Examining FOLFIRINOX Resistance in Pancreatic Cancer through the Creation of Resistant Cell Culture Models

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ABSTRACT

Pancreatic ductal adenocarcinoma (PDAC) is an extremely aggressive form of cancer with a 5-year survival rate of 8.5%. PDAC is often characterized by its early-stage metastasis, resistance to cytotoxic chemotherapy, and extremely poor prognosis. A leading treatment for patients who can tolerate its severe side effects is a cocktail of 4 chemotherapeutic drugs, leucovorin, 5-fluorouricil, irinotecan, and oxaliplatin (FOLFIRINOX). Despite FOLFIRINOX being a leading treatment for patients with pancreatic cancer, the cellular response to the treatment is poorly understood. This investigation focused on chemotherapy resistance to FOLFIRINOX in four PDAC cell lines. To determine initial sensitivity to the drug cocktail, PDAC cells were dosed with FOLFIRINOX at a ratio similar to that used clinically. Cell viability was assessed to calculate GI_{50} concentrations. The PANC 03.27 cell line was found to be the most sensitive to FOLFIRINOX with a GI_{50} concentration of 0.71 mM, while PANC 04.03 was found to have a GI_{50} value of 1.33 mM. These two cell lines were then cultured in FOLFIRINOX to create a clinically relevant model of chemotherapy resistance. To assess resistance, post-treatment GI_{50} assays were performed and demonstrated a 1.6-fold increase in FOLFIRINOX resistance in PANC 03.27 cells. Additionally, RNA Sequencing was performed on untreated control, DMSO control, and FOLFIRINOX-treated cells to identify changes in the transcriptome and possible mechanisms promoting chemotherapy resistance. Changes in gene expression accompanying resistance may lend insight into the underlying mechanism of resistance in FOLFIRINOX experienced by many patients with pancreatic cancer.

Introduction

Overview of Pancreatic Cancer

According to the American Cancer Society, cancer is the second leading cause of death in the United States [1]. Pancreatic cancer accounts for 7.5% of all cancer-related deaths despite only making up around 3% of all diagnoses. Of patients with pancreatic cancer, 95% will be diagnosed with a specific type: pancreatic ductal adenocarcinoma (PDAC). This aggressive form of cancer begins in the ducts of the pancreas but rapidly metastasizes to distant sites, most commonly the abdomen and liver. Despite a tremendous amount of research conducted by the scientific community, the five-year survival rate for all pancreatic cancers remains at 8.5% [2].

The low five-year survival rate is due to a host of unique properties of pancreatic cancer. Due to a lack of symptoms shown in early stages of the disease, PDAC is commonly diagnosed after it has spread, which is known as metastasis [3] and is usually a hallmark of poor prognosis and low survival rates in patients. At the time of diagnosis, 81% of PDAC cases have tumors which have metastasized [4], often making late-stage pancreatic cancer impossible to surgically remove. This aspect of the disease contributes to the overall median survival rate of only six months [5].

Even in cases where pancreatic cancer has not metastasized, patient outcomes remain poor. Despite 15%-20% of patients having resectionable tumors, 80% will relapse within five years of surgery [6]. Worse, over 60% will have relapsed within two years [6, 7, 8]. While most recurrent tumors will reappear at the site of the original tumor, it is possible for the cancer to reappear at distant parts of the body. These two patterns of recurrence contribute to a median survival time of 19 months post-resection [8].

Treatments for Pancreatic Cancer

As PDAC is often diagnosed post-metastasis, chemotherapy remains the best treatment option for many patients. Unfortunately, in addition to a lack of early-stage symptoms and high rates of recurrence, pancreatic cancer is highly resistant to many chemotherapy treatments currently available. One of the first treatment options known to be effective against pancreatic cancer was a drug known as gemcitabine. Gemcitabine was shown to increase the median survival time for patients with pancreatic cancer, especially when compared to other alternative treatments available [9]. Unfortunately, the response rate (RR) of gemcitabine monotherapy was found to be as low as 10%, meaning that only 1 out of every 10 patients would respond to treatment and see a shrinkage in tumor size [10]. When gemcitabine was paired with taxanes like nab-paclitaxel which work to arrest the cell cycle, its efficacy was drastically increased to 23% making it the new standard of care [11]. Although the combined therapy of gemcitabine and nab-paclitaxel was a major discovery in the fight against pancreatic cancer, better patient outcomes have been observed with other chemotherapy agents.

FOLFIRINOX, a combination of four different drugs: 5-fluorouracil, leucovorin, irinotecan, oxaliplatin, is a leading treatment option for pancreatic cancer in patients who can tolerate its severe side effects [12, 13]. The most concentrated drug in FOLFIRINOX is 5-fluorouracil (5-FU), which is an antimetabolite and uracil analog. Once inside of a cell, 5-FU is converted into fluorodeoxyuridine monophosphate (FdUMP). FdUMP then inhibits the enzyme thymidylate synthase (TS) which is necessary for the conversion of uracil to thymine [14]. This inhibition will result in less deoxyuridine monophosphate (dUMP) being converted into deoxythymidine monophosphate (dTMP), subsequently resulting in higher concentrations of deoxyuridine triphosphate (dUTP) and lower concentrations of deoxythymidine triphosphate (dTTP). Additionally, some FdUMP will be phosphorylated into fluorodeoxyuridine triphosphate (FdUTP).

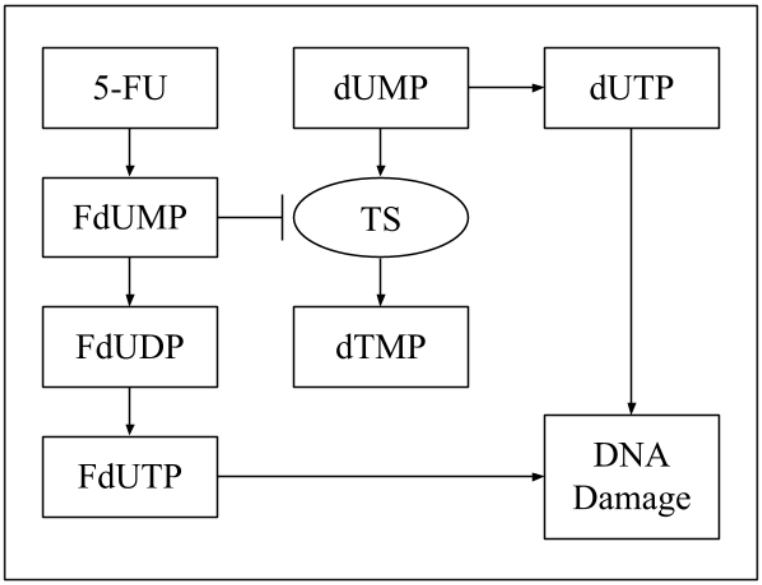

Figure 1: Mechanism of 5-Fluorouricil.

