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Novel Synergistic Combination of Mitotic Arrest and Promotion of Apoptosis for Treatment of Pancreatic Adenocarcinoma

Zhijian Duan, Danielle Chinn, Mei-Juan Tu, Qian-Yu Zhang, Jasmine Huynh, Justin Chen, Philip Mack, Ai-Ming Yu and Edward J. Kim

University of California at Davis Medical Center

Abstract

The BCL-2 family of proteins, including anti-apoptotic members BCL-2, BCL-XL and MCL-1, are part of a complex network that controls apoptosis. BH3-mimetics such as ABT-263 inhibit anti-apoptotic BCL-2 proteins and have been developed as potential cancer therapeutics. Aurora Kinase A (AKA) is over-expressed in pancreatic cancer (PC) and controls G2-M transition during mitosis and AKA inhibitors have been developed that induce mitotic arrest. We hypothesized that mitotic arrest induced by AKA inhibition may sensitize PC to accelerated apoptosis by a BH3-mimetic. Our results demonstrated that ABT-263 plus MLN8237 treatment showed greater activity than either single drug alone, as well as strong synergism, in the inhibition of growth of pancreatic cell lines (AsPC-1, PANC-1, MIA PaCa-2, HPAF-II) and PC patient-derived organoids (PDOs). The higher efficacy of combination treatment was attributable to the higher levels of induction of apoptosis and reduction of MCL-1 in PC cells and PDOs. In addition, combination therapy was more effective than single drug in the suppression of tumor growth in AsPC-1 xenograft mouse models. Together, our findings suggest that combination therapy with ABT-263 and MLN8237 should be considered for further exploration as a novel treatment of deadly PC disease.

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Introduction

Pancreatic cancer is aggressive and is the fourth leading cause of cancer-related death in the United States [1]. Due in part to a lack of an effective screening method, 60% to 70% of patients present with metastatic disease at time of diagnosis. In advanced disease, the median survival rate is 3-4 months without therapy [2]. Surgical resection is the only treatment modality with the potential for cure but the majority of patients present with unresectable or metastatic disease. In advanced disease, treatment consists of chemotherapy with or without palliative radiation therapy. Gemcitabine, a deoxycytidine nucleoside analog, has been a standard-of-care chemotherapeutic agent for advanced pancreatic cancer for the past two decades despite limited efficacy [3]. FOLFIRINOX, a combination regimen of fluorouracil, leucovorin, irinotecan, and oxaliplatin, has proven more efficacious than gemcitabine alone; however median overall survival for newly-diagnosed advanced pancreatic cancer remains under a year and tolerability is limited by toxicity [4,5].

There is a need for novel, more effective, and better-tolerated therapeutics for treatment of pancreatic cancer. Cancer cells exhibit both genetic and epigenetic changes that promote anti-apoptotic over pro-apoptotic signals, thus favoring survival. Modulation of the BCL-2 family of proteins has been well characterized as one mechanism by which cancer cells develop the ability to withstand genotoxic stressors and promote tumor maintenance, metastatic progression, and therapy resistance [6–9]. BH3-domain mimetics have been developed to inhibit select anti-apoptotic BCL-2 family members. Venetoclax, formerly known as ABT-199, is a BH3-mimetic and small molecular inhibitor of BCL-2 which in combination with rituximab has proven successful in treating relapsed chronic lymphocytic leukemia. Further investigations are ongoing in examining its role in other hematologic malignancies including acute myeloid leukemia and non-Hodgkin lymphoma [10–14]. Unfortunately, similar therapies have yielded disappointing results in various solid tumors [15,16]. Increased

Address all correspondence to: Edward J Kim MD, PhD, Associate Professor of Medicine, Division of Hematology and Oncology, UC Davis, 4501 X Street, Suite 3016, Sacramento, CA 95817.
E-mail: jhkim@ucdavis.edu
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understanding of the mechanisms of resistance to BH3-domain mimetics has created opportunities to more strategically design drug combinations. KRAS oncogene mutations are prevalent in multiple cancers including >90% of pancreatic adenocarcinoma. Studies have shown that RAS-mutated cancer cells exhibit a pro-survival balance among the BCL-2 family members [17]. This can be exploited by selective susceptibility of KRAS-mutated cancer cells to combination BH3-mimetics with strategic partner drugs to create synthetic lethality [18,19]. Potent BH3-domain mimetics under clinical development such as ABT-263 (navitoclax) inhibit anti-apoptotic BCL2 family members (BCL2, BCL-Xl and BCL-W) [20,21]; however, they fail to inhibit the Bcl-2 family member MCL-1 whose expression is a potential mechanism of resistance to BCL-2 inhibitors [22,23].

There is evidence that the protein level of MCL-1 decreases during mitotic arrest through degradation. Although cytotoxic chemotherapy drugs like taxanes induce cancer cell death by causing mitotic arrest, they also cause dose-limiting toxicity due to other effects on non-cancer cells unrelated to mitotic arrest such as myelosuppression and peripheral neuropathy. The next generation of mitosis inhibitors have been developed to target proteins specifically expressed during mitosis. Aurora Kinase A (AKA) is a serine/threonine kinase intricately involved in centrosome maturation and spindle assembly and thus is highly expressed during G2 through mitosis and is located on duplicated centrosomes as well as spindle microtubules [24,25]. AKA is overexpressed in multiple malignancies including pancreatic cancer [26,27] and has been shown to cause centrosome amplification, aneuploidy, and chromosomal instability [26,28]. Inhibition of AKA using an siRNA to AKA in pancreatic cancer cells causes increased mitotic arrest [29]. There was also an increase in the sub-G1 fraction suggesting an increase in apoptosis [29]. These effects phenotypically resulted in decreased proliferation and tumorigenicity [29]. Two phase I studies of MLN8237, an oral AKA inhibitor, have demonstrated modest single agent activity in solid tumors [30,31]. Venetoclax has previously been tested in combination with MLN8237 and was found to induce widespread apoptosis in MYCN-amplified neuroblastoma via the reduction of MCL-1 [32]. In this study, we tested the efficacy of ABT-263 combined with MLN8237 as a potential new treatment option for pancreatic cancer.

