

PLK1 inhibition leads to mitotic arrest and triggers apoptosis in cholangiocarcinoma cells

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Abstract. Cholangiocarcinoma (CCA) is a lethal cancer originating from the epithelial cells within the bile duct and ranks as the second most prevalent form of liver cancer in Thailand. Polo-like kinase 1 (PLK1), a protein serine/threonine kinase, regulates a number of steps in cell mitosis and is upregulated in several types of cancer, including CCA. Our previous study identified PLK1 as a biomarker of the C1 subtype, correlating with poor prognosis in intrahepatic CCA. The present study aimed to examine the effect of PLK1 inhibition on CCA cells. Different CCA cell lines developed from Thai patients, HuCCA1, KKU055, KKU100 and KKU213A, were treated with two PLK1 inhibitors, BI2536 and BI6727, and were transfected with small interfering RNA, followed by analysis of cell proliferation, cell cycle distribution and cell apoptosis. It was discovered that BI2536 and BI6727 inhibited cell proliferation and caused G₂/M-phase arrest in CCA cells. Furthermore, the number of total apoptotic cells was increased in PLK1 inhibitor-treated CCA cells. The expression levels of mitotic proteins, aurora kinase A, phosphorylated PLK1 (T210) and cyclin B1, were augmented in PLK1-inhibited CCA cells. Additionally, inhibition of PLK1 led to increased DNA damage, as determined by the upregulated levels of γ H2AX and increased cleavage of poly (ADP-ribose) polymerase, an apoptotic marker. These results suggested that inhibiting PLK1 prolonged mitotic arrest and subsequently triggered cell apoptosis. Validation of the antiproliferative effects of PLK1 inhibition was accomplished through silencing of the PLK1 gene. In conclusion, targeting PLK1 provided promising results for further study as a potential candidate for targeted therapy in CCA.

Introduction

Cholangiocarcinoma (CCA) is a rare malignancy that originates in the epithelial cells within the biliary tract, occurring either within (intrahepatic) or outside (extrahepatic) the liver. CCA is the second most prevalent form of liver cancer, with its highest incidence observed in northeast Thailand (1,2). The major risk factors associated with CCA often occur from chronic biliary inflammation, due to conditions such as liver fluke infection, hepatitis B and C viruses, and primary sclerosing cholangitis (3-5). Diagnosis at advanced stages and the challenging prognosis greatly complicate treatment strategies. While surgical resection remains the primary therapeutic option, its applicability is limited, depending upon the site and stage of the cancer (6,7). To enhance the treatment choices, chemotherapeutic regimens are crucial in patients with CCA. Notably, drugs such as 5-fluorouracil, gemcitabine and cisplatin, either individually or in combination, are frequently employed in the treatment of patients with cancer (8-10). However, long-term drug treatment outcomes remain insufficient, emphasizing the need for innovative treatment approaches in patients with CCA.

Previously published research from Chaisaingmongkol *et al* (11) identified molecular subtypes of intrahepatic CCA from Thai patients through the systematic integration of genomics, transcriptomics and metabolomics data derived from paired tumor and non-tumor tissue specimens. This previous study discovered the C1 subtype, which is associated with a poor prognosis and shows defects of mitotic checkpoint signaling. Importantly, the authors discovered the polo-like kinase 1 (PLK1) gene as a subtype biomarker and also as a molecular target for CCA (11).

PLK1 is a serine/threonine protein kinase involved in various steps in mitotic events. Among other PLK family proteins (PLK1-5), PLK1 has been extensively examined for its role in the control of the cell cycle and cancer (12). PLKs comprise an N-terminal kinase domain, which is responsible for the binding of ATP and activating the enzyme, and a C-terminal polo box domain, involved in phosphopeptide binding (13). PLK1 regulates a number of steps in mitosis, including maturation of the centrosome, initiation of mitosis, establishment of kinetochore-microtubule attachments,

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spindle orientation and the process of cytokinesis (14,15). PLK1 is upregulated in numerous types of cancer, including hepatocellular carcinoma (16), breast cancer (17), colorectal cancer (18), gastric cancer (19), ovarian carcinoma (20), chronic myeloid leukemia (21) and CCA (11). On the other hand, PLK1 expression is mostly absent in normal cells and non-dividing healthy cells (22). This makes PLK1 an appealing target for the enhancement of cancer therapeutic drugs.

A number of reports have revealed that inhibiting PLK1 favors antitumor effects in numerous types of cancer. Depleting PLK1 levels in cancer cells reduces cell proliferation and perturbs the spindle assembly, triggering activation of the mitotic checkpoint, resulting in mitotic arrest and ultimately leading to cell death (23,24). Several PLK1 inhibitors have been developed as cancer therapeutic agents. BI2536 (dihydropteridinone), a highly selective and potent inhibitor, was first developed and has been shown to exert an antitumor effect in several types of cancer (25,26). BI6727, another dihydropyridinone derivative, emerged as an ATP-competitive inhibitor of PLK1 that was later developed as a drug (27). Small molecule inhibitors targeting PLK1 can lead to a decrease in cell proliferation, disruption in cell cycle progression (specifically G₂/M-phase arrest) and the induction of cell apoptosis in a number of cancers, including oral cancer cells (28), melanoma cells (29), lymphoma cells (30) and non-small lung cancer cells (31). To date, there are only a few studies exploring the consequences of inhibiting PLK1 in CCA (32-34).

The present study aimed to explore the effects of inhibiting PLK1 in four distinct CCA cell lines derived from Thai patients. It revealed the antiproliferative effects, G₂/M-phase arrest and apoptosis in CCA cells following the inhibition of PLK1 using both small molecule inhibitors and small interfering (si)RNA.

Materials and methods

Cell culture. In the present study, four different CCA cell lines derived from Thai patients with CCA, HuCCA1, KKU055, KKU100 and KKU213A, from the Japanese Collection of Research Bioresources Cell Bank (JCRB) were employed. KKU100 (cat. no. JCRB1568, RRID: CVCL_3996), KKU055 (JCRB cat. no. JCRB1551, RRID: CVCL_M258) and KKU213A (JCRB cat. no. JCRB1557, RRID: CVCL_M261). The cell passage numbers for KKU055, KKU100, and KKU213A upon purchase were P4, P9, and P9, respectively. HuCCA1 (RRID: CVCL_M255; cell passage 108) was established and generously provided by Dr Stitaya Sirisinha, Department of Microbiology, Faculty of Science, Mahidol University, Thailand (35). The attributes of the CCA cell lines used in this investigation are detailed in Table SI. HuCCA1 originated from a patient with intrahepatic CCA, and the serum of the patient was positive for *Opisthorchis viverrini* (Ov) antigen. KKU055 and KKU213A were also established from patients with intrahepatic CCA (36,37). KKU100 was isolated from the tissue of a patient with extrahepatic CCA (38). KKU100 and KKU213A cell lines were both from patients with Ov in stool samples. The doubling times of the CCA cell lines are as follows: HuCCA1, 55 h (35); KKU100, 72 h (38); KKU055, 23.6 h (39); and KKU213A, 23.4 h (40). Genetic alterations of genes frequently mutated in cancer were also shown in

Table SI (41-43). KKU100, KKU055 and KKU213A cells were cultured in DMEM (cat. no. SH3024302; HyClone; Cytiva), and HuCCA1 cells were maintained in Ham's F12 media (cat. no. SH3002602; HyClone; Cytiva). All cell culture media were supplemented with 10% FBS (cat. no. IIVG3-10270-106; Invitrogen; Thermo Fisher Scientific, Inc.) and 1% penicillin/streptomycin (cat. no. ITFS-ICC-15140122; Invitrogen; Thermo Fisher Scientific, Inc.) and all cells were cultured at 37°C in a 5% CO₂ humidified incubator. All four CCA cell lines used in the present study were sent to CLS Cell Lines Service GmbH (cat. no. 900154) for cell line authentication (report date: August 2023) using highly polymorphic short tandem repeat loci. The analyzed data of the samples perfectly aligned with the DNA profiles of the cell lines HuCCA1, KKU100 and KKU213A with a 100% match. The score for the KKU055 cell line, however, was slightly lower at 93.8%.

Agents and antibodies. PLK1 inhibitors, BI2536 (cat. no. HY-50698) and BI6727 (cat. no. HY12137) were purchased from MedChemExpress. The primary antibodies for various proteins were obtained from Cell Signaling Technology, Inc., unless specified otherwise: Phosphorylated (p)-PLK1 (Thr210; cat. no. 9062; RRID: AB_11127447), aurora kinase A (aurora A/AIK; cat. no. 3092; RRID: AB_2061342), cyclin B1 (cat. no. 4138; RRID: AB_2072132), poly (ADP-ribose) polymerase (PARP; cat. no. 9542; RRID: AB_2160739), p-Histone H2AX (Ser139; γH2AX; cat. no. 9718; RRID: AB_2118009), Histone H2A.X (cat. no. 2595; RRID: AB_10694556), GAPDH (cat. no. 8884; RRID: AB_11129865), α-tubulin (cat. no. 2125; RRID: AB_2619646) and PLK1 (anti-PLK1; clone 35-206; cat. no. 05-844; RRID: AB_310836; Millipore). The secondary antibodies used were HRP-conjugated goat anti-rabbit (cat. no. 7074) and horse anti-mouse (cat. no. 7076). The dilutions of primary antibodies and secondary antibodies were 1:1,000 and 1:2,000, respectively.

