Examining Novel Combination Therapies

for Pancreatic Cancer Treatment

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Worcester Polytechnic Institute Major Qualifying Project

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Abstract

This proposal explored new drug treatment plans for pancreatic cancer. The mTOR protein kinase activates a signaling network that effects cell growth substantially. Research has shown that unregulated mTOR activity is linked with cancer progression. Therefore, finding inhibitors of mTOR could halter cell growth, particularly those of cancer cells. AZD2014 is a selective inhibitor of the mTOR complex. DNA damage stimulators Doxorubicin, Olaparib, and Trametinib in combination treatments can cause serious cell cycle arrest and death in pancreatic cancer cells.

Experiments were completed with pancreatic cancer cells to observe the results of how effective the drug combinations were through p-AKT, total AKT, and Chk-1 signal detection and cell viability. P-AKT and Chk-1 levels indicate the amount of mTOR activity and DNA damage in the cells, respectively.

Introduction

The third leading cause of cancer death in the United States of America is pancreatic cancer. In 2018, an estimated 44,330 deaths were caused by pancreatic cancer. 10.7 individuals per 100,000 is the current pancreatic cancer death rate (Siegel, Miller, & Jemal, 2018). More than 85% of pancreatic tumor cases are pancreatic ductal adenocarcinoma (PDAC) cases. The average survival time of PDAC is less than six months, with only 3-5% of the patients reaching a 5 year survival time. This is caused by the cancer's rapid metastasis to the lymphatic system and other organs. PDAC is characterized by dense stroma of fibroblasts and inflammatory cells in the head of the pancreas. This condition, named desmoplasia, spreads to the surrounding tissues of the lymphatics, spleen, and peritoneal cavity. PDAC will eventually metastasize to the liver and lungs over time (Hezel, 2006). New advances in therapeutic targets and genomic profiles have led to opportunities of new treatments for pancreatic cancer and prolonging a patient's life. Overactive enzyme pathways are some of the current clinically targets for these new cancer treatments.

One such enzyme is the mechanistic Target of Rapamycin (mTOR). mTOR regulates many cell processes, and coordinates cell growth and metabolism (Saxton & Sabatini, 2017). The mTOR protein kinase activates a signaling network that effects cell growth substantially. Research has shown that mTOR overactivity is linked with cancer progression and mTOR inhibition decreases tumorigenesis (Saxton & Sabatini, 2017) (Driscoll-Morten et al., 2016). Therefore, finding inhibitors of mTOR could halter cell growth, particularly those of cancer cells.

mTOR has two main distinct complexes, mTOR Complex 1 and 2 (mTORC1 and mTORC2), shown in Figure 1 (Meng, Frank, & Jewell, 2018). mTORC1 mainly works to promote protein synthesis by phosphorylation to regulate mRNA translation. mTORC2 works to phosphorylate AGC protein kinases to effect proliferation of the cell. mTORC2 also activates AKT, which promotes proliferation and cell

growth. AKT is in the AGC family of kinases. AKT is a downstream effector of KRAS, an important protein in the signaling pathway for cell proliferation and differentiation). Inhibiting mTOR will also disrupt the KRAS pathway. If mTOR were effectively inhibited, p-AKT levels in the cells would decline. Additionally, cell proliferation would decrease due to the inhibition of KRAS (Baines, Xu, & Der, 2011).
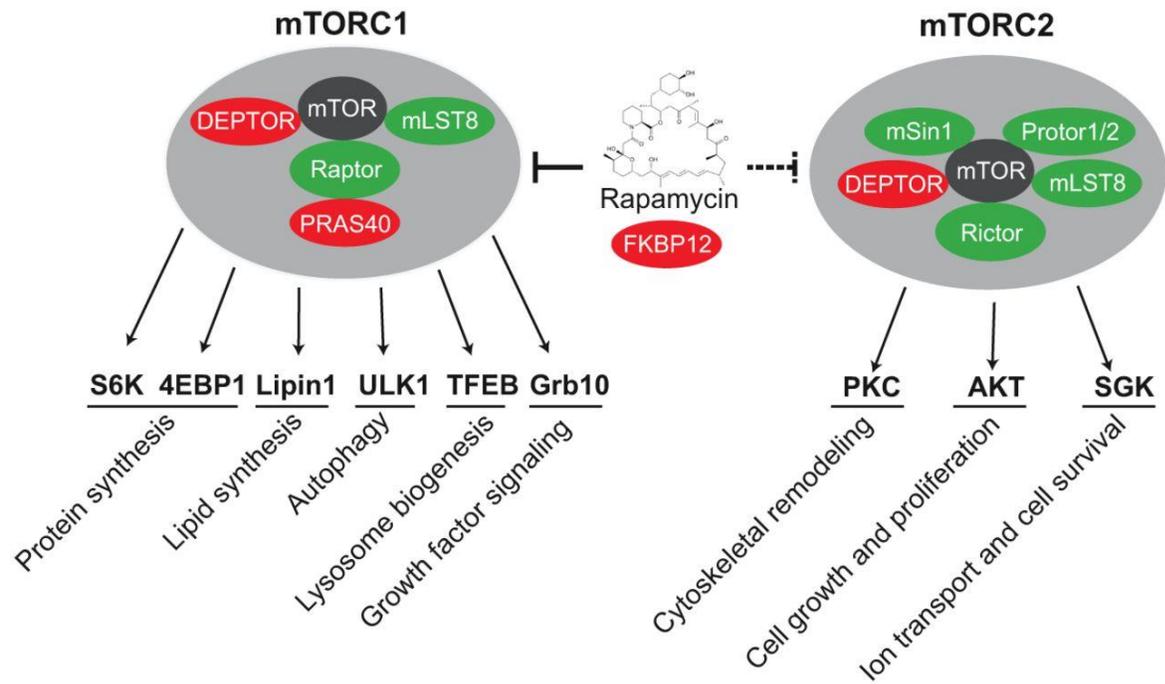

Figure 1. Components of the mTORC1 and mTORC2 complexes. The complexes of mTOR has many effects on cell biology. mTORC2 directly effects AKT which is related to cell growth and proliferation. Other subunits within the complexes have additional functions and can be targeted for gene knockouts. (Meng, Frank, & Jewell, 2018).

