



## Basic Study

# Erlotinib combination with a mitochondria-targeted ubiquinone effectively suppresses pancreatic cancer cell survival

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## Abstract

### BACKGROUND

Pancreatic cancer is a leading cause of cancer-related deaths. Increased activity of the epidermal growth factor receptor (EGFR) is often observed in pancreatic cancer, and the small molecule EGFR inhibitor erlotinib has been approved for pancreatic cancer therapy by the food and drug administration. Nevertheless, erlotinib alone is ineffective and should be combined with other drugs to improve therapeutic outcomes. We previously showed that certain receptor tyrosine kinase inhibitors can increase mitochondrial membrane potential ( $\Delta\psi_m$ ), facilitate tumor cell uptake of  $\Delta\psi_m$ -sensitive agents, disrupt mitochondrial homeostasis, and subsequently trigger tumor cell death. Erlotinib has not been tested for this effect.

### AIM

To determine whether erlotinib can elevate  $\Delta\psi_m$  and increase tumor cell uptake of  $\Delta\psi_m$ -sensitive agents, subsequently triggering tumor cell death.

### METHODS

$\Delta\psi_m$ -sensitive fluorescent dye was used to determine how erlotinib affects  $\Delta\psi_m$  in pancreatic adenocarcinoma (PDAC) cell lines. The viability of conventional and patient-derived primary PDAC cell lines in 2D- and 3D cultures was measured after treating cells sequentially with erlotinib and mitochondria-targeted ubiquinone (MitoQ), a  $\Delta\psi_m$ -sensitive MitoQ. The synergy between erlotinib and MitoQ was then analyzed using SynergyFinder 2.0. The preclinical efficacy of the two-drug combination was determined using immune-compromised nude mice bearing PDAC cell line xenografts.

### RESULTS

Erlotinib elevated  $\Delta\psi_m$  in PDAC cells, facilitating tumor cell uptake and mito-

ondrial enrichment of  $\Delta\psi_m$ -sensitive agents. MitoQ triggered caspase-dependent apoptosis in PDAC cells in culture if used at high doses, while erlotinib pretreatment potentiated low doses of MitoQ. SynergyFinder suggested that these drugs synergistically induced tumor cell lethality. Consistent with *in vitro* data, erlotinib and MitoQ combination suppressed human PDAC cell line xenografts in mice more effectively than single treatments of each agent.

## CONCLUSION

Our findings suggest that a combination of erlotinib and MitoQ has the potential to suppress pancreatic tumor cell viability effectively.

**Key Words:** Pancreatic cancer; Erlotinib; Mitochondria-targeted ubiquinone; Mitochondria; Combination therapy

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**Core Tip:** In this study, we demonstrated that epidermal growth factor receptor inhibitor erlotinib increases mitochondrial membrane potential in pancreatic cancer cells, priming the tumor cells to mitochondria-targeted ubiquinone (MitoQ) sensitivity. Our data show that the combination of erlotinib and MitoQ can effectively and synergistically suppress pancreatic cancer cells *in vitro* and *in vivo*.

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## INTRODUCTION

Pancreatic cancer is a highly aggressive and poorly prognostic disease with an overall five-year survival rate of 12.5% [1, 2]. Many molecular alternations have been identified and evaluated for their potential as a therapeutic target in pancreatic cancer [3]. Nevertheless, there is still an urgent need to develop an effective treatment for pancreatic cancer.

Epidermal growth factor receptor (EGFR) is a receptor tyrosine kinase (RTK) abnormally activated in many epithelial tumors, including non-small cell lung cancer (NSCLC) [4], glioblastoma [5], colon cancer [6], breast cancer [7], and pancreatic cancer [8]. Aberrantly activated EGFR promotes tumor cell proliferation and survival by activating critical signaling pathways such as the Ras/extracellular signal-regulated kinase, phosphatidylinositol-3 kinase/protein kinase B, and mammalian target of rapamycin pathways [9]. EGFR overexpression is a critical process for facilitating pancreatic tumorigenesis [10]. As such, the EGFR inhibitor erlotinib (Tarceva®) has been approved for treating pancreatic cancer. However, unlike its successful use for NSCLC as monotherapy [11], erlotinib is not as effective as monotherapy but is combined with gemcitabine to treat advanced pancreatic cancers [12-14]. Clinical trials have shown that erlotinib, in combination with capecitabine and chemoradiation, can also improve the survival of patients with resected pancreatic cancer [15, 16]. Therefore, it is important to identify a combination therapy strategy to use erlotinib for effective pancreatic cancer therapy.

Certain RTK inhibitors (TKIs) affect mitochondrial activity, and this characteristic can be exploited to design a novel therapeutic strategy. For example, we previously showed that vandetanib and cabozantinib increase mitochondrial membrane potential ( $\Delta\psi_m$ ) in the RTK REarrangement during Transfection-mutated thyroid tumor cells and subsequently facilitate tumor cell uptake and retention of  $\Delta\psi_m$ -sensitive mitochondria-targeted agents, including triphenyl-phosphonium (TPP)-mitochondria-targeted carboxy-proxyl (MitoCP) and mitochondria-targeted ubiquinone (MitoQ) [17]. This resulted in the accumulation of MitoCP and MitoQ in the tumor cell mitochondria, disrupting mitochondrial homeostasis and ultimately causing tumor cell death [17, 18]. Of note, MitoQ is currently used as a dietary supplement due to its beneficial effects on mitochondrial bioenergetics [19, 20] and vascular function [21-23], although this compound has been found to suppress tumor cells derived from the thyroid [17], skin [24], breast [25-27], and pancreas [28]. Therefore, selectively facilitating tumor cell enrichment of MitoQ using a TKI could be an effective strategy for tumor suppression. This strategy has not been tested for erlotinib.

Erlotinib is a TKI that targets multiple RTKs, including EGFR [29]. In this study, we tested a hypothesis that erlotinib increases  $\Delta\psi_m$  in pancreatic tumor cells, hence priming the tumor cells to MitoQ sensitivity. Our data show that the combination of erlotinib and MitoQ can effectively and synergistically suppress pancreatic cancer cells *in vitro* and *in vivo*.

