TAKING ADVANTAGE OF NOVEL VULNERABILITIES FOR THE TREATMENT OF PANCREATIC CANCER

A Thesis

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In Partial Fulfillment of the Requirements for the Degree of

Master of Science

by

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ABSTRACT

A vast majority of pancreatic ductal adenocarcinomas (PDACs) have mutations in KRAS. This type of cancer is also notoriously difficult to treat, often resulting in patient death. Thus, there is a clear need to better understand PDAC progression. Here, I have discovered that the cell survival protein Survivin is highly upregulated in KRAS-driven pancreatic cancer cells and blocking Survivin expression using YM155 causes cell death. Moreover, treating PDAC cells with small doses of YM155 and the heat shock protein 90 (HSP90) inhibitor, Geldanamycin, was found to strongly kill certain PDAC cells, suggesting that drug combinations that include Survivin inhibition may hold promise as treatments for PDAC. Moreover, a YM155-resistant PDAC cell line was generated, which could be a powerful tool to investigate the mechanisms of drug resistance. These findings highlight Survivin as an important player in PDAC, and provide new insights regarding potential treatments for this disease.

BIOGRAPHICAL SKETCH

Yinzhe Liu was born and raised in Beijing China, a modern metropolis with more than three thousand years of history. Because both of his parents are researchers, he has been immersed in the active research atmosphere since his childhood. In his joyful time at The Experimental High School Attached to Beijing Normal University, he became interested in chemistry and biology after seeing fascinating reactions and mechanisms, as well as learning about their broad applications. He then went to The University of Illinois at Urbana-Champaign for his undergraduate education, where he double majored in landscape architecture and chemistry. During this time, he started to appreciate the importance of pharmaceutical science, and how understanding the mechanisms that promote disease progression could be used to develop new treatments to save lives. This was the primary reason he joined Dr. Duxin Sun's group as a summer researcher at The University of Michigan. Dr Sun studies breast cancer stem cells, and how they are resistant to multiple chemotherapy drugs. Later, Yinzhe joined Dr. Douglas Mitchell's group at the University of Illinois as an undergraduate researcher, where he used biochemical approaches to develop potential natural product antibiotics, which could be used to super-bacteria. After receiving his two bachelor's degrees, he was fortunate enough to be accepted into the Chemistry and Chemical Biology Department at Cornell University and joined Dr. Richard Cerione's group. There, he continued his interest in investigating the mechanisms that underlie cancer progression. He primarily focused on a protein called Survivin, which provides cancer cells with multiple survival advantages. Although he had limited time as an M.S. student due to his heavy class load, he learned a lot about cancer biology and experiments, but more importantly, how to think like a scientist. After experiencing the family-like atmosphere in

the lab and being exposed to the exciting and cutting-edge research questions that the Cerione lab pursues, Yinzhe decided to pursue a Ph.D. degree and continue on his research pathway.

致敬爱的父亲母亲

ACKNOWLEDGMENTS

I would like to express my deep gratitude to my parents, who have always been my strongest supporters. They not only provided me with the best educations and helped my interest, but they also gave me much advice regarding “life”. Their care and unconditioned love has always been the driving force that has allowed me to get where I am today.

Looking back on the past year at Cornell, I’m also grateful to Dr. Richard Cerione, who gave me the treasured opportunity to carry out this interesting project in his lab, even under the unprecedented Covid-19 pandemic. He consistently provides scientific inspirations to all his students and creates a fabulous supportive atmosphere in the laboratory. I felt very fortunate to be his student. I would also like to thank Dr. Marc Antonyak, who not only very patiently guided me all the cell biology experiments performed in this thesis, but also put a lot of effort into training me to think critically as a researcher. His friendship and the time we spent together will always remain in my memory.