Materials and Methods

Cell lines, Organoids, and Reagents

Four pancreatic cell lines generously provided by Dr. Richard Bold and Dr. Julie Sutcliffe's laboratories (University of California, Davis, Davis, CA) were studied: AsPC-1, HPAF-II, MIA PaCa-2 and PANC-1. AsPC-1 were maintained in RPMI 1640 media with glutamine (Invitrogen) supplemented with 2% penicillin/streptomycin and 10% heat-inactivated fetal bovine serum (FBS) (JR Scientific). The other cell lines were maintained in Dulbecco's Modified Eagle's High Glucose media with glutamine and sodium pyruvate (DMEM, GE Healthcare Hyclone) supplemented with 1× MEM Vitamin Solution (Invitrogen) and 1× MEM Non-Essential Amino Acids solution (Invitrogen), and 10% FBS. All cell lines were incubated at 37°C in 5% CO_2.

Pancreatic organoids were developed from patient tumor specimens following an established protocol [33]. Briefly, tumor tissue was minced and digested with collagenase II (5 mg/ml, GIBCO) in complete organoid feeding media at 37 degrees for 4 hours. The material was further digested with TrypLE (GIBCO) for 15 minutes at 37 degrees, embedded in growth factor reduced Matrigel, and cultured in complete organoid media. The complete organoid media consisted of the following ingredients: AdDMEM/F12 media supplemented with HEPES (1× Invitrogen), Glutamax (1×, Invitrogen), penicillin/streptomycin (1×, Invitrogen), B27 (1×, Invitrogen), Primocin (1 mg/ml, InvivoGen), N-acetyl-L-cysteine (1 mM, Sigma), Wnt3a-conditioned media (50% v/v), RSPO1-conditioned media (10% v/v), recombinant Noggin protein (0.1 mg/ml, Peprotech), epidermal growth factor (EGF,50 ng/ml, Peprotech), Gastrin (10 nM, Sigma), fibroblast growth factor 10 (FGF10, 100 ng/ml, Peprotech), Nicotinamide (10 mM, Sigma), and A83–01 (0.5 mM, Tocris).

MLN8237 and ABT-263 were purchased from Selleck Chemicals. MLN8237 and ABT-263 were dissolved in dimethyl sulfoxide, stored at −80°C, and further diluted in media for in vitro studies.

Growth Inhibition Assays

Cells were seeded at 2×10^3-5×10^3 cells/well in 96-well flat-bottom plates and allowed to attach overnight prior to treatment. Cells were treated with either MLN8237 (0.001-10 μM) or ABT-263 (0.1-10 μM) for 72 hours in triplicate wells including an untreated control. Cells were then assayed by the Celltiter-Glo Assay (Promega) according to the manufacturer's protocol and dose-response curves for triplicate experiments were generated via Prism software (GraphPad) to assess 50% inhibitory concentration (IC_{50}) values. The MLN8237 + AB-263 combination was explored in the cell proliferation assay using a dose range of MLN8237 (0.01-10 μM) and three fixed doses of ABT-263 (0.1, 1, 10 μM) for the combination and single-agent controls. To assess the interaction of MLN8237 with ABT-263, median-effect analysis was used according to Chou-Talalay method [34]. The AsPC-1, MIA PaCa-2, PANC-1, and HPAF-II cell lines were seeded in black 96-well plates at 2×10^3-5×10^3 cells/well and concurrently exposed to four dose combinations of MLN8237 (MLN) (0.05, 0.25, 0.5, and 2.5 μM) and ABT-263 (ABT) (0.1, 0.5, 1, 5 μM) at a constant ratio of 1:2 for 72 hours. Triplicate wells were used for all treatments and the untreated control. Surviving cell populations were quantified by the Celltiter-Glo Assay according to the manufacturer's protocol and combination indexes (CI) were generated using CompuSyn software (ComboSyn) to determine whether the combination treatments were synergistic (CI< 1). ED_{50} and ED_{75} values were reported for triplicate experiments.

Organoids were used for proliferation assays with either single cells or small organoids. In order to dissociate organoids into single cells, organoids were triturated in media through a fire-polished glass pipette, and then enzymatically dissociated with TrypLE (Life Technologies) until they appeared as single cells under the microscope. Cells were counted, and diluted in a mixture of complete media, Rho Kinase Inhibitor Y-27632 (10.5 mM final concentration, Sigma), and Growth factor reduced Matrigel (GFR-Matrigel, 10% final concentration). The drugs were added after 24 hours and cell viability was measured 72 hours after drug treatment using the CellTiter-Glo assay. Organoids were also passed and plated into 25ul of GFR-Matrigel. Triplicate wells per time point were used. Cell viability was measured every 24 hours after drug treatment using the CellTiter-Glo assay for up to 96 hrs. Luminescence data were analyzed with GraphPad Prism.

Flow Cytometry

Annexin V staining was performed using the FITC Annexin V Apoptosis Detection Kit I (BD Biosciences) to evaluate apoptotic effects following combined treatment with MLN8237 and ABT-263. AsPC-1 cells were plated at 1×10^6-1.8×10^6 cells per 100 mm dish and treated the following day with MLN8237 (0.1 and 0.5 μM) and ABT-263 (1 μM) as single agents and in combination. Cells were harvested 24 hours later with Accutase solution (Millipore) and stained with FITC-Annexin V and propidium iodide according to the manufacturer's protocol. Heat-treated cell samples (incubated at 55 degrees for 20-30 minutes) were included in addition to the standard staining and untreated controls. Annexin V staining was measured and analyzed by a Stratedigm S1400 flow cytometer and Cell CapTure Analysis software (Stratedigm).

Immunoblotting

Cell lysates were prepared by washing once with cold PBS followed by lysis with RIPA buffer with protease inhibitors. Protein concentration was determined using the Pierce BCA protein assay kit (Thermo Scientific) according to the manufacturer's protocol. SDS-PAGE was performed with 20-46 μg of protein loaded for each sample. Protein was transferred to nitrocellulose membranes (Bio-Rad) and probed overnight with primary antibodies at 4°C. Blots were then incubated for 1 hour at room temperature with the HRP-conjugated secondary antibodies, anti-mouse IgG and anti-rabbit IgG (Promega), and visualized by chemiluminescence with Amersham ECL (GE Healthcare). Levels of MCL-1, Bcl-xL (BD Biosciences); Bcl-2, Cleaved Caspase-3, cleaved Caspase-7 (Cell Signaling Technology); and PARP (Santa Cruz Biotechnology) were determined. β-Actin (Sigma-Aldrich) or GAPDH (Santa Cruz Biotech) was used as a loading control.

