination shows promise for developing a targeted therapeutic approach, applicable not only to CCA but also to other types of cancer.

Dysregulation of cell cycle regulators, particularly G1 regulators (Rb, CCND1, CDK4, CDK6 and CDKN2A), has been reported in CCA[11,39,40]. Our results demonstrated higher expression of cyclin D, CDK4, and CDK6 in CCA patients, as obtained from the GSE107943, OEP00115 and TCGA datasets, is particularly associated with shorter survival. These results suggest targeting the cyclin D/CDK4/6 complex as a therapeutic strategy in CCA patients. To examine the effectiveness of CDK4/6 inhibitor, cyclin D and phosphorylated Rb (pRb), a specific target of CDK4/6, were determined in three different CCA cell lines. The intact and functional Rb is essential for cells to respond to CDK4/6 inhibitors. Additionally, loss or mutation in the *RB1* gene (encoding the Rb protein) is a common resistance mechanism to CDK4/6 inhibitors[41]. All tested CCA cell lines expressed both cyclin D and pRb, consistent with previous studies in CCA patients and cell lines[11,42]. Although the lack of correlation between cyclin D1 expression and phospho-Rb (pRb) at basal levels may be attributed to cell population heterogeneity and other regulatory mechanisms influencing pRb[43], CDK4/6 inhibition leads to complete suppression of phospho-Rb. This suggests that CDK4/6 is a key regulator of pRb in CCA cells and that these cells may respond to therapies targeting the CDK4/6 complex. We investigated the effects of CDK4/6 inhibitor palbociclib monotherapy on cell proliferation and its ability to induce cell death in these three CCA cell lines. Our results showed that palbociclib can inhibit cell proliferation, but it was not as effective compared to a representative sensitive breast cancer cell line. The effectiveness of CDK4/6 inhibitors in CCA cell lines varies across different studies. A study by Sittithumcharee *et al*. showed that palbociclib can inhibit cell proliferation and induce senescence in 11 of 15 CCA cell lines expressing pRb[11]. In contrast, another study found that palbociclib had no effects on cell proliferation and cell death[44]. This discrepancy might be explained by differences in treatment conditions, particularly drug exposure time and the methods used to measure cell proliferation. As mentioned earlier, although palbociclib can inhibit cell proliferation in our study, it is not as effective as in a representative sensitive breast cancer cell line, and it has a minimal effect on inducing cell death. Therefore, the expression of pRb alone might not be sufficient for determining the sensitivity of CDK4/6 inhibitors. The limitations of CDK4/6 inhibitor monotherapy are due to different mechanisms, including aberrations in cell

survival signaling[20,45,46]. Additionally, recent findings reported that CDK4/6 inhibitors can induce a senescent-like phenotype[45,47]. The cytostatic effect and senescence-like phenotype of CDK4/6 inhibitors contribute to the low efficiency of CDK4/6 inhibitor treatment and limit their clinical benefits[48,49]. Since the effect of CDK4/6 inhibitors is cell cycle arrest induction but does not induce cell death, intrinsic and acquired resistance frequently occur in clinical practice. Therefore, the combination of CDK4/6 inhibitors with other compounds targeting cell survival and cell death pathways is a rational and necessary strategy to improve their efficacy and prevent resistance. Presently, CDK4/6 inhibitors have been combined with aromatase inhibitors, autophagy inhibitors, and mTOR inhibitors in clinical trials[21,50]. The combination of CDK4/6 inhibitors and mTOR inhibitors, which have limited antiproliferative activities but effectively induce apoptosis, has been reported in CCA[42]. In this study, we sought to identify novel key regulators that might modulate the effectiveness of CDK4/6 inhibitors and aimed to develop a rational combination treatment. Given the complexities of oncogenic signaling in CCA, combination therapy targeting multiple pathways might enhance tumor growth suppression, overcome drug resistance, and reduce drug toxicity due to the lower doses of drugs used compared to monotherapy.

