

Synergistic suppression of cholangiocarcinoma cells via DNA damage response and cell cycle arrest by dual targeting PARP and ATM in DNA damage repair pathway

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ABSTRACT

This study investigated alterations in DNA damage repair (DDR) genes in cholangiocarcinoma (CCA) influencing cellular vulnerability to DDR inhibitors, notably poly (ADP-ribose) polymerase inhibitors (PARPi) and ataxia-telangiectasia mutated inhibitors (ATMi). Genomic DDR alterations in CCA tumors were identified via cBioPortal. The cytotoxic effect of the inhibitors was determined in 8 CCA cell lines. Cell cycle arrest and apoptosis were determined using flow cytometry. The levels of γ -H2A histone family member X (γ -H2AX) were evaluated using an immunofluorescence assay. Proteins associated with the cell cycle and DDR were evaluated using Western blotting. Among these CCA cells, *BRCA*-mutated Kku-023 cells exhibited the highest PARPi sensitivity. Co-treatment with ATMi induced a synthetic homologous recombination deficiency (HRD) state in *BRCA*-proficient CCA cells, thereby sensitizing them to PARPi. This drug combination suppressed CCA cell growth via G2/M phase arrest, downregulated cyclin A and B1 expression, and enhanced apoptosis. Mechanistically, this synergistic cytotoxicity was attributed to increased DNA double-strand breaks, evidenced by elevated γ -H2AX and reduced RAD51 expression, signifying compromised DNA repair. These preclinical findings support the therapeutic potential of DDR inhibitors, individually or in combination, for CCA subsets characterized by DDR gene alterations.

1. Introduction

Cholangiocarcinoma (CCA), a heterogeneous malignancy originating from the biliary epithelium, presents a significant clinical challenge. While its incidence is relatively low in Western countries, CCA poses a substantial and increasing health burden in several Asian countries, including Taiwan, China, South Korea, and Thailand [1]. Curative-intent surgery remains the primary treatment modality; however, it is feasible for only a minority of patients at diagnosis, and disease recurrence at post-resection is common [2]. For unresectable or advanced CCA, palliative chemotherapy, typically comprising gemcitabine (Gem) with or without cisplatin (CDDP), offers limited efficacy, and

the development of chemoresistance is a frequent obstacle [3–5]. Consequently, there is an urgent need to develop novel therapeutic strategies for this aggressive cancer. Targeted therapies, particularly those exploiting specific molecular vulnerabilities such as alterations in DNA damage repair pathways, represent a promising option for improving patient outcomes.

The genetic landscape of CCA, encompassing mutations, copy number variations, and gene rearrangements, is well-characterized and underpins its carcinogenesis and metastatic potential [6,7]. Prevalent somatic mutations in CCA frequently affect genes involved in critical cellular processes such as receptor tyrosine kinase signaling, genome instability (including DNA damage repair), and epigenetic regulation.

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Notable examples include *TP53* (mutated in ~32 % of cases), *ARID1A* (~17.4 %), *KRAS* (~16.5 %), *BAP1* (~8.5 %), *STK11* (~5 %), *BRCA2* (~3.7 %), and *IDH1* (~3.4 %). Among these, alterations in genes such as those encoding fibroblast growth factor receptors (FGFRs), human epidermal growth factor receptor 2 (ERBB2/Her-2), and isocitrate dehydrogenase 1 (IDH1) are recognized as key oncogenic drivers and actionable therapeutic targets, particularly within the intrahepatic CCA (iCCA) subtype [8]. Given that CCA is a highly heterogeneous malignancy often characterized by dysregulation across multiple pathways rather than dependence on a single oncogenic event, a comprehensive understanding of how these pivotal genetic alterations contribute to CCA pathogenesis is essential for developing more effective therapeutic interventions.

Somatic mutations in genes integral to the DNA damage repair (DDR) network are prevalent in CCA, identified in approximately 29 % of patients. These include alterations in key genes such as *BRCA1/2*, *ATM*, *ATR*, *CHEK1/2*, *MLH1*, *MSH2/6*, and *BAP1*, with defects in the homologous recombination repair (HRR) pathway being particularly common [9]. Tumors deficient in HRR (HRD) exhibit heightened sensitivity to poly (ADP-ribose) polymerase inhibitors (PARPi). This therapeutic vulnerability arises from the principle of synthetic lethality, wherein the concurrent loss of two distinct DNA repair pathways—HRR and PARP-mediated base excision repair (which PARPi disrupts)—leads to cell death. Consequently, HRD tumors, notably those harboring *BRCA1/2* mutations, demonstrate marked sensitivity to PARPi [10–13]. Furthermore, the integrity and function of other HRR components, such as ataxia-telangiectasia mutated (ATM), are also implicated in the cellular response to PARP inhibition, suggesting a complex interplay that may influence therapeutic outcomes [14]. Thus, PARP inhibition represents a promising therapeutic strategy for CCA patients whose tumors harbor deleterious HRR pathway alterations.

Currently, several PARP inhibitors (PARPi), including olaparib, niraparib, rucaparib, and talazoparib, have received regulatory approval for treating patients with *BRCA*-mutated ovarian, breast, prostate, and metastatic pancreatic cancers [15–17]. Despite this progress, PARPi have not yet been approved for treating CCA. Preclinical investigations, particularly in pancreatic cancer models harboring *BRCA* and *ATM* deficiencies, have demonstrated the capacity of PARPi to inhibit DNA repair and induce apoptosis both *in vitro* and *in vivo* [18]. However, a significant challenge to PARPi efficacy is the development of acquired resistance, often mediated by the upregulation or reactivation of alternative double-strand break (DSB) repair pathways, including those reliant on ATM and ATR signaling [19]. To address this, combination strategies are being explored. For instance, co-administration of PARPi with other DNA damage response inhibitors (DDRi), such as ATR inhibitors (ATRi) or DNA-dependent protein kinase inhibitors (DNA-PKi), reportedly enabled a substantial 5–10-fold PARPi dose reduction while maintaining anti-tumor activity in *ATM*-deficient murine pancreatic cancer cell lines, potentially mitigating toxicity. Furthermore, direct ATM inhibitor (ATMi) has been shown to reduce cell viability in *ATM*-proficient human pancreatic cancer cell lines, suggesting its potential to overcome resistance or broaden the applicability of DDR-targeted therapies [18].

ATM, a member of the serine/threonine phosphatidylinositol 3-kinase-related kinase (PIKK) family, orchestrates an extensive signaling cascade integral to HRR [20,21]. Upon activation by DNA double-strand breaks (DSBs), ATM phosphorylates a multitude of downstream effector proteins, including p53, CHK2, KAP1, RAD50, SMC1, MDC1, and H2AX (often resulting in γ -H2AX). This ATM-driven signal transduction is critical for mediating cell cycle checkpoints (intra-S-phase, G1/S, and S/G2M) and facilitating the recruitment of DNA repair machinery to damage sites, notably via H2AX and MDC1 [21]. Given its pivotal role in DSB repair, deficiencies in *ATM* sensitize cells to PARPi [22,23].

Preclinical evidence strongly supports this synergy. For instance, the ATMi (AZD0156) potentiated the anti-tumor effects of olaparib across various lung, gastric, and breast cancer cell lines *in vitro* and enhanced

olaparib's efficacy in patient-derived triple-negative breast cancer xenograft models *in vivo*. Mechanistically, AZD0156 was shown to disrupt the repair of olaparib-induced DNA damage, leading to an accumulation of DSBs, subsequent cell cycle arrest, and apoptosis [24]. Consequently, combining PARPi with ATMi represents a rational therapeutic strategy to potentially broaden the clinical utility of PARPi and enhance anti-cancer responses in CCA. Indeed, olaparib, both as a monotherapy and in combination with chemotherapy or other targeted agents, is under active clinical investigation across various malignancies, including ovarian cancer, breast cancer, advanced solid tumors, advanced glioma, and recurrent CCA, highlighting the ongoing efforts to optimize its therapeutic application.

This study investigated the role of the DDR in CCA carcinogenesis, with a particular focus on identifying DDR gene alterations that could predict sensitivity to PARPi. To achieve this, CCA cell lines with characterized genetic alterations in DDR pathways were utilized as *in vitro* models for drug screening of various DDRi. Specifically, the anti-tumor efficacy of PARPi (olaparib and niraparib), administered either as monotherapies or in combination with an ATMi (AZD0156), was evaluated in these CCA cell lines. The findings aim to provide preclinical rationale for the potential therapeutic application of DDRi in subsets of CCA patients whose tumors harbor specific DDR gene mutations.