KRAS is an important gene in oncology. 33% of human cancers have an oncogenic mutation of *RAS* genes. These three genes, KRAS, NRAS, and HRAS, encode for similar proteins. *KRAS* mutations are associated with 21.6% of human cancers and account for 86% of *RAS* mutations (Baines et al., 2011). *KRAS* is the mutated gene in roughly 90% pancreatic cancer cases (Driscoll-Morten et al., 2016). It is believed that *KRAS* causes formation of precursor lesions of PDAC called Pancreatic Intraepithelial Neoplasia (PanINs).

Direct anti-Ras inhibitors have been clinically ineffective. Thus, work with indirect inhibitors of

Ras membrane association and downstream signalers has been expanded (Baines et al., 2011).

MEK/ERK, PI3K/AKT, and RAL are all downstream effectors of KRAS (Dienstmann, Kodon, Serra, &

Tabernero, 2014). PI3K regulates cell growth, cell cycle entry, cell survival, and metabolism (Driscoll-

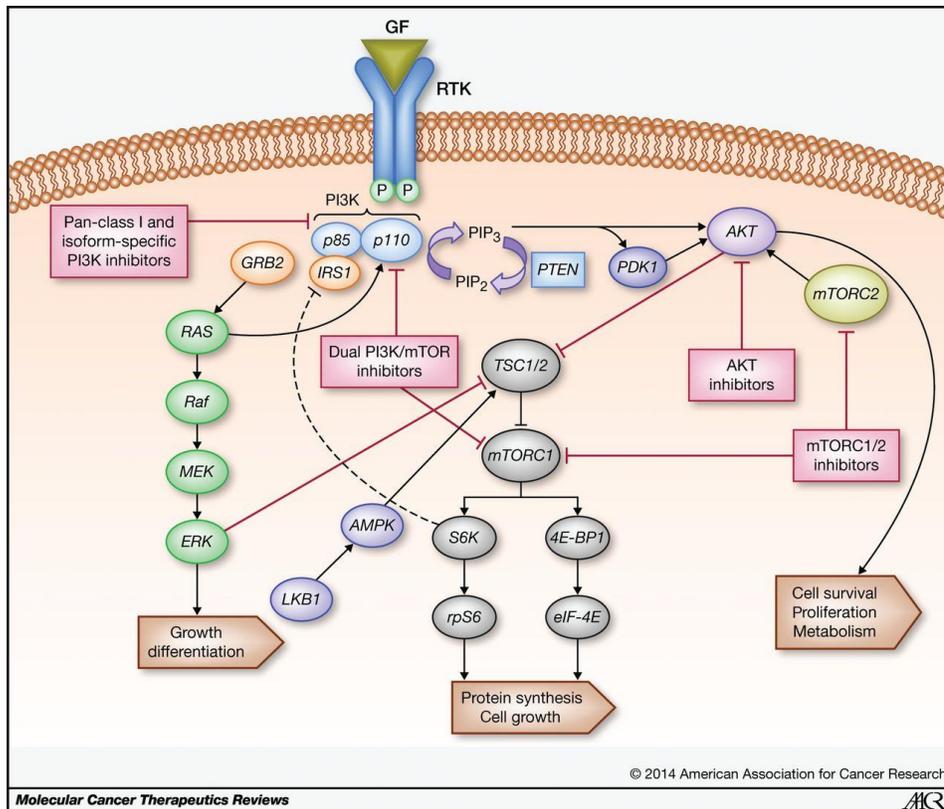Morten et al., 2016). In Figure 2, the relationships between these proteins and others are expressed.

Figure 2. Overview of the PI3K/AKT/mTOR pathway and drug targets. This diagram highlights some of the pathways in relationship with mTOR. The RAS pathway is showcased on the left in green, and the mTORC1 pathway is in the center colored grey. On the right, the relationship between mTORC1/2 inhibitors and the AKT values are shown. Overall these relationships affect the cell's growth, differentiation, protein synthesis, survival, proliferation and metabolism. (Dienstmann, Rodon, Serra, & Tabernero, 2014).

The drug AZD2014 prolongs survival in late stage tumors by inhibiting mTORC1/2. The Driscoll-

Morten group attempted to pair AZD2014 with the common chemotherapy drug, gemcitabine. This test

did not result in prolonged cancer survival, although the treatment worked in mice with earlier stage

tumors. The group began looking at other small molecule drugs to add, such as AZD8186. AZD8186 is a PI3Kβ kinase inhibitor, and therefore should disrupt the pathways shown in Figure 2. The Driscoll-Morten group observed a reduction of cell viability of the tumors, and extended survival in mice with pancreatic cancer. This experiment established evidence that AZD2014 drug combination can be effective treatment against pancreatic cancer (Driscoll-Morten et al., 2016).

Drug cocktails can be dangerous but also effective. Drug combinations can have more side effects, more toxicity in the body, and unknown complications. However, some treatments work tremendously better in combination. For example, one drug could knock down the ability of DNA to replicate, while another works to stop protein transcription. Alone, they might not be efficient enough to stop an illness, but together they could become an effective treatment. Additionally, when using drug combinations, sometimes the individual doses can be lowered due to the partnership chemical effect. Chemotherapy drugs are known to have adverse side effects, therefore lowering the doses of each individual drug would decrease the possibly of such side effects in patients.

This proposal was written with the purpose to explore new drug treatment plans for pancreatic cancer. In combination with current pancreatic cancer drugs, the results of this study will be directed in creating more effective drug combinations, by introducing small molecular inhibitors with AZD2014. This study will be done by cell culturing pancreatic cancer cells, PaO 3C. Tests would completed to observe the results of how effective the inhibitors were through phosphorylation signal detection. Productive inhibitors will be cell cultured with pancreatic cancer cells, for a Western blot analysis and treated with various pathway indicator antibodies.