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TABLE OF CONTENTS

1. Introduction

Pancreatic ductal adenocarcinoma (PDAC), more commonly referred to as simply pancreatic cancer, is considered one of the most aggressive and deadly forms of cancer. In 2021, the estimated incidence of pancreatic cancer in the United States will be 60,400 cases, while nearly 50,000 of these patients are expect to die from the disease, ranking it third among all cancer-related deaths.[1] There are multiple reasons for the high mortality rate of pancreatic cancer patients, including late-stage diagnosis, quick relapse following surgery, the high invasion and metastatic activity of the cancer cells, and therapy resistance.[2,3] Despite extensive efforts to find effective approaches to treat pancreatic cancer, the overall 5-year survival rate has remained extremely low (less than 10%).[1,2] Thus, there is a clear need to better understand the mechanisms that drive pancreatic cancer progression, with the hope that this information can be used develop new ideas regarding disease management and treatment options.

Common traits of Pancreatic Cancer: mutations in KRAS

Pancreatic cancer is an interesting example of a type of cancer where approximately 95% of the cases are known to have a mutation in a single gene, specifically in Kirsten rat sarcoma viral oncogene homolog (KRAS).[4] This gene encodes a protein that is a member of the small guanosine triphosphate (GTP)-binding protein (GTPase) superfamily, that also includes the closely related Neuroblastoma RAS viral oncogene homolog (NRAS) and Harvey rat sarcoma viral oncogene homolog (HRAS), as well as other classes of small GTPases like the Rho, Ran, Arf, and RAB

proteins.[4-6] All of the proteins in this family function as molecular switches (Fig. 1) that cycle between an active guanosine triphosphate (GTP)-bound state, and an inactive guanosine diphosphate (GDP)-bound state.[4-6] In normal cells, the activity of KRAS is tightly controlled. Signals that originate at the cell surface, for example, when a growth factor binds its corresponding receptor expressed on the cell surface, lead to the rapid activation of guanine nucleotide exchange factors (GEFs, Fig. 1, left side). For KRAS, one of the most common GEFs is Son of Sevenless (SOS),[7] which promotes the release of GDP and the subsequent binding of GTP. The GTP bound form of KRAS undergoes conformational changes that allows it to engage and activate effector proteins, many of which promote cell growth, migration and survival.[5,6,8] Perhaps the best known signaling pathway activated by KRAS (as well as the other isoforms of RAS) is the RAS-RAF-MEK-ERK pathway,[5,9] which will be discussed in further detail below.

However, just as rapidly as KRAS is activated, it also has to be inactivated. This is accomplished by GTPase activating proteins (GAPs, Fig. 1, right side), which convert KRAS to its inactive, GDP-bound state. There are several divergent proteins that inactivate KRAS and they include p120 GAP, neurofibromin (NF1), and RAS Protein Activator Like proteins (RASAL).[5,10,11] In cancer cells, mutations in KRAS often cause the protein to preferentially bind GTP and thus render it active.[5,11] The mutated forms of KRAS lead to the increased activation of signaling proteins (including components of the RAS-ERK pathway) that mediate cancer cell proliferation and survival, as well as promote cellular transformation.[5,6,9,11]

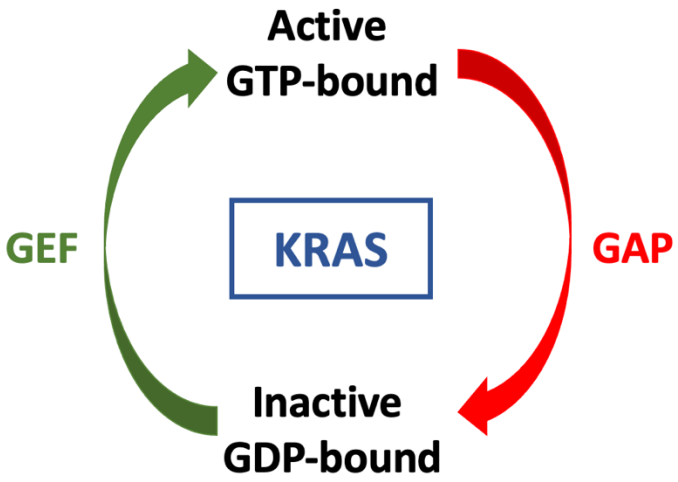

Figure 1. The KRAS GTP/GDP binding cycle and its effects on activity.