2. Materials and methods

2.1. Chemicals and reagents

Ham's F12 medium and 0.25 % trypsin-ethylenediaminetetraacetic acid (EDTA) solution were purchased from Gibco BRL Life Technologies (Grand Island, NY, USA). Fetal bovine serum (FBS) and penicillin-streptomycin solution were purchased from Cytiva HyClone (Kremplstrasse, Pasching, Austria). N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) was purchased from Bio Basic Inc. (Markham, ON, Canada). CellTiter 96® Aqueous one solution cell proliferation (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium [MTS]) assay was purchased from Promega Corporation (Madison, WI, USA). Halt™ protease and phosphatase inhibitor cocktail and radioimmunoprecipitation assay (RIPA) lysis buffer were purchased from Thermo Scientific™ (Waltham, MA, USA). Skim milk powder, Triton X-100, and propidium iodide (PI) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Ribonuclease A was obtained from Vivantis Technologies (Shah Alam, Selangor, Malaysia). The protein assay dye reagent concentrate and 4x Laemmli sample buffer were obtained from Bio-Rad Laboratories (Hercules, CA, USA). Tween 20 was obtained from Amresco® (Solon, OH, USA). Bovine serum albumin (BSA) was obtained from Capricorn Scientific (Ebsdorfergrund, Hesse, Germany). Polyvinylidene fluoride (PVDF) membranes and Luminata™ Forte Western HRP substrates were obtained from Merck Millipore Corporation (Billerica, MA, USA).

2.2. Cell lines and cell cultures

Human CCA cell lines with *BRCA1*-deficient status (harboring *BRCA1* nonsense mutations: KKU-023, KKU-097, and KKU-610), *BRCA1*-missense mutations (KKU-100, KKU-213A, and KKU-213C), and wild-type *BRCA1* (KKU-452 and KKU-466) were established at the Cholangiocarcinoma Research Institute (CARI), Khon Kaen University. All the CCA cell lines used in this study were *ATM*-proficient. Four cell lines (KKU-100, KKU-213A, KKU-452, and KKU-023) were obtained from the Japanese Cancer Research Resources Bank (Ibaraki, Osaka, Japan).

The CCA cell lines were cultured in Ham's F12 nutrient mixture medium supplemented with 10 % (v/v) FBS, 10 mM HEPES pH 7.4, 100 μ g/mL streptomycin, and 100 U/mL penicillin. The cells were maintained in a humidified atmosphere of 5 % CO₂ at 37°C and trypsinized with 0.25 % trypsin-EDTA upon reaching 70–80 % confluence. Routine screening for *Mycoplasma* contamination was performed using a

MycoAlert Plus Mycoplasma® Detection Kit (Lonza, Rockland, ME, USA).

2.3. Drug preparation

Olaparib, niraparib, and AZD0156 were purchased from MedChemExpress (Monmouth Junction, NJ, USA). Each compound was initially dissolved in dimethyl sulfoxide (DMSO) at a concentration of 10 mM. AZD0156 was sonicated at 40 kHz for 30 min to facilitate its solubilization. The dissolved compounds were stored at -80°C and used within six months.

2.4. Genetic-alteration analysis in publicly available databases

Genetic alterations in a cohort of 88,384 tumor samples from 23 studies were analyzed using the cBioPortal platform (<http://www.cbioportal.org>). The analysis focused on genetic alterations, including mutations, structural variants, amplifications, deep deletions, and multiple alterations, in 11 DDR genes: *CHEK1/2*, *RAD51*, *BRCA1/2*, *MLH1*, *MSH2*, *ATM*, *ATR*, *MDC1*, and *PARP1*. Subsequently, mutations in the 11 DDR genes and five epigenetic modification genes, *ARID1A*, *BAP1*, *KMT2C*, *PBRM1*, and *RBI*—were further investigated using publicly available datasets of 1481 CCA samples from eight studies on September 9, 2024.

2.5. Cell viability assay and drug sensitivity analysis

The relative viability of the cells was measured using the MTS assay. Briefly, CCA cells were seeded into 96-well plates at a density of 7.5×10^3 cells per well and treated with various concentrations of olaparib, niraparib, AZD0156 alone, or drug combinations for 72 h. After treatment, 100 μL of MTS reagent, diluted 1:10 in culture medium without FBS, was added to each well and incubated for 0.5–3 h. Absorbance was measured at 492 nm using a microplate reader.

Drug combination sensitivity was analyzed using SynergyFinder (<https://synergyfinder.org/#!/dashboard>), a web application designed to quantify drug interactions based on a Bliss-independent model. The inhibitory dose-response data were submitted to the SynergyFinder interface in a table format, with each row representing an observation in the dose-response study. The expected combination effect was calculated based on the probability of independent events. The synergy score was calculated as the average excess response elicited by drug interactions, with scores interpreted as follows: ≤ -10 indicates antagonism, -10 to 10 indicates an additive effect, and ≥ 10 indicates synergy [25–27]. A three-dimensional plot of the Bliss score was generated using SigmaPlot 10.0.

2.6. Colony-formation assay

The cells were seeded into 6-well plates at a density of 400 cells/well. Twenty-four hours after seeding, the cells were treated with DDRi (niraparib or AZD0156) for 72 h. The cells were then maintained for 14–21 days, allowing colonies of more than 50 cells to form in a medium containing 10 % FBS. The medium was renewed every three days. Subsequently, the colonies were fixed with absolute methanol at 4°C for 30 min, stained with 0.5 % crystal violet in 2 % ethanol for 1 h, washed with tap water to remove excess dye, and air-dried overnight. Colonies, defined as clusters of at least 50 cells, were counted and analyzed using the ImagePro Plus software (Media Cybernetics Inc., Rockville, MD, USA).

2.7. Apoptosis analysis

The apoptosis-inducing effect of the DDRi combination in CCA cells was analyzed using annexin V and PI staining. CCA cells were seeded into 6-well plates at a density of 2×10^5 cells per well and allowed to

grow overnight. The cells were then treated with olaparib, niraparib, AZD0156, or a dual combination in a medium containing 10 % (v/v) FBS for 72 h. After treatment, the cells were harvested and washed with phosphate-buffered saline (PBS) one time, the supernatant was removed, and 5 μL of double staining solution of fluorescein isothiocyanate (FITC) Annexin V and PI was added, in accordance with the protocol specified by the manufacturer (BD Biosciences, San Jose, CA, USA). After 15 min of incubation, the apoptotic cells were examined using a flow cytometer (Becton Dickinson FACSCanto II, BD Biosciences) and analyzed by FlowJo™ software version 10.10.0 (Ashland, OR, USA).

2.8. Intracellular reactive oxygen species measurement

CCA cells were plated at a density of 2×10^5 cells/well in a 6-well plate and incubated overnight. Adherent cells were treated with drugs for 72 h. After the incubation period, the culture medium was discarded. Serum-free medium containing 5 μM H_2DCFDA (MedChemExpress) was added and incubated at 37°C for 30 min in the dark. The stained cells were trypsinized, washed with PBS, and resuspended in PBS. The fluorescence signal of the stained cells was measured by flow cytometry (BD Biosciences) and analyzed using the Floreada.io online analysis tool (<https://floreada.io/>).

2.9. Cell cycle analysis

Cell cycle progression was examined by flow cytometry. Briefly, CCA cells were seeded in a 6-well plate (2×10^5 cells per well) overnight, then treated with single or dual combination in a medium containing 10 % (v/v) FBS. After 72 h of treatment, the cells were harvested by trypsinization, fixed with cold 70 % ethanol to preserve their structure and integrity, and washed with cold PBS. Then, cell pellets were stained with 20 $\mu\text{g}/\text{mL}$ PI in PBS containing 20 $\mu\text{g}/\text{mL}$ RNase and 0.1 % Triton X-100 on ice for 1 h in the dark. The percentages of cells in various cell cycle phases were analyzed using a flow cytometer (BD Biosciences).

2.10. Immunofluorescence

The levels of $\gamma\text{-H2AX}$ were evaluated using an immunofluorescence assay. Briefly, CCA cells were cultured on chamber slides at a density of 3×10^4 cells per well and treated with niraparib, AZD0156, or their combination for 24 h. Treatment with hydroxyurea (HU) 10 mM was performed as a positive control of DNA damage. After treatment, cells were washed with PBS, fixed with 4 % paraformaldehyde in PBS for 10 min, and permeabilized with 0.25 % Triton X 100 in PBS for 10 min. Cells were then blocked with 2 % BSA for 2 h, incubated overnight with primary antibody against $\gamma\text{-H2AX}$ (ab11174, Abcam, Cambridge, UK), diluted 1:400 in PBS containing 2 % BSA, and then incubated with Alexa Fluor® 594-conjugated goat anti-rabbit IgG H&L secondary antibody (ab150080, Abcam), diluted 1:400, for 1 h. The nuclei were counterstained with Vectashield® plus antifade mounting medium with DAPI (Vector Laboratories, Newark, CA, USA). The coverslips were applied and sealed at the rim. Fluorescence images of $\gamma\text{-H2AX}$ foci were captured using a Nikon ECLIPSE Ni-U microscope (Nikon Instruments, Tokyo, Japan).