## MATERIALS AND METHODS

### Cell culture and reagents

The human pancreatic cancer cell line MiaPaCa-2 (ATCC) and pancreatic cancer cells-1 (PANC-1, ATCC) were maintained in Dulbecco's minimal essential medium (DMEM, Gibco, Thermo Fisher Scientific, No. 11965) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Gibco, No. 16000-044) and 100 U/mL of penicillin-streptomycin (Gibco, Thermo Fisher Scientific, No. 15140) per mL. Patient-derived pancreatic cell lines MCW462 and MCW670 were previously described[30,31]. They were maintained in DMEM (Invitrogen, Thermo Fisher Scientific, No. 11330032) supplemented with 6% FBS, 100 U of penicillin-streptomycin, 50  $\mu$ L of 100  $\mu$ g/mL of epidermal growth factor (Thermo Fisher Scientific, No. PHG0311), 2 mL of bovine pituitary extract (Gibco, No. 13028014), 2  $\mu$ g/mL of hydrocortisone (MilliporeSigma, St. Louis, MO, No. H0888) and 70  $\mu$ L of insulin (Gibco, No. 12585014). For organoid culture, 10,000 cells were plated onto 24 well plates precoated with 200  $\mu$ L Matrigel (Corning, Tewksbury, MA, No. 356231) and maintained with the DMEM medium mixed with Matrigel at 10% of final volume (0.8-1.1 mg/mL), as instructed by the manufacturer. Hypoxic cell culture was carried out in a humidified incubator with 1% O<sub>2</sub>, 5% CO<sub>2</sub> and 94% N<sub>2</sub>. All experiments were performed using cells within ten passages from the acquisition point. Erlotinib was purchased from LC Laboratories (Woburn, MA, No. E-4997). Carbobenzoxymethyl-L-alanyl-L-aspartyl-L-[O-methyl]-fluoromethylketone (ZVAD) and gemcitabine were purchased from Selleckchem (Houston, TX, No. S7023 and No. S1714). MitoCP was obtained from Dr. Balaraman Kalyanaraman (Biophysics, Medical College of Wisconsin). MitoQ [(10-(4,5-dimethoxy-2-methyl-3,6-dioxo-1,4-cyclohexadien-1-yl) decyl) triphenyl phosphonium] was obtained from MitoQ Ltd. (Auckland, New Zealand). Tetramethyl-rhodamine methyl ester (TMRM) was purchased from Invitrogen (No. T668). 4%-20% Mini-PROTEAN<sup>®</sup> TGX Stain-Free<sup>™</sup> Protein Gels, 10 well, 30  $\mu$ L were purchased from Bio-Rad (Hercules, CA, No. 4568093).

### Cell viability and cell cycle analyses

The half maximal inhibitory concentration (IC<sub>50</sub>) values and the combination drug effects were determined by crystal violet staining (Fisher Chemical, Thermo Fisher Scientific, No. C58125). Briefly, cells in 96 well-plates were fixed in formaldehyde (Fisher Chemical, No. BP531), stained with 0.05% crystal violet for 30 min, washed with water three times, air-dried, and incubated in 200  $\mu$ L methanol (VWR Chemicals BDH<sup>®</sup>, Radnor, PA, No. BDH1135) for 10 min at room temperature before measuring absorbance at 540 nm. The IC<sub>50</sub> values in organoid cultures were determined using SYTOX<sup>™</sup> Green (Invitrogen, No. S7020) following the manufacturer's instruction. To determine apoptosis, cells were co-stained with annexin V (Invitrogen, No. A35122) and propidium iodide (Invitrogen, No. P1304MP) according to the manufacturer's instruction. Flow cytometry was performed using BD<sup>®</sup> LSR II Flow Cytometer (Franklin Lakes, NJ). FCS data were analyzed by FCS EXPRESS software (De Novo Software, Los Angeles, California).

### Detection of mitochondrial membrane potential using TMRM

Cells were incubated with culture medium with 2 nM TMRM (Invitrogen, No. T668) in 24-well plates for 30 min in a humidified incubator at 37 °C, collected by trypsinization, resuspended in phosphate-buffered saline containing 0.5% bovine serum albumin, and analyzed using Guava EasyCyte flow cytometry system. Data were analyzed by FCS EXPRESS software as described previously[27].

### Immunoblotting

Mitochondrial fractions were extracted using the Mitochondria Isolation Kit for Cultured Cells (Thermo Fisher Scientific, No. 89874). Total cell lysates were prepared by harvesting cells in a lysis buffer containing 62.5 mmol/L Tris-HCl (pH 6.8), 2% sodium dodecyl sulfate (SDS), and the protease and phosphatase inhibitor cocktails 2 and 3 (MilliporeSigma, No. P8340, P5726, and P0044, respectively). Protein concentration was determined using the Pierce<sup>™</sup> BCA Protein Assay Kit (Pierce, Thermo Fisher Scientific, No. 23227). Protein samples were resolved on the SDS-polyacrylamide gel electrophoresis and transferred to the polyvinylidene difluoride membrane filter (Bio-Rad, No. 1620177). After transfer, membranes were blocked in a buffer containing 0.1 M Tris (pH 7.4), 0.9% NaCl, 0.1% Tween 20, and 5% nonfat dry milk for 1 h at 25 °C. Membranes were then incubated with an appropriate antibody overnight at 4 °C with agitation at the following dilutions: Poly (ADP-ribose) polymerase (PARP) (cell signaling, No. 9542) 1: 2000; cleaved lamin A (cell signaling, No. 2035) 1: 1000; Caspase 9 (cell signaling, No. 9502T) 1: 2000; Caspase 3 (cell signaling, No. 14220); E2F1 (Thermo Fisher Scientific, No. MS-879) 1: 2000; pRb (cell signaling, No. 9307); cytochrome c oxidase subunit IV (COX IV, cell signaling, No. 4850) 1: 2000; p21<sup>CIP1</sup> (Santa Cruz Biotechnology, No. sc-56335) 1:1000, p27<sup>KIP1</sup> (Santa Cruz Biotechnology, No. sc-1641) 1:1000; hypoxia-inducible factor 1 subunit alpha (HIF1 $\alpha$ , Cell Signaling, No. 14179) 1:1000 and  $\beta$ -actin (MilliporeSigma, No. A2228) 1:5000. Chemiluminescence signals of immunoblots were visualized by SuperSignal West Pico (No. 34079) and Femto (No. 23227) chemiluminescence kits (Pierce), captured by ChemiDoc XRS+ (Bio-Rad), and analyzed by Image Lab software (Bio-Rad) for densitometry.