Gene expression analysis by reverse transcription-quantitative (RT-q) PCR. Cells (1×10⁶) were initially seeded into 10-cm cell culture plates. The following day, the cells were collected by trypsinization and washed with PBS. The extraction of total RNA was carried out using the RNeasy mini kit (cat. no. 75144; Qiagen GmbH), and its quantification was assessed using the NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Inc.), following the manufacturer's instructions. Subsequently, cDNA synthesis was accomplished using 1 μg total RNA with the SuperScript III First-strand synthesis system (cat. no. IIV02-18080-051; Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. The expression of the PLK1 gene was conducted through qPCR, utilizing the Power SYBR Green PCR Master Mix (cat. no. 4367659, Applied Biosystems; Thermo Fisher Scientific, Inc.) and was analyzed using the Applied Biosystem StepOnePlus Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions. The PCR cycling conditions were as follows: Initial activation at 95°C for 10 min, followed by 40 cycles consisting of denaturation at 95°C for 15 sec, annealing and extension at 60°C for 30 sec. GAPDH expression served as the endogenous control. The primer sequences were as follows: GAPDH forward, 5'-ACAGTCAGCCGCATCTTCTT-3' and reverse, 5'-ACG

ACCAAATCCGTTGACTC-3'; PLK1 forward, 5'-CGTGACCTACATCGACGAGA-3' and reverse, 5'-AGCAGCTTGTCACCACATAGT-3'. The relative mRNA expression levels were analyzed by normalization to the endogenous gene using the $2^{-\Delta C_q}$ method, where $\Delta C_q = C_{q\text{PLK1}} - C_{q\text{GAPDH}}$ (44). The experiments were repeated three times.

Cell viability and colony formation assays. Cell proliferation was evaluated by analyzing cell viability and the ability to form colonies. Cell viability was assessed using MTT assay, a colorimetric method that measures metabolically active cells. In this experiment, 10,000 cells were placed in duplicate into 24-well plates and treated with different concentrations of PLK1 inhibitors dissolved in DMSO for 24, 48 and 72 h. After treatment, the cell culture medium was replaced with a solution containing 5 mg/ml MTT (cat. no. M2128; MilliporeSigma) and the cells were incubated at 37°C for 1.5 h. The medium was then substituted with DMSO, and the absorbance of the resultant solution was measured at 540 nm using the PerkinElmer Victor Nivo plate reader (PerkinElmer, Inc.). The absorbance is directly proportional to the number of viable cells. Cell viability was compared with that of cells treated with a vehicle control, and these experiments were replicated three times.

In the colony formation assay, 3,000 cells were placed in 6-well plates and exposed to PLK1 inhibitors for 48 h at 37°C in a 5% CO₂ humidified incubator. Following this treatment, the culture medium was substituted with drug-free medium, and the cells were allowed to grow for 1 additional week. The resulting colonies were rinsed twice with 1X PBS, fixed with cold methanol for 2 min, air-dried and then stained with a 0.25% aqueous crystal violet solution (cat. no. V5265; MilliporeSigma) at room temperature for 30 min. Subsequently, the plates were rinsed with tap water and left to air dry.

PLK1 gene silencing by small interfering RNA (siRNA). A synthetic siRNA targeting PLK1 (cat. no. 6292; sense: 5'-CCCUCACAGUCCUCAAUAA-3', antisense: 5'-UUAUUGAGGACUGUGAGGG-3') and control siRNA (cat. no. 6568; sense: 5'-CGUACGCGGAUACUUCGA-3', antisense: 5'-UCGAAGUAUUCGCGUACG-3') were purchased from Cell Signaling Technology, Inc. CCA cell lines were transfected with 100 nM PLK1 siRNA or control siRNA (negative control) using Lipofectamine® 3000 reagent (cat. no. L300015; Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. Following a 48-h transfection at 37°C in a 5% CO₂ humidified incubator, the transfected cells were analyzed for cell viability, cell cycle distribution, cell apoptosis and protein detection. PLK1 protein depletion was confirmed through western blotting.

Cell cycle analysis. Cell cycle distribution was examined using a flow cytometer and the Muse cell cycle assay kit (cat. no. MCH100106; Luminex Corporation) following the manufacturer's guidelines. In summary, treated cells were harvested through trypsinization, rinsed with PBS and fixed overnight at -20°C with ice-cold 70% ethanol. After washing with PBS, the cells were centrifuged at 300 x g for 5 min at room temperature, suspended in Muse cell cycle reagent, and then incubated

in the dark at room temperature for 30 min. Subsequently, the samples were analyzed using the Muse cell analyzer (MUSE 0500-3115B; Luminex Corporation) to determine the percentage of cells in each cell cycle phase (G₀/G₁, S and G₂/M) based on their varying DNA content. These experiments were conducted in biological triplicates.

Detection of cell apoptosis. To evaluate the impact of PLK1 inhibition on apoptotic cell death, flow cytometric analysis was performed using the Muse Annexin V and dead cell kit (cat. no. MCH100106; Luminex Corporation), following the manufacturer's guidelines. The assay is based on the binding of Annexin V to phosphatidylserine (PS) on the surface of apoptotic cells. The kit uses a premixed reagent containing fluorescently labeled Annexin V and a dead cell marker known as 7-Aminoactinomycin D (7-AAD) as the second dye. 7-AAD is a fluorescent dye used for evaluating cell viability by binding to DNA through intercalation. Live cells typically exclude 7-AAD due to intact cell membranes, while it readily enters cells with compromised membranes, such as late apoptotic and dead cells. Cells (15x10⁴) were cultured in 6-well plates with 2 ml culture media. After an overnight incubation, the cells were treated with 10 and 100 nM BI2536 or BI6727. Following a 48-h treatment at 37°C in a 5% CO₂ humidified incubator, floating cells were collected and adherent cells were detached through trypsinization. The harvested cells were then centrifuged at 300 x g for 5 min at room temperature, and cell pellets were suspended in culture media with 1% FBS. Equal volumes of cell suspension and Muse Annexin V reagent were mixed and incubated for 20 min at room temperature. The percentages of live, early apoptotic, late apoptotic, total apoptotic and dead cells were determined using the Muse cell analyzer (MUSE 0500-3115B; Luminex Corporation). The experiments were repeated three times.

Western blot analysis. The treated cells were rinsed with cold PBS, and then scraped and collected. They were lysed in a cold buffer (composed of 50 mM Tris-HCl, pH 7.4; 150 mM NaCl; 1% NP40; 0.25% Na-deoxycholate; 1 mM EDTA) containing the Halt protease and phosphatase inhibitor cocktail (cat. no. 78440; MilliporeSigma). After centrifugation of the cell lysates at 13,000 x g for 15 min at 4°C, the protein concentration was determined using Bradford reagent (cat. no. B6916; MilliporeSigma) according to the manufacturer's protocols. Equal amounts of protein (20-30 µg) were separated using 12% polyacrylamide gels and were then transferred onto Immobilon PVDF transfer membranes (MilliporeSigma). The membrane was cut into two or three strips based on the molecular weight of the targeted proteins. Subsequently, the membrane strips were blocked with 5% non-fat dry milk in Tris-buffered saline (TBS) with 0.1% Tween-20 for 1 h at room temperature. Each strip was then probed separately with the specific antibodies. Proteins were identified by incubating with primary antibodies, appropriately diluted in 4% BSA (cat. no. sc-2323; Santa Cruz Biotechnology, Inc.) or 5% dried milk in TBS with 0.1% Tween-20, overnight at 4°C, followed by a 2-h incubation with secondary antibodies at room temperature. Ultimately, the proteins were visualized using the ECL plus western blotting detection system (Cytiva) and a gel western blot imaging system (G: BOX;

Syngene Europe). The relative expression of the protein bands was semi-quantified using ImageJ version 1.53 software (National Institutes of Health).

Statistical analysis. The bar graphs show the mean \pm standard deviation of three independent experiments. The Student's two-tailed unpaired t-test for independent samples was used to analyze the statistically significant differences between treated and control cells. Comparisons among treatment groups were performed by one-way analysis of variance, followed by the Bonferroni post hoc test using SPSS version 26.0 (IBM Corp.). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

PLK1 is upregulated in CCA cell lines. the level of PLK1 was assessed in four different CCA cell lines: HuCCA1, KKU055, KKU100 and KKU213A, by detecting the gene and protein expression levels. These CCA cell lines are widely used in CCA research. PLK1 mRNA expression was quantified by RT-qPCR, with relative mRNA expression normalized to a reference gene (Fig. 1A). PLK1 protein expression was detected by western blotting and the results revealed a substantial presence of PLK1 protein in CCA cell lines (Fig. 1B). Notably, among the four CCA cell lines, KKU100 exhibited the lowest expression of PLK1 at both the mRNA and protein levels.

BI2536 and BI6727 suppress the proliferation of CCA cell lines. To determine the effects of PLK1 inhibition on CCA cells, BI2536 and BI6727, two potent PLK1 inhibitors, were used to treat four CCA cell lines, HuCCA1, KKU055, KKU100 and KKU213A, across a range of increasing concentrations (0-500 nM) and treatment durations (24-72 h). BI2536 (Fig. 2A) and BI6727 (Fig. 2B) exhibited a time-dependent reduction in cell viability among CCA cell lines. A summary of the half maximal inhibitory concentration (IC_{50}) values of both BI2536 and BI6727 in all cell lines tested at each treatment time (24-72 h) is shown in Table SII. Among the four cell lines, KKU055 displayed the highest sensitivity, while HuCCA1 exhibited the lowest sensitivity. The concentrations of PLK1 inhibitors at 10 and 100 nM were selected for subsequent experiments based on the observation that the concentrations higher than 100 nM for both BI2536 and BI6727 did not yield a more pronounced inhibitory effect. Moreover, considering the varying sensitivity to PLK1 inhibitors across the four distinct cell lines, it was decided to employ a broad range of concentrations (10-fold), to capture and analyze the various responses. Additionally, to further investigate the antiproliferative effect of PLK1 inhibitors, the ability of BI2536- and BI6727-treated cells to form colonies was examined. The results, as shown in Fig. 2C, demonstrated an inhibition in the colony-forming capability of the treated cells across all four CCA cell lines.