2.11. Western blot analysis

After treating the cells with the DDRi, they were lysed on ice for 30 min in RIPA buffer containing a protease and phosphatase inhibitor cocktail. The supernatants were collected by centrifugation at 13,000 rpm for 30 min at 4°C . Protein concentrations were determined using a protein assay dye reagent according to the manufacturer's instructions (Bio-Rad). Twenty micrograms of protein were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), subsequently transferred to a PVDF membrane, and blocked with 5 % (w/v) skim milk powder or 5 % BSA for 2 h. After blocking, the

membrane was washed three times with Tris-buffered saline with Tween 20 (TBST) and incubated overnight at 4°C with primary antibodies against targets such as PARP1, pATM, ATM, p53, p21, cyclin A, cyclin B1, RAD51, γ -H2AX, and β -actin (Supplementary Table S1). The membrane was then incubated for 2 h with horseradish peroxidase-conjugated secondary antibodies. Protein expression was visualized using Luminata™ Forte Western HRP substrate, and the protein bands were photographed using the ChemiDoc™ MP imaging system (Bio-Rad). The intensity of the target bands was analyzed using Image Lab software (version 6.0; Bio-Rad).

2.12. Statistical analysis

The experiments were performed independently in triplicate, and the quantitative data are presented as mean $\pm$ standard deviation (SD). The statistical significance of differences between the treatment and

untreated control groups was analyzed using one-way analysis of variance with the Student–Newman–Keuls post-hoc test. The threshold for statistical significance was set at $p < 0.05$.

3. Results

3.1. Genetic alterations in DDR and epigenetic modification genes in CCA

DDR and epigenetic modifications contribute to cancer development. To identify the frequency of genetic alterations in DDR pathways in different types of cancer, we focused on mutations in 11 DDR genes: *BRCA1/2*, *CHEK1/2*, *RAD51*, *ATM*, *ATR*, *MLH1*, *MSH2*, *MDC1*, and *PARP1*. Analysis from the cBioPortal public database revealed that both iCCA and eCCA subtypes exhibited high DDR mutation rates compared with other cancer types, including ovarian, prostate, and breast cancers (Fig. 1A). Specifically, mutations in the DDR and epigenetic

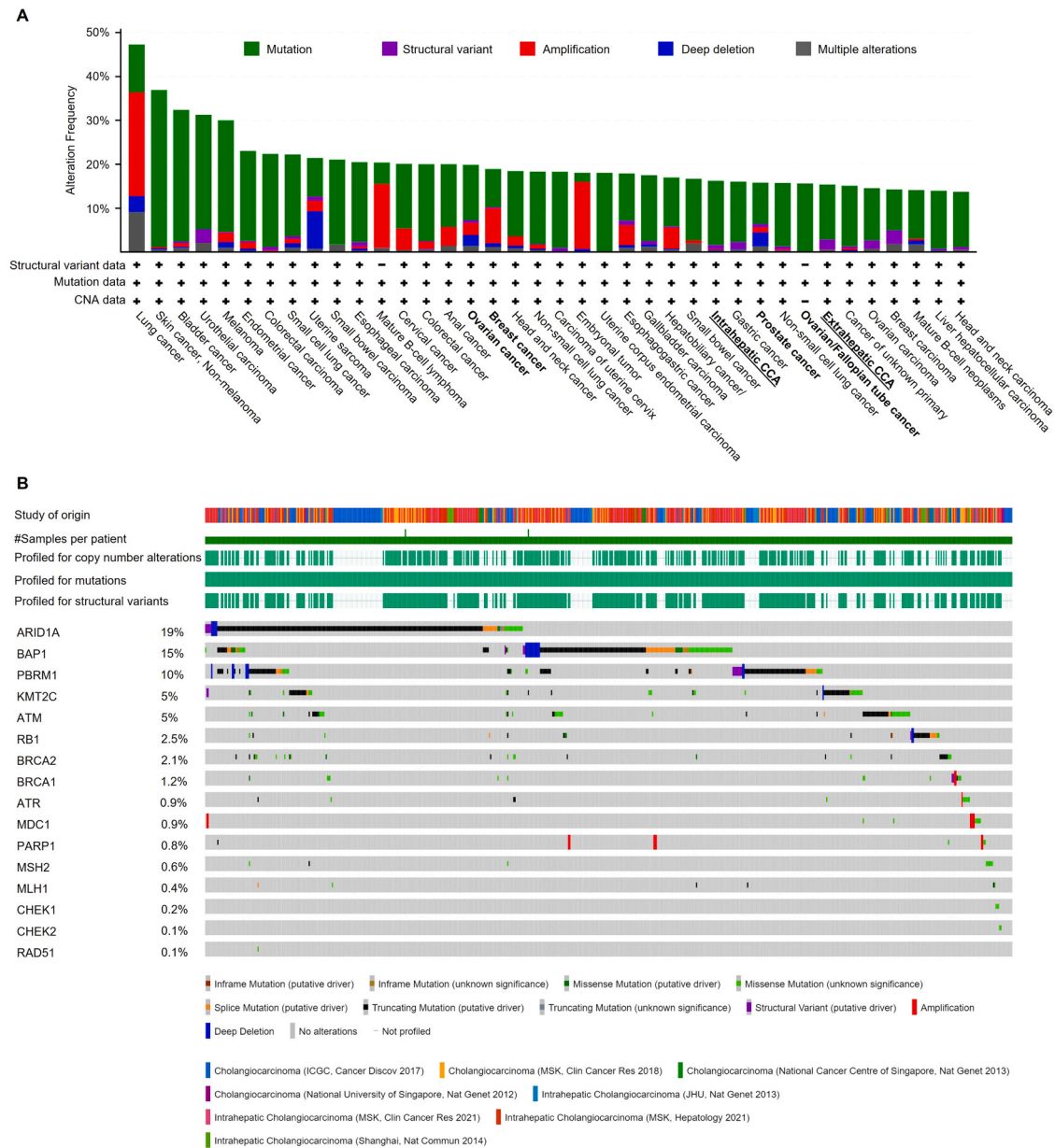


Fig. 1. cBioPortal analysis of genetic alterations in DDR and epigenetic modification genes. (A) Overview of genetic alterations in 11 key DDR genes across a pan-cancer cohort of 88,384 patient samples. Data were visualized using cBioPortal, with alteration types indicated as follows: mutations (green), structural variants (purple), amplifications (red), deep deletions (blue), and multiple alterations (gray). (B) Frequencies of genetic alterations for 16 selected genes involved in DDR and epigenetic modifications in 1481 CCA cases. Data for this CCA cohort analysis were retrieved from cBioPortal on September 9, 2024.

modification genes were identified in 1481 CCA samples. The most frequent alterations were observed in *ARID1A* (19 %), *BAP1* (15 %), *PBRM1* (10 %), *KMT2C* (5 %), *ATM* (5 %), and *BRCA1/2* (3.3 %) (Fig. 1B). These findings suggest that the beneficial effects of DDR-targeted therapies, including treatment with PARPi and ATMi, warrant exploration in a subset of CCA patients carrying DDR alterations.

3.2. PARPi(s) decrease CCA cell viability and proliferation

To explore the antitumor effect of DDRi(s) in CCA cells, we determined the sensitivity of the following eight CCA cell lines to PARPi (olaparib or niraparib): KKU-023, KKU-097, KKU-100, KKU-213A, KKU-213C, KKU-452, KKU-466, and KKU-610. These CCA cell lines exhibited greater sensitivity to niraparib than olaparib, as indicated by a higher maximum killing effect (E_{max}) of more than 60 % (Fig. 2A–C). KKU-023 was the most sensitive to niraparib, with an IC_{50} of $3.97 \pm 0.53 \mu M$. This cell line also showed the greatest sensitivity to olaparib (Fig. 2C). In addition, niraparib treatment led to a dose-dependent increase in reproductive cell death (Fig. 2D–E), highlighting its potential efficacy in inhibiting CCA cell viability and proliferation.

Differential sensitivity to PARPi is observed in cells harboring *BRCA* mutations. The KKU-023 cell line, which carries *BRCA1* nonsense mutations, demonstrated relatively high sensitivity to both PARPi, particularly niraparib. Other cell lines with *BRCA1* nonsense mutations, specifically KKU-097 and KKU-610, exhibited moderate sensitivity to niraparib. Significantly, KKU-023 cells not only displayed the highest sensitivity to PARPi but also harbored a substantial accumulation of mutations in DDR genes, including *BRCA1*, *ATR*, *RAD50*, and *ARID1A*. Conversely, cell lines with *BRCA1* missense mutations—KKU-100, KKU-213A, and KKU-213C—showed variable responses to the inhibitors. In contrast, KKU-466 and KKU-452 cells were characterized by either low efficacy (E_{max}) or relatively high IC_{50} values (Fig. 2F). These results underscore the therapeutic potential of targeting DDR-pathway genes, especially in *BRCA*-mutant backgrounds, to augment PARPi effectiveness.

