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Research article

Dual blockage of PI3K-mTOR and FGFR induced autophagic cell death in cholangiocarcinoma cells

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ABSTRACT

Purpose: To assess the impact of concurrent inhibition of the FGFR and PI3K/mTOR signaling pathways on oncogenic characteristics in cholangiocarcinoma (CCA) cells, including proliferation, autophagy, and cell death.

Materials and methods: KKU-213A, KKU-100, and KKU-213C cells were treated with either infigratinib or PKI-402 alone or in combination. Cell viability and cell death were evaluated using the sulforhodamine B (SRB) assay and acridine orange/ethidium bromide (AO/EB) staining. Cell cycle progression and apoptotic cell death were analyzed by flow cytometry. Western blotting was performed to assess the expression of proteins involved in cell cycle regulation and autophagy. Additionally, AO staining was employed to assess autophagic induction.

Results: The combination of infigratinib and PKI-402 showed a remarked synergistic suppression in cell viability in both CCA cell lines compared to treatment with single inhibitors. This antiproliferative effect was associated with cell cycle arrest in the G2-M phase and a decrease in the expression of cyclin A and cyclin B1 in CCA cells. Furthermore, the combination treatment induced apoptotic cell death to a greater extent than treatment with a single inhibitor. Infigratinib enhanced the induction of autophagy by PKI-402, as evidenced by marked increases of autophagic vacuoles stained acridine orange, levels of LC3B-II and suppression of levels of p-mTOR and. Notably, inhibition of autophagic flux by chloroquine prevented cell death induced by the combination treatment.

Conclusions: This study demonstrated that concurrent inhibition of the key FGFR/PI3K/mTOR pathways in CCA carcinogenesis enhances the suppression of CCA cells. The present findings indicate potential clinical implications for using combination treatment modalities in CCA therapy.

1. Introduction

Advances in oncology research utilizing genomic approaches have identified FGFR (fibroblast growth factor receptor) alterations as promising targets for CCA (cholangiocarcinoma) treatment. However, acquired resistance to FGFR blockade remains a significant challenge, limiting the efficacy of FGFR inhibitors as monotherapy. Selective FGFR inhibitors such as pemigatinib have gained FDA

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approval for the treatment of unresectable, locally advanced, or metastatic CCA with FGFR2 fusion or other FGFR gene rearrangements [1]. Resistance to FGFR inhibitors in CCA and other cancer patients has been associated with gatekeeper mutations, which alter the inhibitor's access to the ATP binding pocket, activation of alternative signaling pathways, and lysosome-mediated sequestration of weakly basic FGFR inhibitors [2,3]. In cases of acquired resistance to FGFR inhibition, reactivation of the downstream PI3K/AKT/mTOR signaling pathway has been identified as a feedback loop promoting cancer cell survival [4]. This resistance mechanism has been observed in urothelial cell carcinoma [5]. Additionally, studies have shown that the combination of FGFR2 and mTOR inhibitors has a synergistic effect on the growth of endometrial cancer cells harboring activating FGFR2 mutations [6]. Thus, the suppression of downstream FGFR signaling is crucial for the efficacy of FGFR inhibitors.

The main targetable aberrations identified in patients with intrahepatic CCA were FGFRs genetic alteration, especially FGFR2 fusions, which are estimated to be 10–15 % of CCA patients [7]. The immunohistochemistry study found that positive FGFR2 protein expression occurred in 100 out of 153 CCA tissues (67.1 %), with high expression in 44 (29.5 %) cases [8]. Our previous report on CCA cells, p-mTOR protein expression was up-regulated following the FGFR inhibitor or gemcitabine treatment [9]. This finding suggested that the mTOR pathway might be one of the key effector proteins in feedback survival response in CCA cells.

The PI3K/mTOR signaling pathway is a key downstream effector in FGFR-dependent tumorigenesis, regulating anti-apoptosis and the development of chemoresistance [4,10]. The sequencing analysis study revealed that 11 (32.4 %) of CCA patients harbor PIK3CA mutations [11]. High expression of PI3K/Akt/mTOR, particularly mTOR, in CCA tissues has been associated with the metastatic stage of CCA [12]. Our recent publication reported that the FGF10 ligand activates FGFR2, mediating metastatic phenotypes in CCA cells via the upregulation of the Akt/mTOR pathway [13]. Dual PI3K/mTOR inhibitors target the three critical nodes of this pathway, including PI3K, mTORC1, and mTORC2. mTORC1 controls cell cycle progression, proliferation, survival, and apoptosis [14]. Furthermore, inhibiting mTORC2 blocks the feedback activation of AKT [15,16]. Currently, numerous PI3K/mTOR inhibitors are undergoing evaluation in clinical trials (phase 2 and 3) for various cancer types [16].

Autophagy is a vital cellular process that occurs under conditions of starvation, low ATP levels, and endoplasmic reticulum stress. The primary function of autophagy is to alleviate stress by removal of damaged organelles and abnormal proteins through autolysosomal degradation, and to maintain energy metabolism under starvation [17]. Thus, autophagy in this context functions as a pro-survival stress response. On the other hand, autophagic cell death (ACD) occurs when cells are exposed to prolonged high autophagic conditions. mTORC1 is a prominent negative regulator of autophagy, acting at the transcriptional and translational levels and directly inhibiting the early process through phosphorylation of ULK1 and ATG13, leading to the blockade of ULK complex activation and autophagosome formation [18,19]. Inhibition of mTORC1 induces autophagy to maintain cellular homeostasis and inhibit cell death. However, when autophagic flux is impaired, autophagosomes accumulate, leading to ACD [20]. Thus, inhibition of mTOR contributes to the induction of autophagy and cell death when autophagic flux is inhibited.

The purpose of this study was to explore the anticancer effects of FGFR inhibitors in combination with PI3K/mTOR inhibitors as a multi-targeted approach. The synergistic effects of the combination drug on antiproliferation, apoptotic induction, and cell cycle regulation were investigated, as well as the mechanisms of cell death through autophagy induction.