BI2536 and BI6727 induce G_2/M -phase arrest in CCA cells. To investigate the effects of PLK1 inhibition on cell cycle distribution, cells were treated with BI2536 and BI6727 for 24 h followed by cell cycle analysis. The population of cells in G_2/M phase was significantly increased in CCA cells treated with both BI2536 (Fig. 3A) and BI6727 (Fig. 3B), as compared

to cells treated with the vehicle control. The induction of G_2/M -phase arrest was significantly pronounced in all four CCA cell lines. Furthermore, it was observed that BI6727 at a concentration of 10 nM displayed a lesser capacity to induce cell cycle arrest when compared with BI2536 at the same concentration.

BI2536- and BI6727-treated CCA cells undergo apoptosis. Next, the effect of PLK1 inhibition on cell apoptosis was determined. The effect of PLK1 inhibitors on cell cycle arrest observed after a 24-h treatment suggested that the inhibitors were able to halt cell cycle progression, the present study thus aimed to determine whether cell apoptosis manifested at a later time point, at 48 h post-treatment. CCA cells were treated with 10 and 100 nM BI2536 (Fig. 4A) and BI6727 (Fig. 4B), followed by Annexin V/7-AAD double staining followed by flow cytometry to detect apoptotic cells. Cells treated with both inhibitors exhibited an increase in total apoptotic cells (early and late apoptotic cells). Furthermore, there was no increase in dead necrotic cells observed with either of the PLK1 inhibitors, as indicated by the absence of significant differences in dead necrotic cells across all treatment conditions. However, it was revealed that the percentages of total apoptotic cells were notably lower in the HuCCA1 cell line in comparison to the other CCA cell lines.

Inhibiting PLK1 causes alterations in mitotic proteins, and induces DNA damage and PARP cleavage. The present study proceeded to evaluate the levels of mitotic proteins in CCA cells subjected to PLK1 inhibition, since CCA cells treated with BI2536 and BI6727 displayed G_2/M arrest. Treatment with BI2536 and BI6727 led to the upregulation of PLK1-related proteins, including aurora kinase A, p-PLK1 (T210), PLK1 and cyclin B1, in CCA cell lines (Fig. 5A). However, treatment with PLK1 inhibitors did not result in an elevation in the expression levels of cyclin B1 in KKU213A cells. The upregulation of these proteins signified the activation of the spindle assembly checkpoint. This confirmed that inhibition of PLK1 initiated a state of mitotic arrest and subsequently induced apoptotic cell death.

Additionally, the effect of PLK1 inhibition on the induction of DNA damage leading to cell death has been previously reported (45). To verify the possible mechanisms of PLK1 inhibitor-induced cell death, the levels of DNA strand breaks were assessed via the detection of phosphorylated histone H2AX at S139 (called γ H2AX), as well as the detection of apoptotic markers, PARP and caspase-9 cleavage, following BI2536 and BI6727 treatment. The present study observed the upregulation of γ H2AX across all four CCA cell lines, thus indicating an increase in DNA damage after PLK1 inhibition (Fig. 5B). Furthermore, both BI2536 and BI6727 caused PARP and caspase-9 cleavage in KKU055, KKU100 and KKU213A cell lines. These results are in line with the percentages of total apoptotic cells shown in Fig. 4B, whereby HuCCA1 cells displayed a comparatively lower count of apoptotic cells in relation to the other cell lines. Similar trends were observed upon treatment with BI6727.

Depletion of the PLK1 gene exhibits antiproliferative effects on CCA cell lines. To further validate the antiproliferative effects of PLK1 inhibitors, PLK1 gene silencing by siRNA

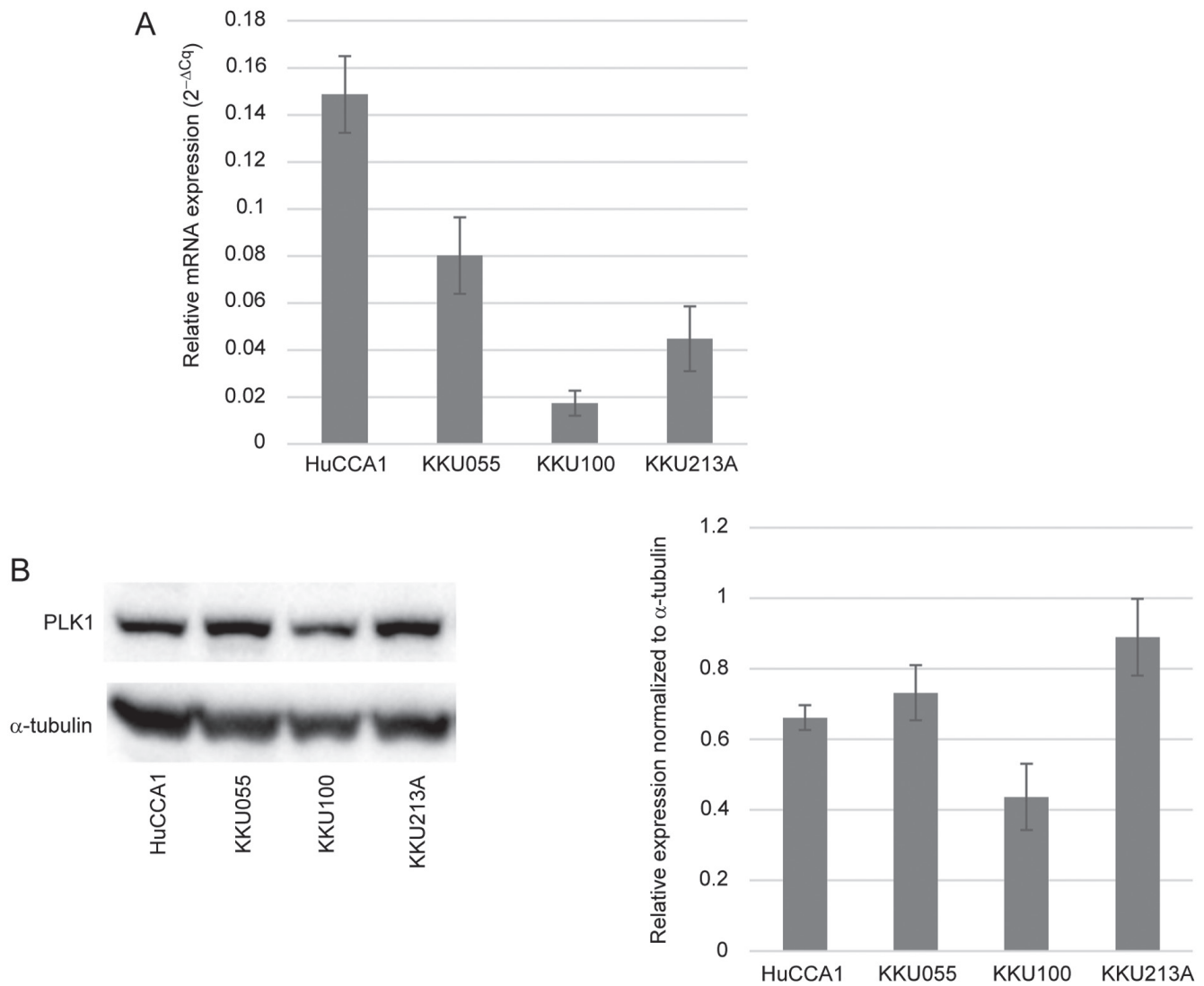


Figure 1. PLK1 expression is increased in CCA cell lines. (A) PLK1 mRNA levels were measured by reverse transcription-quantitative PCR. The relative mRNA expression was normalized to the reference gene GAPDH. The results are presented as the mean \pm SD of three independent experiments. (B) A representative western blot of PLK1 protein expression in CCA cell lines. PLK1, polo-like kinase 1; CCA, cholangiocarcinoma.

was performed in all four CCA cell lines. Cells were transfected with PLK1 siRNA or control siRNA for 48 h, followed by the analysis of cell viability, cell cycle distribution and cell apoptosis. As shown in Fig. 6A, cells transfected with PLK1 siRNA exhibited diminished cell viability in comparison to cells transfected with control siRNA. Silencing of PLK1 induced G₂/M arrest, as demonstrated in Fig. 6B, where representative histograms depicted the distribution of cells across G₀/G₁, S, and G₂/M phases (Fig. S1A). Although the histogram for siPLK1-transfected KKKU055 cells displayed a lower peak in the G₂/M phase, possibly due to the lower number of cells counted by the analyzer compared to siSC-transfected KKKU055 cells, the percentage of cells in the G₂/M phase was higher in siPLK1-transfected cells compared to siSC-transfected cells, as illustrated in Fig. 6B. Moreover, silencing of PLK1 triggered cell apoptosis, particularly notable in KKKU055 and KKKU213A, as shown in Fig. 6C. The representative dot plots showing Annexin V/7-AAD double staining of CCA cells transfected with siSC or siPLK1 were shown in Fig. S1B. There was no significant increase in dead

necrotic cells in siPLK1- and siSC-transfected cells. However, KKKU100 cells transfected with both control siRNA and PLK1 siRNA displayed a substantial population of total apoptotic cells, potentially attributed to the toxicity of the transfection reagent. PLK1 gene depletion was confirmed by the reduction in PLK1 protein expression (Fig. 6D). Moreover, the upregulation of mitotic proteins, including aurora kinase A and cyclin B1, was consistent with the results obtained in response to PLK1 inhibitors. The silencing of PLK1 induced DNA damage and cell apoptosis, as shown by increased γ H2AX and PARP cleavage, respectively. Overall, these results agreed with the effects of BI2536 and BI6727 treatment on CCA cells.