3.3. Synergistic effects of PARPi and ATMi combination therapy

PARPi shows promise in reducing cancer cell viability and proliferation. However, some cancer cells can evade PARPi efficacy by activating alternative DNA repair pathways, such as those involving ATM proteins. To enhance cytotoxic effects and potentially overcome this resistance, we investigated the combination of PARPi with an ATMi (AZD0156) in CCA cells.

Initially, the sensitivity of four niraparib-sensitive cell lines (KKU-023, KKU-100, KKU-097, and KKU-610) to AZD0156 monotherapy was evaluated. This assessment revealed variable responses: KKU-023 and KKU-100 cells exhibited high sensitivity ($E_{max} > 60$ %; IC_{50} values of $2.28 \pm 0.61 \mu M$ and $2.63 \pm 0.30 \mu M$, respectively). KKU-097 cells demonstrated moderate sensitivity to AZD0156, whereas KKU-610 cells were less sensitive ($E_{max} < 50$ %) (Fig. 3A; Supplementary Fig. S1). Notably, despite these varied sensitivities in the viability assay, AZD0156 suppressed all four cell types with comparable efficacy in a reproductive cell death assay (Fig. 3B–C). It should be noted that IC_{50} values of PARPi and ATMi when determined by MTS assay, are higher than the concentration required to inhibit colony formation. The latter assay evaluates agents with ability to cause long-term reproductive cell death, even the initial effect may be subtle toxicities. Therefore, KKU-023 and KKU-100 cells were used as representative cell lines to investigate the effects of drug combinations.

Treatment with a combination of PARPi and AZD0156 resulted in a significantly greater reduction in cell viability than either drug administered alone (Fig. 3D–E, H–I, L–M). This combination showed strong synergistic inhibition of cell proliferation at low doses, achieving a synergy score of ≥ 10 . Specifically, KKU-100 displayed a higher Bliss synergy score (10–60) compared to KKU-023 (10–40) (Fig. 3F–G, J–K).

Moreover, a potent synergistic effect on cell viability was observed when drugs were combined at concentrations below their individual IC_{50} values.

While synergistic effects of PARPi and ATMi were observed in two *BRCA*-mutant CCA cell lines (KKU-023, *BRCA*-nonsense; KKU-100, *BRCA*-missense), our subsequent evaluation in *BRCA* wild-type (KKU-452) and non-cancerous (MMNK-1) cells revealed the influence of genetic heterogeneity. In KKU-452, the combination showed a lower Bliss synergy score, indicating weak synergy or antagonism at certain concentrations (Fig. 3L–O; Supplementary Fig. S2). The non-cancerous MMNK-1 cell line, despite reduced viability, exhibited only limited synergy at low doses and antagonism (synergy scores ≤ -10) at higher doses (Supplementary Fig. S3). These results collectively demonstrate that genetic heterogeneity critically affects the therapeutic efficacy of this combination in CCA cells.

3.4. PARPi and ATMi combination enhances cell apoptosis

Given the synergistic effect of PARPi and ATMi on CCA cells, we further investigated their combined impact on apoptosis using sub- IC_{50} doses. Our findings revealed that the combination treatment significantly induced apoptotic cell death in CCA cells, with niraparib generally demonstrating greater efficacy than olaparib. Specifically, in KKU-023 cells, the combination of olaparib and AZD0156 did not significantly increase apoptosis (Fig. 4A, E). However, combining high-dose niraparib with AZD0156 markedly induced apoptosis in 38 % of these cells (Fig. 4C, G).

Treatment of KKU-100 cells with olaparib or niraparib alone resulted in apoptosis rates below 20 % (Fig. 4B, D, F, H). The addition of AZD0156 to these PARPi substantially elevated apoptosis, reaching levels of approximately 30 %–50 %. It is noteworthy that for the high-dose niraparib combination with AZD0156, apoptosis was somewhat lower than that observed with a lower niraparib concentration (when combined with AZD0156), but this was accompanied by a remarkable increase in cell necrosis (25 %).

We assessed reactive oxygen species (ROS) formation following treatment with niraparib, AZD0156, and their combination. KKU-023 cells generated high levels of ROS, whereas KKU-100 cells showed only a subtle increase. Notably, this ROS generation did not strongly correlate with apoptotic induction, suggesting that ROS formation is likely a secondary effect of the DNA damage response (Supplementary Fig. S4).

3.5. PARPi and ATMi promoted cell cycle arrest in CCA cells

Cell cycle profiling revealed differential sensitivities of KKU-023 and KKU-100 cell lines to PARP inhibition. Olaparib, at concentrations ranging from 1–6 μM , did not significantly perturb cell cycle distribution in KKU-023 cells (Fig. 5A). In contrast, KKU-100 cells exhibited a dose-dependent increase in G2/M phase accumulation following olaparib treatment, reaching 13 % (4 μM) and 26 % (6 μM) (Fig. 5B). Treatment with niraparib alone resulted in significant G2/M arrest, observed as 41 % of KKU-023 cells at 4 μM (Fig. 5C) and 20–35 % of KKU-100 cells at 7.5 μM and 10 μM , respectively (Fig. 5D). The ATMi when administered as a single agent, did not induce significant cell cycle arrest in either cell line. Combination therapy of PARPi and AZD0156 led to more pronounced cell cycle effects. In KKU-023 cells, the combination significantly enhanced G2/M arrest. Notably, co-administration of high-dose niraparib and AZD0156 also increased the sub-G1 fraction, indicative of increased apoptosis (Fig. 5C). In KKU-100 cells, the combined treatment induced arrest at both S and G2/M phases, along with an elevated sub-G1 population (Fig. 5D).

KKU-100 cells treated with the PARPi and AZD0156 combination exhibited distinct morphological changes, including cellular swelling, irregular shapes, and apoptotic features, as observed by bright-field microscopy (Fig. 6A). These findings corroborated the cell cycle