KRAS is a small GTPase that functions as a molecular switch that cycles between an active GTP-bound state and an inactive GDP-bound state. Its activity is tightly controlled by guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs). GEFs catalyze the exchange of GDP for GTP, and thus activate KRAS. GAPs promote the hydrolysis of GTP to GDP and inactivate KRAS.

Mutated forms of KRAS are found in approximately 30% of all human cancer patients, and as mentioned earlier, in as many as 95% of PDAC patients,[4] making KRAS the most commonly mutated oncogene. It is also worth mentioning that the gene encoding KRAS can also be amplified in human cancers, increasing the expression level of the protein.[4] Some of the most common mutations that occur in KRAS pancreatic cancer result in the glycine (G) at position 12 being changed to a cysteine (C), aspartic acid (D) or valine (V), and the G at position 13 to a D. Mutations in glutamine (Q) 61, glutamic acid (E) 6, lysine (K) 117, D119 and Alanine (A) 146 in KRAS also have been reported, but at much lower frequencies.[4,13,14] Nearly all of these mutations perturb or slow the GTPase activation capability of KRAS (i.e. hydrolyzing GTP to GDP), which helps the protein remain in its active conformation.[4,5]

Some studies have shown that mutations in KRAS are initiator events in PDAC, causing cells to undergo transformation or acquire a cancer-like phenotype.[15,16] This idea is reinforced by studies showing that fibroblasts engineered to ectopically express active forms of KRAS acquire several characteristics of cancer cells, including the ability to form colonies in soft agar (i.e. grow under anchorage-independent conditions) and form tumors in nude mice.[15-17] Genetically engineered mouse models of PDAC, where the pancreases in mice have been engineered to express mutant forms of KRAS, were shown to also be sufficient to drive tumor formation, further highlighting the importance of this small GTPase in PDAC initiation.[18]

Targeting RAS and its effector proteins as a cancer treatment

Given the importance of KRAS in human cancer, it would be ideal to develop small molecule drugs that inactivate KRAS. However, for more than 30 years, academic groups and the pharmaceutical industry have been largely unsuccessful in targeting KRAS.[19] This has resulted in KRAS being perceived as an "undruggable target".[19,20] Very recent efforts have led to the identification of a small molecule inhibitor that can specifically bind and inhibit the activation of the $KRAS^{G12C}$ mutant protein.[21,22] This inhibitor, referred to as KRAS (G12C) inhibitor 6, has shown promising results in clinical trials, yet it is only useful in cancers that express $KRAS^{G12C}$.[23]

Another possible way to treat KRAS-driven cancers is to target key effectors or cellular processes that are activated by oncogenic forms of this protein. One example of this is using pharmacological or genetic approaches to inhibit different components of the classic RAS-RAF-MEK-ERK pathway. Treating KRAS transformed cells with the MEK inhibitor (PD98059) or the ERK inhibitor (SCH772984) has been shown to initially block cell growth, survival and migration.[24,25] However, cancer cells often quickly develop resistance to MEK and ERK inhibition.[26,27] Recently, Dr. Kirsten Bryant discovered that inhibiting the RAS-ERK pathway in pancreatic cancer cells increases autophagy, a unique form of protein degradation and recycling.[28] Blocking both ERK activation using SCH772984, and autophagy using hydroxychloroquine can potentially halt pancreatic cancer cell growth both in cell culture and in mice.[28] These findings received a good deal of attention from other PDAC researchers and oncologists, and led to a clinical trial where this drug combination is being evaluated in pancreatic cancer patients.[29]

Although there have been some potentially important recent advances in efforts to target KRAS-driven cancers, there is still a good deal to learn regarding how this oncogene promotes cellular processes related to cancer progression, and then test whether blocking these processes can potentially be used as effective strategies to treat pancreatic cancer. One exciting example that will be the focus of the current thesis is how a protein called Survivin mediates KRAS stimulated cell growth and survival.