Discussion

Alterations in the expression of genes related to the regulation of the cell cycle are frequently observed in various types of cancer. PLK1, a protein kinase that is involved in multiple stages of cell cycle progression, is often overexpressed and is correlated with aggressiveness and unfavorable prognosis

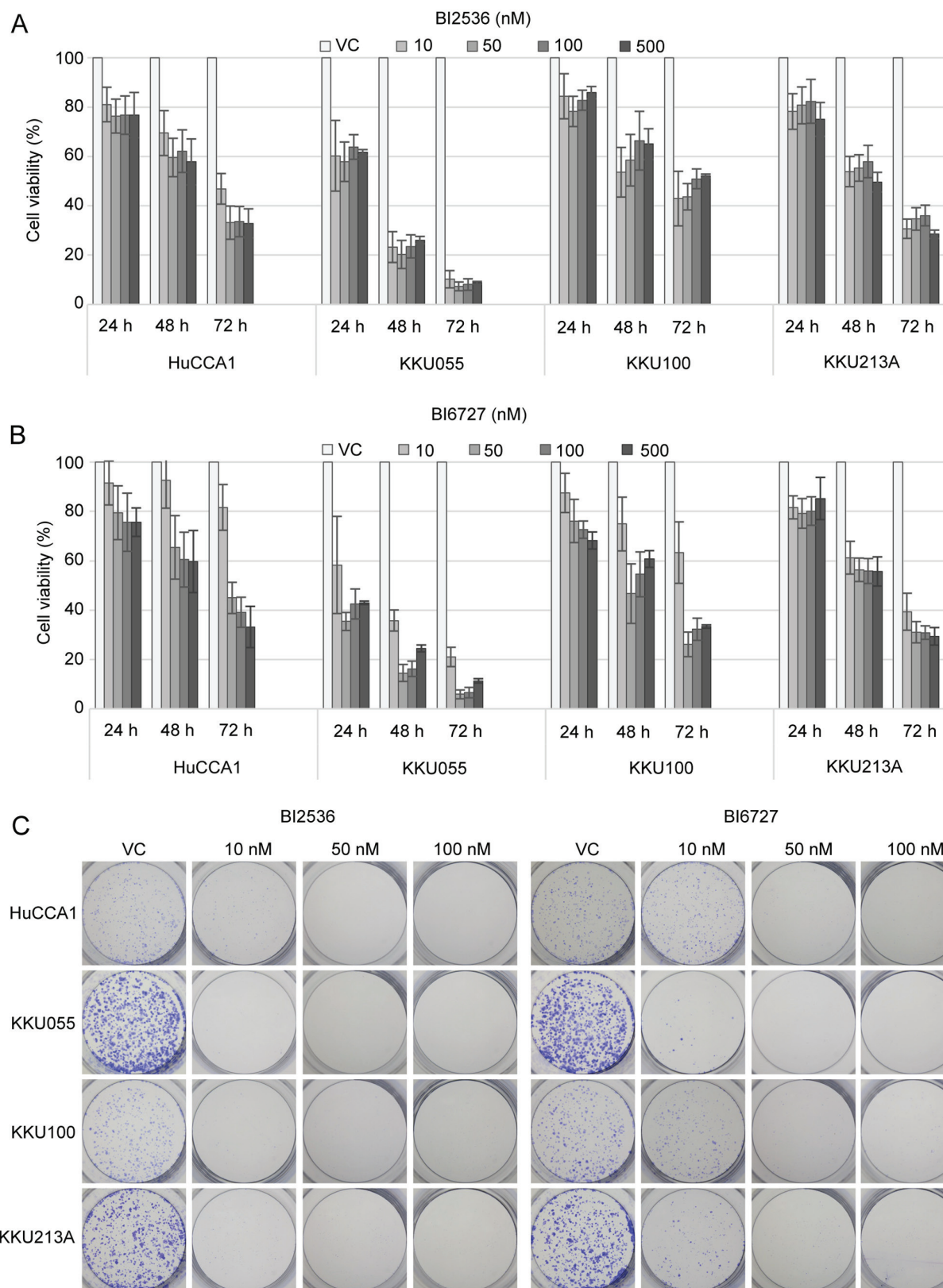


Figure 2. PLK1 inhibitors reduce the proliferation of CCA cell lines. CCA cell lines were treated with 0-500 nM (A) BI2536 and (B) BI6727 compared with the VC DMSO for 24, 48 and 72 h. Cell viability was measured by MTT assay. The bar graphs show normalization to the VC-treated cells and the results are presented as the mean \pm SD of three independent experiments. (C) Colony formation assay of CCA cells treated with BI2536 and BI6727. Representative images of the colony formation assay are shown. PLK1, polo-like kinase 1; CCA, cholangiocarcinoma; VC, vehicle control.

across various types of cancer (12,14). Therefore, inhibiting PLK1 is a potential therapeutic approach for cancer treatment. The present study examined the inhibitory impacts of PLK1

inhibition on four CCA cell lines derived from Thai patients with CCA: HuCCA1, KKKU055, KKKU100 and KKKU213A. These cell lines represented both intrahepatic (HuCCA1,