Chemotherapies Treatments with AZD2014

AZD2014 in conjunction with proven chemotherapy drugs could lead to very interesting results in cell proliferation and cell survival. This proposal will look at the following drugs for treatment:

AZD2014, Doxorubicin, Olaparib, and Trametinib. This section will explore the history of AZD2014 and the possible combination drugs for PDAC treatment.

Drugs for Treatments

mTOR inhibitor drugs have been designed but unfortunately have been found to be less successful than expected. Some of these are rapamycin derivatives called "rapalogs". Rapalogs delay cell aging. Research has suggested than dual treatments of rapalogs and direct mTORC1 inhibition could bring about beneficial results. Rapamycin as a solo treatment is not an effective treatment (Saxton & Sabatini, 2017).

Recent development has shown that small molecule inhibitors might work better than rapalogs. In the paper "mTORC2 signaling drives the development & progression of pancreatic cancer" by Driscoll-Morten et al., research was focused on finding mTOR inhibitors. Small molecule inhibitors target both arms of mTOR and block the kinase activity. They had found that using just mTORC1 inhibitors failed to increase survival rate of mice with pancreatic cancer. When the gene of mTORC2 subunit *Rictor* was deleted, delayed tumorigenesis was observed. Treatment of a combination of rapamycin and a mTORC1/2 inhibitor led to suppressed tumorigenesis, but alone rapamycin was not effective. It was concluded that the combination of mTORC1/2 and PI3K inhibitors led to increased survival in late-stage pancreatic cancer in mice (Driscoll-Morten et al., 2016).

Small molecule inhibitors target both arms of mTOR and block the mTOR kinase activity. AZD2014 inhibits the phosphorylation of AKT (p-AKT), p-S6, and other proteins (Selleck Chemicals, 2013). The inhibition occurs by suppressing phosphorylation of both mTORC1 and mTORC2, the latter being more efficiently suppressed. The drug also blocks signaling downstream of mTOR because of increased concentration of nuclear p27 and pERK (Selleck Chemicals, 2013).

AZD2014 (Vistusertib) has a molecular weight of 462.54 g/mol and a solubility of 92 mg/mL in

DMSO (U.S. National Library of Medicine). AZD2014 is a selective inhibitor of mTOR kinase, and

compares to rapamycin, it has a greater effect on inhibition activity of mTORC1. AZD2014 has been

proved to stop cell growth and cause cell death in breast cancer cells, and stops cell proliferation in

many other cell lines. p-AKT (ser473) and p-NDRG1, mTORC2 biomarkers, levels are decreased through

AZD2014 treatment (Selleck Chemicals, 2013). This drug was selected by the Driscoll-Morten et al. group

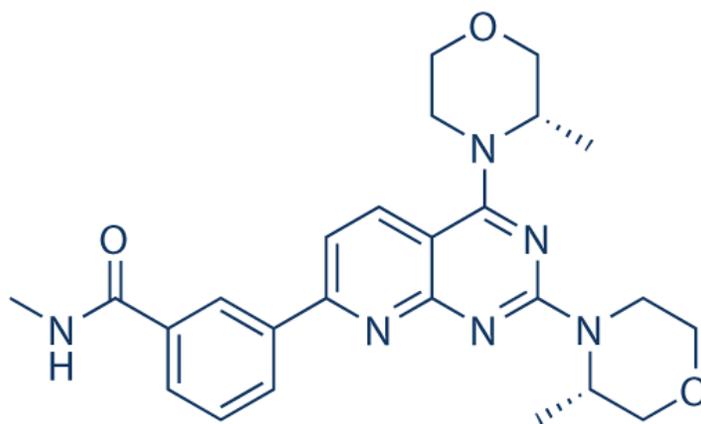do to its stability and past knowledge of its proficiency to inhibit mTOR (Driscoll-Morten et al., 2016).

Figure 3. Vistusertib (AZD2014). A molecular drawing of the structure of AZD2014. (Selleck Chemicals, 2013).

While AZD2014 works to impair cell proliferation, it does not fully stop tumor progression. Other

chemotherapy drugs can cause the PDAC cells to be more susceptible to AZD2014's treatment. AZD2014

increases the sensitivity of the cells, and when paired with a DNA damage drug, it could lead to decrease

cell viability. A variety of DNA damage causation drugs were examined in this primary research.

Doxorubicin and Olaparib are two working chemotherapy drugs. Trametinib is in the beginning stage of

human treatments. These drugs were chosen because of their already stable condition, known side-

effects, and their current state in clinical trials. These drugs all cause damage to DNA or permit the

replication of damaged DNA. DNA damage greatly reduces the chance of cancer cell survival. These
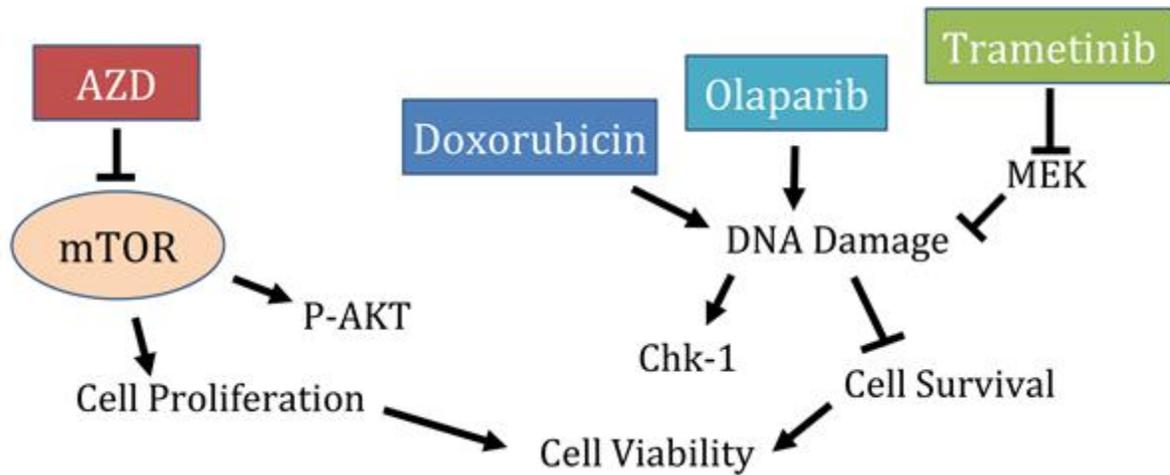
relationships are shown below in Figure 4.