Survivin and its cellular functions

Survivin, also referred to as baculovirus IAP repeat-containing protein 5 (BIRC5), is a small protein with 142 amino acids residues that has a molecular weight of approximately 16.5 kDa.[30] It is a member of a family of proteins referred to as the inhibitor of apoptosis (IAP) family.[30,31] X-ray crystallography studies of Survivin revealed that it is composed of two major domains: a baculovirus inhibitor of apoptosis repeat (BIR) domain, followed by an extended coiled-coil domain at its C-terminus (Fig. 2A and Fig. 2B).[32] The BIR domain is a zinc finger domain (Fig. 2A, orange domain) that binds and inhibits the apoptotic machinery in cells, specifically caspases.[32-35] The coiled-coil domain is an α-helical structure that promotes the dimerization of Survivin. (Fig. 2A, blue domain).[32,33] The crystal structure of Survivin, highlighting these major domains, is shown in Fig. 2B.

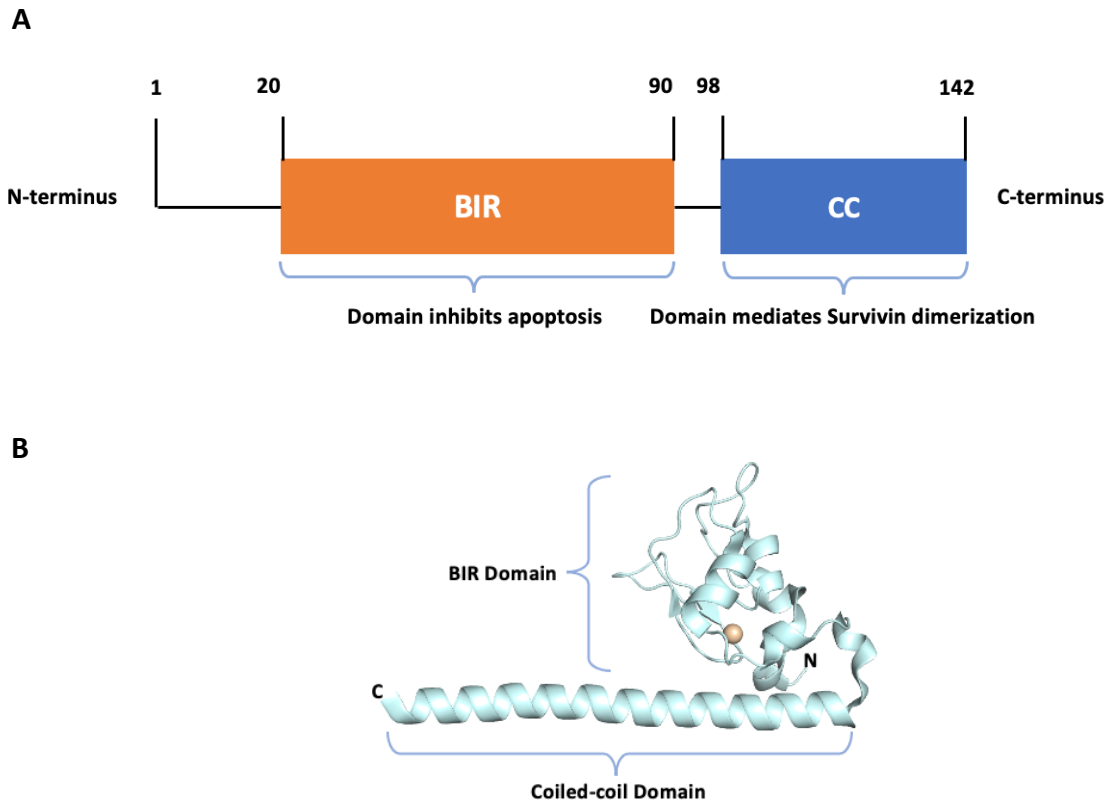

Figure 2. The structure of Survivin.