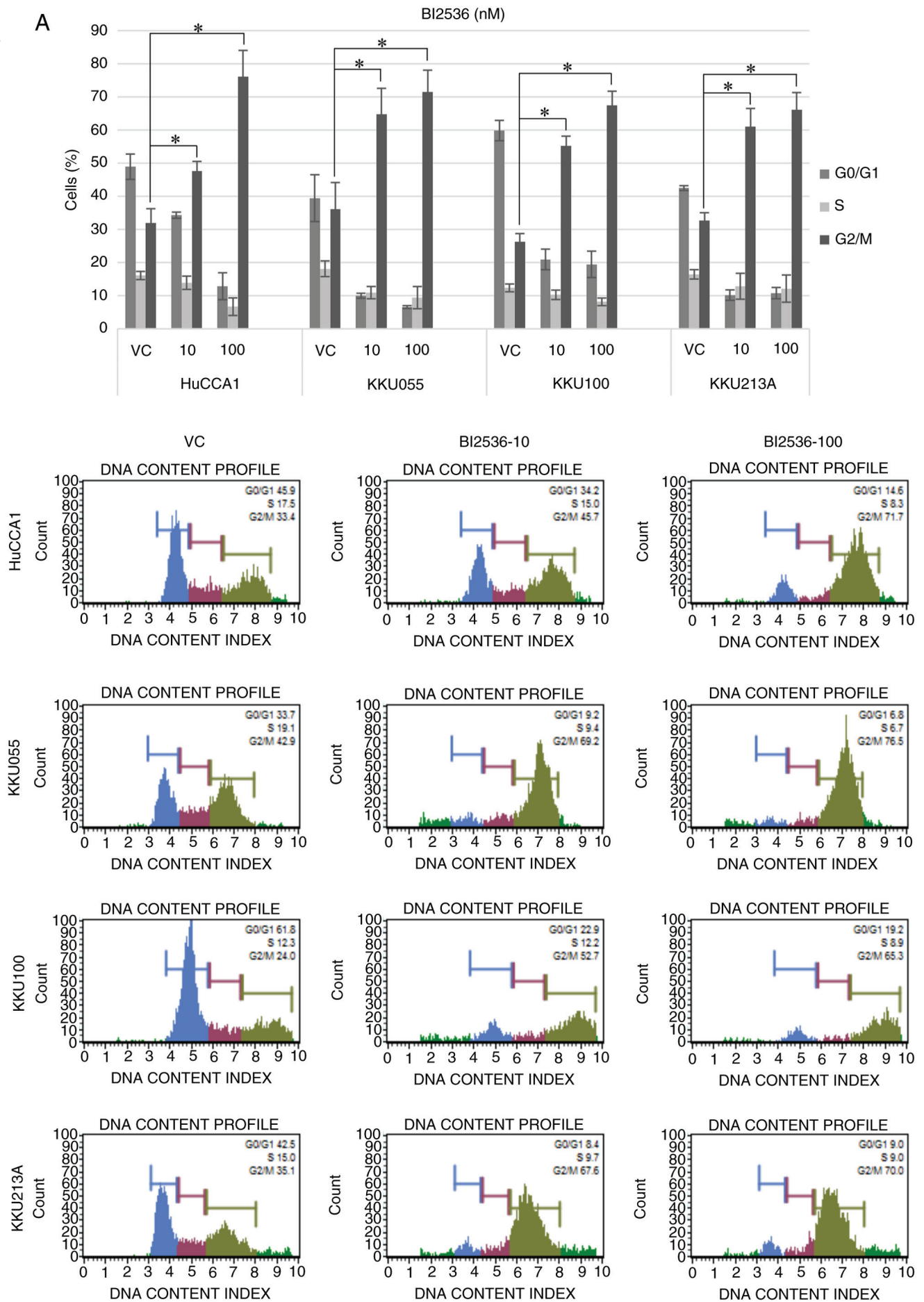


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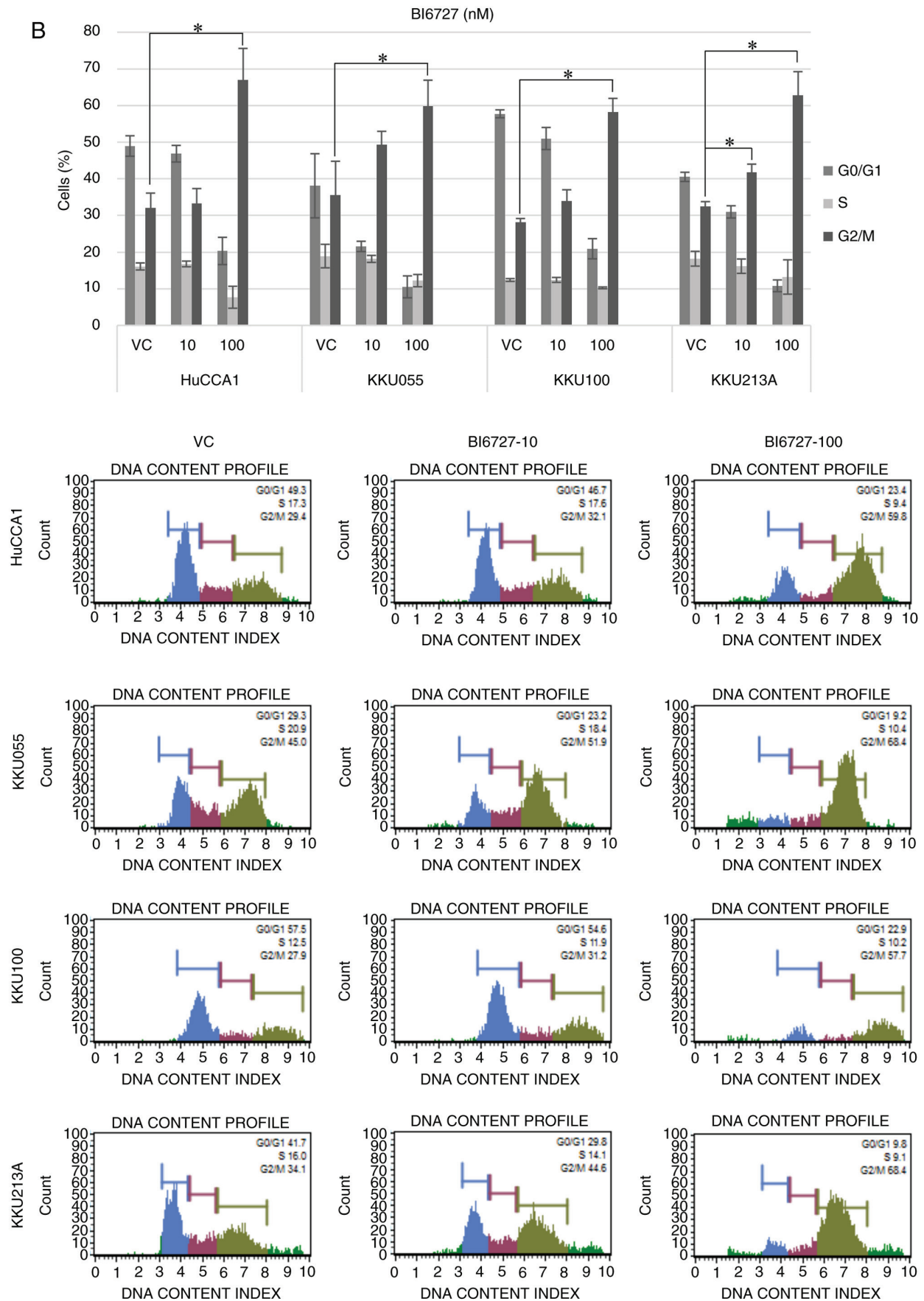


Figure 3. PLK1 inhibitors induce G₂/M-phase arrest in CCA cell lines. CCA cells were treated with VC, 10 and 100 nM (A) BI2536 and (B) BI6727 for 24 h. Cell cycle distribution was analyzed by flow cytometry using the Muse cell cycle assay kit. The percentages of cells in each phase of the cell cycle are shown and the results are presented as the mean \pm SD of three independent experiments. A significant difference was observed when comparing treated cells with control cells (* P <0.05). Representative cell cycle distribution of CCA cells treated with VC, BI2536, and BI6727 are shown below the graphs. PLK1, polo-like kinase 1; CCA, cholangiocarcinoma; VC, vehicle control.