2. Materials and methods

2.1. Cell culture

Human CCA cells, including KKU-100 (JCRB1568), KKU-213A (JCRB1557), KKU-213C cells [21], were employed in this study based on our previous report in which these cells showed high FGFR2 protein expression [9]. These CCA cells were developed at the Cholangiocarcinoma Research Institute, Khon Kaen University, and deposited in the Japanese Cancer Research Resources Bank (JCRB, Ibaraki city, Osaka, Japan). The CCA cell lines were cultured in Ham's F12 media (21700075, Thermo Fisher Scientific Inc, MA, USA), supplemented with 10 % FBS (10100147, Thermo Fisher Scientific Inc, MA, USA), 10,000 units/mL penicillin-gentamicin, and 10 mM HEPES. All cell lines were incubated at 37 °C in a 5 % CO_2 incubator, mycoplasma-negative and used within 10 passages.

2.2. Sulforhodamine B (SRB) assay

Infigratinib (HY-13311) and PKI-402 (HY-10683, MedChemexpress, NJ, USA) were prepared to make stock solutions in DMSO. Both agents were then dissolved in 1X phosphate buffer saline (1X PBS) in cell culture treatment conditions. CCA cells were seeded at a 5000 cells/well density in 96-well plates overnight. Then, the cells were treated with various concentrations of each agent for 72 h. Cell viability was assessed using the SRB (3520-42-1, Sigma-Aldrich Pte Ltd, MO, USA) assay, and the absorbance was measured at 540 nm. Control cells were cultured in full media with 0.05 % DMSO for the entire culture process. The IC50 of cytotoxicity was evaluated using GraphPad Prism version 8.0 (La Jolla, CA, USA).

2.3. Acridine orange – ethidium bromide (AO/EB) staining

The cells were seeded at a density of 5000 cells/well in 96-well plates overnight, followed by treatment with the individual drugs alone or the drug combinations for 72 h. Live and dead cells were stained with the double dye AO (10127-02-3) and EB (1239-45-8, Sigma-Aldrich Pte Ltd, MO, USA) staining method as previously described [22]. The number of living and apoptotic cells were examined under a Nikon Eclipse TS100 inverted fluorescence microscope (at 20X magnification) with a B2A filter. The viable cells were colored green with normal nuclei. Necrotic and late apoptotic cells were stained bright orange, whereas early apoptotic cells were