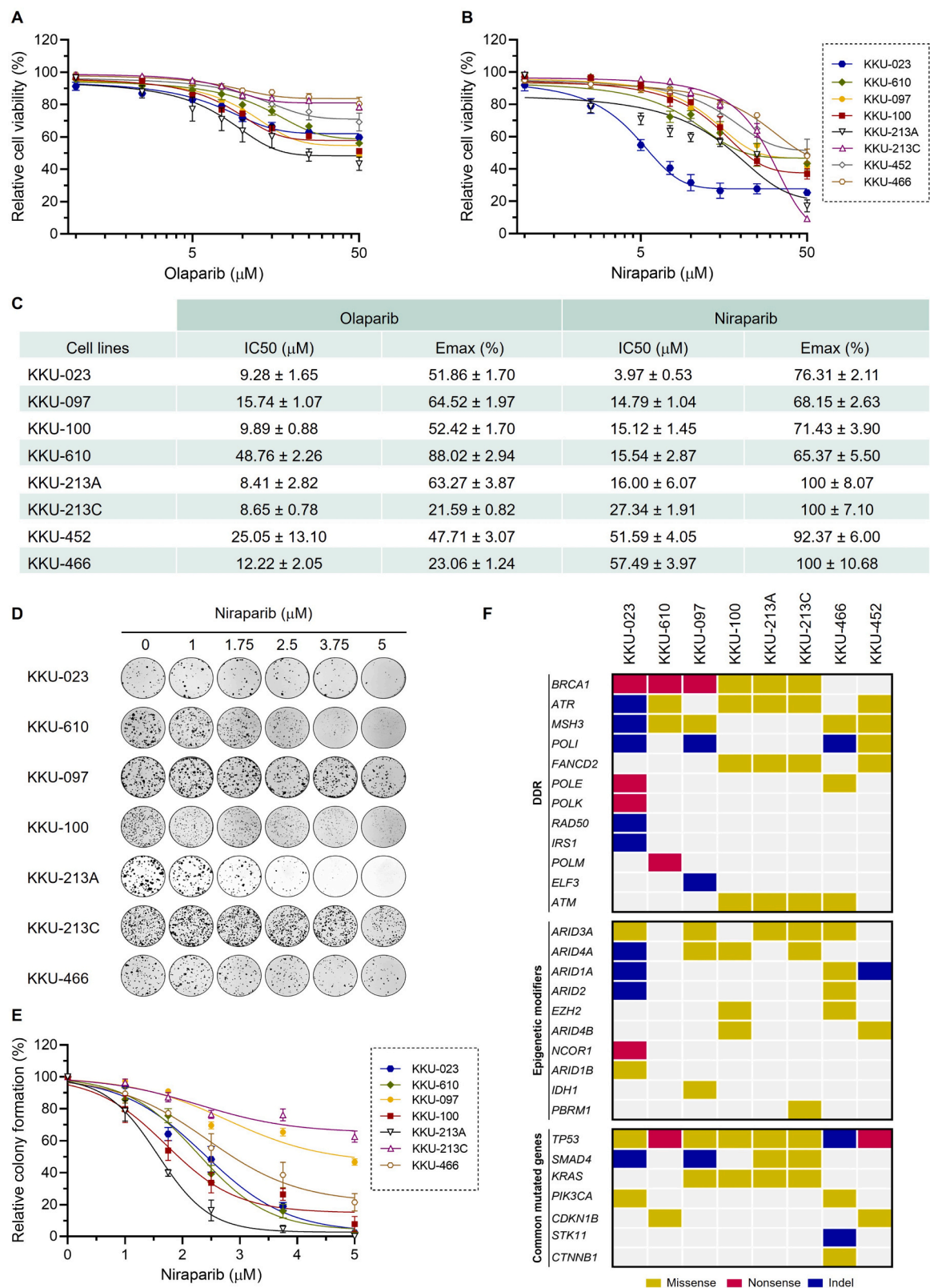


Fig. 2. PARPi decrease viability and clonogenic survival of CCA cell lines. Eight CCA cell lines were treated with PARPi (olaparib or niraparib). (A-B) Cell viability was assessed by MTS assay after 72 h treatment with increasing concentrations (0–50 μM). Data are presented as mean ± SD from three independent experiments (n = 3). (C) IC50 values for olaparib and niraparib were calculated from the dose-response curves using a sigmoidal dose-response equation. (D) Representative images of colony formation in CCA cells treated with niraparib (0–5 μM) for 72 h. (E) Quantification of colony formation, expressed as mean ± SD (n = 3). (F) Mutational landscape of genes involved in DDR, epigenetic modification, and other commonly mutated cancer genes across the eight CCA cell lines.

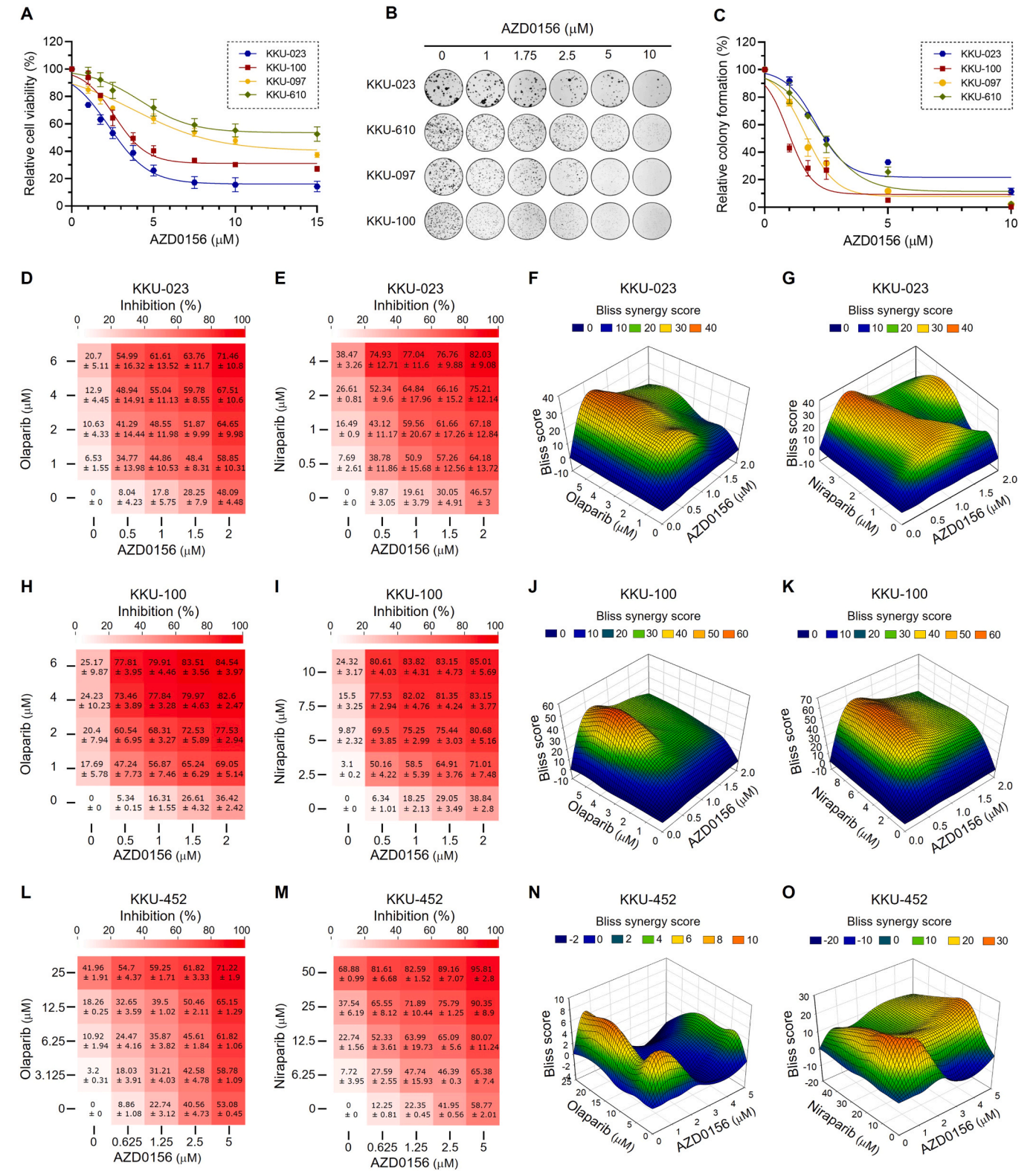


Fig. 3. Synergistic effects of PARPi and AZD0156 in CCA cell lines. (A) Dose-response curves obtained using the MTS assay in niraparib-sensitive CCA cell lines after treatment with AZD0156 at concentrations of 0–15 μM for 72 h. (B–C) Colony-formation assay of CCA cells after treatment with AZD0156 for 72 h. (D–E) Two-dimensional synergy plots showing the combined inhibitory effects of PARPi and AZD0156 in KKKU-023 cells; (H–I) KKKU-100 cells; (L–M) KKKU-452 cells. (F–G, J–K, N–O) Bliss synergy scores for each combination: scores ≤ −10 indicate antagonism, −10 to 10 indicate an additive effect, and ≥ 10 indicate synergy in KKKU-023, KKKU-100, and KKKU-452, respectively. All data are presented as mean ± SD (n = 3).