Figure 4. Drug effects on cell viability. AZD2014 inhibits cell proliferation, and Doxorubicin, Olaparib, and Trametinib inhibit cell survival. Together, these drug work in combination to decrease cell viability.

Doxorubicin is a common cancer treatment drug (Cleveland Clinic Cancer Center). It is already

well used in human treatments for a variety of cancers. The drug works as a DNA topoisomerase

inhibitor. Topoisomerase relaxes DNA from its supercoiled form for transcription to occur. Doxorubicin

prevents the DNA helix from resealing, and therefore stops replication (Cleveland Clinic Cancer Center).

Olaparib is also a chemotherapy drug. It is commonly used with patients with the BRCA1 and

BRCA2 mutations (Prasad-Narayan et al., 2017). Olaparib inhibits PARP (poly ADP ribose polymerase),

which repairs single strand DNA breaks. Without PARP, DNA that has been mutated cannot replicate.

Any DNA that is mutated, and therefore does not match its strand counterpart, cannot replicate. This is

beneficially for stopping cancer cells, which have many mutations.

Trametinib is a MEK inhibitor. This drug works to stop mitogen-activated protein kinase kinase

enzymes MEK1 and/or MEK2 (Schräder-Ludwig et al., 2018). MEK is involved with cell proliferation and

transcription regulation. Inhibiting MEK can allow damaged DNA to be replicated. Trametinib also

affects the MAPK/ERK pathway, which is downstream MEK and Ras, and therefore can be unregulated in some cancers. There has been success with treatment of Trametinib in BRAF/KRAS mutation cancers (Schräder-Ludwig et al., 2018).

AZD2014 will impair proliferation and increase the sensitivity of the cells to survival, and along with Doxorubicin, Olaparib, and Trametinib can cause serious cell cycle arrest and death. This proposal's goal is to uncover how AZD2014 works with cell DNA damage drugs to lower proliferation of PDAC cells.

Approach

As seen in *mTORC2 signaling drives the development and progression of pancreatic cancer* by Driscoll-Morten et al., different drug combinations can be more effective than a single drug treatment. Small molecule screening facility can test hundreds of drugs and small molecules rapidly in a 96-well plate assay. Cell viability is tested using fluoresce through GFP tagging. Rapid pace drug combinations can be assayed, with and without AZD2014, at the Small Molecule Screen Facility at UMass Medical School. Other chemotherapy drugs can also be used to work in combination with AZD2014. Effective combinations would be ones that inhibit different pathways downstream of Ras, mTOR, and Chk-1 (a marker for DNA damage), and work with AZD2014—the mTOR inhibitor—to control cancer cell growth.

Using immunoblots, p-AKT, AKT, and Chk-1 protein levels can be isolated. The amount of p-AKT is relative to the amount of mTOR. In order to analyze phosphorylation levels, controlling for total AKT levels is necessary. Downregulation of mTOR dos not affect the amount of AKT present, rather, it decreases the phosphorylation of AKT. Therefore, the levels of AKT are expected to be equal regardless of the drug treatment and are used to compare phosphorylation levels. Chk-1, also known as Checkpoint

kinase 1, which is activated by DNA damage. Cell survival is inversely related to the amount of Chk-1, because damaged DNA decreases the cell's ability to survive.
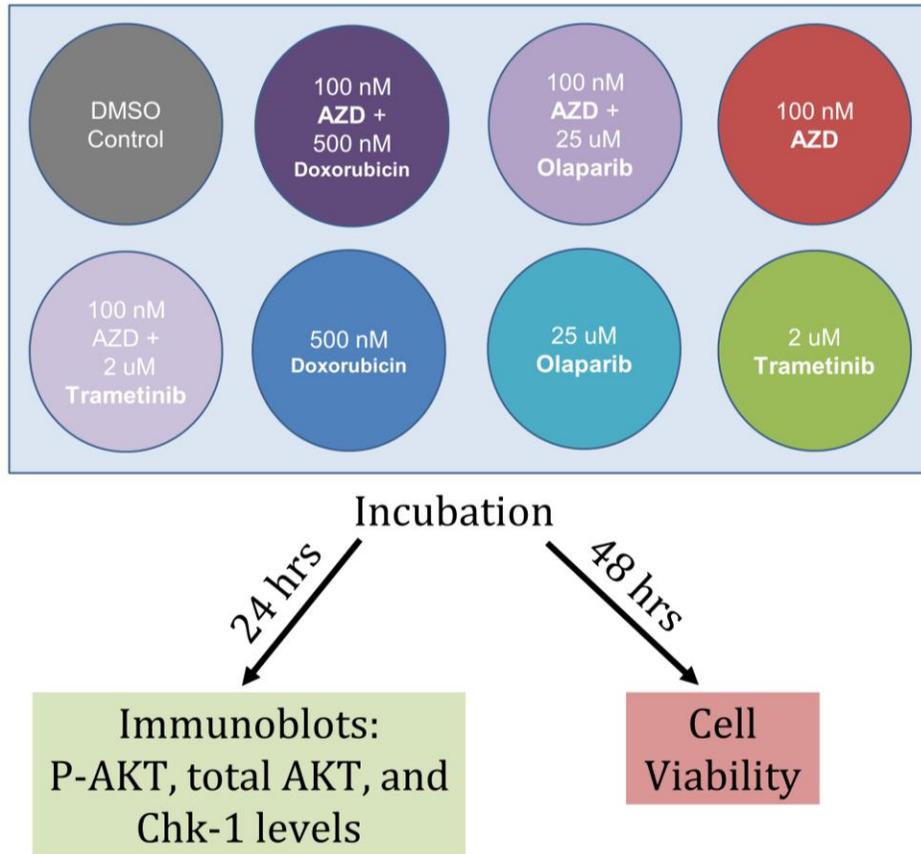