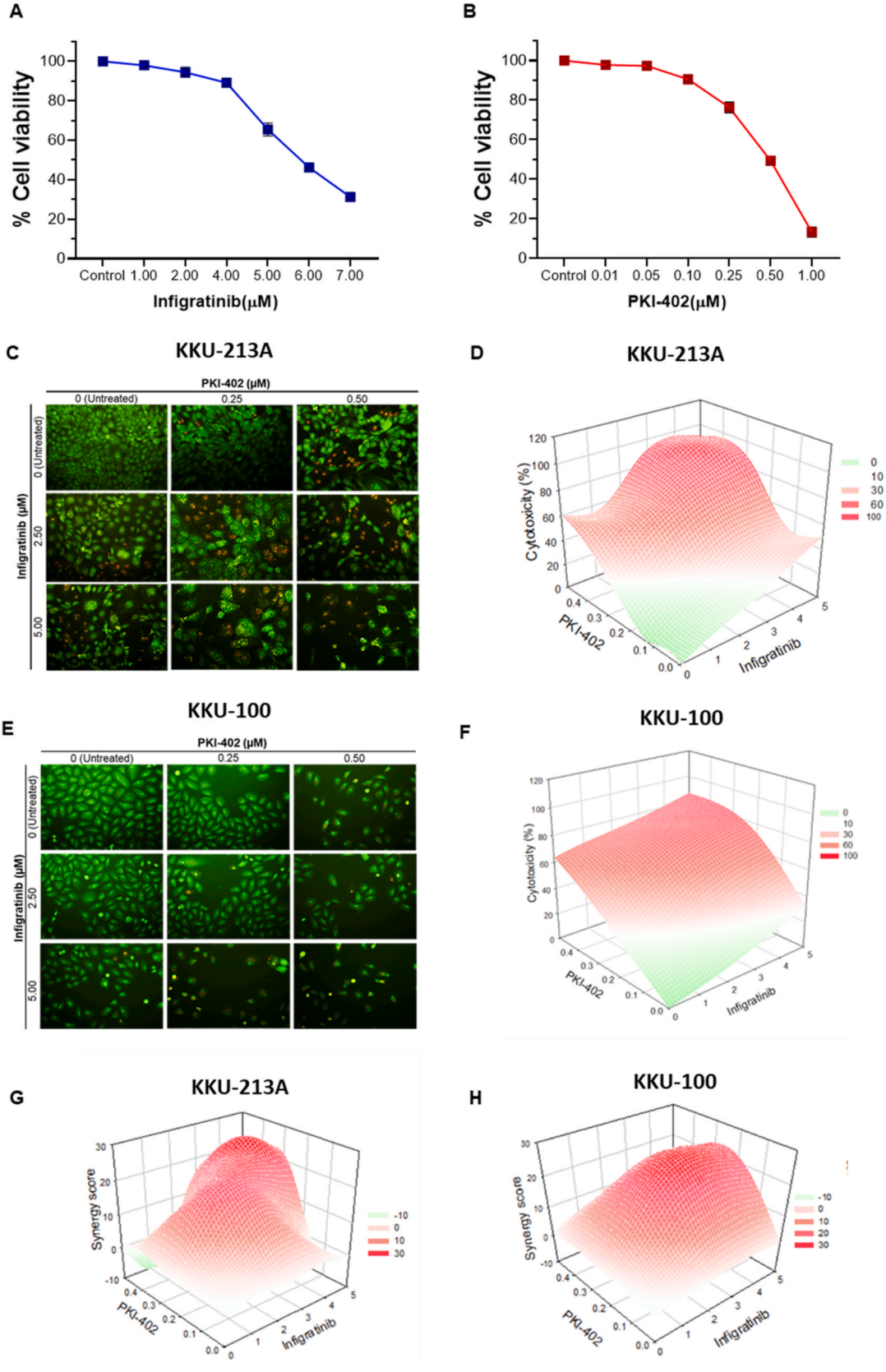

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Fig. 1. Effect of the combination of infigratinib and PKI-402 on CCA cell viability. KKU-213A cells were treated with infigratinib (A) or PKI-402 (B) with wide concentration ranges for 72 h. Cell viability was determined by the SRB method. The effects of the combinations of infigratinib (1.5–5 μM) and PKI-402 (0.15–0.5 μM) on cell cytotoxicity was determined using an AO/EB staining assay in KKU-213A and KKU-100 cells. Images of some combinations are shown (C, E). Three-dimensional (3D) surface plots demonstrating the enhanced cytotoxicity were shown (D, F). Drug synergy evaluated by the Bliss-independent model, an interactive 3D surface plot of both cells is shown (G, H), where the Bliss synergy score (z-axis) was plotted against PKI-402 (x-axis) and infigratinib concentrations (y-axis).

characterized by bright green nuclear staining with nuclear fragmentation.

2.4. Western blotting analysis

Whole cells were lysed in RIPA buffer with protease and phosphatase inhibitors. The clear supernatant of cell lysates was mixed with loading buffer to be loaded in SDS−PAGE. Protein bands were transferred to PVDF membranes. Then, the membranes were blocked with 5 % skim milk in 1X TBST, followed by incubation with primary antibodies against β-actin (sc-1616), cyclin A (sc-751), cyclin B1 (sc-245, Santa Cruz Biotechnology, CA, USA), phospho-mTOR (5536s), mTOR (2983s), and LC3A/B (4108s, Cell Signaling, MA, USA), overnight at 4 °C. The membranes were then incubated with HRP-conjugated secondary antibodies. ECL reagent was used to generate chemiluminescence signals, the protein bands were photographed by a ChemiDoc™ MP Imaging system, and the intensity of the target protein bands was analyzed using Image Lab 6.0 software (Bio-Rad, Hercules, CA, USA).

2.5. Cell cycle analysis

Propidium iodide (PI, 1 μg/mL) (25535-16-4, Sigma-Aldrich Pte Ltd, MO, USA) staining was performed for cell cycle analysis. The cells were seeded at a density of 200,000 cells/well in 6-well plates overnight and were treated with specific treatment conditions as indicated by the experiment. The cells were harvested and stained with PI and then analyzed by flow cytometry (BD FACSCANTO II, BD Biosciences, CA, USA). The percentage of cells in each phase of the cell cycle was analyzed using BD FACSDiva™ Software.

2.6. Cell apoptosis assay

FITC Annexin V/PI double staining kit (556547, BD Biosciences, CA, USA) was used to measure apoptotic cell death according to the manufacturer's protocol. Briefly, KKU-213A cells were treated with specific treatment conditions for 48 h, and then the cells were collected and stained with FITC Annexin V/PI for 15 min. Subsequently, the proportion of apoptotic cells was detected by flow cytometry (BD FACSCANTO II, BD Biosciences, CA, USA) and analyzed by using BD FACSDiva™ Software.

2.7. AO fluorescence staining

Acridine orange (AO) staining was performed to detect acidic vesicular organelles (AVOs) of autophagosomes. KKU-213A cells were seeded at a density of 5000 cells/well in 96-well plates overnight and then treated with drug alone or the drug combinations for 48 h. Subsequently, the medium was removed, and the cells were stained with AO (4.75 μg/ml) for 5 min before images were captured under a Nikon Eclipse TS100 inverted fluorescence microscope (200X magnification) with a B2A filter. The red fluorescence of AVOs was measured for intensity and area by ImagePro Plus software (Media Cybernetics Inc., MD, USA).

2.8. Synergistic assay

To evaluate the potential synergy of drug combination, concentrations of individual drugs and drug combinations were analyzed using SynergyFinder, a web base application (https://synergyfinder.org/#!/). Bliss-independent model was employed to quantify the degree of synergy [23]. The synergy score is interpreted as the average excess response due to drug interactions, whereas scores of ≤ -10, -10 to 10 and ≥ 10 are likely to be antagonistic, additive and synergistic, respectively.

2.9. Statistical analysis

All experimental data represent at least 3 independent experiments and are presented as the mean ± standard deviation (SD) and were analyzed using one-way analysis of variance (ANOVA) with Duncan's post hoc test. $P < 0.05$ was considered statistically significant. Statistical analysis was performed with GraphPad Prism 8.0 (La Jolla, CA).

3. Results

3.1. Combination of infigratinib with PKI-402 enhances cell cytotoxicity

Given that the PI3K/mTOR pathway is a critical downstream effector of FGFR, the simultaneous inhibition of multiple targets by infigratinib and PKI-402 offers strategic advantages in suppressing cell growth [24]. KKU-213A cells were exposed to the inhibitors