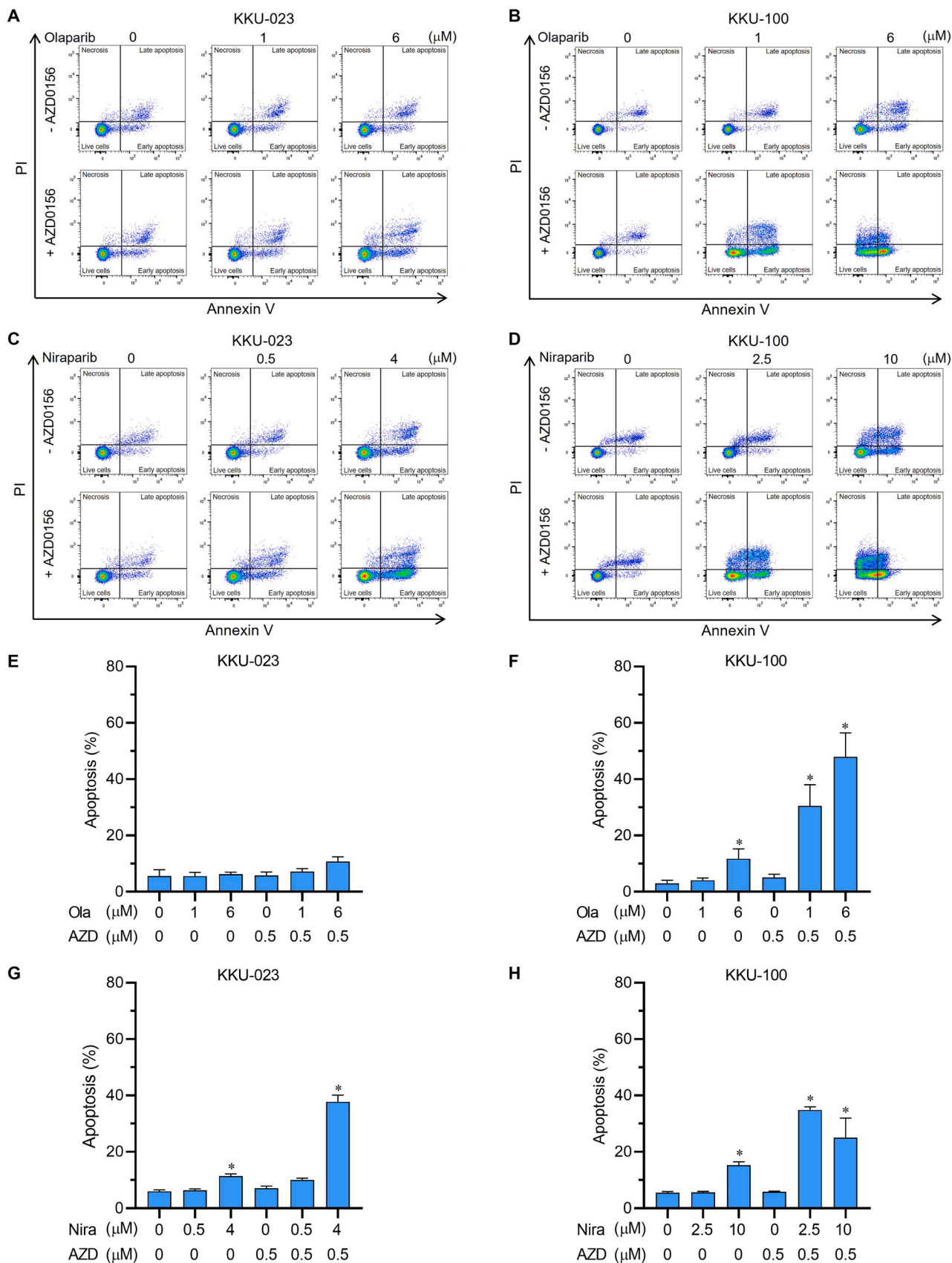


Fig. 4. The combination of PARPi and ATMi enhances cell apoptosis in CCA cell lines. KKKU-023 and KKKU-100 cells were treated with single and combined inhibitor therapy for 72 h. Apoptosis was quantified by flow cytometry using annexin V-FITC/PI staining. Flow cytometry plots illustrate the proportion of apoptotic cells after treatment with (A-B) olaparib and (C-D) niraparib. Each bar graph represented the proportion of apoptotic cells from DDRi treatments in (E, G) KKKU-023 and (F, H) KKKU-100. Data are expressed as mean $\pm$ SD (n = 3). * p < 0.05 compared to untreated control.

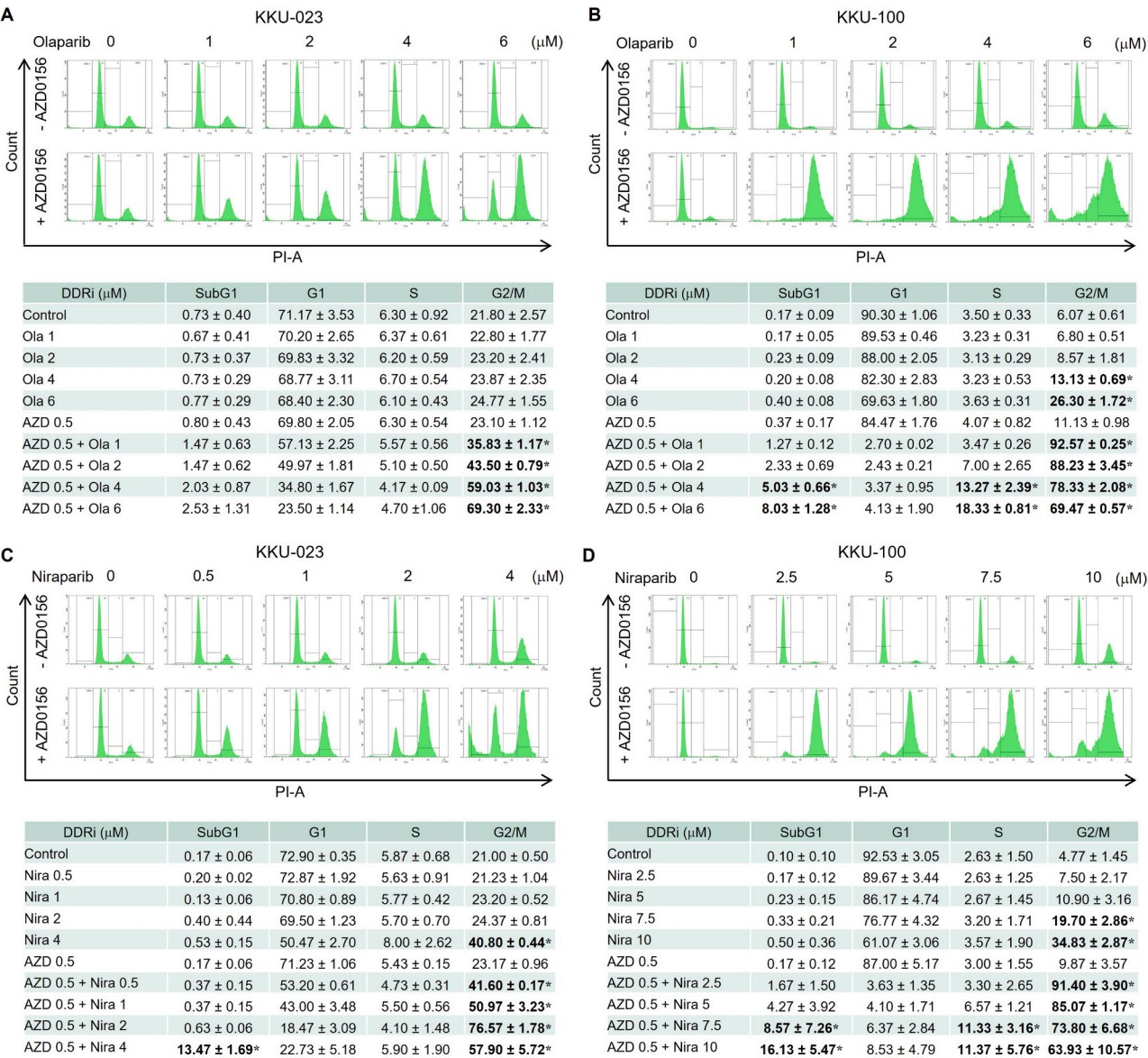


Fig. 5. A combination of PARPi and ATMi promoted cell cycle arrest in CCA cells. (A, C) KKKU-023 and (B, D) KKKU-100 cells were treated with PARPi alone or PARPi combined with AZD0156 for 72 h. Histogram plots represent cell cycle analysis and percentage of cells in each phase of the cell cycle (SubG1, G1, S, G2/M). Data are presented as mean ± SD (n = 3). **p* < 0.05 versus untreated control.

analysis, indicating significant induction of antiproliferation and apoptosis by the drug combination in these cells. Investigation into the molecular basis for these observations revealed alterations in key cell cycle regulatory proteins (Fig. 6B). While niraparib alone increased PARP1 and phosphorylated ATM (pATM) expression, this effect was diminished upon combination with AZD0156. Conversely, the combined treatment significantly increased p53 and p21 protein levels. Moreover, a pronounced, dose-dependent reduction in cyclin A and cyclin B1 expression was observed with the combination treatment (Fig. 6B-I), an effect greater than that seen with either drug alone. These data indicate that the combination treatment potentiates anti-proliferative effects by inducing G2/M arrest, likely driven by the pronounced downregulation of cyclin A and cyclin B1.

3.6. PARPi and ATMi combination enhances DNA damage

The induction of DNA damage was assessed by quantifying γ-H2AX foci. Treatment with niraparib alone resulted in a moderate increase in γ-H2AX foci, yielding an average of 12 and 19 foci per cell at 2.5 μM and

10 μM, respectively. In contrast, co-treatment of niraparib with AZD0156 significantly augmented γ-H2AX foci formation, leading to 26 and 38 foci per cell, respectively (Fig. 7A-B). These findings demonstrate that combined PARP and ATM inhibition leads to a greater accumulation of DNA damage than PARP inhibition alone, suggesting enhanced efficacy of the dual-inhibitor approach.