Figure 5. A visual diagram of the experimental design. The cells were grown in 6-well plates and treated with the above drug concentrations. After 24 hours and 48 hours of incubation, the cells were collected for immunoblot protein probing and cell viability assays respectively.

The following protocols were followed to investigate this proposal.

PDAC Cell Culture

PaO 3C Cell lines were cultured in a sterile cell culture hood room and incubator. The media used was a DMEM (Dulbecco's Modification of Eagle's Medium) with 4.5 g/L glucose and L-glutamine without sodium pyruvate made by Corning. 10% FBS was added to the media, along with a 1% penicillin/streptomycin. Plates were cultured and split by standard protocol. For example, the plate would be placed in the cell culture hood with clean standard protocol. The media was removed using

suction and the plate was washed with ~4-5 mL of PBS. 1 mL of 37°C warm 0.05% Trypsin-EDTA (made by GIBCO) was added to the plate. The plate was then placed back into the incubator for ~12 minutes until the cells had fully detached from the plate. The trypsin treated cells were collected by adding 5 mL of media to the plate. The 6 mL were put in a tube and centrifuged at 400 rpm for 3 minutes at 22°C. The tube was placed back in the sterile environment and the supernatant was removed using suction. The pellet was suspended in media (2-8 mL depending on number of dishes the cells were being split onto) and 2 mL of cell media were put in a single dish, along with 8 mL of fresh media. These dishes were then stored in a 37°C, 5% CO2 incubator. Doubling time of PDAC cells is relatively slow, from 10% to full competency in 5 days.

Protein Extraction – Whole Cell Lysate

The cell dish was brought to the bench. The media was suctioned out and the dish was washed with ~3-4 mL of PBS. The dish was placed at a 45-degree angle to get collect all the PBS by suction. Radio Immuno Precipitation Assay (RIPA) lysis buffer (50 mM pH 7.4 TRIS-HCl, 150 nM NaCl, 1% NP-40, 0.5% Sodium deoxycholate, 0.1% SDS with protease inhibitor) by Boston BioProducts was used for cell lysis. Situationally, 150 μL for a 10 cm, and 18 μL for each well in a 6-well plate. The dish was scraped to collect cells at the bottom of the dish. The solution was then collected and put into a centrifuge tube.

Each tube was put on the cold room rotator for 30 minutes. The tubes were then spun at 13.2 rpm for 10 minutes in the cold room. The supernatant was collected and placed in a new tube, which was then put in the -80°C freezer.

Protein Concentration Analysis

Protein concentration was measured by spectrophotometer. A 1:5 dilution of red protein assay solution was made. 1 μL of thawed cell lysate was inserted into 1 mL of red protein assay solution. Protein concentration was found using the following equation:

$$\frac{A \ value}{0.425} * 10 = Protein \ concentration \ (\frac{\mu g}{\mu L})$$

Western Blot – SDS-PAGE

Western blot gels were made to ProtoGel 10% separation instructions. 15 µg of protein were loaded into each well and run with 1x SDS-PAGE buffer. Concentration of the protein sample was factored in to correctly mix µL of protein with equal amount of 2x blue dye, and raised with 1x blue dye until the total volume was 10 µL. For example, if the protein concentration was 14.965 µL, the amount of protein added was 15/14.965 = ~1 µL. 1 µL of 2x blue dye and 8 µL of 1x blue dye were added to make a 15 µg in 10 µL solution.

The gel was run with 1x SDS-PAGE buffer and then transferred into a sandwich with an immunoblot membrane. The sandwich was placed in a 1x transfer buffer tank with an ice pack and run at 100 V for 1 hour. The membrane was then collected for antibody treatment.

Immunoblot Antibody Treatment

The membrane was blocked with 5% milk in TBST solution for 1 hour. The membrane was then treated with the 1° antibody solution in milk overnight on the cold room shaker. The membrane was then washed three times with 1x TBST for 5 minutes on a shaker. The membrane was then treated with the 2° antibody solution, anti-rabbit or mouse depending on 1° antibody, in milk for 1 hour on a shaker. The 1x TBST washes were repeated. 600 µL of ECL were prepared and put on top of the membrane in a tray. After three minutes incubation, the membrane was placed in a sheet protector and read by a ChemiDoc touch Imaging Sensor. Before the next treatment of antibodies, the membrane was treated with Western Blot Stripping Buffer for 15 minutes and then had 3 washes of 1x TBST for 15 minutes each on the shaker. The membrane was then blocked with 5% milk for 1 hour on the shaker.

For p-AKT analysis, the 1° antibody was α- rabbit-IgG HRP p-AKT 1:1000 dilution in 5% milk, and the 2° antibody was α-rabbit-IgG HRP p-AKT 1:2000 dilution in 5% milk. The ChemiDoc was scanned for a Chemiluscence run for 15 images over 1-120 seconds, and Colormetric run with auto rapid exposure. These images were overlaid to get a clear image of the ladder and protein samples bands.