The 5-fluorouricil is converted into fluorodeoxyuridine monophosphate (FdUMP), an inhibitor of thymidine synthase (TS). Through the inhibition of TS, the incellular concentrations of fluorodeoxyuridine triphosphate (FdUTP) and deoxyuridine triphosphate (dUTP) increases and the concentration of deoxythymidine triphosphate (dTTP) decreases. This increases the likelihood of uridine base misincorporation into DNA, resulting in DNA damage [14].

Due to the inhibition of TS, the intercellular ratio FdUTP and dUTP to dTTP increases, increasing the likelihood of misincorporation into DNA. At a high enough ratio, FdUTP/dUTP misincorporation can overwhelm DNA systems leading to DNA breaks and cell death. The mechanism for 5-FU is summarized in Figure 1. Leucovorin is also often given with 5-FU to increase its binding affinity to TS and to reduce side effects [14] and these drugs work in conjunction with the other 2 drugs in the cocktail, oxaliplatin and irinotecan. Oxaliplatin has the ability to bind and crosslink DNA [15] which alters the structure of DNA promoting strand breaks and preventing DNA repair. Oxaliplatin works in conjunction with irinotecan, an inhibitor of topoisomerase I (TOP1). TOP1 is a key enzyme in DNA replication responsible for the relaxation of DNA supercoils [16]. Without the relaxation, DNA strand breaks occur leading to cell death. All four drugs in the FOLFIRINOX treatment are designed to produce irreparable damage to the cell. This damage results in cells undergoing programmed cell death, known as apoptosis.

FOLFIRINOX been shown to increase patient median survival to over 11 months, the best outcome of any available chemotherapy treatment. It is also reported to have a RR of 31.6%, a drastic increase from both gemcitabine monotherapy and gemcitabine nab-paclitaxel [10, 11]. Recent clinical trials directly comparing FOLFIRINOX to gemcitabine bound nab-paclitaxel have shown that FOLFIRINOX is associated with higher rates of partial response using the Response Evaluation Criteria in Solid Tumors (RESIST) guidelines [17]. Due to the increase in RR, significantly more patients were able to undergo a pancreatectomy to remove their tumor; however, overall survival rates between FOLFIRINOX and gemcitabine bound nab-paclitaxel remained similar [17]. For these reasons, FOLFIRINOX remains the preferred treatment for patients who can tolerate its increased toxicity. Patients given FOLFIRINOX experienced significant increases in grade 3 or 4 adverse events, such as neutropenia, thrombocytopenia, diarrhea, and sensory neuropathy, when compared to patients receiving gemcitabine [10]. Due to a drastic increase in toxicity associated with this treatment method, it is only recommended to who are able to tolerate its toxicity [18, 10].

Research Focus

Despite FOLFIRINOX being the current preferred first line treatment, a gap in the current literature exists. While multiple clinical trials have been conducted, very few scholars have examined the cellular response of PDAC cells to FOLFIRINOX in a laboratory setting. Given that the response rate of FOLFIRNIX is only 31.6% and that even the third of patients who do respond to the therapy ultimately stop responding as resistance develops, understanding how this chemotherapy regimen works on the molecular level is needed. This research project aimed to investigate the cellular response generate in PDAC tumors when exposed to the first-line therapy FOLFIRINOX, as well as gain insights into the mechanism of chemotherapy resistance post-exposure.

To accomplish these goals, this study generated a clinically relevant in-vitro model of PDAC chemotherapy resistance across multiple cell lines. PDAC cell lines were exposed to FOLFIRINOX to promote resistance. CellTiter Glo (CTG) cell viability assays were used to measure cell viability post-exposure to FOLFIRINOX. CTG reagent uses ATP as a co-factor in a luciferase reaction [19]. The enzyme luciferase acts on luciferin, Mg^{+2}, and ATP within a population of cells to produce oxyluciferin and luminesce. As ATP is a cofactor required for this reaction, CTG effectively measures the concentration of ATP within a population of cells as a stand in for the viability of a population. From the luminesce assay, the growth inhibition factor 50 (GI_{50}) can be obtained. The GI_{50} concentration is a measurement of the concentration of drug necessary to inhibit the growth of a population of cells by 50% which correlates with the sensitivity of the cell line to drug. Large GI_{50} concentrations indicate more resistance to FOLFIRINOX.

PDAC cell lines were treated with FOLFIRINOX repeatedly in order to promote the development of chemotherapy resistance. Post-treatment cell viability assays were then performed in order to quantify the increase in

chemotherapy resistance. Once resistance was achieved, cell lines underwent RNA sequencing (RNA Seq.) RNA seq. is a revolutionary technique which measures the presence and quantity of RNA in a population of cells. RNA seq. also sheds light on post-transcriptional modifications to transcripts, alternative splicing patterns, and mutations such as single nucleotide polymorphisms [20]. In addition to quantifying the transcription, RNA seq. data can also be used to compare two different populations. Through the comparison of FOLFIRINOX treated and control cells, the cellular response generated by the treatment can be measured.

This study has generated multiple PDAC cell lines resistant to FOLFINOX which will be made readily available to the scientific community for future investigation. Additionally, the data from viability assays and RNA Seq. may aid clinicians and researchers in subsequent investigations. This study also sheds light on how PDAC cells respond to the current standard of care, which may lead to new, targeted treatment options in the future.