Establishment of Pancreatic Xenograft Model with AsPC-1 Cells for Combination Therapy

Four-week-old female athymic mice were purchased from The Jackson Lab. In order to establish tumors in mice, 2 x 10^6 cells were suspended in total of 100 μL PBS/Matrigel (1:1) and implanted subcutaneously into the dorsal flank on both sides of the mice. We chose to test six or more per cohort to provide sufficient statistic power based on our power calculation. When the tumor volume was approximately 80 mm^3, the mice were randomized and divided into four groups for treatments: vehicle control, MLN8237 (20 mg/kg), ABT-263 (100 mg/kg), MLN8237 (20 mg/kg) combined with ABT-263 (100 mg/kg). ABT-263 was formulated in 10% ethanol, 30% polyethylene glycol 400, and 60% Phosal 50 PG. MLN8237 was formulated in 10% 2-hydroxypropyl-β-cyclodextrin with 1% sodium bicarbonate. The drugs were given by oral gavage. MLN8237 was given once daily for 3 days on, 4 days off (days 1-3 of 7-day cycle) for 3 cycles. ABT-263 was given once daily for 5 days on, 2 days off (days 1-5 of 7-day cycle) for 3 cycles. Tumor growth was monitored by calipers with volume calculated using the equation: (1/2 × (length × $width^2$)). Body weight during the study was also monitored. At the end of the studies mice were sacrificed and tumors were dissected and weighed. Tumors were harvested fixed in formalin and embedded in paraffin prior to sectioning.

H&E Staining and IHC of Pancreatic Organoids

The organoids were fixed with 10% neutral buffered formalin, embedded in paraffin, and sectioned onto slides. Slides were subjected

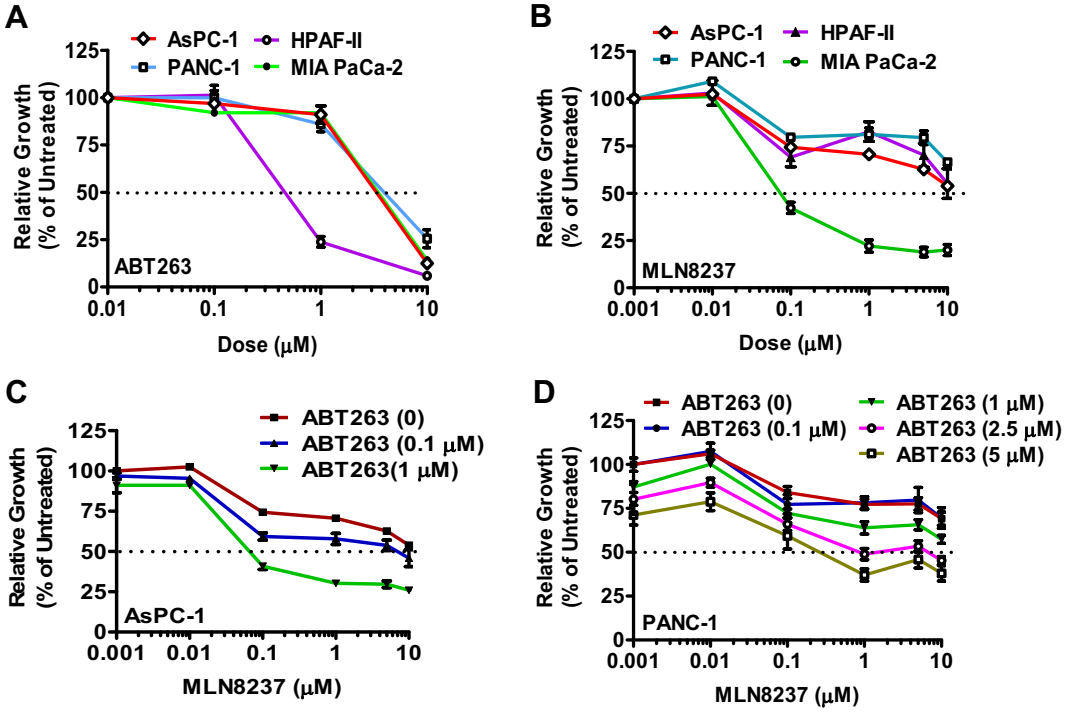

Figure 1. ABT-263 combined with MLN8237 shows greater cell growth inhibition and synergistic cell death of pancreatic cancer cells than single drug treatment.Pancreatic cancer cell lines AsPC-1, PANC-1, HPAF-II and MIA PaCa-2 cells were treated with ABT-263 (A), MLN8237 (B), or combination (C,D) for 72 hours and then analyzed for cell viability. All four cell lines were treated with ABT-263 alone given in a dose range of 0.1-10 μM (A) and MLN8237 alone given in a dose range of 0.001-10 μM (B). AsPC-1 (C) and PANC-1 (D) cells were treated with MLN8237 in a dose range of 0.01-10 μM and fixed ABT-263 doses of 0, 0.1 μM, 1 μM, 2.5 μM or 5 μM.

to hematoxylin and eosin (H&E) staining or immunohistochemistry (IHC) staining following standard protocols. For IHC staining, the samples were incubated with the primary anti-Ki67 antibody (Cell Signaling Technology). Images were then taken with an Olympus microscope (100×).

Statistics

Cell culture-based experiments were performed three times or more unless indicated otherwise. The data are presented as mean values ± SD from three independent experiments. Statistical analysis was performed using one-way or two-way ANOVA using GraphPad Prism. Difference was considered significant when P value was less than 0.05 ($P < .05$).