AZD2014 Concentration Experiment

AZD2014 (Vistusertib) was ordered from Selleckchem and received as a 10 mg powder. Using 4.3240 mL of DMSO, the powder was suspended into a 5mM concentration and placed in the freezer for storage. Aliquots of 100 mM concentration were created and frozen for easy treatment usage. Once the Pao 3C cells in the 6-well plate reached 95-100% competency, the following concentrations in 3 mL of medium was put in the wells, so to make two wells of each concentration: 0.001% DMSO in media, 50 nM AZD2014 in media, and 100 nM AZD2014 in media. The cells were treated for 2 hours, and then the cell lysate was extracted and froze for later analysis. The protein samples were run in a Western blot and the membrane was treated with p-AKT & AKT antibodies.

This experiment was repeated with an additional 200 nM concentration of AZD2014 dose. The protein samples were run in a Western blot and the membrane was treated with p-AKT antibodies.

Drug Combinations Experiment

The chemotherapy drugs Doxorubicin and Olaparib, and Trametinib, a MEK inhibitor, were ordered and received as powders. The powders were suspended in DMSO to make the following concentrations for cell culture experiments: 500 nM Doxorubicin, 25 uM Olaparib, and 2uM Trametinib. The doses were selected from the perspective sources on the drugs (Prasad-Narayan et al., 2017) (Schräder-Ludwig et al., 2018).

8 wells, in triplicate, were plated with PAO 3C cells. Once the PAO 3C cells in the 6-well plate reached 95-100% competency, the following concentrations were added. First, the AZD or DMSO control

media was added to the well. Then after two hours of incubation, the additional drug was added. After

20 hours post-secondary drug, the cells were put on ice and lysed.

Table 1: Drug Combination Experiment Protocol

Well (Number and Abbv.)	Time = 0 hours addition	Time = 2 hours addition
1 – C	3 mL of media with 3 μL DMSO	n/a
2 – A, Dox	3 mL of media with 100 nM AZD2014	500 nM Doxorubicin
3 – A, Ol	3 mL of media with 100 nM AZD2014	25/50 μM Olaparib
4 – A	3 mL of media with 100 nM AZD2014	n/a
5 – A, M	3 mL of media with 100 nM AZD2014	2 μM Trametinib
6 – Dox	3 mL of media with 3 μL DMSO	500 nM Doxorubicin
7 – Ol	3 mL of media with 3 μL DMSO	25/50 μM Olaparib
8 – M	3 mL of media with 3 μL DMSO	2 μM Trametinib

This table presents the information of the amount of media added to each well. At time = 0 hours, the media was changed to the above type. After two hours, the second additional drug was added. N/A means that no additional drug was added after 2 hours of incubation.

Table 2: Drug Combination Experiment Doses

Drug	A blot	B blot	C blot
DMSO Control	n/a (equal volume to AZD)	n/a	n/a
AZD + Doxorubicin	100 nM, 500 nM	100 nM, 500 nM	100 nM, 500 nM
AZD + Olaparib	100 nM, 50 uM	100 nM, 50 uM	100 nM, 25 uM
AZD	100 nM	100 nM	100 nM
AZD + Tris	100 nM, 2 uM	100 nM, 2 uM	100 nM, 2 uM
Doxorubicin	500 nM	500 nM	500 nM
Olaparib	50 uM	50 uM	25 uM
Trametinib	2 uM	2 uM	2 uM

This table presents the drug doses that were in each well of each trial of the experiment.

The cells were lysed with 25 μL lysis buffer for the first two trials. The third trial was done so the

suspended media was additionally spun down with the tryspinized plate, to collect all the cells in the

well. This pellet was lysed with 80 μL lysis buffer. Proper whole cell lysate protocol was implemented.

The protein samples were run in a Western blot and the membrane was treated with p-AKT antibodies. The first two trials were run in a 10% gel, and the third trial was run in a 7.5% gel.

Cell Viability with GFP Infected Cells

PaO 3C cells were infected with a GFP virus and grown in a 0.2 mg/mL puromycin concentration in media. Once cells were at 100% confluency in a 10 cm plate, 1/25 of the cells were plated into each well of 2 6-well plates. These plates were given 24 hours to adhere to the plates. Then the cells were treated with the same doses of AZD2014, Doxorubicin, Olaparib, and Trametinib as Experiment C. Additionally, the AZD2014 was treated to two wells.

After 48 hours of drug incubation, the plates were tryspinized and the cells were collected via centrifugation. The cells were resuspended with 500 uL of Cell Titer Blue. Cell viability was measured by a Bio-Rad TC20 Automated Cell Counter.

Expected results

It was expected that cells treated with AZD2014 would have inhibition of mTOR and therefore lower values of p-AKT in the cells. Additionally, cells that were treated with either Doxorubicin, Olaparib, or Trametinib would have increased DNA damage and therefore have higher amounts of CHK-1 in the cells. And finally, it was expected that the combination of AZD2014 and a DNA damage drug would decrease cell viability. These relationships are shown below in Figure 6.
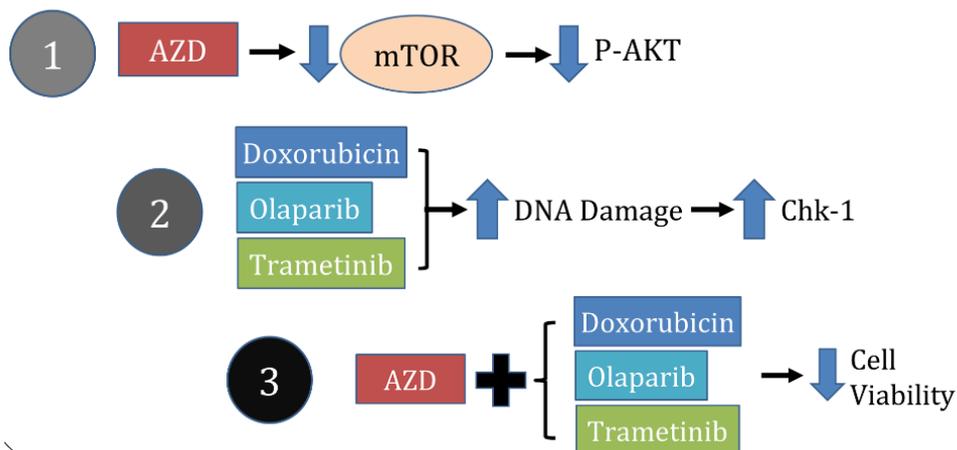
Figure 6. The expected results from the drug dose experiment. 1) AZD2014 addition will lead to decreased levels of p-AKT. 2) Doxorubicin, Olaparib, and Trametinib additions will lead to increased levels of Chk-1. 3) Combination of AZD2014 with Doxorubicin, Olaparib, or Trametinib will lead to decreased cell viability.