Methods

Cell Culture

Four PDAC cell lines (PANC 02.03, 03.27, 04.03, 05.04) were generously donated from the Wood Lab at Duke University. The obtained cell lines were all extracted from the head of the pancreases in patients with PDAC in 1995 from different patients. Information on the obtained lines is readily available online [21]. Cell lines were cultured in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM F12) (Fisher Scientific, DMEM F12, 1:1, 1x) with 10% fetal bovine serum (Gemini Biological), 1% penicillin-streptomycin (Caisson Labs, 100x 1:1), and 1% L-alanine L-glutamine (Caisson Labs, 43.4 mg/mL, 0.85% saline). Cells were cultured in a humidified incubator at 37°**C with** 5% carbon dioxide**. Cells were split when they appeared to be between 70% and 100% confluent. Untreated cells were harvested to split using 0.25% trypsin solution** (Fisher Scientific, 1x). When storage was necessary, cells were frozen in Recovery cell culture freezing medium (Fisher Scientific) and stored in a -80°**C freezer until moved to long term storage in liquid nitrogen.**

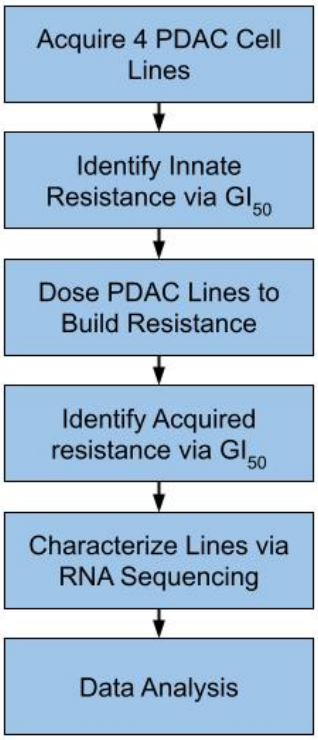

Figure 2 Summary of Methods. After acquiring 4 PDAC cell lines, GI_{50} assays were performed to determine innate resistance to FOLFIRINOX. Select cell lines were then dosed with FOLFIRINOX in order to promote chemotherapy resistance. After multiple rounds of treatment, GI_{50} assays were reperformed to quantify the change in resistance. Cell lines were then characterized via RNA sequencing.

Drug Preparation

In order to keep the experiment clinically relevant, drug dosages were kept at clinical ratios based on the current protocols [22]. The ratio of drugs can be found in table 1. The prepared stock solution was created based on the maximum concentration of 5-FU soluble in dimethyl sulfoxide (DMSO). **The final concentrations of the FOLFIRINOX stock solution are** 193.72 mM 5-FU (Selleck Chemicals), 1.81 mM oxaliplatin (Selleck Chemicals), 5.48 mM leucovorin (Selleck Chemicals), and 2.39 mM irinotecan (Selleck Chemicals). 5-FU, oxaliplatin, and irinotecan were dissolved in DMSO, while leucovorin was dissolved in water due to solubility concerns.

Table 1: FOLFIRINOX Drug Concentrations

Drug Name	Molar Mass (g/mol)	Clinical Dosage (kg/m^2)	Ratio	Concentration in Stock Solution
5-Flurouricil	130	2400	1:1	193.72 mM
Oxaliplatin	397	85	1:35	1.81 mM
Irinotecan	677	180	1:14	2.39 mM
Leucovorin	601	400	1:7	5.48 mM

Determination of Initial Drug Cytotoxicity

Confluent $25cm^3$ (T25) or $75cm^3$ (T75) flasks were used to conduct drug cytotoxicity assays. Cells were harvested using 0.25% trypsin and transferred to a 15 mL centrifuge tube (CellTreat). The walls of the flask were rinsed well

with media prior to transfer. Cells were centrifuged in a Sorvall ST40R Centrifuge at 1200x gravity for 5 minutes. Media was aspirated off and a single cell suspension in 3mL of DMEM-F12 was achieved. 100 uL of cell solution was then removed, placed in a counting cup, and diluted with 5 mL of diluent (Beckman Culter). The resulting solution was then counted in a Z1 Culture Particle Counter (Beckman Culter) to obtain the concentration of cells in the solution.

In order to perform the cell cytotoxicity assay, PDAC cell lines were seeded into white walled, clear bottom 96-well assay plates (Nest Scientific). Each well requires 5000 cells in 90 uL of DMEM-F12. In order to achieve the desired number of cells per well, a dilution of the cell solution was performed using Equation 1 The concentration of each solution, [Cell Solution], was obtained from the particle counter (cells/mL). V_{added} was the volume of cell solution added to achieve a total volume, $V_{totol.}$ The final volume was 10-12 mL in order to provide enough solution to fill the 96-well plate.

Equation 1 $$[Cell\ Solution] \cdot V_{Added} = \frac{5000\ cells}{0.09\ mL} \cdot V_{Total}$$

In order to treat cells, a drug plate was made in a 96-well clear V-bottom plate (Corning). A 1:10 serial dilution occurred down each column in the plate, giving a range of treatment dosages from 194 mM of 5-FU to 19.4 nM of 5-FU, with other drugs being scaled down to be delivered at their clinical ratio. A media plate was then generated by moving 10 uL of solution from the drug plate to a corresponding well filled with 90 uL of DMEM-F12 media. This resulted in a 1:10 dilution. The concentration of FOLFIRINOX in the media plate ranged from 19.4 mM 5-FU to 1.94 nM 5-FU, with other drugs being delivered at their clinical ratio. In addition to treatment dosages, each media plate also contained a column of DMSO control wells and a column of non-treatment wells to act as controls.

After a 24-hour waiting period which allowed cells to adhere to the 96-well plate, cells were treated with 10 uL of FOLFIRINOX solution from the media plate. This resulted in a second 1:10 dilution of FOLFIRINOX. The final dose given to cells ranged from 1.94 mM 5-FU to 0.194 nM 5-FU, with other drugs being delivered at their clinical ratio. Cells were treated for 72 hours in a humidified 37°**C incubator with** 5% carbon dioxide.

Next, a 15 uL aliquot of CTG reagent (Promega) was added to each well of the treated 96-well plate and stored in a dark cabinet for 10 minutes. The plate was then run on a EnSpire multimode plate reader (Perkin Elmer) in order to measure cell viability. All CTG assays were ran in triplicate. A ratio of the luminesce between the DMSO control well and each treated we then plotted against the concentration of 5-FU in each well in order to generate a GI_{50} plots. Preliminary GI_{50} plots can be found in figure 3. GI_{50} values were interpolated from each plot using a line of best fit drawn between the two points between which relative cell viability crosses 0.5. All GI_{50} values were run at least three times and then averaged to calculate the GI_{50} concentration for each cell line. GI_{50} values can be found in table 2.