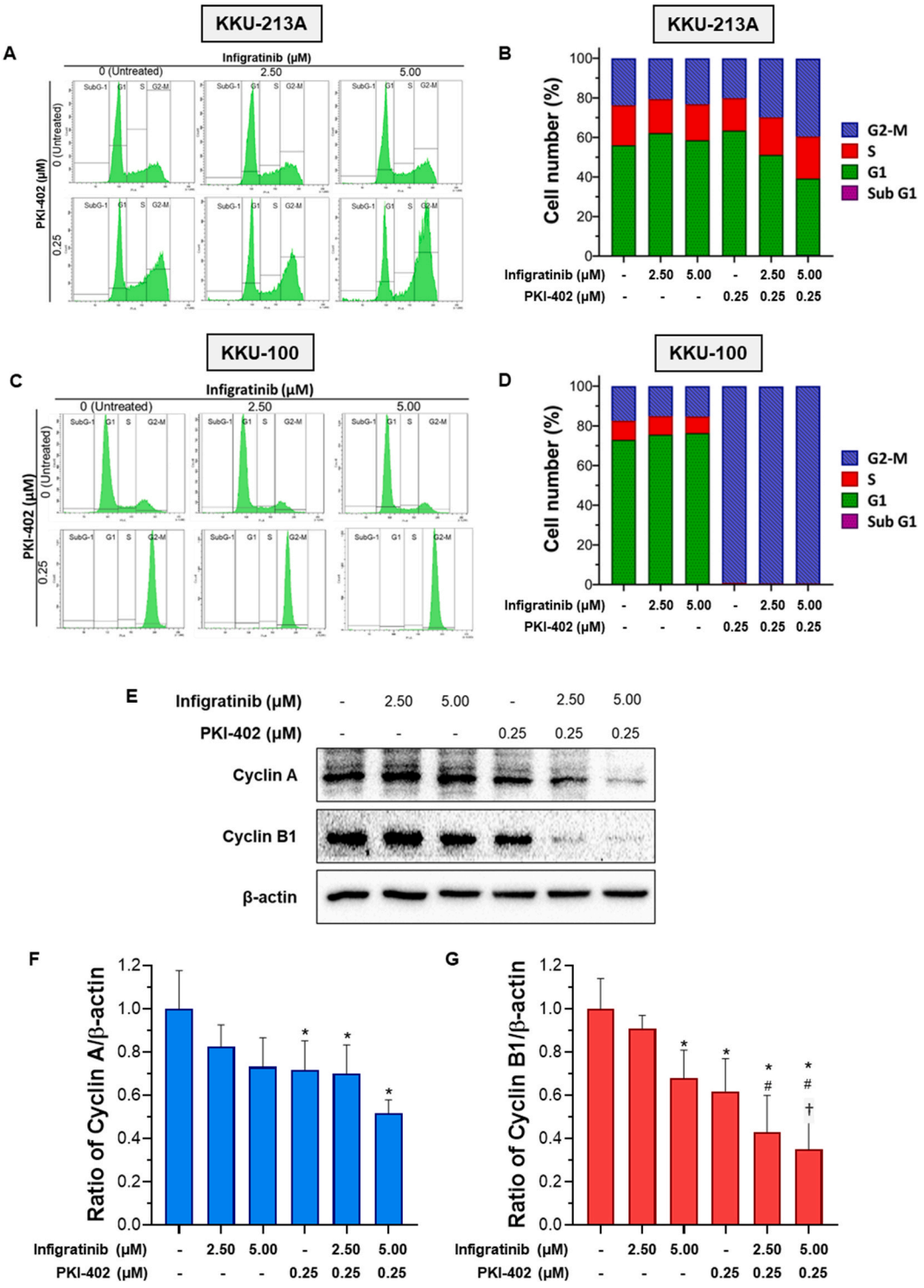

Fig. 2. Infigratinib in combination with PKI-402 caused cell cycle arrest. KKU-213A and KKU-100 cells were treated with single and combined inhibitors for 48 h, and the effect on cell cycle progression was determined by flow cytometry (A, C). The bar graphs show the percentage of cells in each cell phase (B, D). Expression of cyclin A and cyclin B1 in KKU-213A cells was analyzed by Western blot analysis (E, Supplementary Fig. S4 Images of original blot 2E) and presented as the relative intensity of the target protein normalized to β-actin (F, G). Data are expressed as the mean ± SD of three independent experiments. *$p < 0.05$ versus control group (DMSO), $^{\#}p < 0.05$ versus infigratinib alone, $^{\dagger}p < 0.05$ versus PKI-402 alone.