p-AKT and total AKT

p-AKT has a molecular weight of 60 kDA. The three immunoblots were labeled A, B, and C. As per table one, the protocol for the drug incubation and cell culture were all identical expect for the Olaparib concentration was lowered for blot C. AKT also has a molecular weight around 60 kDA, although the addition of the phosphonate group would make p-AKT slightly heavier. The immunoblots were probed for p-AKT and then total AKT and had the following resolution:

Figure 7. The p-AKT and total AKT immunoblots of the drug combination treatments. The experiment was done in triplicate and each blot A, B, C were done separately. P-AKT probing is the top image, and the total AKT probing is underneath it.
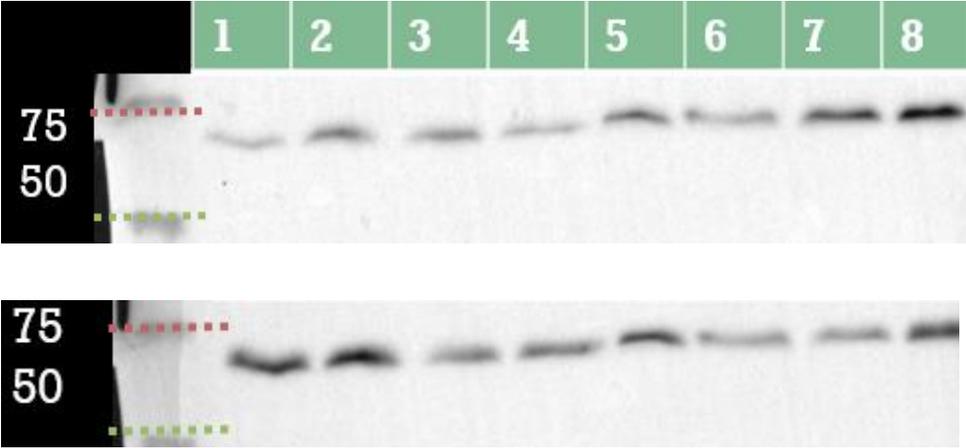
(A)

(B)

(C)

The figure is laid out so the p-AKT values are on top, and the total AKT values are on the bottom of each pair. As mentioned above, the columns are the following treatments: (1) DMSO Control (2) AZD + Doxorubicin (3) AZD + Olaparib (4) AZD alone (5) AZD + Tremetinib (6) Doxorubicin (7) Olaparib (8) Trametinib.

p-AKT amounts are indicators of the mTOR inhibition, which is caused directly by AZD2014.

Since there was not a cell standard used, it is hard to interpret the results for these immunoblots. In

blots (A) and (B) and bands were extremely faint for the AZD + Olaparib, and Olaparib alone treatments;

at first glance it might seem that the p-AKT values are low, but really the amount of general protein is

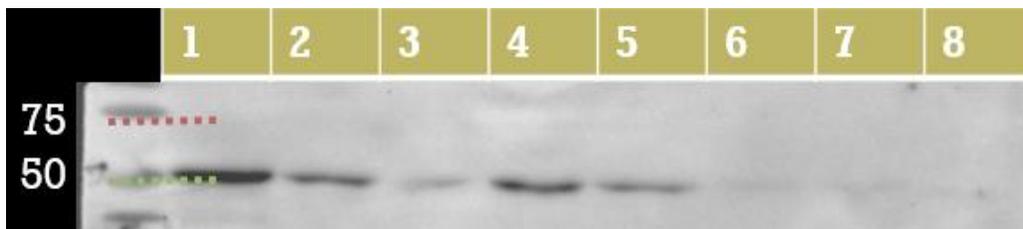lower than the other wells probably due to a lower cell count.

CHK-1

CHK-1 is 54kDA in weight. The three immunoblots were probed for CHK-1 and had the following resolution:

Figure 8. Immunoblots of CHK-1 . The experiment was done in triplicate and each blot A, B, C were done separately.
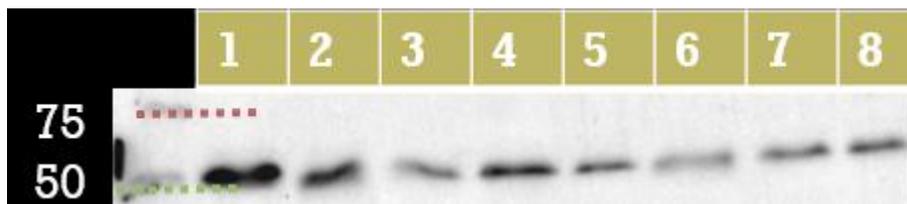
(A)

(B)

(C)

In Figure 8, the columns are the following treatments: (1) DMSO Control (2) AZD + Doxorubicin (3) AZD + Olaparib (4) AZD alone (5) AZD + Tremetinib (6) Doxorubicin (7) Olaparib (8) Trametinib.

CHK-1 values are lower in all treatments compared to the DMSO control dose. Inconclusively, the cells treated with DNA damage drugs had lower amounts of Chk-1 than cells without DNA damage drugs.

Cell Viability

Table 3: Cell Viability of PDAC cells after incubation in drug combinations.