To further investigate the interplay between DNA damage and repair mechanisms under PARP and ATM inhibition, the expression levels of RAD51 (DNA repair marker) and γ-H2AX (DNA damage marker) were assessed in KKKU-100 cells. Following 24 h of treatment, niraparib monotherapy suppressed RAD51 expression in a dose-dependent manner. Conversely, combined treatment with niraparib and AZD0156 resulted in a slight increase in RAD51 expression at this time point (Fig. 7C-D). However, this transient elevation of RAD51 was not sustained, as its expression levels were reduced by 72 h of combination treatment (Fig. 7F-G). Concurrently, γ-H2AX protein levels, indicative of DNA damage, were elevated after 24 h of combination treatment (Fig. 7C-E) but subsequently declined by 72 h (Fig. 7F-H). These results suggest that while an initial attempt at DNA repair may occur, the

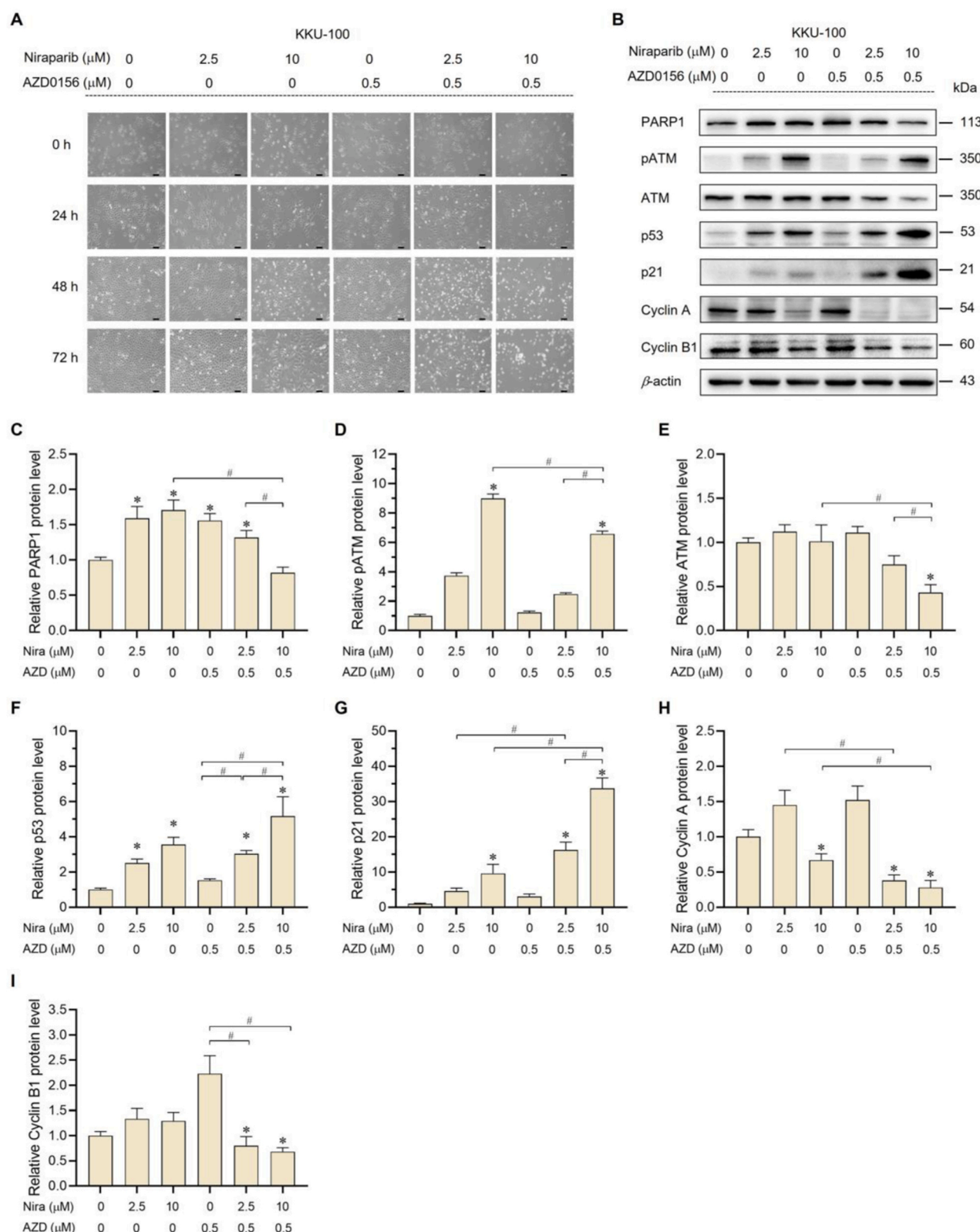


Fig. 6. Effect of PARPi (niraparib) and AZD0156 on cellular morphology and expression of key cell cycle regulatory proteins in KKU-100 cells. (A) Bright-field images (10x magnification, scale bars = 100 μm) illustrate the cellular morphology of KKU-100 cells after 0–72 h treatment with PARPi and AZD0156. (B) KKU-100 was treated with a single PARPi and combined with AZD0156 for 72 h. Representative western blots show expression of key cell cycle regulatory proteins. Protein expression levels were analyzed by Western blot. Bar graphs showing quantification of relative protein levels: (C) PARP1, (D) pATM, (E) ATM, (F) p53, (G) p21, (H) cyclin A, and (I) cyclin B1. Data are presented as mean $\pm$ SD ($n = 3$). * $p < 0.05$ versus untreated control; $p < 0.05$ versus treatment group.

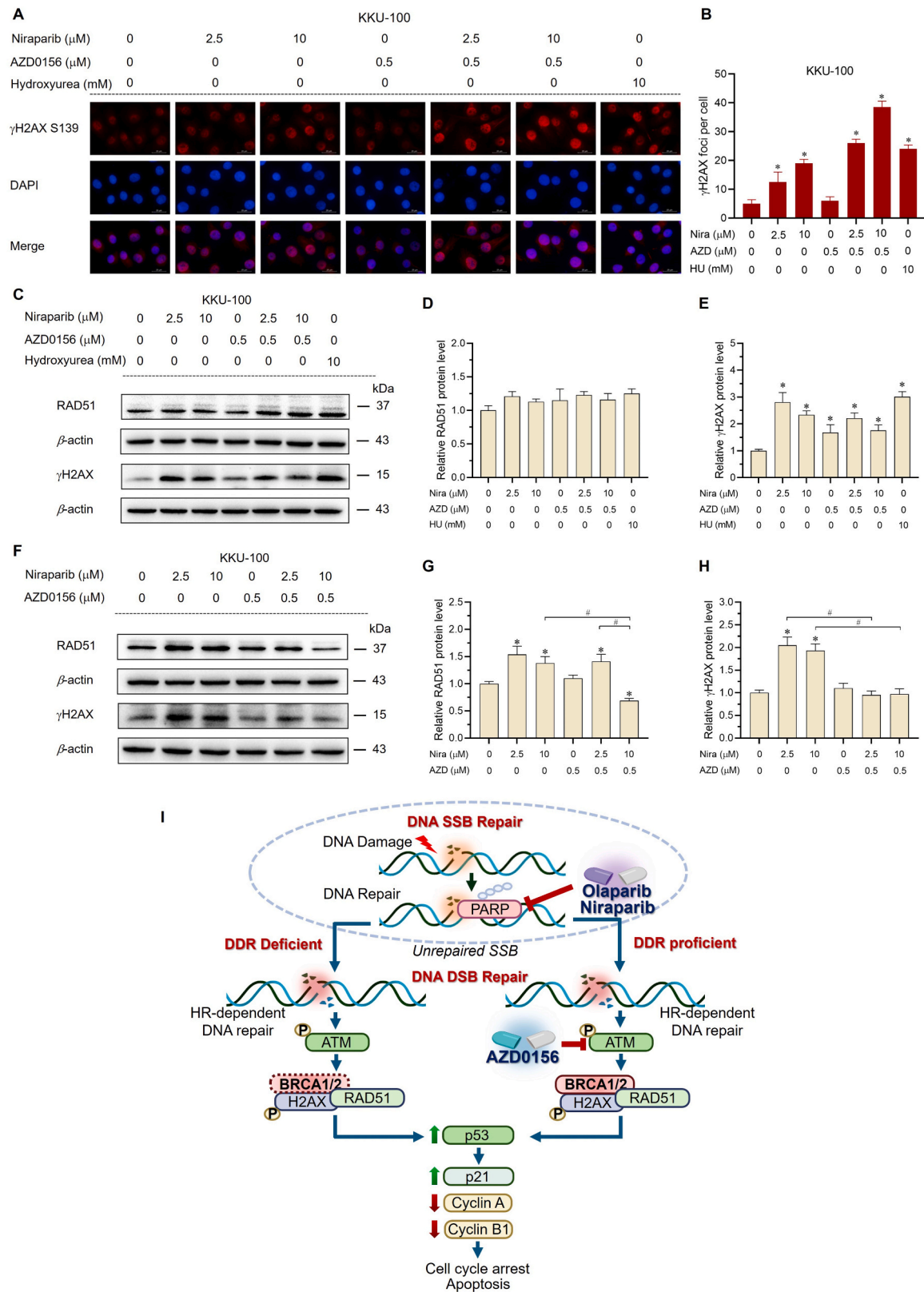


Fig. 7. The combination of PARPi and ATMi treatment enhances DNA damage in CCA cells. KKU-100 was treated with single and combined inhibitors for 24 h. DNA damage was analyzed by immunofluorescence. Hydroxyurea (HU) was used as a positive control for DNA damage induction (3 h treatment). (A) Representative immunofluorescence images of KKU-100 cells treated for 24 h, stained for DNA with DAPI (blue) and for γ -H2AX foci (red) (scale bars = 25 μ m). (B) Quantification of γ -H2AX-positive cells treated with DDRis, single agents, or their combination. Western blot analysis shows the expression of key DNA damage (γ -H2AX) and DNA repair (RAD51) proteins after (C-E) 24 h and (F-H) 72 h treatment in KKU-100 cells. Data are presented as mean $\pm$ SD (n = 3). * p < 0.05 versus untreated control; # p < 0.05 versus treatment group. (I) The proposed mechanism by which combined PARPi and AZD0156 treatment suppresses the DDR machinery in CCA cells.

combination of niraparib and AZD0156 ultimately leads to persistent DNA damage and compromises the DNA repair capacity in KKKU-100 cells.