Dysregulation of cell survival and cell death is common in many cancers, including CCA[26]. A previous study demonstrated that all NF-κB subunits (p50, p52, and p65) were highly expressed in hyperplastic, dysplastic, and CCA tissues but not in normal bile duct tissues[26]. Activated NF-κB promotes the expression of over 150 target genes involved in cell proliferation, suppression of apoptosis, and chemotherapeutic drug resistance[51]. The cellular inhibitors of apoptotic protein (cIAP) 1/2, key apoptosis regulators, are also target genes of NF-κB[52]. Therefore, it is possible that the upregulated basal level of cIAP1/2 in primary CCA tissues might result from the dysregulation of NF-κB. Multiomics profiling and GEO2R bioinformatic analysis revealed that cIAP1/2 was upregulated after breast cancer cell lines were exposed to CDK4/6 inhibitors[24]. This data is consistent with our study, which shows that cIAP1 and cIAP2 were upregulated following palbociclib treatment. As mentioned earlier, the non-canonical function of CDK4/6 inhibitors is to induce a senescence-associated secretory phenotype (SASP), which releases many types of cytokines such as interleukins and chemokines[53]. These cytokines act in a paracrine and autocrine manner, creating a positive feedback loop that triggers NF-κB signaling and might upregulate cIAP1/2[54]. Following palbociclib treatment, palbociclib increased the phosphorylation of p65 and its nuclear translocation, correlating with the upregulation of cIAP1/2 in CCA cells. Previous studies have reported that hyperphosphorylation of Rb by cyclin D-CDK4/6 facilitates specific interactions between Rb and p65, thereby inhibiting the transcription of NF-κB target genes. Following palbociclib treatment, which prevents phosphorylation of Rb at S249/T252, the release of p65 can activate the transcription of NF-κB target genes such as cIAP1/2[25,55]. However, our study does not directly confirm that palbociclib activates NF-κB signaling, resulting in cIAP1/2 upregulation in CCA cells, which might contribute to the responsiveness to CDK4/6 inhibitors in CCA. Further experimental validation is required to prove this point. The proposed mechanism of how palbociclib activates NF-κB signaling and cIAP1/2 is depicted in (Fig. 8).

Smac mimetics, cIAP1/2 antagonists, have been developed to degrade cIAP1/2 through proteasomal degradation[56]. Smac mimetic LCL161, which is currently in clinical trials for use both as a monotherapy and in combination therapy for various cancers, has favorable pharmacological properties[38]. In addition, recent studies have explored the use of IAP inhibitors in CCA treatment. A study by Prasopporn, *et al.* demonstrated that the combination of Smac mimetic, LCL161 with Gemcitabine and Cisplatin could be an effective strategy for treating CCA by inhibiting tumor growth and preventing the development of multidrug resistance[32]. Similarly, a study by Fingas, *et al.* showed that Smac mimetics reduce TRAIL-induced invasion and metastasis of CCA cells, suggesting the potential of combining Smac mimetics with TRAIL as a strategy to suppress cancer metastasis[57]. Based on these studies, the potential of combining Smac mimetics in CCA therapy is highlighted, supporting the rationale for further exploration of this therapeutic strategy. Therefore, palbociclib, at concentrations that inhibit cell proliferation and induce minimal cell death, was combined with Smac mimetic LCL161 at a concentration that effectively degrades cIAP1 and cIAP2, while only inducing minimal cell death[30,31,45]. Our study is the first to demonstrate that the combination treatment of the CDK4/6 inhibitor palbociclib and Smac mimetic LCL161 synergistically inhibited cell proliferation and induced cell death in both 2D monolayer cell culture and 3D spheroid tumor models, which having the less effect on non-tumor cholangiocytes and human peripheral blood mononuclear cells (PBMCs). Since LCL161 is well known for inducing apoptosis by targeting the degradation of cIAP1/2, the observed synergistic effects may be mediated through apoptosis. Moreover, the possibility that other cellular mechanisms are involved cannot be excluded. One possible mechanism is that LCL161 may eliminate senescent cells induced by palbociclib, as previous studies have shown that palbociclib can induce senescence, and these senescent cells may escape and contribute to cancer recurrence[48,49]. Additionally, senescent cells have been reported to evade apoptosis by upregulating anti-apoptotic proteins, such as cIAP1 and cIAP2, suggesting that targeting these proteins may induce apoptosis in senescent cells[58]. While LCL161 has not been directly reported as a senolytic effect, other IAP inhibitors have demonstrated senolytic activity[58,59]. Another possible mechanism is that LCL161 may induce other forms of cell death apart from apoptosis, such as necroptosis, which has been reported in some cancers[29–31,60–62]. However, further studies are needed to elucidate the underlying mechanisms of CDK4/6 inhibitor and Smac mimetic combination treatments in inducing cell death. Additionally, *in vivo* studies using animal models are necessary before the combination treatment of palbociclib and Smac mimetic can be translated into clinical applications for CCA patients, with the goal of developing a more effective targeted therapy. Taken together, we show that cyclin D, CDK4, and CDK6 are overexpressed in CCA. Therefore, the CDK4/6 inhibitor palbociclib can inhibit the cyclin D/CDK4/6 complex, resulting in unphosphorylated Rb and disruption of the Rb-E2F complex, leading to inhibition of cell proliferation. We propose that this effect of the CDK4/6 inhibitor also results in the release of p65 from unphosphorylated Rb, allowing p65 to activate cIAP1/2, NF-κB target gene transcription. Additionally, cIAP1 and cIAP2 are also overexpressed in CCA. Therefore, the Smac mimetic was used to degrade cIAP1/2 in combination with the CDK4/6 inhibitor. Therefore, the antiproliferative effect of the CDK4/6 inhibitor and the cell death-inducing activity of the Smac mimetic can