Results

ABT-263 Combined with MLN8237 Shows Synergistic Cell Growth Inhibition In Vitro and Suppresses Tumor Xenograft Growth In Vivo

Among the 4 pancreatic cancer cell lines treated with ABT-263, HPAF-II was the most sensitive with an IC_{50} between 0.5-1 μM (Figure 1*A*). AsPC-1 and MIA PaCa-2 cell lines were moderately sensitive with IC_{50} values ranging from 1-5 μM, while the IC_{50} for PANC-1 cells fell between 5-10 μM (Figure 1*A*). In the same 4 pancreatic cancer lines treated with MLN8237, MIA PaCa-2 cells exhibited the greatest response to treatment with an IC_{50} ranging from 0.05-0.1 μM while the other three cell lines AsPC-1, PANC-1 and HPFA-II required a dose of 10 μM or higher (Figure 1*B*). The cells were treated concurrently at a dose range for MLN8237 (0.01-10 μM) and fixed doses for ABT-263 (0.1, 1, 2.5 and 5 μM) for 72 hours. In AsPC-1 cells, ABT-263 combined with MLN8237 enhanced growth inhibition with 1 μM of ABT-263 with 0.1 μM MLN8237 leading to more than 50% percent inhibition (Figure 1*C*). PANC-1 cells also showed enhanced sensitivity to the combination treatments (Figure 1*D*).

To assess the synergistic activity of ABT-263 and MLN8237, the four pancreatic cancer cell lines were exposed to single agent doses and four concurrent treatments of MLN8237 and ABT-263 at a constant ratio of 1:2 for a period of 72 hours. Combination index (CI) values were formally generated using CompuSyn. The CI values for both actual and projected effect levels were calculated based on these results (Supplementary Table 1). All four cell lines tested demonstrated synergistic activity (CI< 1) for the combined treatment at ED_{50} and ED_{75}, although PANC-1 exhibited greatest synergy at both the ED_{50}

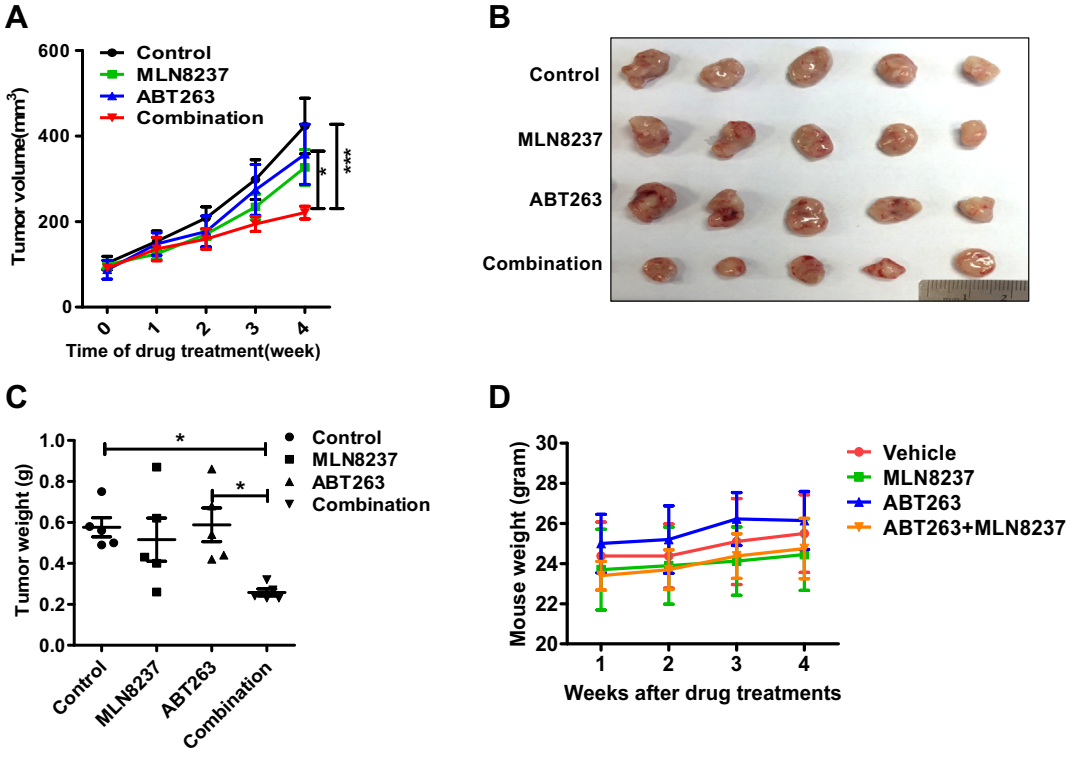

Figure 2. Combination of ABT-263 and MLN8237 suppresses pancreatic xenograft tumor growth.AsPC-1 cells were injected subcutaneously into nude mice to establish mouse xenograft tumors. Mice were randomly assigned to one of four treatment groups and treated with either vehicle control, ABT-263 alone, MLN8237 alone, or combination of ABT-263 plus MLN8237 for 4 weeks. A) Tumor volume was measured by digital caliper weekly and revealed statistically significant reduction in growth of tumors in the combination group (***$P < .001$, control vs. combination; **$P < .05$ ABT-263 vs. combination; two-way ANOVA with Bonferroni's multiple comparison tests). Values are mean ± SD (N = 5 per group). B) At the end of 4 weeks, tumors harvested and imaged under bright light with reference ruler showing decreased size of tumors in the combination group. C) Quantification of the final tumor weights show statistically significant decrease in weight of tumors from the combination group (*$P < .05$; one-way ANOVA with Bonferroni post-tests). C and D) To evaluate safety of the different treatments, mouse weight was monitored during treatment and there were no significant differences between the treatment groups with overall increase in body weight in all treatment groups.