Development of an In-Vitro model of FOLFIRINOX Resistance

Based on the GI50 assay results, PANC 03.27 and PANC 04.03 were chosen for future experimentation. Multiple models were employed in order to achieve multidrug resistance to FOLFIRINOX, although only the standard pulse method was ultimately successful.

Clinical Pulse Treatment

In clinical settings, FOLFIRINOX is given over a two-day period. Oxaliplatin (85 mg/m^2), leucovorin (400 mg/m^2), and irinotecan (180 mg/m^2) are given intravenously over a 2-hour period. 5-FU (400 mg/m^2) is then given on day two through an intravenous push lasting from 3 to 5 minutes, followed by an intravenous drip of 5-FU (2400 mg/m^2) for 46 hours post-push. In order to mimic clinical administration of FOLFIRINOX and keep results as clinically relevant as possible, a clinical pulse method was used. Cell lines were treated at their GI_{30} concentration of oxaliplatin, leucovorin, and irinotecan based on their clinical ratios to 5-FU. After 24 hours, treatment media was replaced with a 5-FU

treatment media for 48 hours. Treated cells were then allowed to recover in FOLFIRINOX-free media until confluent. Treatment was repeated when confluency was reached.

Extended Exposure Treatment

Confluent T75 flasks were treated and maintained at GI_{10} concentrations. All four drugs in FOLFIRINOX were given simultaneously throughout this treatment process. Treatment media was changed as needed throughout the experiment. Once confluent, cells were split and allowed three days to recover in non-treatment media before again being exposed to GI_{10} treatment media.

Standard Pulse Treatment

Both cell lines were exposed simultaneously to all four drugs in FOLFIRINOX at the GI_{10} concentration for 72 hours. The treatment for PANC 03.27 was reduced to its GI_5 value due to complications with cell viability post-treatment. Both cell lines were in 20 mL of DMEM F-12 in a T75 flask at the time of treatment. Following a three-day treatment period, cells were harvested using Accutase or (Thermofisher Scientific) or TripLE (Thermofisher Scientific, 1x) after 0.25% trypsin proved to reduce cell viability during harvesting post-treatment. Cells were then moved to a T25 flask. A Gelatin-Fibronectin (Sigma Aldrich, Thermofisher Scientific) pretreatment was later added to the T25 flask to future increase cell viability post-treatment. In order to apply the G/F pretreatment, a 3 mL aliquot of G/F was added to each flask and allowed to incubate at 37C for 1 hour. After 1-hour, excess G/F solution was removed and the flask was washed with phosphate buffered saline (GE Healthcare, Ca-/Mg-) 3 times before cells were added to the flask. Treated cell lines received media refreshes as needed to remove cells killed by the FOLFIRINOX treatment. Once a confluency of 70%-100% was reached, cells were split using Accutase or TripLE. The treatment process was repeated three times for PANC 03.27 and twice for PANC 04.03.

DMSO Control Treatment

Many FOLFIRINOX drugs were dissolved in DMSO during treatment. As DMSO is cytotoxic, DMSO control cells were generated in order to ensure that DMSO did not significantly alter RNA expression. In order to generated solvent control flasks, T75 flasks of untreated PANC 03.27 and 04.03 were dosed with DMSO. The dosage of DMSO was dependent on the volume of solvent delivered at each cell lines GI_5 or GI_{10} concentration. Following a three-day treatment period, cells were harvested using Accutase or TripLE. Cells were then moved to a T25 flask and allowed to grow to confluence. A Gelatin-Fibronectin pretreatment was added to more accurately mimic the standard pulse treatment method. Once confluency was reached, the treatment process was repeated. PANC 03.27 DMSO control cells were dosed three times with DMSO, while PANC 04.03 DMSO control cells were dosed twice with DMSO.

Determination of Post-Treatment Drug Cytotoxicity

FOLFIRINOX treatment, untreated control, DMSO control, and FOLFIRINOX treatment cells underwent CTG cell viability assays. In order to gain a more accurate GI_{50} concentration, the dosages across each 96 well plate were changed. Unlike the 1:10 serial dilution down the 96 well plate used preliminary assays, post treatment GI50 assays used a 1:2 serial dilution. Additionally, a 1:5 dilution occurred between the drug plate and media plate, as opposed to a 1:10 dilution in preliminary treatments. This resulted in the top dose being 3.87 mM of 5FU to 3.02 uM 5FU, with all other drugs being given at their clinical ratio. Post treatment GI_{50} plots were then generated in order to compare untreated control, DMSO control, and FOLFIRINOX treatment cells.

Fold Resistance Calculations

Following the final treatment and generation of DMSO controls, CTG cell viability assays were repeated in order to calculate the change in GI_{50} concentrations post treatment. Assays were performed on PANC 03.27 and 04.03 DMSO

control cells and treated cells, and were rerun on untreated cells. Based on the results, the fold resistance was then calculated using equation 2.

Equation 2 $$\frac{GI_{50}\ Concentration\ (Treated\ cells)}{GI_{50}\ Concentration\ (Untreated\ cells)} = Fold\ Resistance$$

RNA Sequencing and Data Analysis

In order to assess the molecular changes responsible for resistance to FOLFIRINOX, RNA sequencing (RNA seq.) was utilized. In order to perform RNA seq, cell lines were sent to GENEWIZ. GENEWIZ is a company which specializes in RNA seq. instrumentation and analysis. Treated, untreated, and DMSO control cells in a T75 flask were harvested using 0.25% trypsin, resuspended in phosphate buffered saline and snap frozen in liquid nitrogen. Cells were then shipped on dry ice to GENEWIZ where RNA seq. was performed. The generated data was then analyzed. The differences between treated, untreated, and DMSO control cells were analyzed for each cell line in order to identify changes in gene regulation potentially responsible for the development of chemotherapy resistance. Additionally, analysis was conducted across cell lines in order to gain a more robust understanding of the differences between PANC 03.27 and PANC 04.03.