(A) Linear schematic depicting the major domains in Survivin. The protein is 142 amino acids (aa) in length. The baculovirus IAP repeat (BIR) domain (aa 20-80) is highlighted in orange and is known to inhibit apoptosis (i.e. cell death) by blocking caspase activation. The coiled-coil (CC) domain (aa 98-142) of Survivin is shown in blue and is important for promoting the dimerization of the protein.

(B) Structural representation of Survivin.[33] (PDB:1F3H, resolution: 2.58 Å). The BIR and coiled-coil domains are indicated and the Zn^{2+} ion is shown as a brown sphere. The N- and C- termini of the protein are denoted with an "N" or "C".

Survivin is thought to have two major functions in cells that can directly impact cancer progression: inhibiting cell death, and maintaining cell stemness.[30] Survivin expression is frequently up-regulated in cancer, where it blocks cell death when its BIR domain binds to specific caspases 3 and 7, and prevents their activation.[35] Since caspase activation promotes program cell death, the ability of Survivin to block caspase activation provides the cell with survival advantages.[35] The ectopic expression of Survivin in several different cell types has been shown to be sufficient to inhibit cell death caused by a wide-variety of intrinsic or extrinsic stimuli. For example, the ectopic expression of Survivin in breast cancer cells was shown to be sufficient to promote resistance to the cancer drug Paclitaxel.[36,37] On the other hand, cancer cells, like the human non-small cell lung cancer cell line H460 and the human breast cancer cell line JIMT-1, inherently express Survivin at high levels.[38,39] Depleting these cells of Survivin using siRNA or shRNA, or the small molecule YM155, caused them to become sensitive to drug treatments and radiotherapy.[40,41]

Survivin is also known to be involved in the maintenance of cell stemness.[30] Pluripotent or stem cells have two characteristics that distinguish them from all other cell types: 1) the ability to self-renew indefinitely and 2) the ability to differentiate into multiple cell lineages.[42] Survivin expression in stem/progenitor cells is quite high, and blocking its expression causes the cells to lose their pluripotency.[43] Importantly, Survivin is also expressed in cancer stem cells (CSCs).[44,45] CSCs refer to a discrete cell population within tumors that retain stem-like characteristics, and are believed to be able to give rise to the entire tumor.[42] These cells have also been shown to have a

strong correlation with therapy resistance and tumor recurrence.[42] Thus, Survivin expression in CSCs contributes to the strong tumor promoting traits of these cells.

Targeting Survivin as a cancer therapy

Given that Survivin expression is frequently up-regulated in conventional cancer cells and in CSCs, combined with its pivotal roles in promoting cell viability and stemness, it is not surprising that Survivin has become an attractive target for cancer drug discovery.[46] Moreover, it is noteworthy that Survivin expression in normal, differentiated cells and adult tissues is quite low.[46] Thus, drugs that selectively target Survivin should theoretically have almost no effect on normal cells, thereby minimizing the side effects that are often associated with cancer treatments.

However, since Survivin lacks intrinsic catalytic activity, and has almost no traditional binding pockets, conventional approaches used to design small-molecule antagonists that bind and inhibit the protein have failed.[20] Against this backdrop, the best Survivin inhibitor that has been generated is called YM155 (Fig. 3A). YM155 binds to transcription factors (TFs) that associate with the promoter region of the gene that encodes Survivin (BIRC5) and prevents it from being transcribed (Fig. 3B). Specifically, YM155 has been shown to interfere with transcription promoting activities of ILF3 and NF110.[47] In effect, treating cells with YM155 causes Survivin levels to decrease. The toxicity and effectiveness of YM155 have already been evaluated in phase I and phase II clinical trials, with it showing manageable toxicity but not having a significant effect on inhibiting tumor growth or extending patient survival.[48,49]