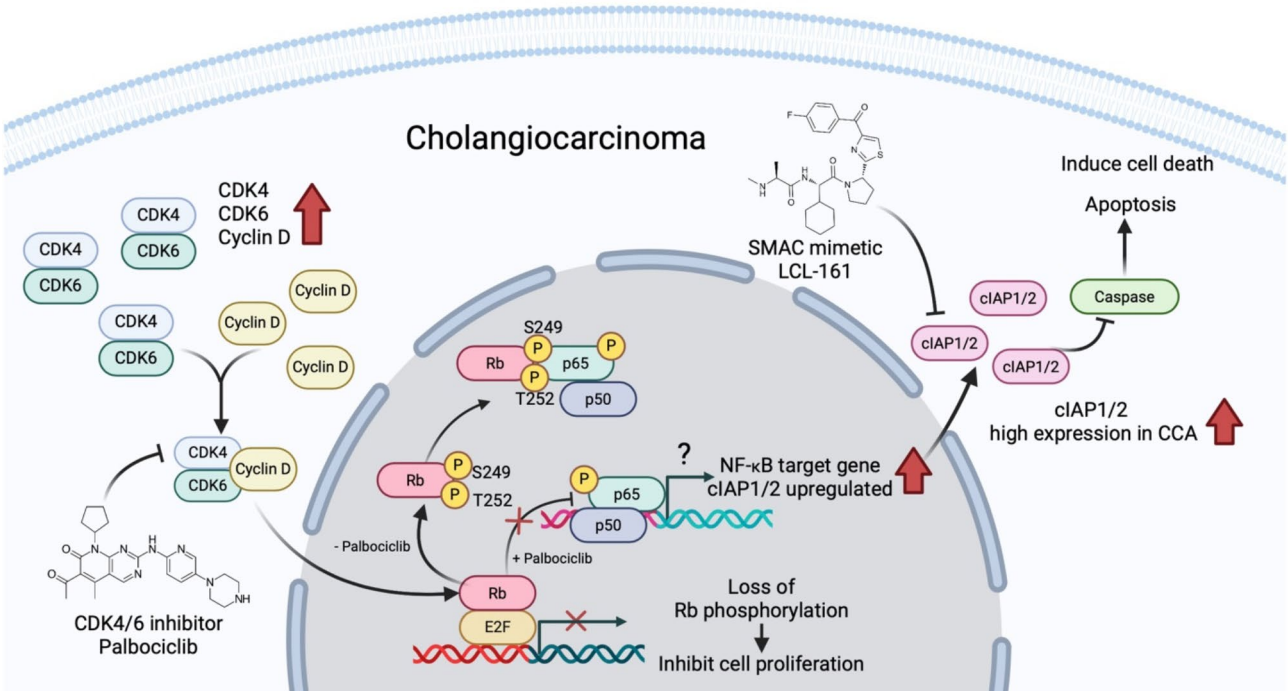


Fig. 8. Schematic proposed mechanism of palbociclib and Smac mimetic combination treatment in CCA cells. Cyclin D, CDK4, and CDK6 are overexpressed in CCA. Therefore, the CDK4/6 inhibitor palbociclib can inhibit the Cyclin D/CDK4/6 complex, resulting in unphosphorylated Rb and disruption of the Rb-E2F complex, leading to inhibition of cell proliferation. We propose that this effect of the CDK4/6 inhibitor also results in the release of p65 from unphosphorylated Rb, allowing p65 to activate cIAP1/2, NF-κB target gene transcription. Additionally, cIAP1 and cIAP2 are also overexpressed in CCA. Therefore, the Smac mimetic was used to degrade cIAP1/2 in combination with the CDK4/6 inhibitor. The antiproliferative effect of the CDK4/6 inhibitor and the cell death-inducing activity of the Smac mimetic can synergistically inhibit cell proliferation and induce cell death in CCA, making this combination approach a novel and promising targeted therapy for CCA patients. This figure was created using BioRender. Jitkaew, S. (2025). Retrieved from https://BioRender.com/x20i079.

synergistically inhibit cell proliferation and induce cell death in CCA, making this combination approach a novel and promising targeted therapy for CCA patients (Fig. 8).

Conclusions

Our findings demonstrate that the combination treatment of Smac mimetics and CDK4/6 inhibitors is a promising targeted therapy for CCA patients. This strategy enhances the CDK4/6 inhibitor response and shows potential for effective treatment with minimal impact on normal cells.

Data availability

All datasets in this study can be found in public databases, including GSE107943 from the Gene Expression Omnibus (GEO) database (https://www.ncbi.nlm.nih.gov/geo/), TCGA-CHOL downloaded from the University of California at Santa Cruz (UCSC) Xena platform (https://xena.ucsc.edu/), and OEP001105 from the National Omics Data Encyclopedia (NODE) database (https://www.biosino.org/node/project/detail/OEP001105).