Results and Discussion

Innate FOLFIRINOX Resistance

CellTiter Glo cell viability assays were used to measure the initial sensitivity to FOLFIRINOX in 4 PDAC cell lines. The results of this experiment can be found in table 2. Preliminary GI_{50} plots can be found in figure 3. PANC 03.27 was found to be the most sensitive to the FOLFIRINOX treatment, with a preliminary GI_{50} concentration of 0.7 mM while PANC 05.04 was found to be the most resistant with a GI_{50} concentration of 1.5 mM. PANC 02.03 and 04.03 had GI_{50} concentrations of 1.26 mM and 1.33 mM respectively. Based on preliminary results and observations about cell growth, PANC 03.27 and 04.03 were chosen for subsequent analysis. It is important to note that these were preliminary GI_{50} concentrations, and the cell viability assays were rerun post-treatment in order to more accurately measure FOLFIRINOX resistance.

Table 2: Preliminary GI50 Values for 4 PANC Cell Lines

Cell Line	Preliminary GI_{50} Concentration (mM)
PANC 02.03	1.26
PANC 03.27	0.70
PANC 04.03	1.33
PANC 05.04	1.50

FOLFIRINOX Resistance Models

Clinical Pulse Treatment

Multiple methods were used to achieve FOLFIRINOX resistance, although only the standard pulse method was ultimately successful. The clinical pulse method, despite being the closest to clinical administration, suffered from a lack of cell viability. Although cells would rarely apoptosis in the treatment flask, the addition of trypsin post-treatment was often enough to cause widespread apoptosis throughout the cell population. FOLFIRINOX was essentially found to prime cells for apoptosis, and trypsin stressed on the population to promote cell death. It is possible that lowering

the dosages, as was done in the standard pulse method, may have produced resistant and more clinically relevant cell lines.

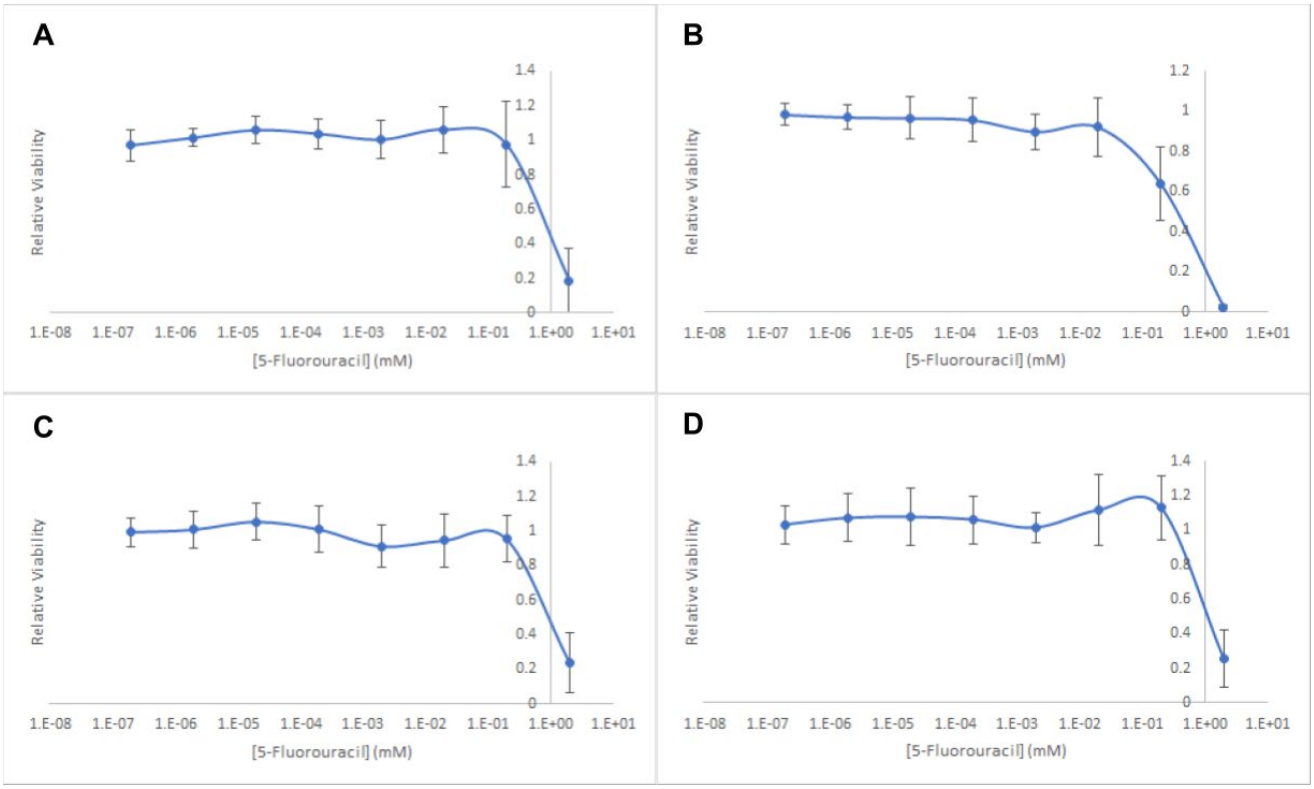

Figure 3 Preliminary GI50 Plots for 4 PDAC Cell Lines. PANC 02.03 (A) 03.27 (B) 04.03 (C) and 05.04 (D) underwent Celltiter Glo cell viability assays to assess initial sensitivity to the FOLFIRINOX treatment regimen. From the results, GI_{50} plots were generated and GI_{50} concentrations were obtained. PANC 02.03 and PANC 03.27 had GI_{50} concentrations of 1.26 mM and 0.70, while PANC 04.03 and 05.04 had GI50 concentrations of 1.33 mM and 1.5 mM.

Extended Exposure Treatment

A similar but more exaggerated problem was observed in the extended exposure method. Although numerous scholars have generated resistant cell lines through extended exposure to monotherapies, few have done so with multidrug treatments. Although cells would rarely apoptosis in the treatment flask, little to no cell growth was observed after treatment with low levels of FOLFIRINOX. If cells were split, trypsin was found to kill large portions of the population. Although this study was ultimately unsuccessful at generating resistant cell lines through extended exposure to FOLFIRINOX, it is possible that a lower dose and longer treatment times may generate resistance. Cells were treated at their GI_{10} concentration, the same dosage used in the successful pulse treatment method. Lowering the dosages may have significantly increased the likelihood of successfully generating resistant cells.