4. Discussion

CCA remains a challenging and malignant biliary tract cancer with limited curative pharmacological options [28]. Although genetic alterations driving CCA pathogenesis have been reported [29,30], only a limited number of targeted molecular therapies have been developed, including those targeting *FGFR*, *IDH1*, and *HER2* [31]. However, these approved drugs show unsatisfactory responses owing to acquired resistance [32,33]. Our results demonstrate that the combination of PARPi and ATMi produces a potent synergistic effect in suppressing CCA cell growth by inducing DNA damage and cell cycle arrest. This finding provides preclinical evidence for the possibility of using DDRi(s) in a subset of CCA patients.

Genomic profiling is crucial for identifying targetable oncogenic alterations across diverse tumor types [34]. Interrogation of publicly available databases revealed that CCA patient samples harbor mutations in various epigenetic and DDR-related genes, suggesting potential sensitivity to PARPi. In the present study, CCA cell lines with a higher burden of mutations in these pathways demonstrated markedly greater sensitivity to niraparib (IC50 range 4–25 μ M; Emax > 50 %) than to olaparib (IC50 >50 μ M; Emax < 50 %). Specifically, among the eight CCA cell lines tested, KKKU-023, KKKU-097, and KKKU-610, all harboring *BRCA1* nonsense mutations, exhibited relatively high sensitivity to niraparib. The KKKU-023 cell line, which displayed the most pronounced sensitivity to PARPi (particularly niraparib), possessed multiple DDR pathway mutations, including alterations in *BRCA1*, *RAD50*, *ATR*, and *ARID1A*. The observed lower potency of olaparib in some CCA contexts (IC50 >50 μ M) is comparable to its reported efficacy in certain prostate cancer cell lines, such as DU145 (olaparib IC50: 73.20 μ M) and PC3 (olaparib IC50: 64 μ M) [35]. Conversely, the potent niraparib efficacy (IC50 range 4–25 μ M) identified in our sensitive CCA lines aligns with reported IC50 values for other DDR-mutated CCA cell lines. For instance, KMCH-1 cells (harboring *ATM*-mutant) are susceptible to niraparib with an IC50 of 3 μ M, and HuCCT1 cells (harboring *BRCA2* and *POLE2*-mutant) show a niraparib IC50 of 8 μ M [36].

The principle of synthetic lethality between *BRCA1/2* mutations and PARP inhibition underpins a key therapeutic strategy for *BRCA*-mutant tumors. This approach is supported by clinical evidence: olaparib treatment in patients with *BRCA1*- or *BRCA2*-mutated breast cancer resulted in significantly improved invasive disease-free survival (85.9 % vs. 77.1 % with placebo) and distant disease-free survival (87.5 % vs. 80.4 % with placebo) [37]. Similarly, in metastatic castration-resistant prostate cancer (mCRPC) patients harboring alterations in *BRCA1*, *BRCA2*, or *ATM*, olaparib monotherapy achieved significantly longer imaging-based progression-free survival compared to standard therapy (7.4 vs. 3.6 months) [38]. Preclinical evidence further supports PARPi efficacy, as metastatic CCA patient-derived xenograft (PDX) models with *BRCA2* mutations demonstrated therapeutic benefit from PARPi [39]. Moreover, in *ATM*-deficient murine and human pancreatic ductal adenocarcinoma (PDAC) models, synthetic lethality has been observed with inhibitors of PARP, ATR, or DNA-PKcs [18]. Collectively, these findings suggest that combining PARPi with ATMi may represent a promising therapeutic strategy for cancers characterized by DDR pathway deficiencies.

Combination treatment with PARPi and ATMi, especially involving niraparib, substantially increased cytotoxicity in CCA cells, reflected in diminished cell viability and elevated synergy scores. This effect was markedly greater in *BRCA*-mutant CCA cell lines (both nonsense and missense mutations) compared to *BRCA* wild-type or non-cancerous cells, where weak synergy or antagonism was observed, highlighting *BRCA* mutational status as a key determinant of response. PARPi efficacy is predicated on pre-existing HRD, often due to inactivating mutations in

HR pathway genes such as *BRCA1/2*, *ATM*, or *PALB2* [40]. The observed synergy in *BRCA*-missense cells (considered proficient in *BRCA* function), which may not be fully HR-deficient, implies that ATMi co-treatment can create a synthetic HRD state, thereby sensitizing these cells to PARPi. In contrast, the antagonistic effect in *BRCA*-wild-type CCA cells suggests that proficient HR activity in these cells mitigates the impact of combined PARP and ATM inhibition. Our findings are consistent with earlier studies demonstrating that ATM inhibition mediates the enhanced antitumor effects of combined PARP and ATM inhibition. Notably, AZD0156 was previously reported to augment the antitumor activity of olaparib in diverse cancer cell lines (lung, gastric, and breast) *in vitro* and to improve olaparib efficacy in patient-derived triple-negative breast cancer xenografts [24].

Niraparib treatment significantly induced DNA damage, as evidenced by a marked increase in γ -H2AX foci. This DNA damage was further potentiated upon combination treatment with AZD0156. Activation of the DDR was also apparent, characterized by the upregulation of RAD51, a key protein in the homologous recombination DNA repair pathway [41]. ROS formation was observed in this study. While ROS are known to directly damage cellular components such as DNA, proteins, and lipids, potentially leading to cell death, it is also established that DNA damage can activate DDR pathways that subsequently increase ROS generation, creating a positive feedback loop that may further exacerbate DNA damage [42]. However, the weak correlation observed between ROS levels and apoptotic cell death in our experiments suggests that ROS may play a subordinate role in directly mediating apoptosis under these conditions, or that its production is predominantly a secondary consequence of DDR activation.

The observed suppression of CCA cell growth was attributed to both the induction of apoptotic cell death and the inhibition of cell division. The latter was evidenced by a profound G2/M phase cell cycle arrest induced by the drug combination in both cell lines. This cell cycle arrest was associated with markedly upregulated levels of p53 and p21, accompanied by decreased expression of their downstream targets, cyclin A and cyclin B1. Consistent with this, the drug combination led to a significant increase in the proportion of γ -H2AX-positive cells, indicative of DNA damage, and disrupted DNA repair mechanisms, as evidenced by RAD51 suppression.

5. Conclusion

This study provides preclinical evidence supporting the efficacy of a DDRi-based approach for CCA treatment. Specifically, PARPi effectively induced anticancer responses in *BRCA*-deficient CCA cells, suggesting the utilization of synthetic lethal therapeutic strategies for CCA treatment characterized by DDR mutations. ATMi co-treatment can create a synthetic HRD state in *BRCA*-proficient CCA cells, thereby sensitizing these cells to PARPi.

CRediT authorship contribution statement

Rattanaporn Jaidee: Writing – review & editing, Software, Formal analysis, Data curation. **Piman Pocasap:** Validation, Supervision, Software, Resources, Methodology. **Apinya Jusakul:** Writing – review & editing, Resources, Conceptualization. **Laddawan Senggunprai:** Validation, Supervision, Resources, Methodology. **Auemduan Prawan:** Validation, Supervision, Resources, Methodology. **Jing Han Hong:** Software, Formal analysis, Data curation. **Hong Lee Heng:** Software, Formal analysis, Data curation. **Veerapol Kukongviriyapan:** Writing – review & editing, Validation. **Bin Tean Teh:** Software, Resources, Methodology. **Kongpetch Sarinya:** Writing – review & editing, Visualization, Validation, Supervision, Resources, Methodology, Funding acquisition, Data curation, Conceptualization.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.biopha.2025.118273](https://doi.org/10.1016/j.biopha.2025.118273).

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