Received: 6 October 2024; Accepted: 17 February 2025
Published online: 25 February 2025

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Acknowledgements

PM gratefully acknowledges the scholarship from the Graduate School, Chulalongkorn University to commemorate the 72nd anniversary of his Majesty King Bhumibol Adulyadej. ND acknowledges Second Century Fund (C2F), Chulalongkorn University and the Royal Golden Jubilee Ph.D. Programme Scholarship from National Research Council of Thailand (NRCT) (N41A670190). AS-F acknowledges the Royal Golden Jubilee Ph.D. Programme Scholarship from National Research Council of Thailand (NRCT) (NRCT5-RGJ63001-026). SS acknowledges Second Century Fund (C2F), Chulalongkorn University and the Royal Golden Jubilee Ph.D. Programme Scholarship from National Research Council of Thailand (NRCT) (NRCT5-RGJ63001-023).

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SJ; Supervision, Conceptualization, Funding acquisition, Validation, Formal analysis, Writing—review & editing. PM; Methodology, Validation, Investigation, Visualization, Formal analysis, Writing—original draft. ND; Methodology, Investigation, Visualization. AS-F; Methodology, Investigation, Visualization, SS; Methodology, Investigation, Visualization.

Funding

This research is funded by the Office of National Higher Education Science Research and Innovation Policy Council by Program Management Unit for Human Resources and Institutional Development, Research and Innovation (PMU-B); grant number B05F640067 and the Thailand Science Research and Innovation Fund Chulalongkorn University (HEA663700097) to SJ and the 90th Anniversary Chulalongkorn University Fund (Ratchadaphiseksomphot Endowment Fund; Grant Number GCUGR1125662073M) to PM.

Declarations

Competing interests

The authors declare no competing interests.

Additional information

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1038/s41598-025-90997-y.

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