Standard Pulse Treatment

The standard pulse treatment method was ultimately successful at generating resistant PDAC cell lines. Through repeated exposure to GI_5/GI_{10} concentrations of FOLFIRINOX, resistance was achieved. This method also suffered problems with cell viability which were overcome using multiple methods. Compared to the clinical pulse treatment method, dosages were dropped significantly. Additionally, cells were plated onto a gelatin/fibronectin mixture to promote cell survival and growth. Numerous flasks were also generated for each round of treatment and merged to create a stable population.

Acquired FOLFIRINOX Resistance

GI_{50} assays were reassessed post-treatment in order to quantify acquired resistance. As DMSO was used to dissolve multiple drugs in FOLFIRINOX, solvent controls were also generated based on the concentration of DMSO when generating resistance. Cell viability assays were used to identify GI_{50} concentrations for PANC 03.27 and 04.03 untreated, DMSO control, and treated cell lines. The results of these assays can be found in table 3. GI_{50} plots for PANC 03.27 cells can be found in figure 4.

Table 3: GI50 Values for Untreated, DMSO treated, and FOLFIRINOX Treated PANC Cells

Cell Line (# of Treatments)	GI_{50} Concentration (mM)
03.27 Untreated	0.23
03.27 DMSO Treated (x3)	0.26
03.27 FOLFIRINOX Treated (x2)	0.37
03.27 FOLFIRINOX Treated (x3)	0.42

The GI_{50} concentration indicate resistance was successfully created using the pulse treatment method. PANC 03.27 cells were found to have a GI_{50} concentration of 0.26 mM for DMSO control cells, 0.37 mM for cells treated twice with FOLFIRINOX, and 0.42 mM for cells treated three times with FOLFIRINOX. This corresponds to a 1.6-fold increase in resistance. On average, each treatment increased resistance by 0.05 mM.

Although FOLFIRINOX was found to be effective at inhibiting the growth of PDAC cell lines, it is possible that errors in measuring innate GI_{50} concentrations led to significant difficulties throughout the study. Cell viabilities reassessed the end of the experiment using different dosages to more accurately measure the GI_{50} concentration showed that GI_{50} values were only 25-50% of preliminary values. As a result, treatment dosages when building resistance were higher than expected. When using GI_{50} concentrations measured at the end of the experiment, treatment dosages were actually GI_{10} for PANC 03.27 instead of GI_5 as originally believed. Although higher dosages of FOLFIRINOX are more effective at building resistant cell lines, it possibly resulted in the problems maintaining cell viability post-treatment observed in the study.

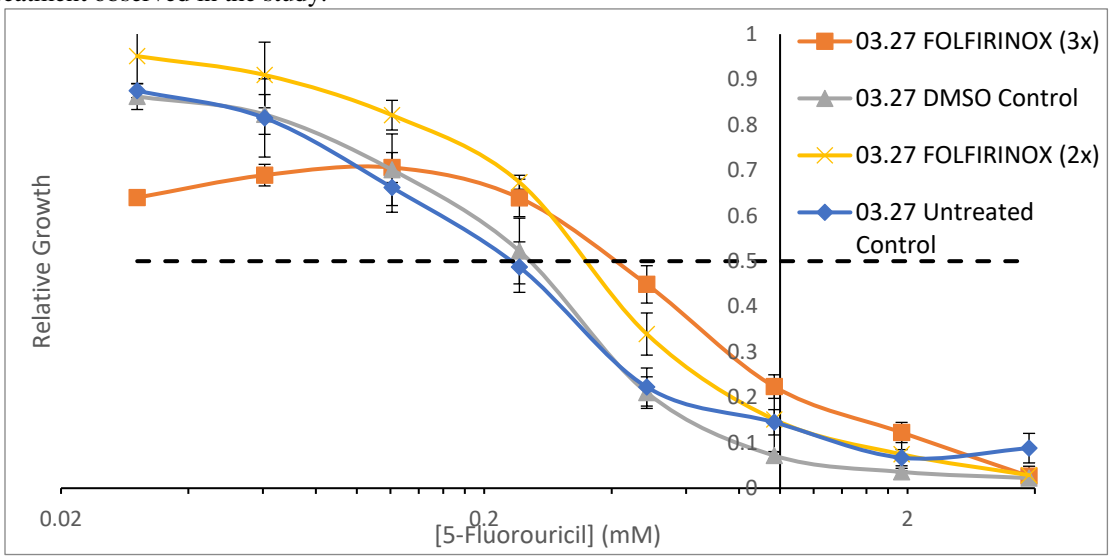

Figure 4 Control and Treatment GI50 assays for the PANC 03.27 Cell Line. PANC 03.27 untreated control, DMSO control, 2x FOLFIRINOX treated, and 3x FOLFIRINOX treated cells lines underwent Celltiter Glo cell viability assays to assess initial and required resistance to the FOLFIRINOX. Untreated and DMSO control lines were found to have similar GI50 concentrations at 0.23 and 0.26 mM respectively. Twice treated cells had a GI_{50} concentration of 0.37 mM while three times treated cells had a GI50 value of 0.42 mM.

RNA Sequencing

Base Reads and Quality Control

RNA sequencing revealed many important differences in RNA expression and the transcriptome between untreated, DMSO control, and treated cells. RNA sequencing of untreated cells led to 395 million base reads with a quality score of 35.86. This quality score indicates an accuracy of over 99.9% for RNA base calls. RNA sequencing data was mapped onto the GRCh38 reference genome which is available on ENSEMBL. The STAR aligner was used to detect spice junctions and help align reads to the reference genome. An average of 98.89% of reads were successfully mapped to the reference genome for untreated cells. A summary of this RNA quality control can be found in table 5.