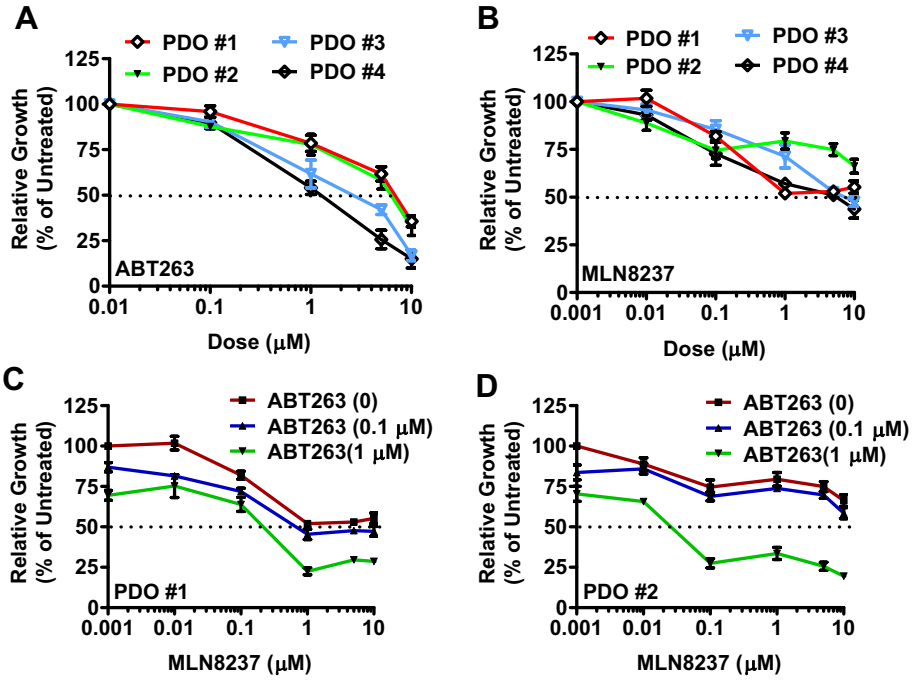

Figure 3. ABT-263 combined with MLN8237 shows greater cell growth inhibition in pancreatic cancer organoids. Four patient-derived pancreatic cancer organoid (PDO) models were treated with ABT-263 (A), MLN8237 (B), or combination (C, D) for 72 hours and then analyzed for cell viability. All four PDO models were treated with ABT-263 alone given in a dose range of 0.1-10 μM (A) and MLN8237 alone given in a dose range of 0.001-10 μM (B). PDO #1 (C) and PDO #2 (D) were treated with MLN8237 in a dose range of 0.01-10 μM and fixed ABT-263 doses of 0, 0.1 μM, or 1 μM. Compared to either drug alone (A, B), combination treatment in both PDO #1 and #2 shows greater inhibition of cell growth.

and ED_{75} (CI = 0.01564 and CI = 0.000668) (Supplementary Figure 1).

To test the effect of the combination on tumor growth in vivo, AsPC-1 cells were injected into nude mice to establish a pancreatic xenograft model. Combination treatment with both ABT-263 and MLN8237 showed slower tumor growth resulting in small tumor weight and volume compared to single treatment with either drug alone (Figure 2). Mice body weights remained similar despite treatment (Figure 2*D*). Blood biochemistry profiles were also examined, and no difference was observed (data not shown).

Combined Treatments of ABT-263 and MLN8237 Show Greater Inhibition on Cell Growth and Colony Formation in Pancreatic Cancer Organoids

In order to test the efficacy of the combination therapy in patient tumor specimens grown in 3-dimensional space, we utilized four distinct pancreatic organoid lines established from patients. Single drug studies demonstrated those organoids had a low sensitivity to ABT-263 similar to the pancreatic cancer cell lines AsPC-1 and PANC-1 cells, with an IC_{50} between 5-10 μM (Figure 3*A*). Pancreatic cancer organoids were also resistant to MLN8237 with an IC_{50} greater than 10 μM (Figure 3*B*). However, both pancreatic cancer organoids tested were sensitive to 1 μM ABT-263 combined with MLN8237 (>0.1 μM) (Figure 3, *C* and *D*). The sensitivity pattern of the organoids was similar to pancreatic cancer cell lines. Growth inhibition was also evaluated qualitatively using microscopy over a time-course. The combination of 1 μM ABT-263 and 0.1 μM MLN8237 greatly inhibited the cell growth of the organoids as early as 48 hours with sustained effect at 72 and 96 hours of treatment whereas single drug could not inhibit the cell growth even after 72 hours. Based on visual inspection under microscopy, there appeared to be more unhealthy cells in the dual treatment cohort compared with controls (Figure 4).

Combination of MLN8237 and ABT-263 Induces Activation of Apoptosis in Pancreatic Cancer Cells and Disruption of Organoid Formation

Based on the mechanism of action of the studied drugs, we hypothesized that the effect of cell growth inhibition from the drug combination was a result of enhanced apoptosis. Immunoblots for apoptosis markers cleaved Caspase 3 or 7 were conducted following the drug treatments in both pancreatic cancer cells lines and pancreatic cancer organoids. Caspase-3 cleavage was tested in AsPC-1, MIA PaCa-2, PANC-1 and HPAF-II cells, and increased cleaved caspase-3 was seen with combined treatment relative to the single agent controls in all cell lines (Figure 5*A*). In the organoids, the drug combination also induced apoptosis as demonstrated by increased expression of cleaved caspase-7 compared with single drug treatment (Figure 6*A*). Annexin V staining was performed on AsPC-1 (Figure 5*B*). The population of early apoptotic cells increased significantly in the AsPC-1 cell line following combined treatments for 24 hours (17.16% vs. 2%-5%) compared to the single agent controls (Figure 5*B*). Taking advantage of the morphology of organoids, the pancreatic organoids were processed for histology studies after drug treatment. The combination treatment resulted in a collapsed lumen structure in organoids when evaluated using H + E staining which was consistent with the morphology changes viewed by light microscopy (Figure 4). These results were consistent with apparent increased induction of apoptosis as measured by induction of cleavage of PARP and Caspase-3 (Figure 6*A*). Using the 3-