### Tumor xenografts

A total of 5  $\times$  10<sup>6</sup> PANC-1 cells suspended in 100  $\mu$ L of Hank's balanced salt solution (Gibco, No. 14025) mixed with Extracellular Matrix Gel (MilliporeSigma, No. E6909) at 1:1 ratio was inoculated subcutaneously into the rear flanks of 6-week-old female athymic nude (*nu/nu*) mice (The Jackson Laboratory, Bar Harbour, ME, No. 007850). Once palpable, tumors were measured using Vernier calipers twice a week. Tumor volumes were calculated using the formula: Length  $\times$  width  $\times$  height  $\times$  0.5236. When tumor volumes reached 50 mm<sup>3</sup>, mice were sorted into 4 groups of 7 animals to achieve equal tumor size distribution in all treatment groups. Mice were treated with vehicle, MitoQ, erlotinib, and the combination of two compounds (combo), respectively. Drugs dissolved in 100  $\mu$ L vehicle (1:12 mixture of Dimethyl

sulfoxide /15%  $\beta$ -cyclodextrin) were orally administered by gavage daily for 4 d, followed by one day break. Four cycles of this treatment were conducted. The control group received only the vehicle, the MitoQ group received 20 mg drug/kg body weight/dose, the erlotinib group received 25 mg drug/kg body weight/dose, and the combination group received two doses of erlotinib followed by two doses of MitoQ in a cycle. Ethical endpoints were when tumor size reached 2000 mm<sup>3</sup>. At the end of the experiments, animals were euthanized by CO<sub>2</sub> asphyxiation, and tumor tissues were harvested. All animal studies were performed according to protocols approved by the Institutional Animal Care and Use Committee at the Medical College of Wisconsin, No. AUA00001327.

### Statistical analysis

Unless otherwise specified, all graphs represent the mean  $\pm$  the standard error of mean from biological replicates ( $N \geq 3$ ). Statistical significance was determined by one-way or two-way analysis of variance with Bonferroni post-tests and two-tailed unpaired Student's *t*-test using PRISM (Graph-Pad Software, La Jolla, CA). IC<sub>50</sub> was determined by PRISM. *P* values of  $< 0.05$  were considered statistically significant.

## RESULTS

### MitoQ can suppress the viability of pancreatic ductal adenocarcinoma cells

We determined how MitoQ affects pancreatic adenocarcinoma (PDAC) cell viability in a cell line panel that included the conventional PDAC cell lines PANC-1 and MiaPaCa-2 and the patient-derived PDAC cell lines MCW462 and MCW670. As determined by the crystal violet viability assay, 48 h MitoQ treatment significantly decreased cell viability in the two-dimensional cell culture with the IC<sub>50</sub> values determined below 1  $\mu$ M in all cell lines (Figure 1A and B). In contrast, the functional moiety of MitoQ CoQ10 (ubiquinone) did not suppress cell viability, while the vehicle moiety TPP decreased cell viability mildly only at higher doses (Figure 1A). The organoid culture model is more physiologically relevant[32], and we also examined MitoQ responsiveness of MCW462 cells in organoid cultures. As determined by Sytox Green viability assay, MitoQ, but not CoQ10 and TPP, consistently decreased the viability of MCW462 cells in the organoid culture (Figure 1C). However, the IC<sub>50</sub> value was higher in the three-dimensional culture. These data demonstrate that MitoQ can suppress PDAC cell viability, for which the mitochondrial targeting of its functional moiety might be critical.

### MitoQ can induce caspase-dependent apoptosis in pancreatic ductal adenocarcinoma cells

To determine the molecular mechanism by which MitoQ suppresses PDAC cell viability, we performed the annexin V/propidium iodide co-staining assay. We found that, if used at a high dose, MitoQ increased annexin V positive population in MiaPaCa-2 and MCW670 cultures (Figure 2A). However, the control CoQ10 and TPP did not show similar effects. This effect of MitoQ was significantly blocked by the pan-caspase inhibitor ZVAD (Figure 2A). Moreover, Western blot analysis of total lysates of these cells revealed that MitoQ, but not TPP or CoQ10, notably increased the cleavages of PARP and lamin A in a dose-dependent manner (Figure 2B). The cleavage of these proteins is a bonafide marker of caspase-dependent apoptosis[33]. Indeed, 5  $\mu$ M MitoQ increased the cleavage of caspases 9 and 3 in MiaPaCa-2 cells while depleting these enzymes in MCW670 cells (Figure 2B). We also found that MitoQ decreased E2F1, the S-phase transcription factor[34], and the phosphorylation of its regulator Rb while increasing the cyclin-dependent kinase inhibitor p27<sup>KIP1</sup> without notably affecting p21<sup>CIP1</sup> levels in these cells (Figure 2B). These data suggest that MitoQ can suppress PDAC cell viability by inducing caspase-dependent apoptosis and partly by suppressing a few critical regulators for cell cycle progression.

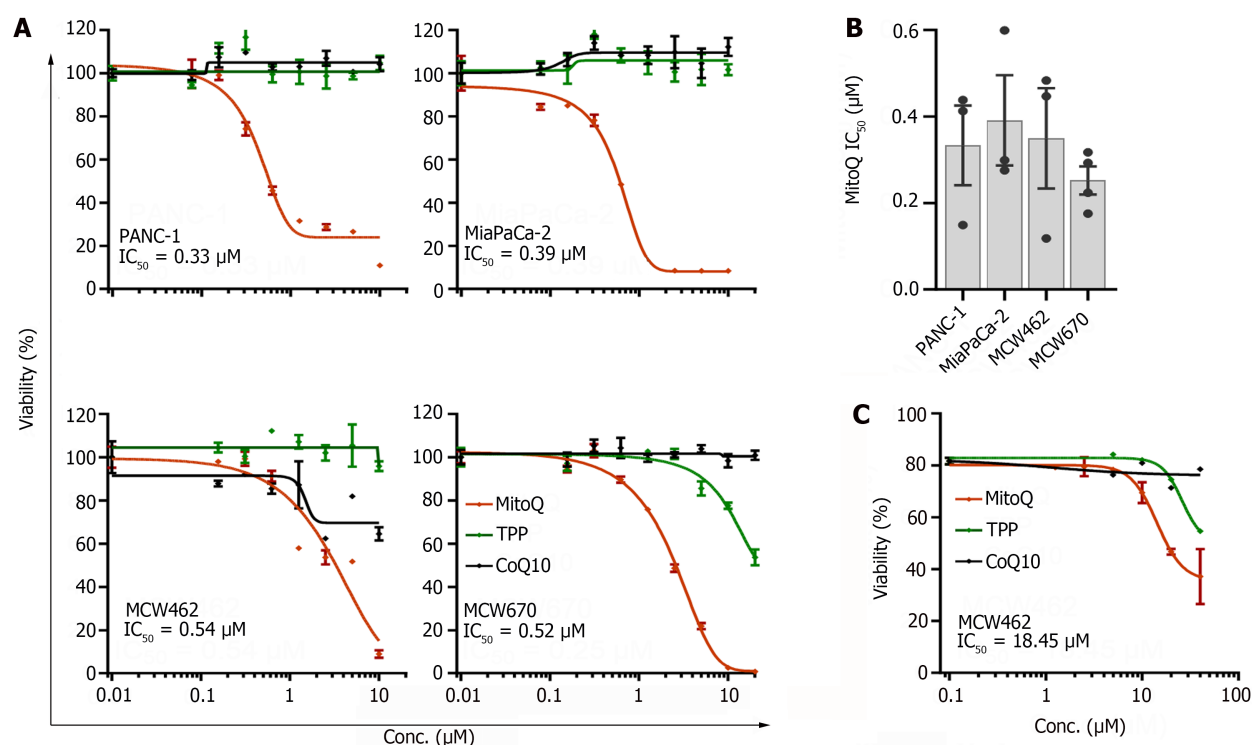
### Erlotinib can increase mitochondrial membrane potential and facilitate mitochondrial enrichment of mitochondrial membrane potential-sensitive agents in pancreatic ductal adenocarcinoma cells