Drug	Percent cell viability
DMSO Control	98%
AZD	100%
AZD	100%
AZD; Doxorubicin	N.D.*
AZD; Olaparib	N.D.
AZD; Trametinib	92%
Doxorubicin	N.D.
Olaparib	N.D.
Trametinib	100%

This table shows the cell viability of the cells collected after 48 incubation post drug injection. *N.D. = all dead. The cell count was below the threshold of machine.
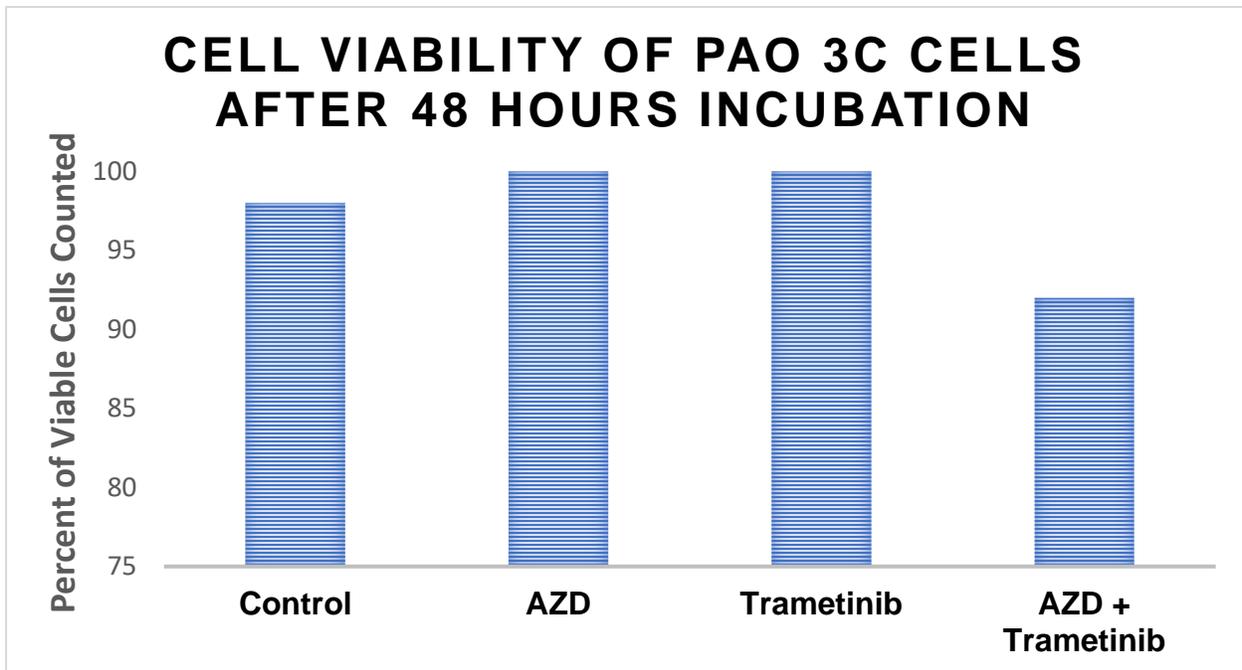
Figure 9. Cell Viability of PaO 3C cells after 48 hours of incubation with drug combinations. Shown are the four treatments of DMSO Control, AZD2014, Trametinib alone, and AZD2014 + Trametinib. Since the other treatments had a N.D. cell viability, there data is not shown above. N=1, AZD alone N=2.

Some cell counts were so low, that the machine could not read the percent of viable cells, due to the fact a majority of the cells were dead. Of the cell viability counts available, the AZD2014 and Trametinib combination yielded the lowest percent.

Appearance of the cells were cataloged. Cells incubated with Olaparib showed a great amount of disadheration after 24 hours. This is most likely due to the death of the cells, and therefore they become detached from the plate.

Overall, Trametinib in conjunction with AZD2014 could possibility be a good drug treatment plan for pancreatic cancer, due to the possibility of mTOR inhibition and decrease of cell viability.

Discussion: potential pitfalls

Every experiment can be expedited, and the best have low p-values. To receive conclusive data, I recommended the experiments being repeated. Additionally, there are other potential pitfalls that I believe should be addressed.

The immunoblots were executed without a cell standard to proper quantify the number of cells and protein in the plate. A good loading control of above 15 ug/ul would also be useful. Also, drug uptake into the cells was not measured.

It is possible the dose or volume of Olaparib was too high for the PDAC cells. The Olaparib was in quite a high volume of DMSO for a 6 well plate. The amount of disadhersion after 24 hours was much more for Olaparib treated cells than the other treatments. I recommend repeating the experiment with a lower dose of Olaparib. Perhaps the inhibition will still occur but be less dramatic.

Discussion: alternate approaches

ATM/ATR is a kinase that regulates Chk-1 and 2 during the cell cycle. ATM becomes activated by DNA damage. The kinase acts as a mediator through phosphorylation, recognizing double strand breaks and replication stress. When ATM is inhibited, unregulated cell reproduction occurs. If the cell goes through with cell replication, the damaged DNA gets passed on. It is hard for a cell to survive with quantifiable DNA damage. Focused experiments on ATM/ATR inhibitors could yield interesting cell survival data, especially in junction of AZD2014.

Receiving data from the Small Molecule Screening Facility could provide insight on other drugs to complete trials with AZD2014. Other cell proliferation and survival effectors, or more DNA damage causing drugs could yield interesting results.

As work with Driscoll-Morten et al. showed, gene editing could work well in conjunction with AZD2014. Genetic engineering for Rictor or parts of mTOR knockout. Additionally, gene editing out the genes for Chk-1. CRISPR could yield some interesting results when attempted to knock out Chk-1.

Conclusion

This proposal's goal was to uncover how AZD2014 works with cell DNA damage drugs to lower proliferation of PDAC cells. AZD2014 impairs proliferation and increases sensitivity to chemodrugs such as Doxorubicin, Olaparib, and Trametinib. With more time invested in different approaches and drugs combination, these treatments could prove effective against pancreatic cancer cells.

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