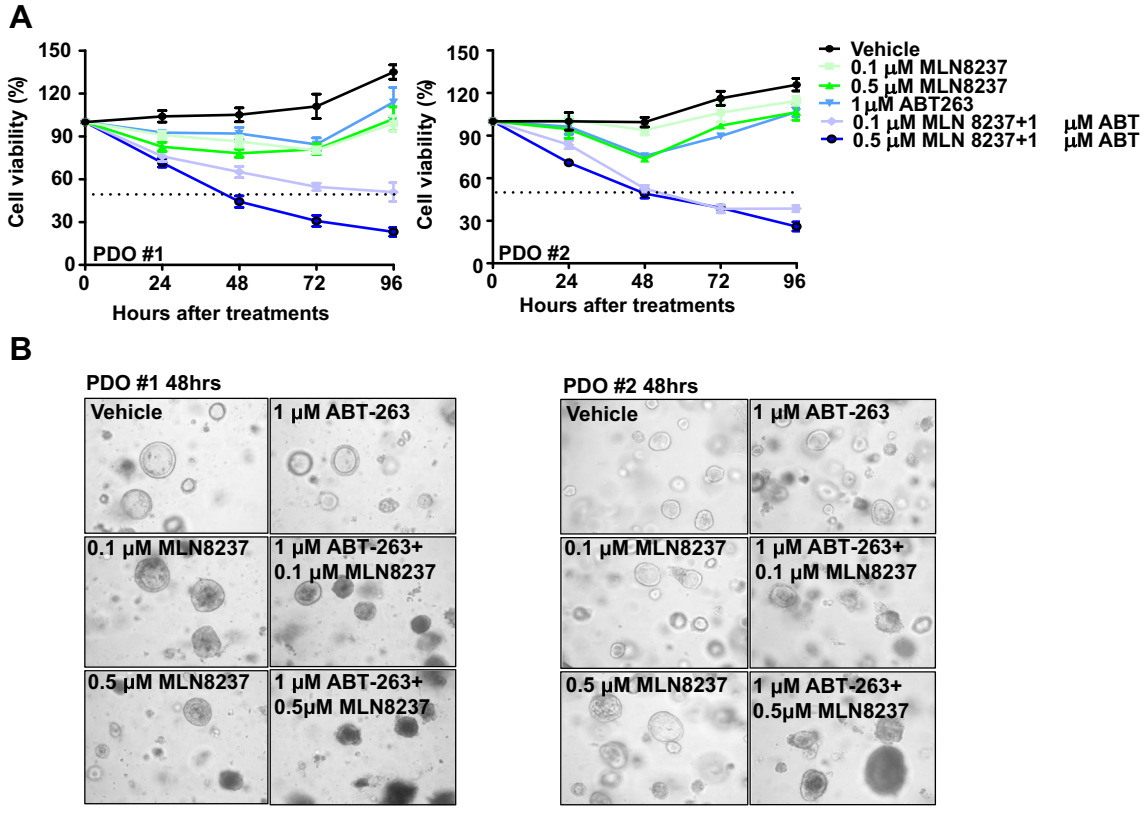

Figure 4. Combined treatments of MLN8237 and ABT-263 suppresses organoid proliferation and colony formation in pancreatic cancer organoids. PDO #1 and #2 were treated with vehicle control, ABT-263 alone, MLN8237 alone, or combination. Cell viability was measured at 24-, 48-, 72- and 96-hour time points. Greatest inhibition of cell viability was achieved with combination in both PDO #1 and #2 B) Representative images of PDO #1 and #2 were taken by a digital camera under 10× microscopy and after 48 hours of treatment which revealed increased necrotic appearing organoids treated with combination.

dimensional organoid model also allowed us to determine that there appeared to be persistent Ki67 staining in the periphery of the organoids suggesting differential response within the organoid structure (Figure 6*B*).

MLN8237 Enhances ABT-263 Activity Through Modulation of BCL2 Proteins in Organoid Pancreatic Cancer Cells

Treatment with MLN8237 alone decreased MCL-1 but had variable effect on BCL-xL. One potential mechanism of resistance to ABT-263 is through up-regulation of BCL-2 proteins including BCL-xL and MCL-1 [35]. PDO-1 and PDO-2 both demonstrated increased MCL-1 and to a lesser extent BCL-xl expression after treatment with 1 μM ABT-263 (Figure 7*A*). This up-regulation of MCL-1 induced by ABT-263 was abrogated by addition of MLN8237. Degradation of MCL-1 can be induced by mitotic arrest and MLN8237 alone appeared to cause cell cycle arrest in PDO-2, as evidenced by accumulation of Cyclin A/B (Figure 7*B*), and G2/M cell cycle arrest in pancreatic cancer cell lines (Supplementary Figure 2).

Discussion

Pancreatic cancer continues to be one of the deadliest cancers. It is projected to become the second-most common cause of cancer-related death in the United States by the year 2020. Although there have been successful new multi-drug regimens approved, these have been limited to chemotherapy drugs with frequently overlapping toxicities. Targeted therapies combined with chemotherapy have largely been unsuccessful in improving outcomes compared to chemotherapy alone. There has not always been clear preclinical rationale to combining specific targeted therapies with chemotherapy and trials have largely failed to capture specific insight into why the addition of particular targeted therapies has failed. Simply adding targeted therapies to chemotherapy also fails to address the standard toxicities associated with chemotherapy. However, targeted therapies given without chemotherapy has also been challenging as there are multiple examples of targeted therapies yielding no signal in early phase clinical trials when tested as monotherapy.

We chose in this study to approach the problem by testing our hypothesis that pancreatic cancer cells are vulnerable to a rational combination of two non-chemotherapy drugs that work synergistically by inducing mitotic arrest and accelerating apoptosis. One of the most recently approved chemotherapy drugs for pancreatic cancer is the taxane nab-paclitaxel that targets rapidly dividing cancer cells by causing mitotic arrest through stabilization of microtubules. Dose-limiting toxicity associated with this drug is sensory neuropathy and myelosuppression which is attributable to the nonspecific effects of the drug on microtubules beyond mitosis. Unfortunately, sensory neuropathy is also the dose-limiting toxicity of oxaliplatin which is part of the standard of care first line treatment regimen FOLFIRINOX. Therefore, many patients who receive FOLFIRINOX in the front line are not candidates to receive nab-paclitaxel at time of progression. We chose to study inhibition of aurora kinase A as one of several new approaches to induction of mitotic arrest similar to taxanes. AKA is specifically expressed and present during mitosis and is critical to progression of cancer cells through mitosis. Therefore,