We determined whether erlotinib can increase  $\Delta\psi_m$  in PDAC cells. In the cultures of multiple PDAC cell lines, erlotinib treatment significantly increased cells stained with TMRM, a  $\Delta\psi_m$ -sensitive fluorescent dye, in a dose-dependent manner within 48 h (Figure 3A and B). Given this data, we determined whether erlotinib can increase the mitochondrial enrichment of a  $\Delta\psi_m$ -sensitive agent using the TPP-conjugated superoxide dismutase mimetic MitoCP as a tool compound. The CP moiety of MitoCP is a 5-membered nitroxide free radical that can form a covalent conjugate with thiol proteins, which can be detected by a TPP-specific antibody[17,35]. When the mitochondrial extracts from MitoCP-treated PANC-1 and MCW462 cells were analyzed for the effects of 24 h erlotinib pretreatment, western blotting revealed much higher levels of TPP-protein adducts in erlotinib-pretreated cells (Figure 3C). These data suggest that erlotinib can increase  $\Delta\psi_m$  and, subsequently, mitochondrial enrichment of a  $\Delta\psi_m$ -sensitive agent in PDAC cells.

### Erlotinib and MitoQ can synergistically suppress the viability of pancreatic ductal adenocarcinoma cells in culture

Given the data above, we asked whether erlotinib can synergize with MitoQ to suppress PDAC cell viability. As determined in PANC-1, MCW462, and MCW670 cells, a robust viability loss was induced when these cells were sequentially treated with erlotinib for the first 24 h and MitoQ for the subsequent 48 h (Figure 4A). Of note, our analysis using SynergyFinder 2.0[36] suggested that these two agents synergistically induced the viability loss (Figure 4B). Importantly, tumor cells in a physiological microenvironment are generally under a hypoxic condition[37]. We, therefore, determined whether the erlotinib-MitoQ combination can produce a similar synergistic effect under a hypoxic culture condition. To this end, we used PANC-1 cells maintained in the human plasma-like medium with 1% O<sub>2</sub>, under which condition HIF1 $\alpha$  expression substantially increased (Figure 4C). Under this hypoxic culture condition, the combination of erlotinib and





**Figure 1** Mitochondria-targeted ubiquinone can suppress the viability of pancreatic adenocarcinoma cells. **A:** pancreatic adenocarcinoma (PDAC) cells in 12 well plates were treated with increasing doses of mitochondria-targeted ubiquinone (MitoQ), CoQ10, and triphenyl-phosphonium (TPP) for 48 h prior to determining cell viability by crystal violet staining. CoQ10 is the functional moiety of MitoQ and TPP is the vehicle moiety. 462 and 670 denote MCW462 and MCW670 cell lines, respectively; **B** Summary of the half maximal inhibitory concentration values determined for different PDAC cells treated and analyzed as described; **C:** MCW462 cells in organoid cultures were treated with increasing doses of MitoQ, CoQ10, and TPP for 48 h prior to determining cell viability by Sytox Green assays. Data (mean  $\pm$  SEM,  $n \geq 3$ ) are expressed as the percentage of untreated controls. TPP: Triphenyl-phosphonium; MitoQ: Mitochondria-targeted ubiquinone;  $\text{IC}_{50}$ : Half maximal inhibitory concentration.

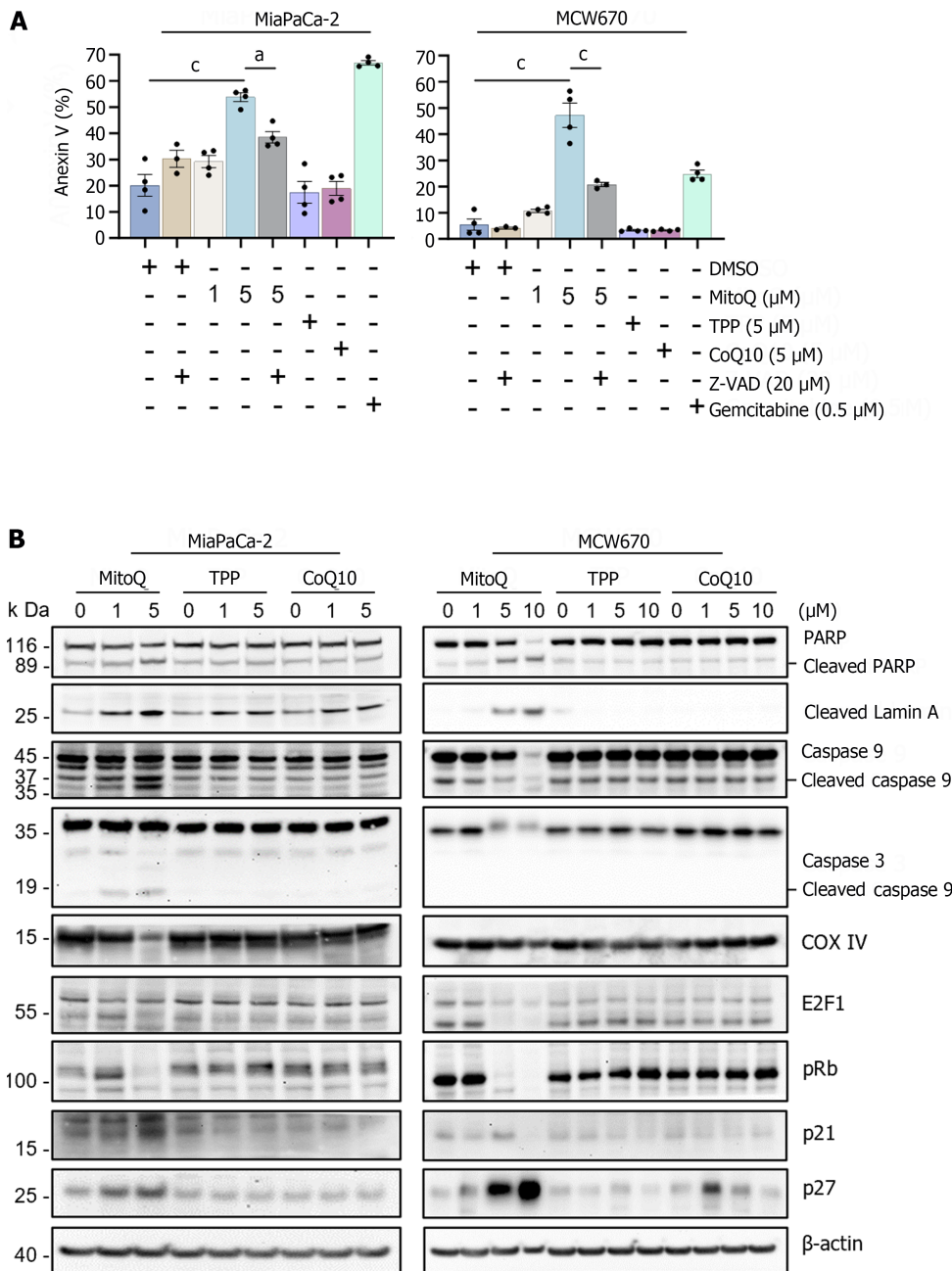
MitoQ consistently suppressed the viability of PANC-1 cells (Figure 4D), through a synergistic effect as suggested by SynergyFinder 2.0 (Figure 4E). These data strongly suggest that erlotinib can synergize with MitoQ to suppress PDAC cells.