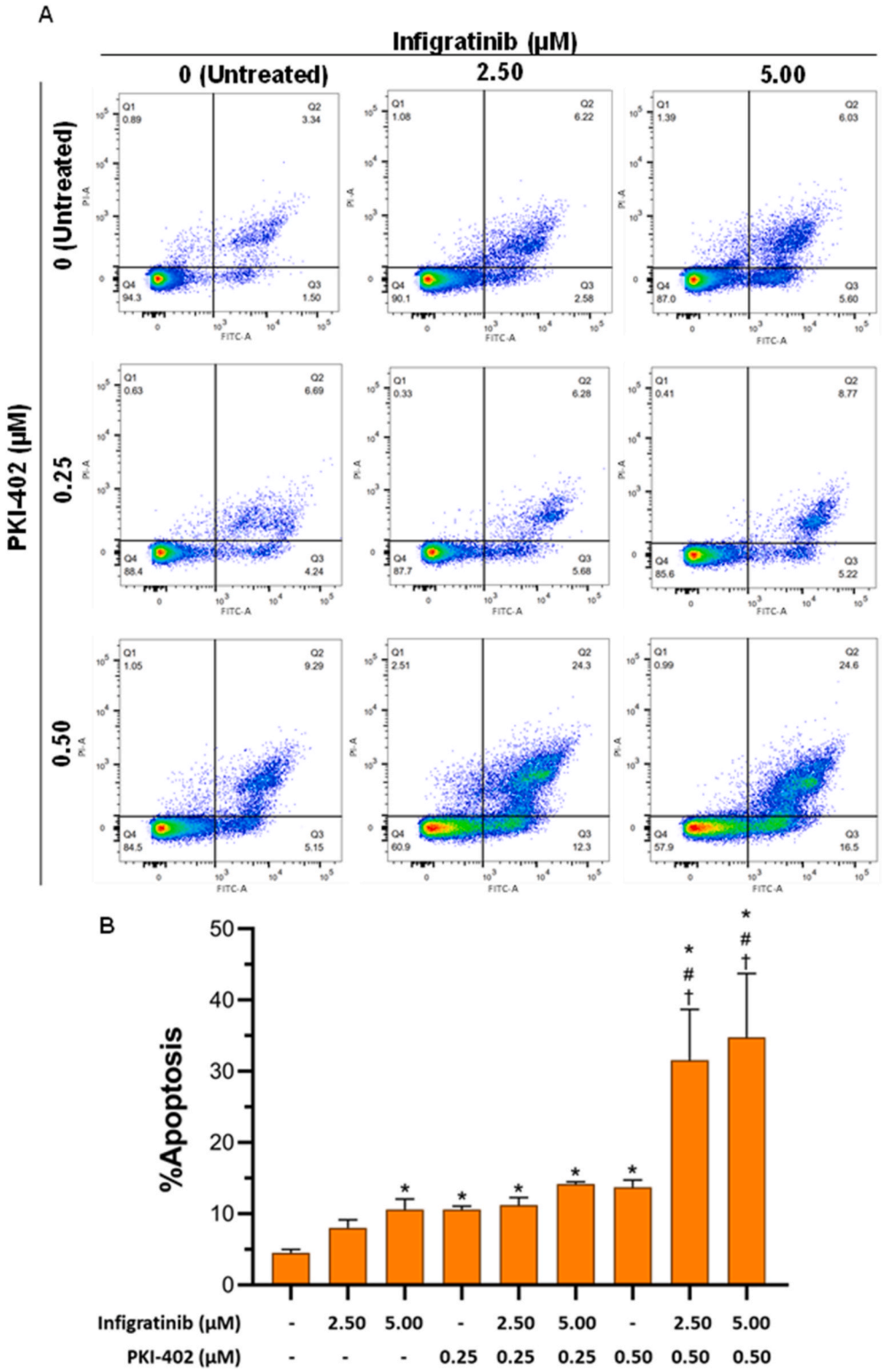

Fig. 3. Apoptotic-inducing effect of the combination of infigratinib and PKI-402. KKU-213A cells were treated with infigratinib alone or in combination with PKI-402 for 48 h, followed by staining with annexin V-FITC/propidium iodide (PI). Representative images of flow cytometric analysis for various drug combination groups are shown (A). The bar graphs show the proportion of apoptotic cells from various treatments (B). Data are expressed as the mean ± SD of three independent experiments. *$p < 0.05$ versus control group (DMSO), $^{\#}p < 0.05$ versus infigratinib alone, $^{\dagger}p < 0.05$ versus PKI-402 alone.

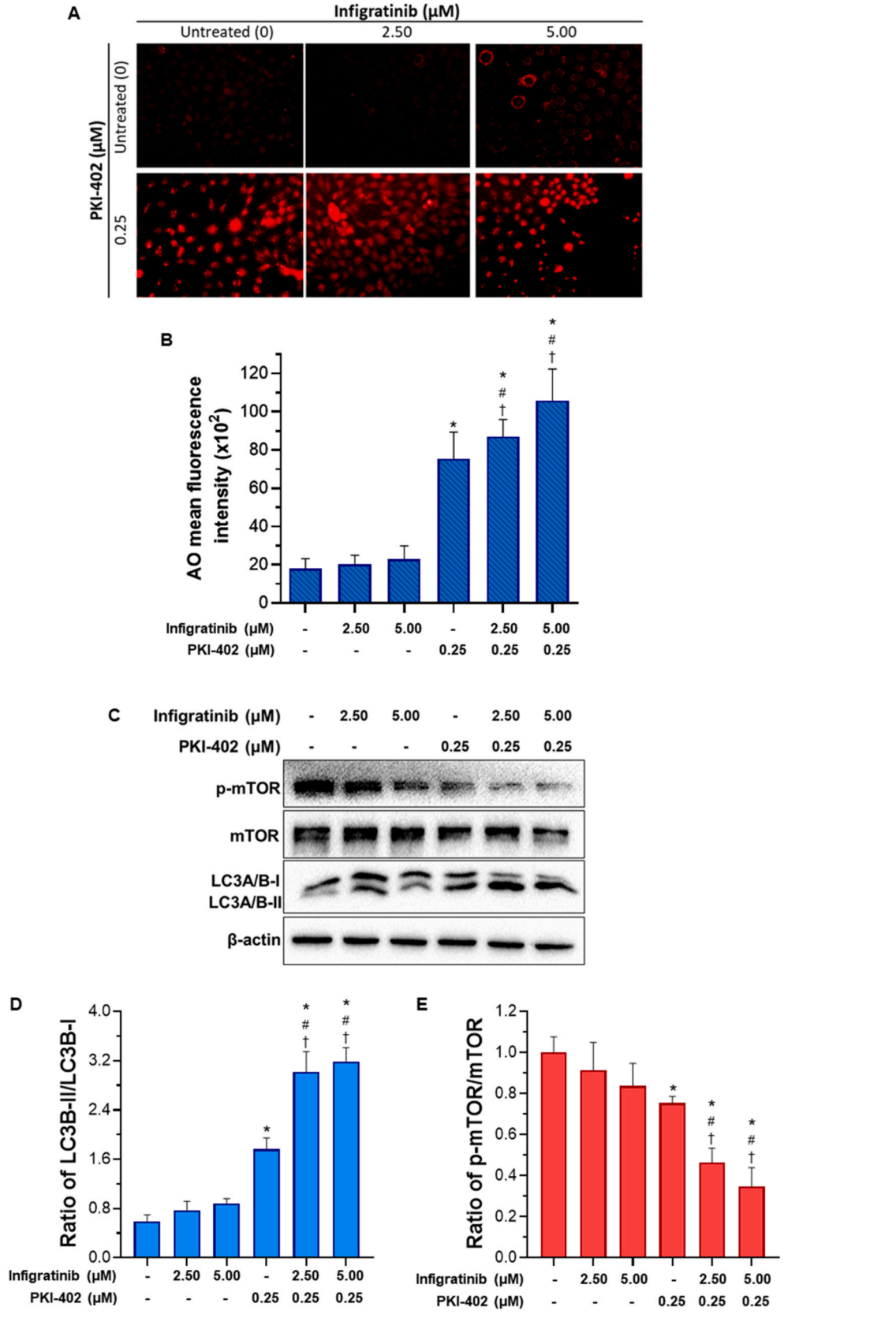

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Fig. 4. Effect of PKI-402 and infigratinib on autophagy formation. KKU-213A cells were treated with infigratinib and PKI-402 for 48 h. The cells were stained with acridine orange dye to observe autophagic vacuoles (AVOs) (A), and protein lysates of the cells were subjected to Western blot analysis for mTOR and LC3A/B (C, Supplementary Fig. S5 Images of original blot 4C). The percentage of red fluorescent intensity is represented in the bar graph (B). The expression of mTOR, phosphorylated-mTOR (p-mTOR) and LC3BII/I proteins is shown as the ratio of p-mTOR/mTOR (D) and the ratio of LC3B-II/LC3B–I (E) after normalization to β-actin as a loading control. Data are expressed as the mean ± SD of three independent experiments. *$p < 0.05$ versus control group (DMSO), $^{\#}p < 0.05$ versus infigratinib alone, $^{\dagger}p < 0.05$ versus PKI-402 alone.