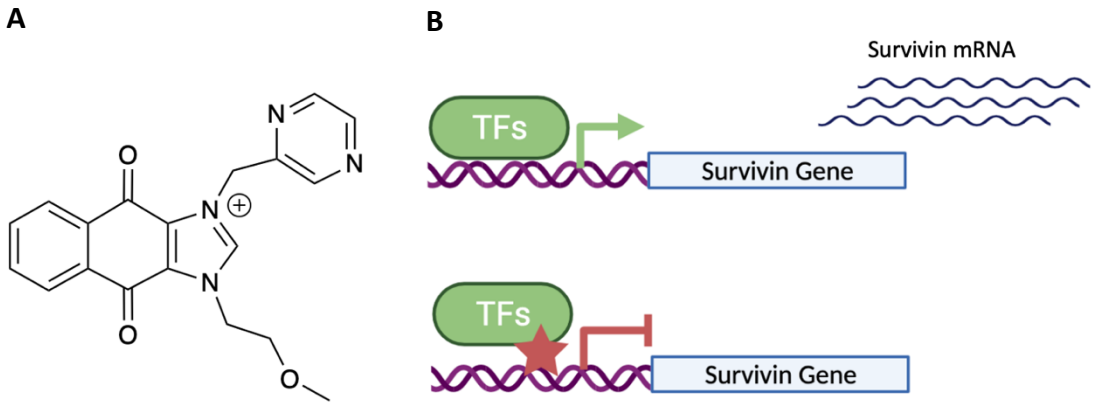

Figure 3. YM155

(A) The chemical structure of YM155

(B) Diagrams showing the mechanism by which YM155 inhibits Survivin expression. Survivin expression is increased when transcription factors (TFs), (i.e. ILF3/NF110) bind to its promotor region (purple) and promote gene transcription, generating Survivin mRNA (top panel). YM155 (red star) binds to ILF3 and NF110 and disrupts their ability to properly associate with the Survivin promotor. This suppresses the transcription of the gene and inhibits Survivin expression (bottom panel).

Overall goals

One of the commonly used approaches to improve the efficacy of cancer treatments is combination therapy, that is, combining two or more therapeutic drugs targeting different signaling pathways. As introduced above, KRAS and its effectors, including Survivin, play major roles in pancreatic cancer progression and thus could be potential targets for combination therapy. Here, I examined how targeting Survivin and other proteins that are known to be important in cancer progression, for example, the chaperone protein Heat shock protein 90 (HSP90) and the non-receptor tyrosine kinase focal adhesion kinase (FAK), might show additive or synergistic effects.[50-52]

By testing rational drug combinations that include targeting Survivin, this thesis aims to better understand the importance of Survivin in pancreatic cancer and hopes to identify a novel treatment that potentially more effective than current approaches used during pancreatic cancer treatments.

2. Material and methods

2.1 Cell Culture

All cells were cultured in an incubator at 37 °C and 5% CO_2. The mouse embryonic fibroblasts (MEFs) which stably express an inducible form of HA-tagged KRAS were generated as described previously, and cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco)[17]. The human pancreatic cancer cell lines, PANC-1 and MIA PaCa-2, were obtained from the ATCC and maintained in DMEM supplemented with 10%

FBS. Where indicated, cells were treated with 100 nM Doxycycline, 100 nM YM155, 150 nM Geldanamycin, 7.0 μM FAK inhibitor III, and/or 2.0 μM SCH772984. To generate the YM155-resistant MIA PaCa-2 pancreatic cancer cell line (MIA PaCa-2/R), cells were treated weekly with increasing concentrations of YM155, starting with 5 nM and ending at 120 nM in DMEM containing 10% FBS.

2.2 Cell treatments and survival assay using trypan blue

Cells grown in six-well plates to 50-70% confluency were washed 5 times with 2 mL of serum-free DMEM or sterile phosphate buffered saline (PBS) before being maintained in DMEM containing 1.5% FBS (i.e. low serum conditions) and nothing (i.e. control or untreated), or the indicated combinations of YM155, Geldanamycin, FAK inhibitor III and SCH772984. After 24 and 40 hours of treatment, the attached and floating cells were collected, treated with a 1:1 ratio of 0.04% trypan blue (Gibco), and counted using a hemocytometer. Non-viable cells stain blue, while viable cells do not take up the dye. To determine the percentage of cell death for each condition examined, the number of non-viable cells was divided by the total number of cells. The results of the survival assays were plotted as the percent change in viability, compared to the percent of cell death determined for the control cells (i.e. the cells with no treatment).