### A combination of erlotinib and MitoQ effectively suppresses the growth of pancreatic cancer cells xenografts in mice

PANC-1 cells express relatively higher EGFR[38]. Given this information and the relatively high synergy score in this cell line (Figure 4), we used immune-compromised nude mice bearing PANC-1 xenografts to determine the preclinical efficacy of the erlotinib and MitoQ combination. These mice were subjected to 4 cycles of treatments in which erlotinib and MitoQ were orally administered singly or in combination (depicted in Figure 5A). For the drug combination, a cycle consisted of 2 d erlotinib treatment followed by 2 d MitoQ treatment and one drug holiday (Figure 5A), which is similar as the schedule that we previously used for preclinical evaluation of the vandetanib and MitoCP combination for thyroid cancer[17]. To compare mono- and combination therapies, we used erlotinib at 25 mg/kg/dose, a lower dose than the highly potent doses (50 mg/kg once daily) in the other preclinical models[39,40]. Likewise, we used MitoQ at 20 mg/kg/dose, which did not significantly suppress tumor growth in a preclinical breast cancer model, albeit decreasing tumor metastasis[26]. Consistent with the *in vitro* data above, the sequential administration of erlotinib and MitoQ suppressed the growth of PANC-1 xenografts more effectively than the monotherapy using each agent, as determined by monitoring tumor volume changes (Figure 5B) and by measuring the sizes and weight of tumors harvested at the end of the treatment (Figure 5C and D). In contrast, these treatments did not cause a significant difference in animal body weights (Figure 5E). These data suggest that erlotinib can synergize with MitoQ *in vivo* to suppress the growth of PDAC cells.