individually or in combinations for 72 h to determine the appropriate concentration ranges for drug combination. Cell viability was assessed using the SRB assay. The IC50 values for infigratinib and PKI-402 were found to be 5.31 ± 0.2 μM (Fig. 1A) and 0.497 ± 0.01 μM (Fig. 1B), respectively. The antitumor effect of combined treatment was screened in KKU-M213A using cell viability test with SRB assay. The cells were treated with drug alone and drug combination for 48 and 72 h. The result of cell viability assay at 48 h was consistent with that at 72 h. In addition, the effect of combined treatment was more pronounced at 72 h compared to 48 h (Supplementary Fig. S1).

The effect of drug combinations was assessed the synergy of cytotoxic effect. Two CCA cell lines, KKU-213A and KKU-100, were treated with the concentrations of infigratinib and PKI-402, which corresponded to approximately IC20 to IC50 values, i.e. infigratinib (0, 1.5, 2.5, and 5 μM) and PKI-402 (0, 0.15, 0.25, and 0.5 μM) using AO-EB fluorescent method. Images of the cell staining at some combinations are shown in Fig. 1C and E for KKU-213A and KKU-100 cells, respectively. Treatment with each agent alone at defined concentrations caused cytotoxicity less than 40 % for PKI-402 and 60 % for infigratinib in both cells. However, when these drugs were used in combinations, it led to an increase in cell cytotoxicity shown by 3D surface plots up to 80%–90 % in many drug combinations and in both cell lines compared to the treatment with infigratinib or PKI-402 alone (Fig. 1D and F).

The remarkably enhanced cytotoxicity of drug combination was apparent using infigratinib and PKI-402 at low to moderate concentrations. Bliss synergy scores were about 20 in most combinations of the two drugs in both cells (Fig. 1G and H). The high synergy scores indicate a high level of synergistic effect between the two drug combinations. Other CCA cells, including KKU-213C, were evaluated for the effect of the drug combination. It was found that the interaction of infigratinib and PKI-402 resulted in only an additive effect, no synergy was found in this cell line (Supplementary Fig. S2). This drug combinations which achieved optimal synergism and cytotoxicity in KKU-213A and KKU-100 cells were investigated the effects on cell cycle progression.

3.2. Combining inhibitors induces cell cycle arrest

To examine the effects of the combination of FGFR and PI3K/mTOR inhibitors on cell cycle progression, we utilized infigratinib at concentrations of 2.5–5.0 μM and PKI-402 at 0.25 μM. Infigratinib did not significantly affect any phase of the cell cycle in KKU-213A cells (Fig. 2A–D). In contrast, PKI-402 alone displayed a trend toward increasing the G2/M phase while the combinations with infigratinib notably increased the accumulation of cells in the G2/M phase, accompanied by a corresponding decrease in the G0/G1 phase (Fig. 2A and B). The effect on KKU-100 cells was similar to KKU-213A cells in that infigratinib had almost no effect on cell cycle progression, whereas PKI-402 alone or in combinations with infigratinib remarkably increased G2/M phase (Fig. 2C and D). These results suggest that the antiproliferative effect of the combined inhibitors is associated with G2/M cell cycle arrest, especially in KKU-213A cells.

Furthermore, to investigate the molecular events underlying cell cycle arrest, we examined the expression of proteins that regulate the G2/M phases in KKU-213A cells, including cyclin A and cyclin B1, through immunoblotting. Infigratinib and PKI-402 both decreased the expression of cyclin A and cyclin B1, with infigratinib having a more pronounced effect at high concentrations (Fig. 2E, F, G). In the presence of the drug combinations, the reduction in cyclin A and B1 expression was significantly greater than that observed with single inhibitor treatments. These findings suggest that the combination treatment enhances the suppression of cell proliferation by inducing G2/M cell cycle arrest, which is associated with reduced cyclin A and cyclin B1 expression.

3.3. Combination of drugs induces apoptotic cell death

To further investigate the ability of the drug combination to induce apoptosis in KKU-213A cells, cells were treated with infigratinib alone (2.5 and 5 μM) or in combination with PKI-402 (0.25 and 0.5 μM) for 48 h. Infigratinib alone induced apoptotic cell death in approximately 10 % and 12 % of cells at low and moderate concentrations, respectively, while PKI-402 alone induced apoptosis in approximately 12 % and 15 % of cells at low and moderate concentrations, respectively. With the combination of low concentrations of PKI-402 and infigratinib, apoptotic cells showed a slight increase to 13–15 % (Fig. 3A and B). Interestingly, when a moderate concentration of PKI-402 was combined with infigratinib, there was a marked increase in apoptotic cells to approximately 40 %, approximately two times higher than the effect of any single agent. These results demonstrate that a moderate concentration of PKI-402 enhances the apoptosis-inducing effect of infigratinib, leading to cell death in CCA cells.