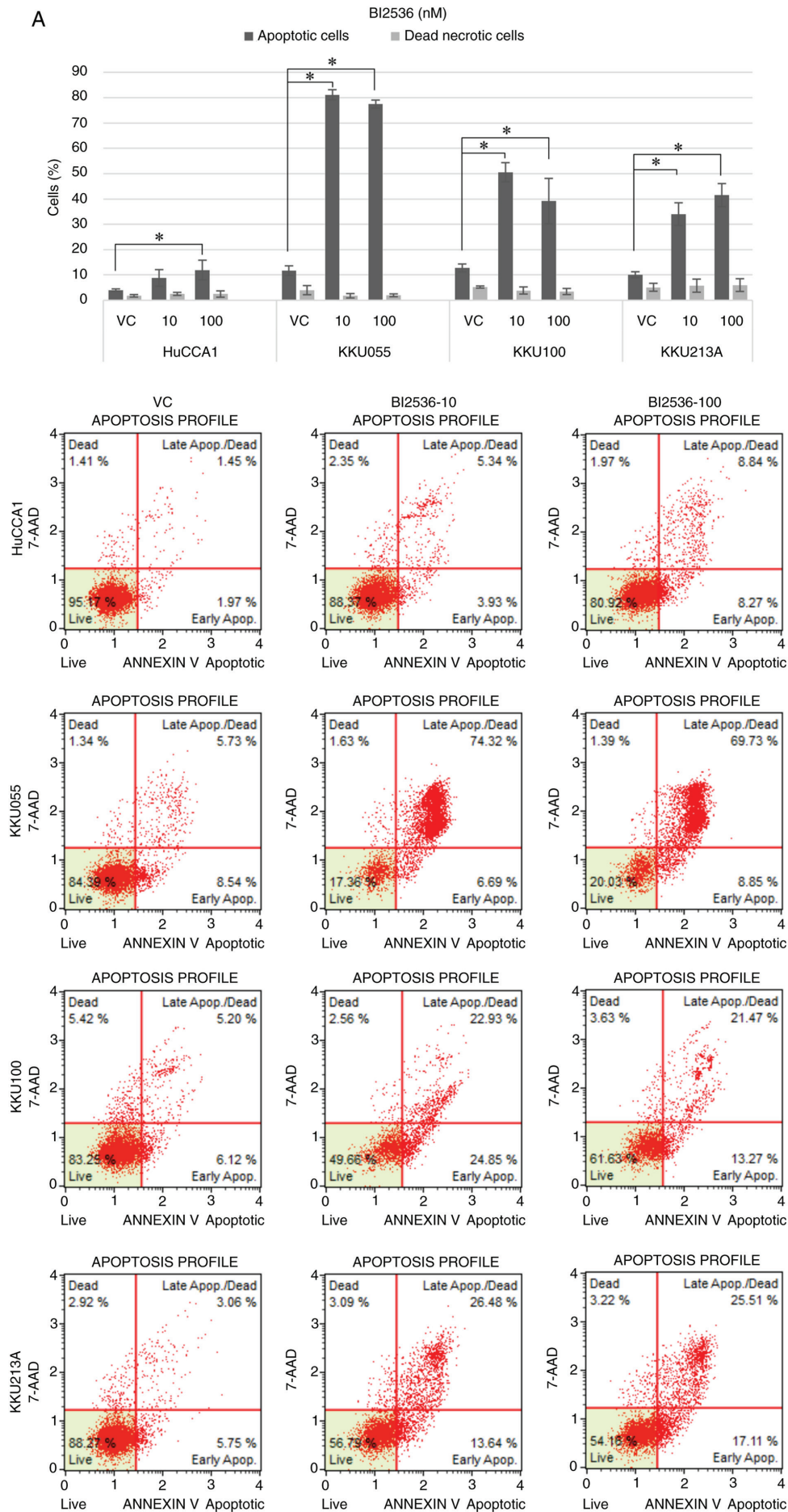


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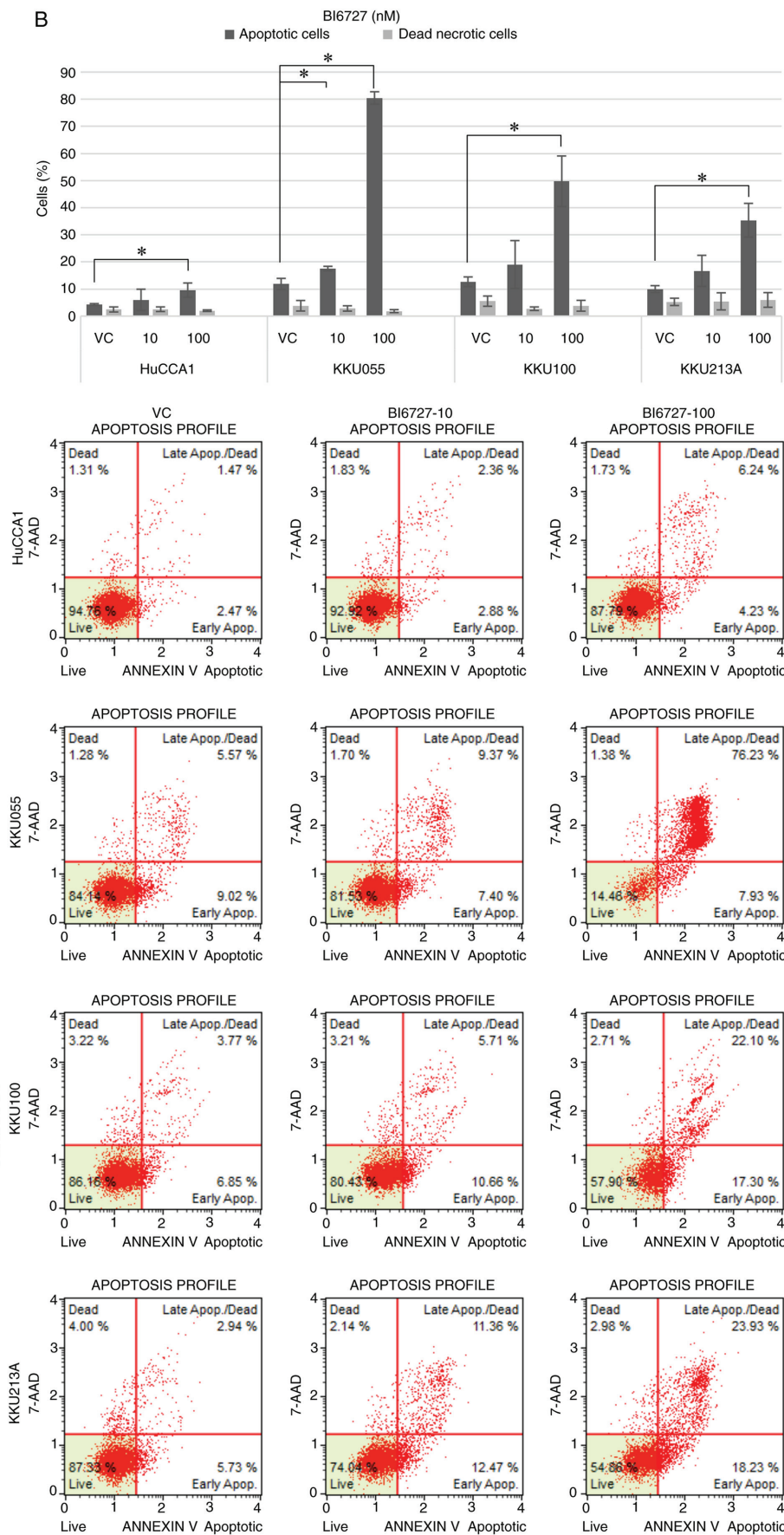


Figure 4. BI2536 and BI6727 induce the apoptosis of CCA cells. Cells were treated with VC, 10 and 100 nM (A) BI2536 and (B) BI6727 for 48 h. Total apoptotic cells were determined by flow cytometry using the Muse Annexin-V and Dead cell kit. The percentages of total apoptotic and dead necrotic cells are shown and the results are presented as the mean \pm SD of at least three independent experiments. A significant difference was observed when comparing treated cells with control cells ($P < 0.05$). Representative plots showing Annexin V/7-AAD double staining of CCA cells treated with VC, BI2536, and BI6727 are shown below the graphs. CCA, cholangiocarcinoma; VC, vehicle control.

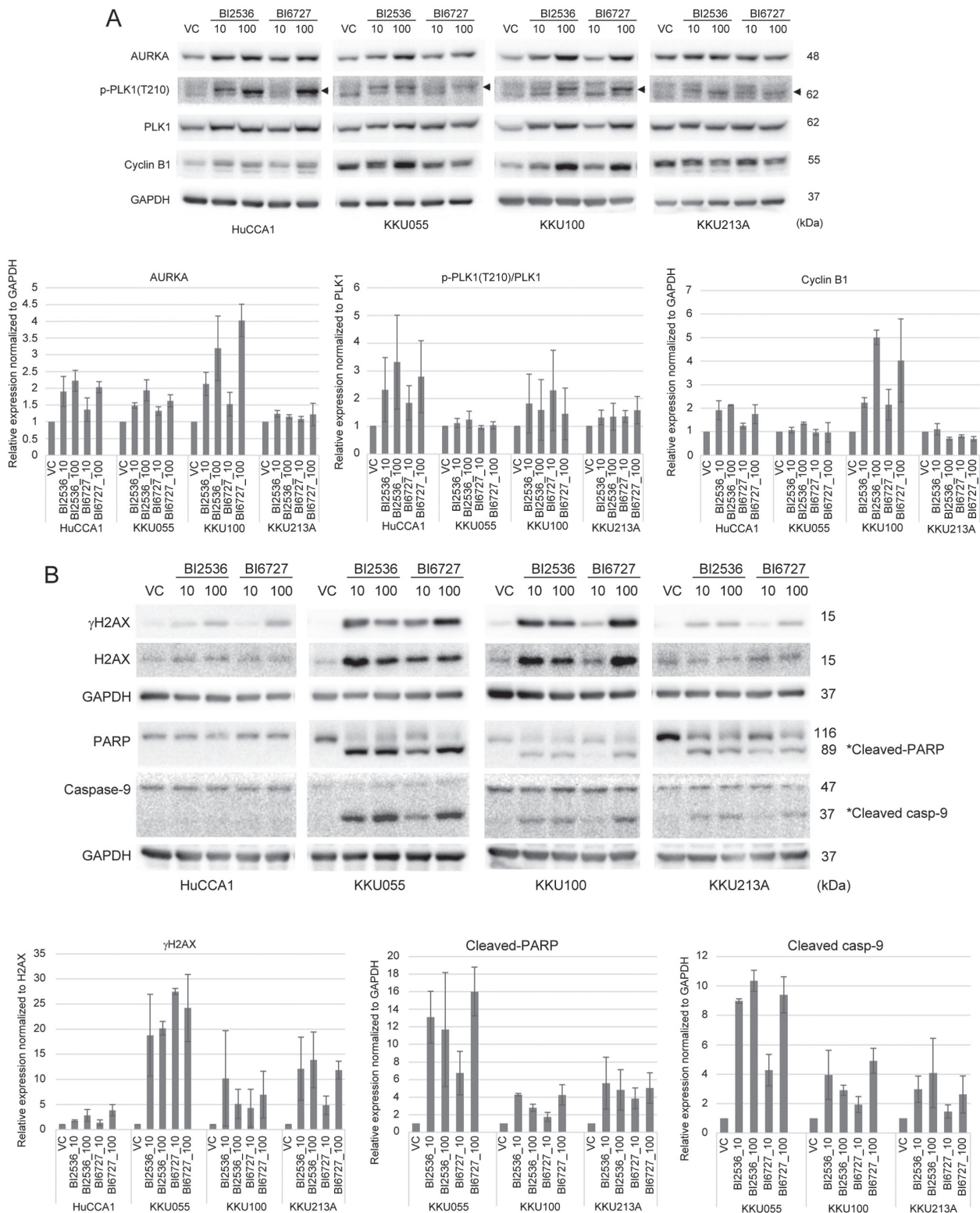


Figure 5. BI2536 and BI6727 upregulate the expression of (A) mitotic proteins and (B) induce DNA damage, as well as cleavage of PARP and caspase-9. CCA cells were treated with VC, or 10 and 100 nM BI2536 and BI6727 for 24 h. The expression of AURKA, p-PLK1 (T210), PLK1, cyclin B1, gH2AX, H2AX, PARP, caspase-9 and GAPDH proteins were detected by western blot analysis. Representative western blot images are shown. The bar graphs demonstrate the relative expression of the proteins normalized to GAPDH and VC-treated cells. The relative expression of p-PLK1 (T210) was normalized to total PLK1 and VC-treated cells. The relative expression of gH2AX was normalized to total H2AX and VC-treated cells. The relative protein expression was calculated from three independent experiments. PARP, poly (ADP-ribose) polymerase; CCA, cholangiocarcinoma; VC, vehicle control; AURKA, aurora kinase A; p-, phosphorylated; PLK1, polo-like kinase 1; H2AX, histone H2AX.

KKU055, KKKU213A) and extrahepatic (KKU100) CCA, ensuring a comprehensive examination of different subtypes of the disease within the Thai population.

The expression of PLK1 was initially detected in the four CCA cell lines. The analysis revealed that three out of the four cell lines exhibited substantial PLK1 expression. KKKU100