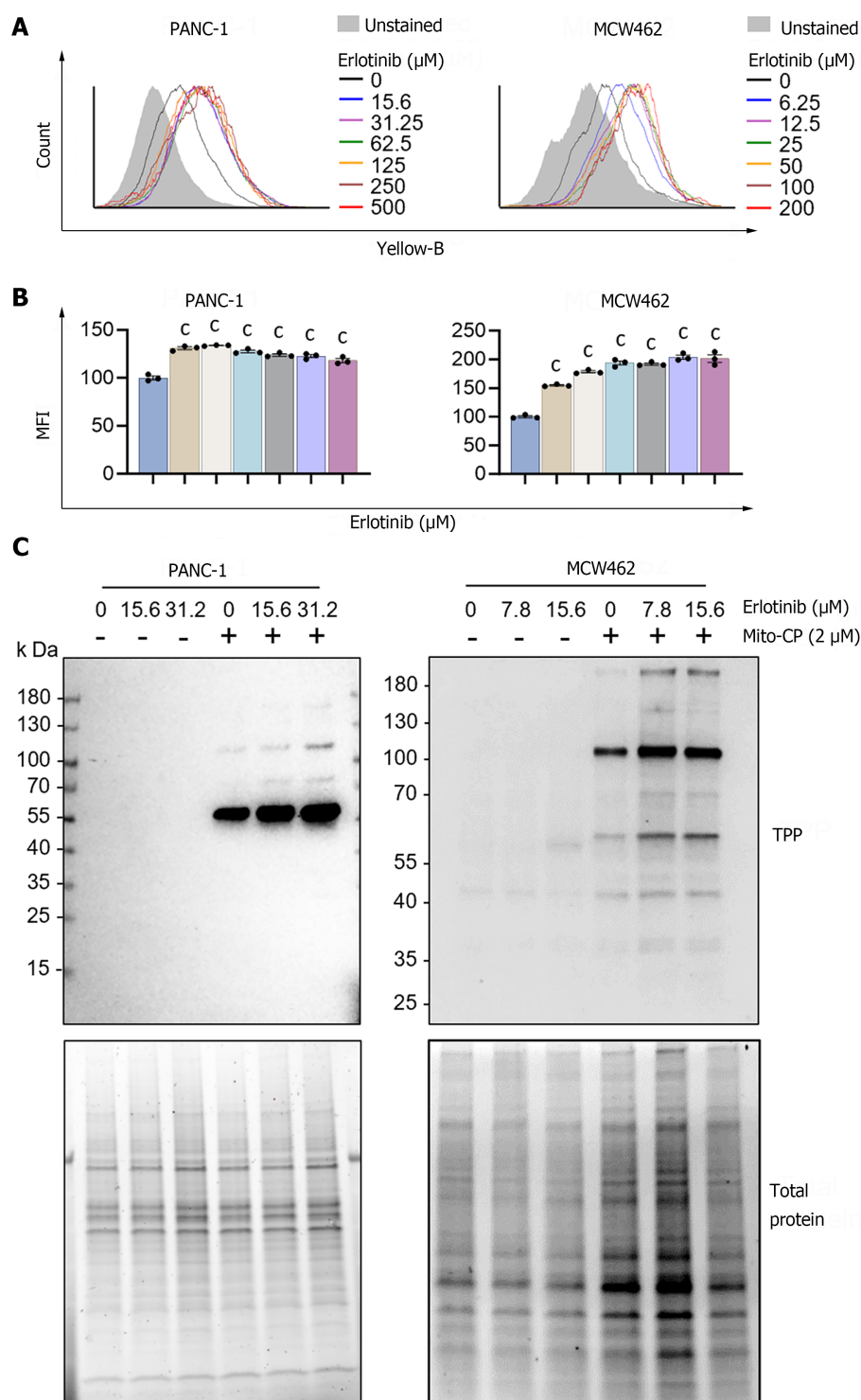
## DISCUSSION

Our data show that MitoQ can suppress PDAC cell survival if used at an effective dose. Similarly, tumor cells derived from thyroid, skin, and breast cancers have shown sensitivity to MitoQ[24,25,27]. Many tumor cells exhibit elevated steady-state  $\Delta\psi_m$ , which is required for adaptation to hypoxia, escape from anoikis, and enhanced invasiveness[41,42]. For this reason, MitoQ would accumulate higher in the mitochondria of tumor cells than those of most normal cell types[23], which might increase the chance for MitoQ to exert any adverse effect on tumor cells. Our data suggest that the property of erlotinib to increase  $\Delta\psi_m$  in PDAC cells can be exploited to drive the mitochondrial enrichment of MitoQ in PDAC cells.



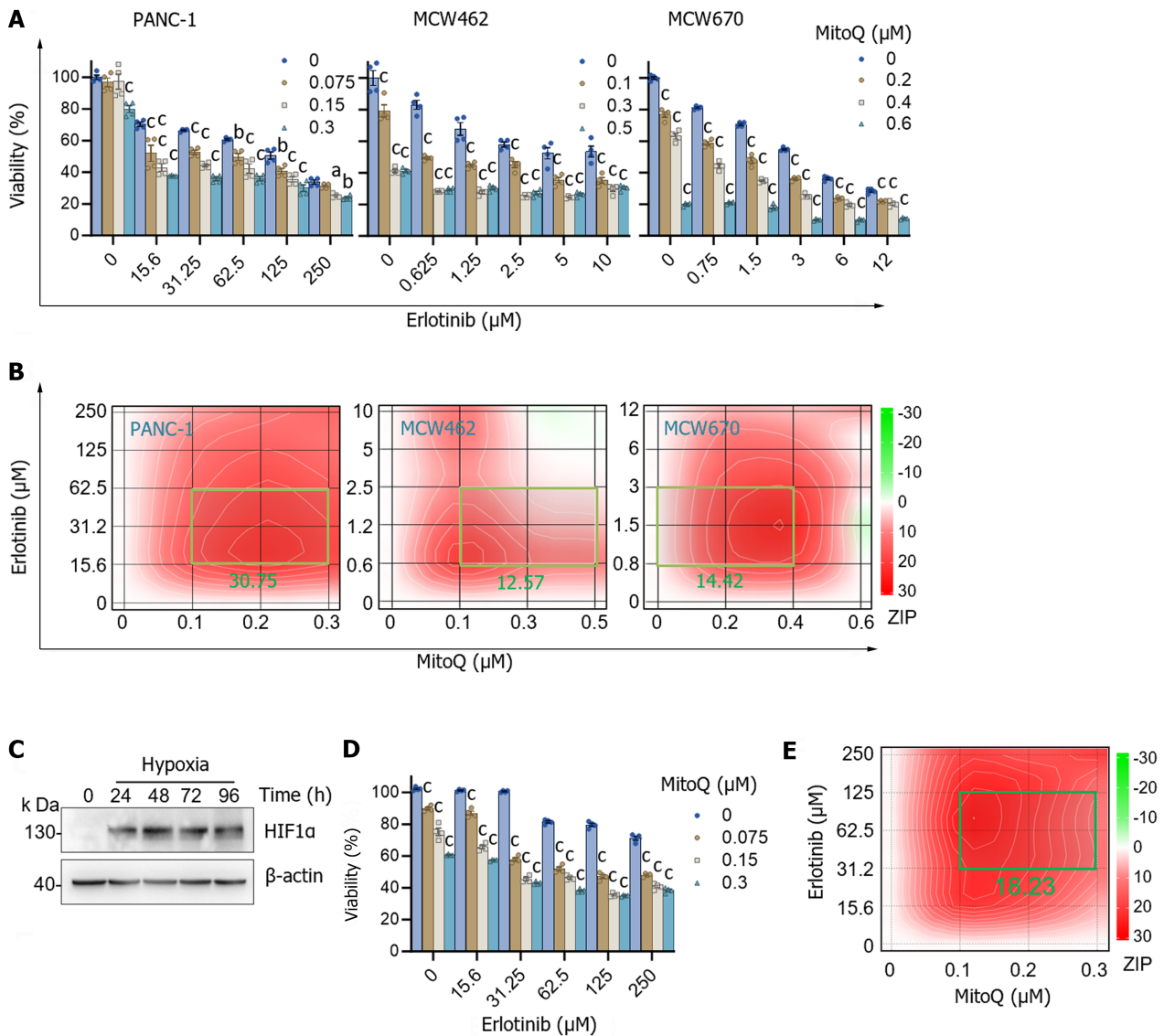
**Figure 2 Mitochondria-targeted ubiquinone can induce caspase-dependent apoptosis in pancreatic adenocarcinoma cells.** A: Apoptosis analysis of cells treated with mitochondria-targeted ubiquinone (MitoQ), with or without Carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]-fluoromethylketone, for 24 h. CoQ10 is the functional moiety of MitoQ and triphenyl-phosphonium is the vehicle moiety. Gemcitabine is the positive control for apoptosis induction. Data (mean  $\pm$  SEM,  $N \geq 3$ ) are expressed as the percentage of untreated controls. One-way ANOVA with Bonferroni post-tests; B: Western blot analysis of total lysates of cells treated as described.  $\beta$ -actin is the control for equal amounts of protein loading.  $^aP < 0.05$ .  $^cP < 0.001$ . MitoQ: Mitochondria-targeted ubiquinone; TPP: Triphenyl-phosphonium.

How does MitoQ kill tumor cells? Reactive oxygen species (ROS) produced in tumor cells are supposed to promote tumorigenesis, angiogenesis, metastasis, and chemoresistance[43,44]. Given this, tumor cells may be more susceptible to ROS scavenging. Indeed, an oxidized form of ubiquinone (BPM31510) is currently in clinical trial to treat solid tumors with a high ROS burden[45], and MitoQ may act in a similar fashion. In support of this notion, MitoQ can decrease ROS generation through the NRF 2/NAD(P)H quinone oxidoreductase 1 pathway, a key endogenous antioxidant defense mechanism[27,46,47]. Moreover, MitoQ was shown to inhibit redox signaling to prevent PDAC metastasis in mice[28]. However, in contrast, studies using bovine aortic endothelial cells demonstrated that MitoQ can also increase ROS generation in mitochondria[48,49]. Mechanistically, MitoQ can be reduced by complex II, but not by complexes I and III due to the bulkiness and positive charge of its TPP moiety that sterically hindered access to the proteins[50,51]. Therefore, MitoQ is anti- as well as pro-oxidant, depending on cell types. In either scenario, MitoQ over-enrichment would harm cells.