3.4. Infigratinib sensitizes PKI-402 to induce autophagic cell death

To evaluate the apoptotic mechanism, we performed an immunoblot analysis to determine the protein expression of BCL-2 and cytochrome C. It was apparent that individual drugs inducing cell death may be associated with the increased release of cytochrome *c*. However, the combination of infigratinib and PKI-402 did not alter cytochrome *c* levels (Supplementary Fig. S3). We, therefore, further

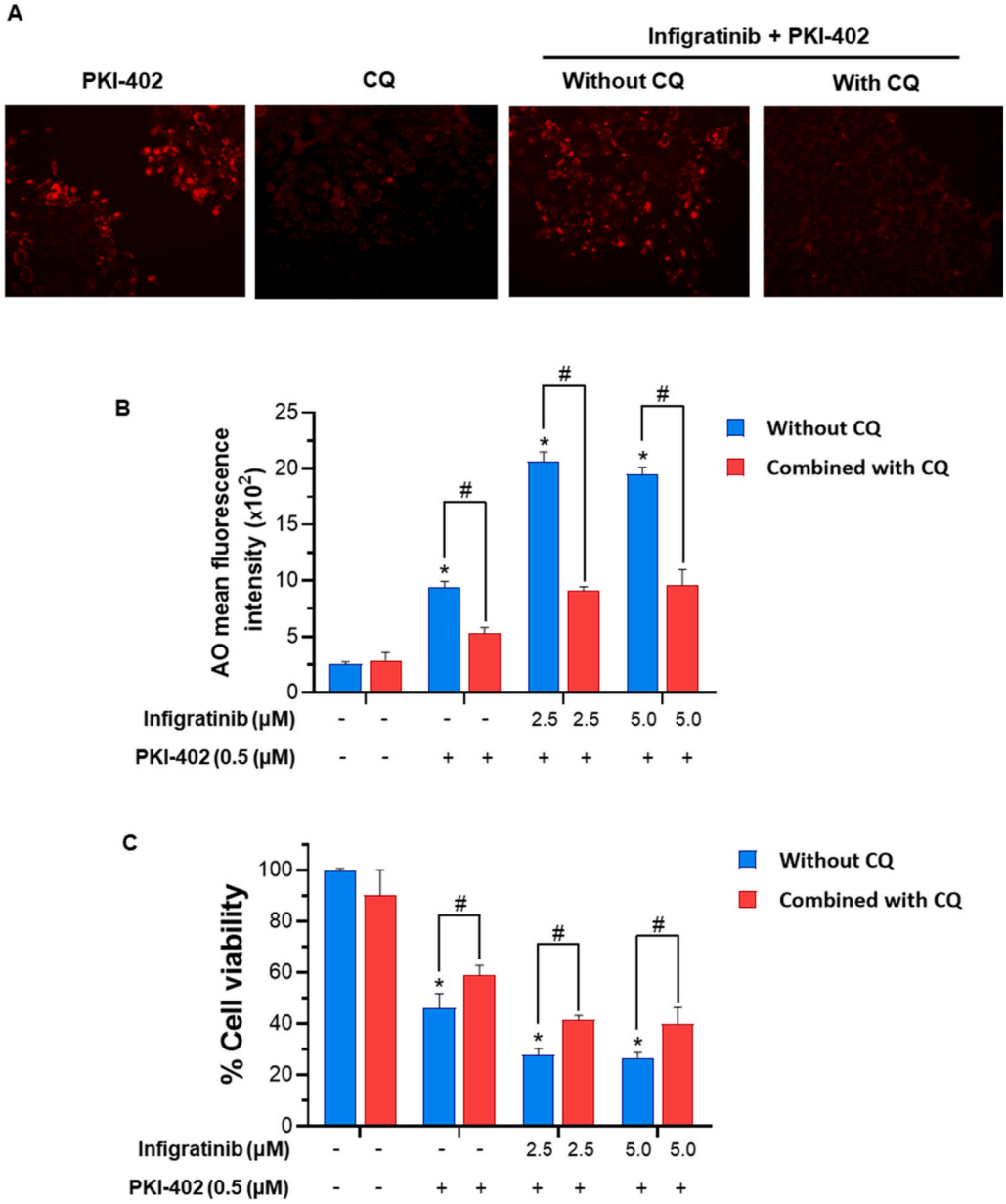

Fig. 5. Chloroquine suppressed autophagy in association with improved cell viability. KKU-213A cells were treated with combinations of infigratinib and PKI-402 with or without chloroquine for 48 h. The cells were stained with acridine orange to observe the stained AVOs in cultured cells (A). The percentage of red fluorescent intensity is demonstrated in a bar graph (B). The cells treated with the drug combination with or without chloroquine were assessed for cell viability (C). Data are expressed as the mean ± SD of three independent *$p < 0.05$ versus control group (DMSO) $^{\#}p < 0.05$ versus the treatment without chloroquine (CQ).

explored another mechanism of enhanced cell death through the autophagic process. We examined whether the synergy between infigratinib and PKI-402 in inducing cell death was indeed associated with the process of autophagic cell death. KKU-213A cells were treated with the individual drugs and drug combinations, followed by the detection of bright red fluorescence representing acidic autophagic vacuoles (AVOs) in autophagosomes. Treatment with infigratinib alone produced very low signals, while PKI-402 alone significantly increased the fluorescence signals. The combination of PKI-402 and infigratinib further increased AVOs compared to PKI-402 treatment alone (Fig. 4A and B), indicating enhanced autophagosome formation due to infigratinib.

To gain insight into the mechanism of this drug combination, we analyzed proteins involved in the regulation of autophagy. Infigratinib alone at moderate concentrations slightly suppressed p-mTOR and slightly increased LC3A/B-II, whereas the combination

with PKI-402 strongly suppressed p-mTOR and markedly increased LC3A/B-II (Fig. 4 C, D, E). These effects suggest an increase in autophagic flux induced by the drug combination.

Since autophagy can act as a cellular protective mechanism and on the other hand, autophagy can induce autophagic cell death (ACD), we assessed whether the synergy between infigratinib and PKI-402 in causing cell death involved an enhanced ACD due to alterations in autophagic flux. Chloroquine was employed as an autophagy inhibitor to assess the role of autophagy formation whether for protection or induction of cell death. It was apparent that CQ inhibited autophagy by suppression of the formation of AVOs (Fig. 5A and B) and it was in association with the protection of cell death (Fig. 5C). This suggests the drug combination enhanced cell death is causally associated with autophagic formation.