Table 5: Summary of RNA Sequencing Quality Control

Cell Line	Number of Samples	Total Number of Reads	Average Quality Score	Average % Mapped
03.27 Untreated Control	3	242,382,561	35.88	98.87
04.03 Untreated Control	2	153,371,811	35.82	98.94

Untreated RNA Seq. Comparisons

Assessment of RNA sequencing results showed significant differences in expression between PANC 03.27 and PANC 04.03. Although both cell lines are KRAS driven primary pancreatic tumors, a total of 1510 genes were found to be significantly differentially expressed. For PANC 04.03, 475 genes were found to be over expressed and 1035 genes were found to be under expressed when compared to PANC 03.27. The number of genes found to be differently expressed was expected. Although PANC 03.27 and 04.03 are both primary PDAC tumors, they were extracted from different patients. Differences in genetic expression are expected between different individuals in the population. A volcano plot of the difference between PANC 04.03 and 03.27 can be found in figure 5. Gene ontology (GO) was used to analyze differences in patterns of expression. GO analysis clusters genes according to their biological process. GO analysis revealed that the largest difference between untreated PANC 03.27 and PANC 04.03 were in genes regulating extracellular matrix organization, positive regulation of GTPase activity, and positive regulation of cell proliferation. Over 40 GO genes sets were found to have significant differences ($p<0.05$) between the untreated cell lines (Figure 6).

Limitations

There are a few limitations of this study which should be noted. First, this study attempted to create a clinically relevant model of chemotherapy resistance by dosing cells with the ratio of drugs used clinically. This concentration ratio does not account for the actual concentrations found at the site of the tumor or within a tumor. Blood serum concentrations of the FOLFIRINOX cocktail may be considerably different than the treatment dosages used in this study. Additionally, this study loses the timing of drug dosages given clinically. In a clinic, each drug in FOLFIRINOX is given at different concentrations of varying amounts of time. Although this study did attempt to replicate this timing of drugs, the final treatment protocol did not include a timing piece.

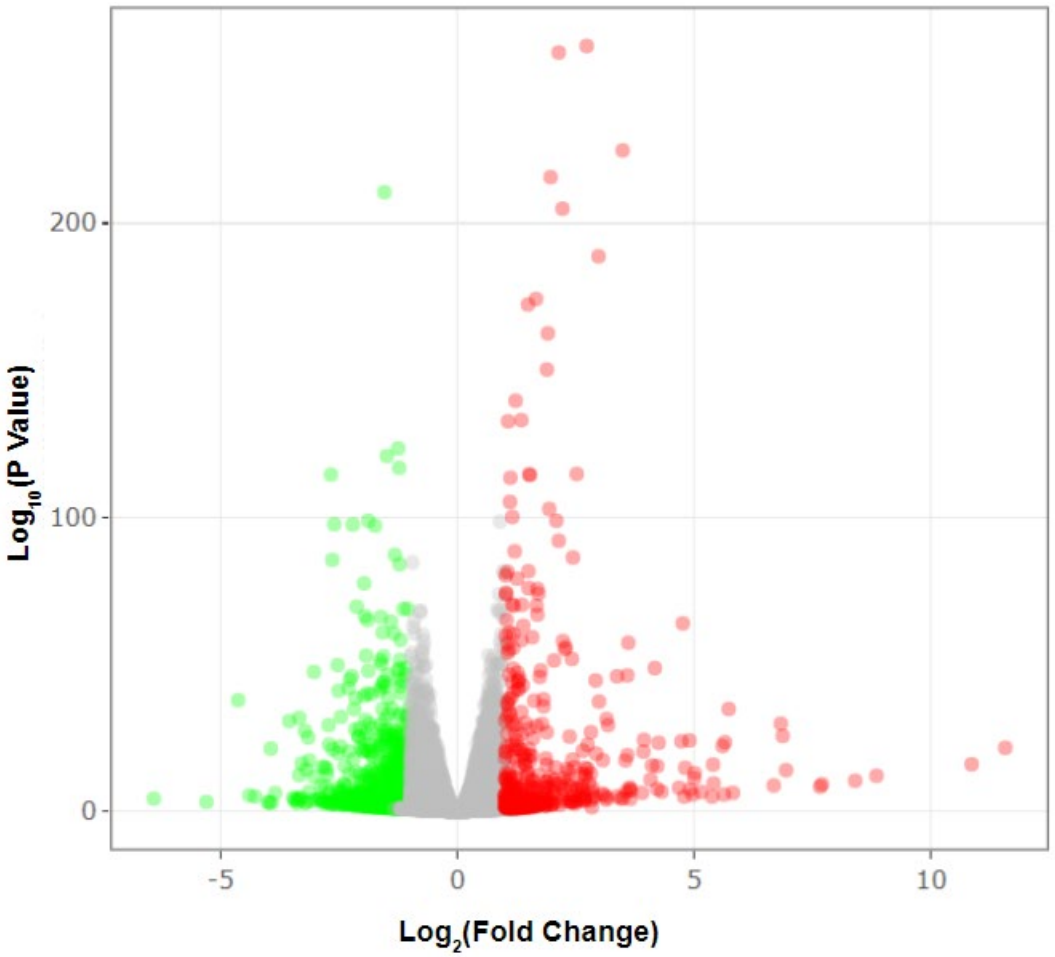

Figure 5 RNA Sequencing Volcano Plot for Untreated PANC 03.27 vs. Untreated PANC 04.03. Analysis of RNA Sequencing data for PANC 03.27 and PANC 04.03 is expressed in the volcano plot. Green dots represent the 475 genes found to be under expressed in PANC 04.03 ($p<0.1$) and red dots represent the 1035 genes found to be over expressed in PANC 04.03 ($p<0.1$).