**Figure 3 Erlotinib can increase mitochondrial membrane potential in pancreatic adenocarcinoma cells.** A: Cells were treated with increasing concentrations of erlotinib for 2 d prior to staining with tetramethyl-rhodamine methyl ester (TMRM). Cellular TMRM retention was analyzed by flow cytometry measuring yellow fluorescence; B: Mean fluorescence intensities of TMRM-stained cells quantified by FCS Express software. Data are mean  $\pm$  SEM ( $n \geq 3$ ). One-way ANOVA with Bonferroni post-tests; C: Cells pretreated with erlotinib for 48 h were treated with 2  $\mu\text{M}$  mitochondria-targeted carboxy-proxyl (MitoCP) for 1 h. Mitochondrial extracts of these cells were analyzed by Western blotting to detect the formation of MitoCP adducts using the antibody specific to the triphenyl-phosphonium moiety of MitoCP. Total proteins in the extracts were visualized in the stain-free gel as the control for equal protein loading.  $^{\circ}P < 0.001$ . MitoCP: Mitochondria-targeted carboxy-proxyl; TPP: Triphenyl-phosphonium.

How does erlotinib increase  $\Delta\Psi_m$ ? Although not widely known, EGFR can translocate to mitochondria and regulate different mitochondria processes[52-55]. For example, EGFR translocation to the outer membrane of mitochondria promotes mitochondrial fission by disturbing the polymerization of the mitochondria fusion protein mitofusin 1 through direct interaction, which promotes the metastatic potential of NSCLC cells[55]. EGFR can directly bind to cytochrome c oxidase subunit II (COXII) in mitochondria[54], which leads to COXII phosphorylation and activity loss[56,57]. Increased COXII activity is implicated in hyperpolarization of mitochondria under conditions such as ischemia-reperfusion[58].



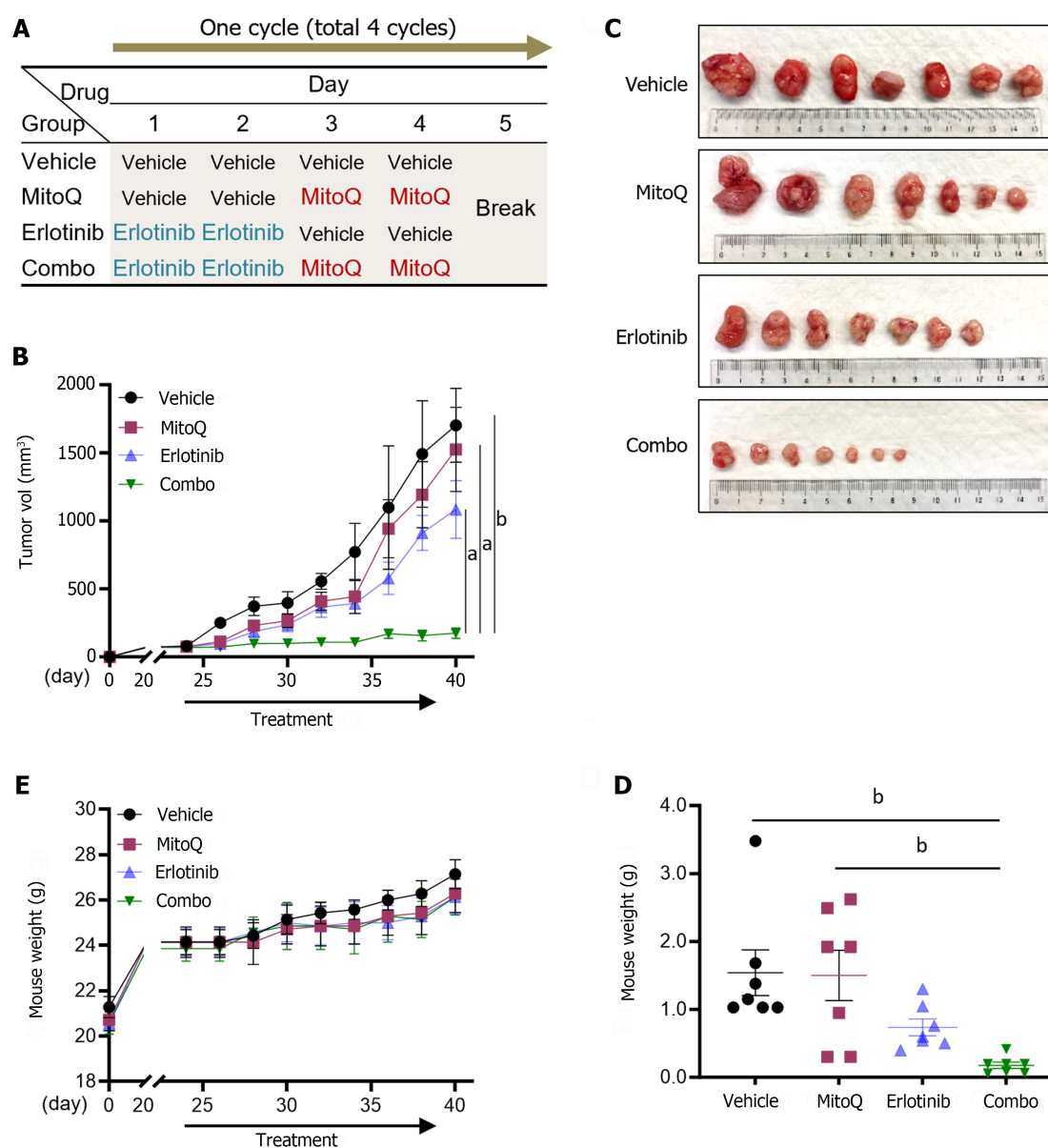
**Figure 4** Erlotinib can synergize with mitochondria-targeted ubiquinone to suppress the viability of pancreatic adenocarcinoma cells. **A:** Cells, pretreated with different concentrations of erlotinib, were treated with different doses of mitochondria-targeted ubiquinone (MitoQ) for 48 h prior to crystal violet viability assay. Data (mean  $\pm$  SEM,  $n \geq 3$ ) are expressed as the percentage of untreated controls; **B:** SynergyFinder plotting of the viability data. The ZIP (zero interaction potency) scores are indicated for the most synergistic areas; **C:** Western blot analysis of PANC-1 cells maintained in the human plasma-like medium with 1%  $O_2$ .  $\beta$ -actin is the control for equal protein loading; **D:** PANC-1 cells pretreated with erlotinib for 24 h were treated with MitoQ for 48 h in the human plasma-like medium with 1%  $O_2$  prior to crystal violet viability assay. Data (mean  $\pm$  SEM,  $n \geq 3$ ) are expressed as the percentage of untreated controls; **E:** SynergyFinder plotting of the viability data. The ZIP score is indicated for the most synergistic area.  $^aP < 0.05$ .  $^bP < 0.005$ .  $^cP < 0.001$ , Two-way ANOVA with Bonferroni post-tests. MitoQ: Mitochondria-targeted ubiquinone.