2.3 Western blot analysis

Cells were treated as indicated and then lysed using cell lysis buffer (25 mM Tris, 100 mM

NaCl, 1 mM EDTA, 1 mM DTT, 1 mM NaVO4, 1 mM β-glycerol phosphate, 1% Triton X-100, 1 μg/mL aprotinin, and 1 μg/mL leupeptin). The protein concentration of each cell lysate was determined using the Bradford Assay (Bio-Rad). The lysates were normalized based on protein concentration, resolved on 4-20% gradient SDS-PAGE gels (Invitrogen), and the proteins were transferred to PVDF membranes (Thermo Fisher). The membranes were blocked for 1 hour using 10% bovine serum albumin (BSA) dissolved in TBST (19 mM Tris, 2.7 mM KCl, 137 mM NaCl, and 0.5 % Tween-20), and then were incubated overnight at 4^{o}C with one of the following antibodies (all from Cell Signaling Technology) diluted 1:1000 in TBST; HSP90 (catalog #4877), Survivin (catalog #2080), Vinculin (catalog #13901), phospho ERK 1/2 (T202/Y204, catalog #9101). The following day, the membranes were incubated with anti-rabbit IgG, or anti-mouse IgG, HRP-linked antibodies (catalog #7074 and catalog #7076) for one hour, washed 3×5 minutes with TBST, and exposed to ECL (Perkin Elmer) reagent. The membrane was exposed to HyBlot CL® Autoradiography Film (Thomas Scientific), and the film was developed using a Konica Minolta SRX-101A.

2.4 Data analysis

All experiments were performed at least three independent times. Quantitative data are presented as means ± standard error. Statistical significance of the experiments was determined using Student's t-tests; ***; $p < 0.001$, **; $p < 0.01$, *; $p < 0.05$.

3. Results

Mutant KRAS promotes Survivin expression

Since the vast majority (95%) of pancreatic cancers have mutations in the KRAS oncogene, and they also overexpress Survivin,[4,30] it is crucial to determine whether there is a functional relationship between these two proteins. Mouse embryonic fibroblasts (MEFs) that had been previously generated to stably express an inducible HA-tagged form of activated KRAS, specifically HA-tagged $KRAS^{G12D}$ (HA-$KRAS^{G12D}$), were used for this purpose.[17] Their expression of HA-$KRAS^{G12D}$ is tightly controlled by doxycycline, such that when these cells are cultured in growth medium (DMEM supplemented with 10% FBS) containing 100 nM doxycycline, little to no expression of HA-$KRAS^{G12D}$ can be detected by Western blot analysis using an HA antibody (Fig. 4, uninduced (-) lane). However, when doxycycline is removed from medium for 48 hours, HA-$KRAS^{G12D}$ expression strongly increased (Fig. 4, induced (+) lane). Probing the same lysates with a Survivin antibody revealed that the expression level of Survivin correlated with HA-tagged $KRAS^{G12D}$ expression. Specifically, the expression of Survivin in the uninduced MEFs was nearly undetected, but increased upon the induction of HA-tagged $KRAS^{G12D}$ expression (Fig. 4 middle panels).

Two KRAS-driven human pancreatic cancer cell lines, PANC-1 and MIA PaCa-2, were similarly evaluated for Survivin expression levels. The Western blots shown in the top panel in Fig. 4B highlight that Survivin can be readily detected in the lysates derived from each of these pancreatic cancer cell lines. These finding suggest that activated forms of KRAS can promote the

expression of Survivin and that pancreatic cancer cell lines express high amounts of this protein.