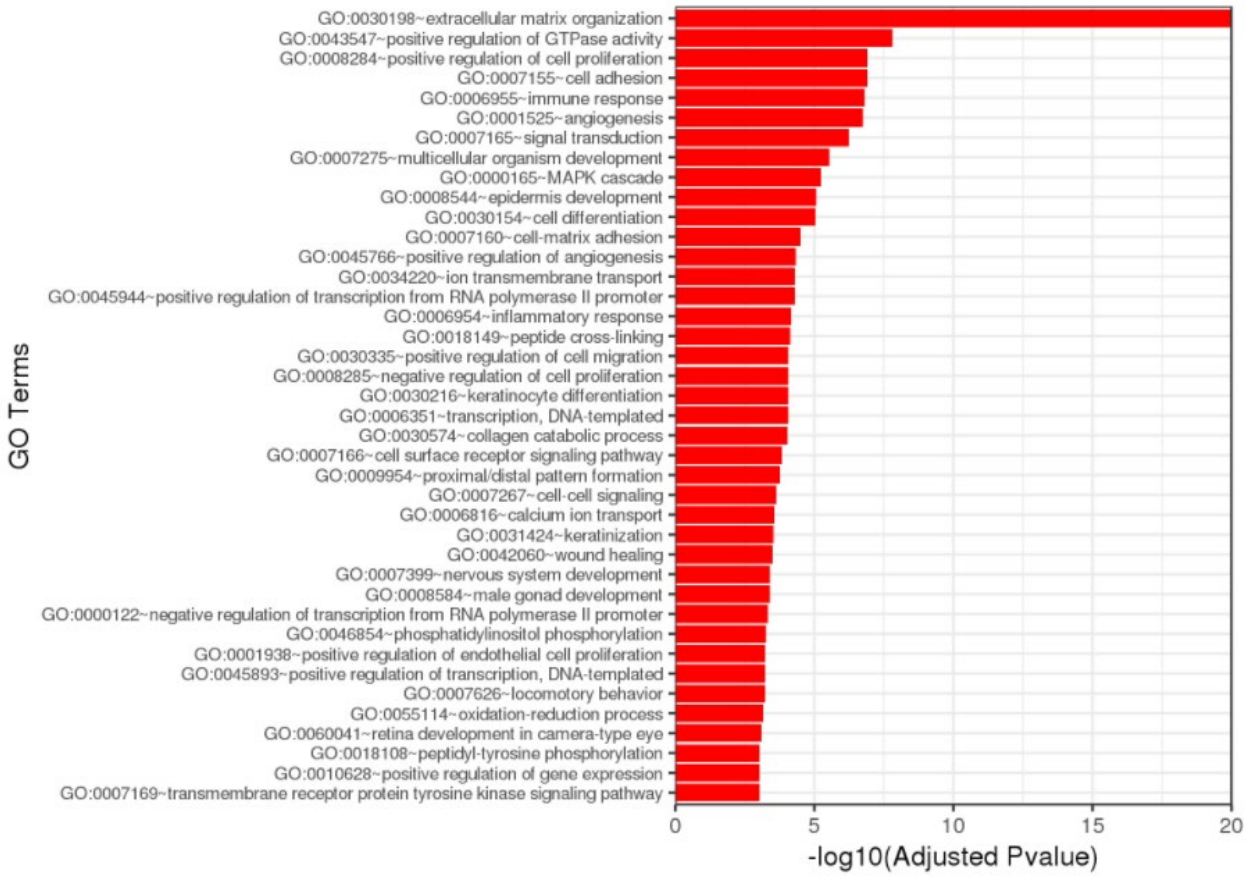

Figure 6 RNA Sequencing Gene Ontology Analysis for Untreated PANC 03.27 vs. Untreated PANC 04.03. Significantly differentially expressed genes were grouped by their gene ontology terms. A Fisher exact test was used to determine significant differences between gene ontology clusters. The 40 most significant differently expressed clusters ($p<0.05$ minimum). Genes encoding extracellular matrix organization, positive regulation of GTPase activity, and positive regulation of cell proliferation were the most significantly differentially expressed.

Conclusion & Future Directions

This study was successful in generating two resistance PDAC cell lines through treatment with FOLFIRINOX. Pre- and Post-treatment GI_{50} assays revealed a 1.6-fold increase in GI_{50} concentrations between DMSO-control and FOLFIRINOX treated cells. RNA Seq. between PANC 03.27 and 04.03 cells showed significant differences between untreated control cell populations. Although large genetic difference between the two cell lines were expected, it does show how tumors considered homogeneous can still have significant genetic variation.

This study hopes to find key differences in expression between DMSO control and FOLFIRINOX treated cells. There are many recorded mechanisms for the development of chemotherapy resistance. Some mechanisms involve decreasing the uptake of cytotoxic drugs, or otherwise increasing the efflux of drugs from the cell. Drugs have a variety of pathways into and out of= the cell which can be altered in order to limit their effectiveness. If changes to drug influx or efflux are observed, we may expect changes in a variety of known membrane transporters. Changes in the expression of ATP-binding cassette (ABC) transporters, for example, may be observed. ABC transporters are one of the largest superfamilies of membrane transporters and have been found to contribute to chemotherapy resistance in some tumors. The enhancement of DNA repair systems has also been seen in some tumors. Many drugs, such as

oxaliplatin in FOLFIRINOX, damage DNA in an attempt to cause apoptosis. If an upregulation of genes responsible for key DNA repair pathways is observed, it may indicate one possible mechanism of chemotherapy resistance.

Another major cause of resistance may be observed in proteins responsible for apoptosis. Some tumors will block the pathways which promote apoptosis, preventing cell death observed in many chemotherapies. Targeting or changing the expression of proteins such as FAS, TNF, BCL2, Bax, or Bak may prevent apoptosis from occurring despite damage caused by treatment. Other mechanisms include changing drug targets or drug metabolism, the amplification of genes which promote drug resistance, and miRNA targeting increasing resistance to chemotherapies. Each of these changes would result in a difference in gene expression between DMSO control and FOLFIRINOX treat cells.

Following analysis of RNA Seq. data, CRISPR/Cas9 gene knock out can be performed in order to confirm which genes may promote chemotherapy resistance. CRISPR/Cas9 gene knockout is a revolutionary technique which allows researchers to alter the genome. Silencing gene targets with CRISPR and confirming the genes were silenced with western blot analysis may restore sensitivity to FOLFIRINOX in resistant cell lines.

The resistant cell lines used in this study will be made available to the scientific community for future investigation. RNA Seq. is currently being performed on DMSO-control and FOLFIRINOX treatment cells. Comparisons between control and treatment cells will shed light on the cellular response generated by FOLFIRINOX. Future investigations may examine RNA Seq. data and investigate possible mechanisms of chemotherapy resistance present in PDAC tumors dosed with FOLFIRINOX.

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