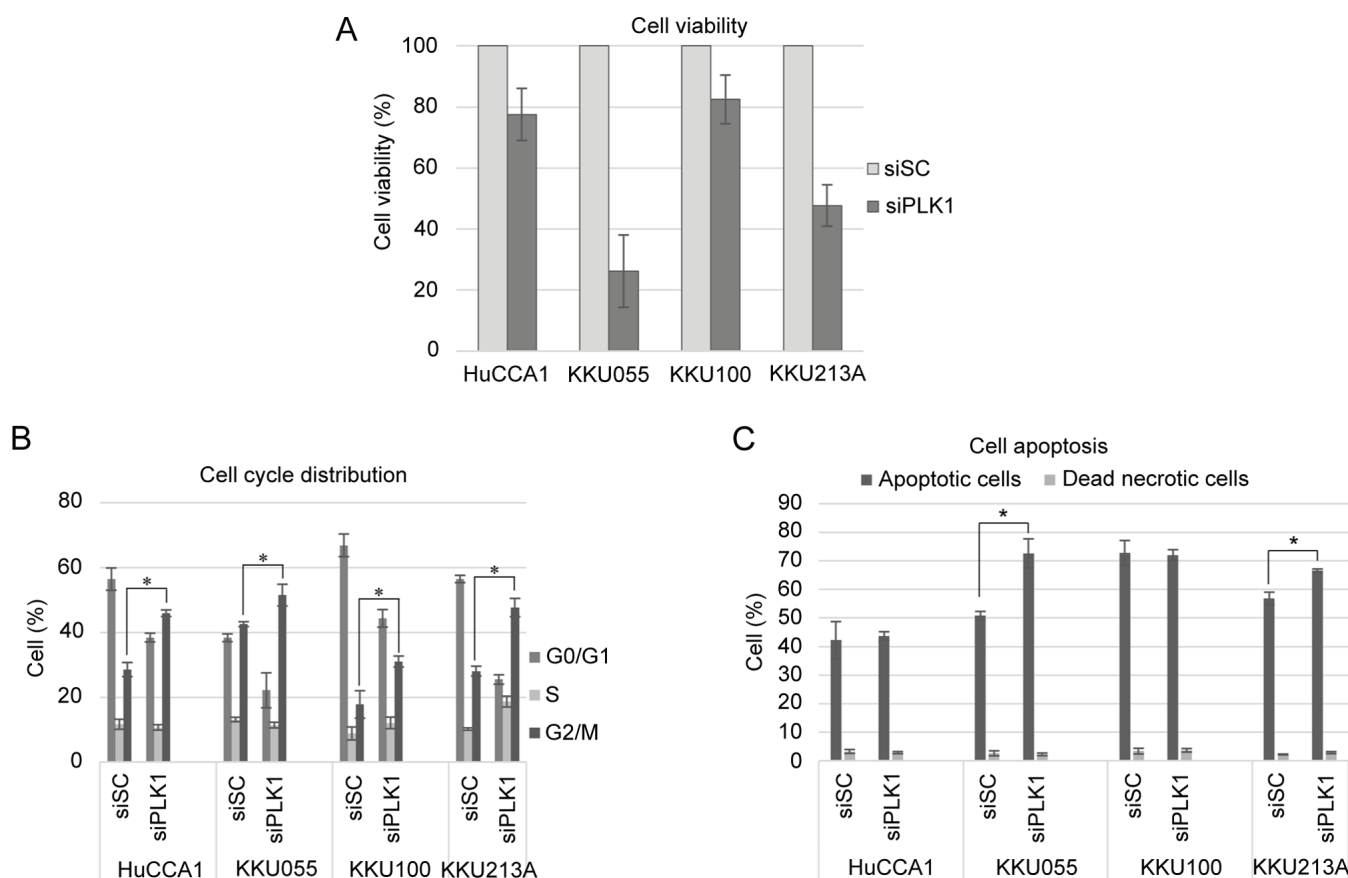


Figure 6. Continued.

demonstrated the lowest levels of PLK1 expression at both the mRNA and protein levels. BI2536 and BI6727, two potent PLK1 inhibitors, inhibited cell viability and colony formation in all four CCA cell lines, with slightly different sensitivity. The most sensitive cell line was KKKU055, which agreed with the findings of Tepsiri *et al* (37) showing that KKKU055 was the most sensitive cell toward chemotherapeutic drugs, such as cisplatin, gemcitabine and 5-fluorouracil. Notably, it was discovered that the expression levels of PLK1 did not determine the sensitivity to PLK1 inhibition. Despite KKKU100 cells exhibiting the lowest PLK1 expression, they were not the most sensitive to PLK1 inhibition. This observation suggested the involvement of other factors, such as DNA damage response and DNA repair mechanisms, in modulating the response to PLK1 inhibition. Investigation into these factors should be extended.

Following the cell proliferation assay, 10 and 100 nM concentrations of PLK1 inhibitors were chosen for subsequent experiments. The IC_{50} values were not directly used in subsequent experiments because the present study aimed to explore the concentrations and IC_{50} values to capture a broader range of responses and consider the different sensitivities to PLK1 inhibitions among cell lines; using IC_{50} might overlook potential differences in their responsiveness. While IC_{50} values provide valuable insights into the inhibitory potency, they may not be consistent with each experiment setup. The present study opted to conduct subsequent experiments, at either 24 or 48 h post-treatment due to significant cell death occurring at 72 h

after treatment, especially in the case of KKKU055 cells. PLK1 inhibition via BI2536, BI6727 and siRNA markedly induced G₂/M-phase arrest. These results are similar to those previously reported in several types of cancer, such as glioblastoma, non-small cell lung cancer and cervical cancer (31,46,47). Several studies have explored the effects of a 100 nM concentration of PLK1 inhibitor (BI2536) across different cell types. Steegmaier *et al* (25) demonstrated that this concentration arrested HeLa cells with monopolar spindles, characteristic of PLK1 inhibition. Similarly, Pezuk *et al* (46) and Lu *et al* (48) found that 100 nM BI2536 induced G₂/M arrest of glioblastoma cells and rat cardiac fibroblast, respectively. However, it remains difficult to entirely dismiss the possibility of off-target effects at this concentration. Similarly, the present study was unable to definitively exclude the potential for off-target effects. Mitotic arrest is one of the effects of PLK1 inhibition and this event leads to increased DNA damage followed by cell death. The present study also observed increasing DNA damage, as revealed by upregulation of γ H2AX, a marker of DNA double-strand breaks (49), in all cell lines. Moreover, both PLK1 inhibitor treatments and PLK1 silencing induced apoptotic cell death. Cleavage of PARP by caspase enzymes serves as a hallmark of apoptosis, whereas depletion of NAD⁺ and ATP leads to induction of necrosis (50). The present study observed the cleavage of both PARP and caspase 9 following PLK1 inhibition, using both PLK1 inhibitors and PLK1 gene depletion. Notably, Hecceg and Wang (50) demonstrated that PARP cleavage, mediated by caspase activation, prevents

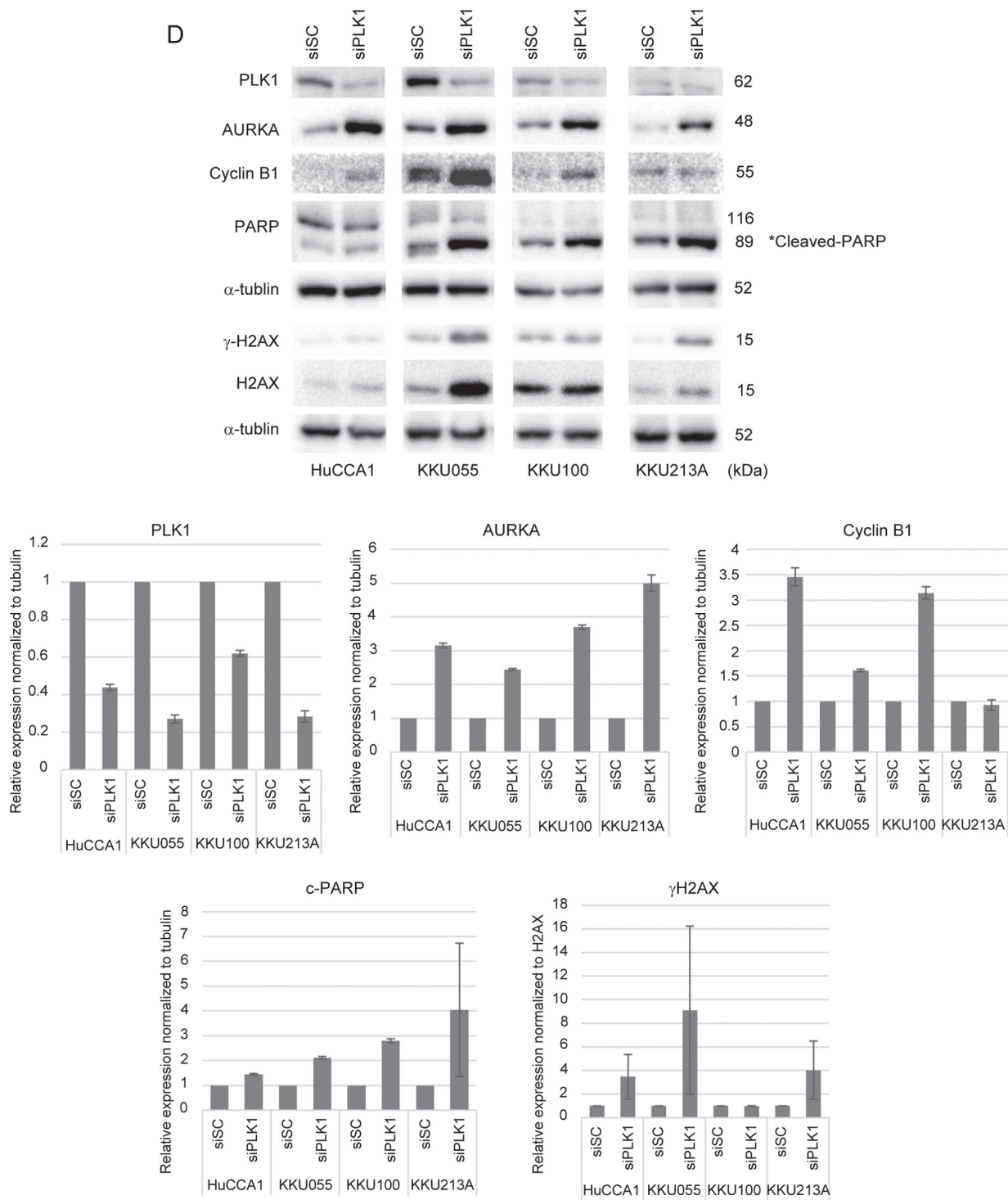


Figure 6. Silencing PLK1 shows antiproliferative effects on CCA cell lines. (A) CCA cells were transfected with 100 nM siSC or siPLK1 for 48 h and cell viability was measured by MTT assay. The bar graphs show the normalization to siSC-transfected cells. (B) Cell cycle analysis was performed by flow cytometry using the Muse cell cycle assay kit. The percentages of cells in each phase of the cell cycle are shown. Representative cell cycle distribution of CCA cells transfected with siSC or siPLK1 were shown in Fig. S1A. (C) Total apoptotic cells were determined by flow cytometry using the Muse Annexin-V and Dead cell kit. The percentages of total apoptotic and dead necrotic cells are shown. The results are presented as the mean \pm SD of at least three independent experiments. Representative plots showing Annexin V/7-AAD double staining of CCA cells transfected with siSC or siPLK1 are shown in Fig. S1B. A significant difference was observed when comparing siPLK1-transfected cells with siSC-transfected cells (* P <0.05). (D) Protein levels of AURKA, PLK1, cyclin B1, γ H2AX, H2AX, PARP and α -tubulin were examined by western blotting. Representative western blot images are shown. PLK1, polo-like kinase 1; CCA, cholangiocarcinoma; si, short interfering; SC, negative control; AURKA, aurora kinase A; PLK1, polo-like kinase 1; H2AX, histone H2AX; PARP, poly (ADP-ribose) polymerase.