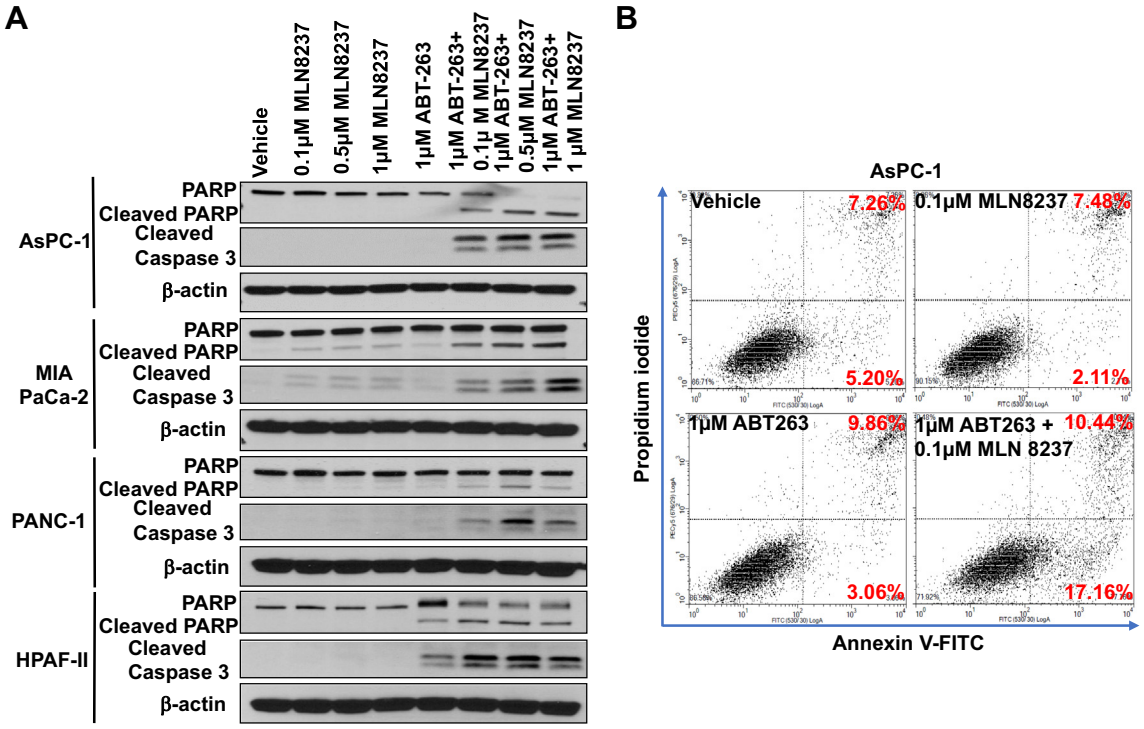

Figure 5. Combination of MLN8237 and ABT-263 induces activation of apoptosis in pancreatic cancer cells. A) Pancreatic cancer cell lines AsPC-1, MIA PaCa-2, PANC-1, and HPAF-II and cells were treated with vehicle, MLN8237, ABT-263 or combination of ABT-263 plus MLN8237 for 24 hours and then harvested. Protein extracts from the cells were subjected to western blotting with cleaved caspase-3 and PARP antibodies revealing consistent activation of apoptosis in combination treated cells. B) AsPC-1 cells were treated with vehicle, MLN8237, ABT-263 or combination of ABT-263 plus MLN8237 for 24 hours. The treated cells were collected and stained with Annexin V-FITC and propidium iodide and analyzed by flow cytometry to quantify percentage of cells undergoing apoptosis which was greatest with combination treatment.

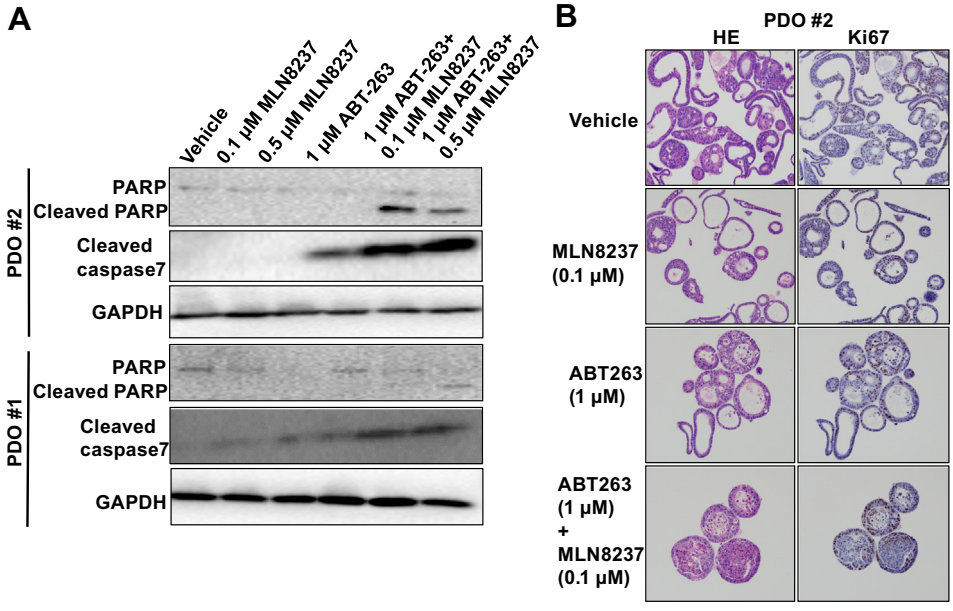

Figure 6. Combination of MLN8237 and ABT-263 induces activation of apoptosis and disruption of organoid formation in pancreatic cancer organoids. A) PDO #1 and #2 were treated with vehicle, MLN8237, ABT-263 or combination of ABT-263 plus MLN8237 for 48 hours and subjected to western blotting with cleaved caspase-7 antibody and PARP antibody. B) PDO #2 was treated with vehicle, MLN8237, ABT-263, or combination of ABT-263 plus MLN8237 for 48 hours. The organoids were then collected and formalin-fixed prior to embedding in paraffin. Sections from the four treatment groups were stained with hematoxylin and eosin or Ki-67 antibody. Representative images were taken under 100× magnification using an Olympus microscope.