Because erlotinib abolished COXII inhibition by EGFR[59], a COXII regulation may underlie erlotinib-induced elevation of  $\Delta\Psi_m$  in PDAC cells. Alternatively, EGFR may use a signaling pathway to regulate  $\Delta\Psi_m$ . For example, a deficiency of mechanistic target of rapamycin (mTOR) complex 2 and mTOR inhibition can cause  $\Delta\Psi_m$  elevation in different cell types, including lung and breast tumor cells[60–62]. Because EGFR regulates mTOR[63,64], it may be possible that erlotinib affects  $\Delta\Psi_m$  *via* the mTOR pathway. Although we hypothesize that  $\Delta\Psi_m$ -dependent mitochondrial over-enrichment of MitoQ is the primary mechanism underlying the synergy between MitoQ and erlotinib, we also appreciate additional possibilities. For example, MitoQ can induce mitophagy *via* the PTEN-induced kinase 1/Parkin RBR E3 ubiquitin-protein ligase pathway[65,66], and erlotinib can induce autophagy through p53 nuclear translocation, AMP-activated protein kinase activation, and mTOR suppression[67,68]. Therefore, it is also possible that MitoQ and erlotinib synergize in the context of autophagic cell death. Interrogation of these mechanisms remains as future studies.

## CONCLUSION

Although MitoQ has been tested for its clinical potential for different diseases[21–23], it has not been tested for cancer therapy in a clinical trial setting. Based on the data in this study, we suggest that MitoQ can potentially suppress PDAC





**Figure 5 An erlotinib and mitochondria-targeted ubiquinone combination effectively suppresses PANC-1 xenografts in mice.** A: Treatment schedule. Detailed information is provided in Materials and Method; B: Changes in tumor size (mean  $\pm$  SEM,  $N = 7$ ). Two-way ANOVA with Bonferroni post-tests; C: Images of tumors collected at the end of treatment; D: Weights of tumors; E: Body weight changes (mean  $\pm$  SEM,  $N = 7$ ) monitored during the treatment. <sup>a</sup> $P < 0.05$ . <sup>b</sup> $P < 0.005$ . MitoQ: Mitochondria-targeted ubiquinone.

cell survival and that its combination with erlotinib is a candidate strategy to target PDAC cells selectively. MitoQ has additional potential benefits because antioxidants can support the physical fitness of patients undergoing chemotherapy [69,70]. In support of this notion, MitoQ improved muscle atrophy and weakness in colon cancer cell line tumor-bearing mice [70]. Studies have shown that TKIs, including ponatinib, regorafenib, sunitinib, imatinib, and sorafenib, can also affect  $\Delta\Psi_m$ , although their effects vary depending upon cell types [61,71-73]. Our findings lay a foundation to evaluate these TKIs in a similar context for the combination therapy concept.

## ARTICLE HIGHLIGHTS

### Research background

We previously showed that receptor tyrosine kinase inhibitors such as cabozantinib and vandetanib can increase mitochondrial membrane potential ( $\Delta\Psi_m$ ) in tumor cells and, thus, facilitate mitochondrial enrichment of  $\Delta\Psi_m$ -sensitive agents in tumor cells. This effect can disrupt mitochondrial homeostasis and trigger tumor cell death.

### Research motivation

Pancreatic cancer is one of the most lethal tumors, demanding highly effective molecular therapies. Although the

epidermal growth factor receptor inhibitor erlotinib has been approved for pancreatic adenocarcinoma (PDAC), its efficacy is limited as monotherapy, and it is often used in combination with other drugs. We sought to determine whether erlotinib can be combined with a mitochondria-targeted agent for PDAC suppression.

### Research methods

We measured cell viability by performing cell death assays in 2D and 3D cultures of conventional and patient-derived primary PDAC cell lines. We determined how erlotinib affects  $\Delta\psi_m$  in PDAC cells using  $\Delta\psi_m$ -sensitive fluorescent dyes and by measuring protein adduct formation with mitochondria-targeted carboxy-proxyl (MitoCP) in mitochondria. We examined the effect of erlotinib and mitochondria-targeted ubiquinone (MitoQ) combination by measuring cell viability and analyzing synergy. We determined the preclinical efficacy and physiological relevance of the drug combination using immune-compromised nude mice bearing PDAC cell line xenografts.

### Research results

Erlotinib elevated  $\Delta\psi_m$  in PDAC cells and facilitated mitochondrial enrichment of the triphenyl-phosphonium (TPP)-conjugated agents. While MitoQ single treatment triggered caspase-dependent apoptosis in PDAC cells, its combination with erlotinib synergistically induced PDAC cell death. Consistent with these data, the drug combination suppressed human PDAC cell line xenografts in mice more effectively than a single treatment of each agent.

### Research conclusions

These data suggest that erlotinib elevated  $\Delta\psi_m$  in PDAC cells and facilitated mitochondrial enrichment of the TPP-conjugated agents. The drug combination synergistically suppressed PDAC cells.

### Research objectives

We aimed to determine whether erlotinib elevates  $\Delta\psi_m$  in PDAC cells, increases tumor cell uptake of the TPP cation-conjugated ubiquinone MitoQ, and subsequently causes tumor cell death.

### Research perspectives

These data suggest that the erlotinib and MitoQ combination may have therapeutic potential for pancreatic cancer.

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## FOOTNOTES

**Co-first authors:** Pui-Yin Leung and Wenjing Chen.

**Author contributions:** Leung PY designed and performed research, and analyzed data; Chen W designed and performed research, analyzed data, and wrote the paper; Sari AN performed research and analyzed data; Wu PK developed methodology; Sitaram P performed research and analyzed data; Tsai S secured funding for this study; Park JI conceived and designed research, wrote the paper, secured funding, and supervised the project. All authors were involved in the critical review of the results and have contributed to, read, and approved the final manuscript. Leung PY and Chen W contributed equally to this work as co-first authors. Designating these two authors as co-first authors accurately reflects the distribution of responsibilities and burdens associated with the time and effort required to complete the study and the resultant paper.

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