necrosis induction during apoptosis. Although changes in necrosis markers, NAD⁺ and ATP depletion, were not measured, the findings of the present study, including the

cleavage of caspase 9 and PARP, along with the results from Annexin V/7-AAD double staining via flow cytometry and the absence of an increase in dead necrotic cells, suggested

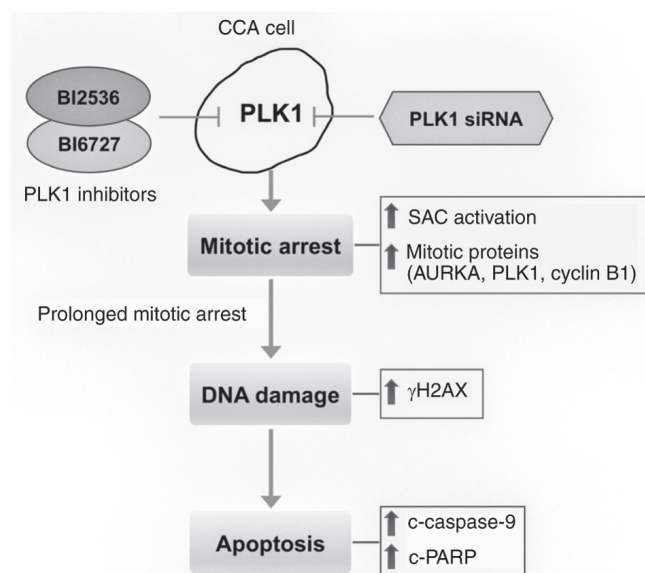


Figure 7. The proposed mechanism illustrates how PLK1 inhibition mediates apoptosis in CCA cells. Inhibiting PLK1 triggers mitotic arrest, activates the SAC, increases DNA damage and ultimately induces apoptosis. PLK1, polo-like kinase 1; CCA, cholangiocarcinoma; SAC, spindle assembly checkpoint; AURKA, aurora kinase A; c-, cleaved; PARP, poly (ADP-ribose) polymerase.

that PLK1 inhibition leads to apoptosis rather than necrosis. However, it may not be possible to confirm the effect of PLK1 silencing by siRNA on cell apoptosis of the KKU100 cell line, due to the toxicity of the transfection procedure in this cell type. Among the four CCA cell lines, HuCCA1 cells did not show a markedly increased population of apoptotic cells and cleavage of PARP protein compared with the other cell lines, despite mitotic arrest and DNA damage being observed. This result indicated that increasing DNA damage can occur due to prolonged mitotic arrest, irrespective of the apoptotic pathway. A deeper study of the molecular mechanism should be conducted to pursue a more comprehensive understanding of these effects. The varying responses to PLK1 inhibition across the four CCA cell lines were studied, particularly the induction of apoptosis alongside the observed mitotic arrest in all cell lines. It is planned to explore the mechanisms involved in these differences in sensitivity following PLK1 inhibition in CCA cells, for instance, the involvement of DNA damage response and DNA repair machinery that could play a role in this matter.

The spindle assembly checkpoint (SAC), also referred to as the mitotic checkpoint, is recognized for stopping mitotic progression during cell cycle dysfunction, ensuring the accurate alignment of chromosomes before segregation. The SAC primarily functions to postpone the transition to anaphase until there is proper attachment of kinetochores to microtubules (51). PLK1 participates in this process, with its activity notably elevated on unattached kinetochores, indicating a potential role for PLK1 in the regulation of kinetochore attachment or SAC (52). In the present study, the detection of mitotic proteins following treatment with BI2536 and BI6727 showed upregulated aurora kinase A, p-PLK1 (T210), PLK1 and cyclin B1 in the CCA cell lines. These proteins have been found to be stimulated in

mitotic-arrested cells and SAC-activated cells upon PLK1 inhibition (28,31,53). The CDK1-cyclin B1 complex is an important component of the SAC (54,55). Aurora kinase A is an upstream regulator of PLK1, which phosphorylates and activates PLK1 at Thr210 (56). The present study agreed with the findings from Choi *et al* (31), showing that inhibition of PLK1 activity with BI2536 preserves the absence of kinetochore tension, leading to prolonged activation of the SAC. This event activates phosphorylation of PLK1 at Thr210 by aurora kinase A. Choi *et al* (31) also show that BI2536-treated non-small lung cancer cells experience arrest at prometaphase and monopolar spindles are formed. Furthermore, BI2536 treatment induces mitotic arrest by impeding the attachment of kinetochores to microtubules, a process dependent on PLK1 activity (31). In addition, a study from Steegmaier *et al* (25) demonstrates that BI2536-treated cells are arrested in prometaphase and contain aberrant mitotic spindles. BI6727 has also been shown to accumulate mitotic cells with monopolar spindles (27). On the other hand, the accumulation of PLK1 protein levels in BI2536- and BI6727-treated cells observed in the present study was in contrast to the reduced accumulation of PLK1 observed in BI6727-treated chronic myeloid leukemia (57) and Burkitt lymphoma cells (30). These studies report that BI6727 can reduce activated PLK1.

PLK1 is primarily recognized for its pivotal role in several events during mitosis (14). However, emerging evidence suggests its involvement in the DNA damage response. DNA damage occurring during mitosis triggers the induction of mitotic arrest to avoid the generation of abnormal daughter cells (58). Studies have demonstrated that PLK1 kinase regulates several proteins involved in DNA damage response, particularly the ATM/ATR/Chk pathway (58,59). PLK1 can phosphorylate and inhibit Chk2, compromising the DNA damage checkpoint and facilitating cell cycle progression despite the presence of DNA damage (60). Additionally, PLK1 has been shown to interact with and phosphorylate p53, influencing its activity (61). Tamura *et al* (62) revealed the function of PLK1 in phosphorylating and modulating the activity of Bcl-2 family proteins, key regulators of apoptosis. These findings underscore the role of PLK1 in cellular processes, including its effect on the response to various cellular stresses, such as DNA damage.

While PLK1 inhibition has demonstrated anti-proliferative effects on cancer cells, concerns have been raised regarding its potential side effects on normal cells. In a study by Liu *et al* (63), the depletion of PLK1 in normal hTERT-RPE1 and MCF10A cell lines did not affect cell proliferation or cell cycle progression. Lu (48) *et al* investigated the effects of BI2536 on primary cardiac fibroblast and cardiomyocytes. While BI2536 did not generate adverse effects in cardiomyocytes, which are differentiated cells, it affected primary fibroblast by inducing mitotic arrest with monopolar spindles, leading to cell death after prolonged arrest. A number of PLK1 inhibitors have been developed and tested in clinical trials (12). BI2536, for instance, has undergone evaluation in NSCLC patients, both as a monotherapy and in combination therapy (64,65). BI6727 has reached phase III clinical trial for AML (66). However, in a number of cases, these inhibitors

have shown limited efficacy and high toxicity. Efforts to explore strategies for limiting PLK1 activity in cancer cells continue. The combined use of PLK1 inhibitors with conventional chemotherapeutics has shown promise and warrants further investigation. These aspects of study remain relatively limited in cholangiocarcinoma research.

While preparing the present manuscript, a recent study by Riantana *et al* (34) was published regarding the effects of two potent PLK1 inhibitors, BI6727 and GSK461364A, on inducing G₂/M arrest and apoptosis in two CCA cell lines (KKU100 and KKU213A). Some of the results presented in the present study agree with this recent publication. The levels of PLK1 protein in KKU100 and KKU213A cells are consistent with the present findings. Furthermore, similar effects of BI6727 on inhibiting cell proliferation were observed, inducing G₂/M cell arrest and triggering apoptosis (as indicated by PARP cleavage). However, there is enhanced support for our current findings by using siRNA to inhibit PLK1 expression and by detecting alterations in mitotic proteins following PLK1 inhibition.

In conclusion, the results of the present study indicated that inhibition of PLK1 by small molecule inhibitors, BI2536 and BI6727, and via the siRNA method, can suppress cell proliferation, induce mitotic arrest and DNA damage, and trigger cell apoptosis in CCA cells (Fig. 7). Regarding CCA, the present study demonstrated convincing results by using both inhibitors and siRNA to explore the effects of PLK1 inhibition. The strong induction of apoptotic cell death did not occur in all cell lines. While the present study used four different CCA cell lines, their representation of the heterogeneity found in human CCA is a valid concern. The present study recognized that the results obtained from different cell lines may vary, emphasizing the need to validate across a broader range of cases. Further research is needed to understand the specific mechanisms involved in determining the response to PLK1 inhibition and to identify potential biomarkers that can predict sensitivity to PLK1 inhibitors. The present study identified PLK1 as a promising treatment target for CCA cells. To validate its effectiveness, future plans will involve both animal experiments and clinical research.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

BM and MR conceptualized and designed the study. BM developed the methodology, conducted the experiments and drafted the manuscript. BM, JC and PS analyzed and interpreted the data. BM, JC, PS and MR reviewed and revised the manuscript. BM and PS confirm the authenticity of all the raw data. All authors read and approved the final version of the 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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