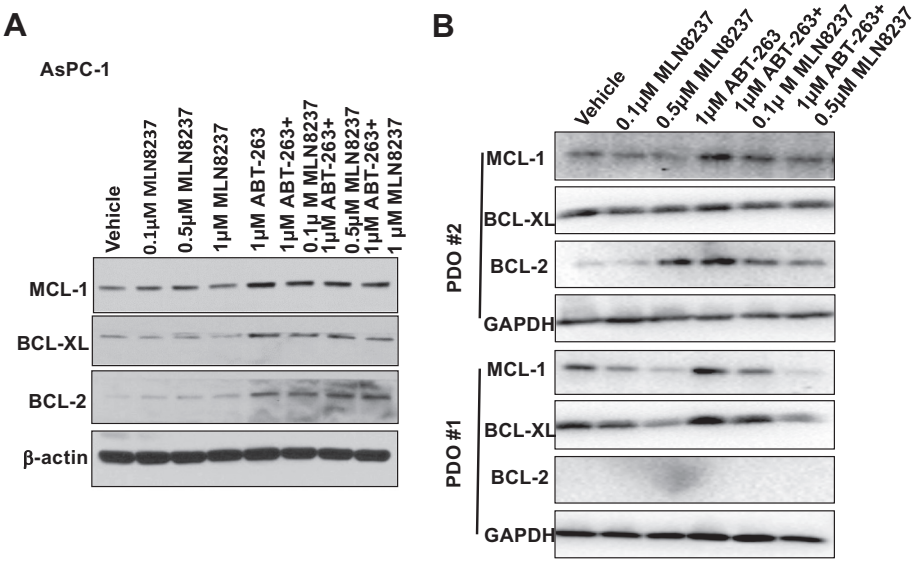

Figure 7. MLN8237 enhances the ABT263 activity through reduction of BCL-XL and MCL-1 in pancreatic cancer organoids. A) AsPC-1 cells were treated with vehicle, MLN8237, ABT263 or ABT263 plus MLN8237 for 24 hours. Protein extracts from the treated cells were subjected to western blotting with antibodies against BCL-2, BCL-XL and MCL-1. A) PDO #1 and #2 were treated with vehicle, MLN8237, ABT263 or ABT263 plus MLN8237 for 48 hours. Protein extracts from the treated cells were subjected to western blotting with antibodies against BCL-2, BCL-XL and MCL-1.

drugs like MLN8237 which inhibit AKA specifically can achieve the same therapeutic intent of mitotic arrest as taxanes with less systemic toxicity. Although some cancer cells that undergo mitotic arrest subsequently undergo apoptosis, other cancer cells can escape mitotic arrest. This is one mechanism of resistance to drugs that induce mitotic arrest as their sole mechanism of action. We hypothesized that we could increase the percentage of cancer cells that undergo apoptosis after mitotic arrest by also exposing cancer cells to the BH3-mimetic ABT263, which blocks anti-apoptotic members of the Bcl2 family of proteins. Like many other drugs in the same class of BH3-mimetics, ABT263 fails to inhibit all of the anti-apoptotic Bcl2-proteins, notably MCL-1 which is hypothesized as a pathway of resistance to ABT263 monotherapy. Mitotic arrest with MLN8237 leads to degradation of MCL-1 thus bypassing this potential resistance mechanism.

Our in vitro results reveal overall poor sensitivity to monotherapy with either MLN8237 or ABT263. Unsurprisingly, there is variability in sensitivity between cell lines tested with Mia PaCa-2 cells demonstrating greatest sensitivity to MLN8237 whereas HPAF-II cells were the most sensitive to ABT-263. However, all four pancreatic cancer cell lines tested demonstrated an increased sensitivity to the combination of both MLN8237 and ABT-263 compared to monotherapy and this effect appeared to be due to synergy between the two drugs. This observed effect of combination therapy was preserved in in vivo tests of AsPC-1 xenografts with greater tumor growth inhibition in the mice treated with both drugs compared to either drug alone. Furthermore, the therapeutic combination appeared safe and well-tolerated based upon the mice blood biochemistry profiles. We further confirmed this enhanced activity of combination MLN8237 with ABT-263 in a patient-derived model system using patient-derived pancreatic cancer organoids. Similar to the pancreatic cancer cell lines, we observed heterogeneity in sensitivity of the PDOs to the drugs given individually and again observed that combination of MLN8237 and ABT-263 induced greater cell growth inhibition than single drug treatment. Despite clear synergy in the cell line assays as assessed by Chou-Talalay method, the response to combination therapy in the in vivo mouse model and in vitro organoid model were less robust.

Our hypothesized mechanism of action for the observed cell growth inhibition was an increase in apoptosis. We observed increased apoptosis in both pancreatic cancer cell lines and the PDOs using qualitative analysis of increased presence of apoptosis markers like cleaved Caspase 3/7 as well as quantitative analysis for the percentage of apoptotic cells using Annexin V staining. Light microscopy of the PDOs allowed us to reveal differential regions of induction of apoptosis within the organoid structure. These experiments highlight some of the unique potential opportunities provided by the PDO model system to not only capture inter- and intra-tumoral heterogeneity but also to observe 3-dimensional alterations in cell organization and structure of patient-derived cancer cells grown as organoids. Our results demonstrate the utility of patient-derived organoids as a clinically relevant model system to study pancreatic cancer [33,36].

Lastly, we investigated the role of the individual BCL2 proteins in the observed synergy between treatment with an AKA inhibitor and BH3-mimetic. Although we did observe MCL-1 decrease in response to treatment with MLN8237, it was not consistently associated with confirmation of mitotic arrest. The potential explanations for this include 1) technical issues of measuring MCL-1 globally when the cells induced in mitotic arrest is not uniform and 2) MLN8237 may affect MCL-1 via a mechanism independent of mitotic arrest. Bcl-2 inhibition in other clinical settings, notably hematologic malignancies, have also proven effective when combined with other therapies such as rituximab, bendamustine, and hypomethylating agents [11,12,14]. Furthermore, the combination Bcl-2 inhibition with MLN8237 appears efficacious for double hit lymphoma in vitro [37]. Thus, dual therapy of Bcl-2 inhibitors with a second therapeutic class, including AKA inhibitors, are promising for a variety of malignancies including pancreatic cancer.

Conclusion

Current systemic therapies such as gemcitabine-based regiments and FOLFIRINOX for metastatic pancreatic cancer are limited by toxicity, and median survival is still under 1 year. Thus, there is a need for identifying targeted and better tolerated agents. Single agent BH3 mimetics that inhibit Bcl-2 family members appear to have limited efficacy in treating solid organ tumors, potentially in part due to failure to inhibit MCL-1 which is a potential resistance mechanism. Our studies show that addition of an AKA inhibitor to BH3 mimetic leads to mitotic arrest and degradation of MCL-1 resulting in synergistic cell death in pancreatic cell lines with confirmation of increased efficacy in both patient-derived pancreatic organoid models and mouse xenografts.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.tranon.2019.01.009.

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