4. Discussion

FGFR inhibitors have emerged as a new class of drugs targeting the fusion FGFR2 gene, specifically in intrahepatic CCA. Despite the favorable initial clinical response and disease control, most patients eventually develop acquired resistance [3]. Recently, downstream signaling of FGFR, particularly the PI3K/AKT/mTOR signaling pathway, has been identified as a feedback activation mechanism in response to FGFR inhibition, leading to resistance to FGFR inhibitors in CCA patients [2,4]. In this study, we demonstrated a novel treatment approach using a multitarget strategy with PKI-402 and infigratinib to inhibit PI3K-mTOR and FGFR, respectively in CCA cells. The combination of both agents showed synergy in suppressing cell proliferation, inducing apoptotic cell death, and triggering autophagic cell death in CCA cells.

A combination of FGFR and mTOR blockade using infigratinib and rapamycin was previously showed a synergistic effect on orthotopic Huh-7 hepatoma cell growth in mice [25]. In present study infigratinib and PI3K/mTOR inhibitor conferred synergistic effect on CCA cells. Cytotoxic effect of the drug combination was also increased to the maximum killing, whereas concentrations of both drugs were used at low to moderate concentrations. This drug combination might be translated to produce high anti-tumor effect with low adverse events in the body. Furthermore, it is interesting whether this drug combination exerted synergy effect in CCA cells as a class of tumor. In our preliminary study with another CCA cell line; KKU-213C, the drug combination produced only an additive effect on tumor cell inhibition. Further study on differences in molecular profiling of two groups of CCA cells may merit to shed some light for discerning the highly sensitive cells for optimal treatment.

The PI3K/mTOR signaling pathway is well established for its role in regulating cell survival and antiapoptotic pathways [12]. Our findings indicate that the combined inhibitors, infigratinib and PKI-402, have a potent effect on inhibiting CCA cell growth, as demonstrated by the AO/EB assay. Additionally, this combined treatment effectively induced cell cycle arrest at the G2-M phase. Consistent with our results, a previous study involving combined treatment with infigratinib and rapamycin (an mTOR inhibitor) exhibited a strong inhibitory effect on growth and induced remarkable cell cycle arrest in ovarian cancer cells (OCCs) with high expression of FGFR2 variants. Furthermore, the reduction in tumor size in xenografted OCCs animals was more pronounced following the combined treatment compared to individual drugs [26]. It should be noted that in the apoptosis assay, the combination treatment demonstrated a synergistic effect on apoptotic cell death, particularly only moderate concentrations of both infigratinib and PKI-402 were employed. We examined the mechanism of synergy over the effects of cell cycle progression and autophagy.

The PI3K-Akt-mTOR pathway is one of the FGFR signaling pathways and plays a crucial role in regulating cell proliferation, autophagy, and apoptosis [3]. Inhibition of this pathway leads to suppression of cell growth and induces autophagy. However, our data suggest that infigratinib's effects on cell cycle progression are negligible, implying that infigratinib probably does not mediate its effects through the PI3K-mTOR pathway. Therefore, the enhanced effects of PKI-402 on cell cycle arrest by infigratinib likely occur through the inhibition of alternative pathways such as Ras-MEK1/2-ERK1/2 and JAK/STAT. It is speculated that if resistance to FGFR inhibitors is caused by dysregulation of downstream pathways to PI3K-Akt-mTOR, the use of PI3K-mTOR inhibitors, for instance, PKI-402, in combination with FGFR inhibitors could potentially overcome this resistance.

Autophagy plays a role in regulating both cancer cell survival and inducing autophagic cell death [15,20]. Our results show that the combined drug treatment enhances autophagy and induces autophagic cell death. However, the effect was not apparent in the infigratinib-only groups, possibly because the drug does not inhibit the PI3K-mTOR pathway, which plays central roles in autophagy [16]. The autophagy-sensitizing effect of infigratinib on PKI-402 may be mediated through other pathways, including the epithelial mesenchymal transition (EMT). Our previous study on the FGFR2 signaling in KKU-213 & KKU-100 cells showed inhibition of vimentin in EMT process is prevail [9]. EMT is an important contributor to cancer growth and has the crosstalk between EMT and autophagy. Effects of EMT, which mediate several signaling pathways, including integrins, TGF-b and NF-kB, play crucial role in autophagy [27]. This aligns with the observed synergism of both agents on cell cycle arrest and autophagic cell death, as mentioned earlier.

To confirm the observed increase in autophagosome formation whether to promote an adaptive survival, or to initiate autophagic cell death [28]. Our experiments explore this event further. The use of an autophagy inhibitor, chloroquine, partially prevented cell death, and the decrease in autophagosomes was associated with improved cell survival in the combination drug treatment and PKI-402 alone; this suggests that increased autophagy is a mechanism by which the combination of drugs enhanced cell death.

In conclusion, this study demonstrates that combined inhibition of the FGFR-PI3K/mTOR pathway enhances antiproliferation and interrupts cell growth through G2-M arrest in CCA cells. Moreover, the drug combination induces autophagic cell death, indicating a potential therapeutic strategy for CCA. Our findings warrant further elucidation of the characteristics of CCA cells in association with cell vulnerability to certain drug combinations.

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Data availability statement

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

CRediT authorship contribution statement

Narumon Mahaamnad: Methodology. **Piman Pocasap:** Resources. **Veerapol Kukongviriyapan:** Writing – original draft, Formal analysis. **Laddawan Senggunprai:** Resources, Conceptualization. **Auemduan Prawan:** Resources, Conceptualization. **Sarinya Kongpetch:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:Sarinya Kongpetch reports financial support was provided by NSRF under the Basic Research Fund of Khon Kaen University. Narumon Mahaamnad reports financial support was provided by the Faculty of Medicine, Khon Kaen University, Thailand. Narumon Mahaamnad reports financial support was provided by a Postgraduate Study Support Grant of Faculty of Medicine, Khon Kaen University.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e31112.

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