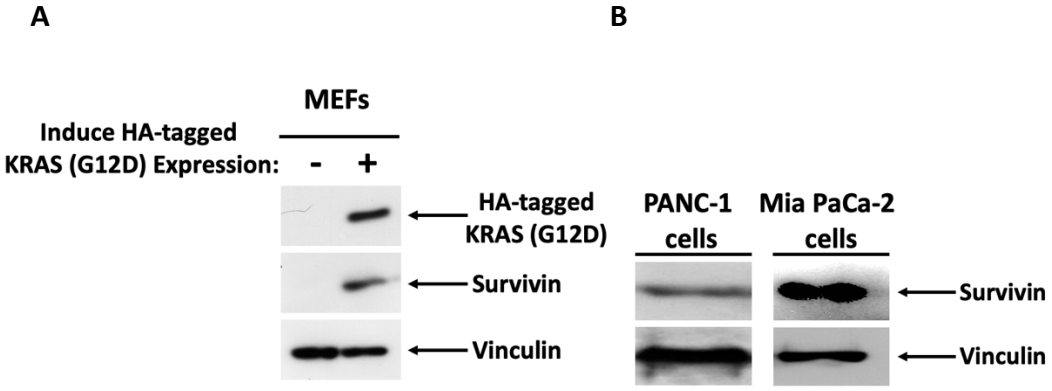

Figure 4. MEFs and pancreatic cancer cells that express mutant forms of KRAS have increased Survivin expression.

(A) Western blot analysis using HA and Survivin antibodies was performed on mouse embryonic fibroblasts (MEFs) that were engineered to express a HA-tagged $KRAS^{G12D}$ construct that is under the control of doxycycline. Specifically, when the MEFs are cultured in the presence of 100 nM doxycycline, $KRAS^{G12D}$ expression is inhibited (lane 1, top panel), while its removal from the culture medium for 48 hours induces $KRAS^{G12D}$ expression (lane 2, top panel). Note that Survivin expression is increased in MEFs ectopically expressing the small GTPase (lane 2, middle panel).Vinculin was used as the loading control.

(B) Western blot analysis using a Survivin antibody was performed on two different human pancreatic cancer cell lines, PANC-1 and MIA PaCa-2 cells (top panels). Vinculin was used as the loading control. (bottom panels).

Treatment of pancreatic cancer cell lines with YM155

The overall 5-year survival rate for PDAC patients has remained low (around 10%) for the past 30 years, despite the best efforts of oncologists.[1,2] This has largely been attributed to the high invasion and metastatic activity of PDAC cells, as well as the resistance of these cell types to theraputics.[2,3] As described previously in the Introduction, Survivin has been shown to play vital roles in mediating both cancer cell aggressiveness, as well as drug resistance.[30] Thus, it is interesting to determine whether Survivin inhibition could be used as part of a regimen for the treatment of human pancreatic cancer.

YM155 is a small molecule that suppresses Survivin expression by binding to specific transcription factors and preventing them from associating with the Survivin promoter.[47] This inhibitor is used to examine the importance of Survivin in the same two human pancreatic cell lines, PANC-1 and MIA PaCa-2. The PANC-1 cells were treated without (-) or with 50 nM YM155, while MIA PaCa-2 cell cells were treated without (-) or with 100 nM YM155. Approximately 24 hours later, the cells were lysed and Survivin expression levels were determined by Western blot analysis. The top panels in Fig. 5 show again that Survivin is expressed in both of these cell types (see lanes not treated (-) with YM155). However, YM155 treatment of both PANC-1 and MIA PaCa-2 cells, dramatically reduced Survivin levels (See lanes treated (+) with YM155), demonstrating that YM155 is sufficient to inhibit Survivin expression in these cells.

To evaluate how inhibiting Survivin expression using YM155 would affect human pancreatic cancer cells, Trypan blue cell viability assays were carried out on PANC-1 and MIA PaCa-2 cells

treated with increasing concentrations of YM155 from 50 nM to 200 nM. About 40 hours later, the cells were collected and stained with Trypan Blue. This dye can only be taken up by cells that are dying (i.e. they stain blue), because their cell membranes have been compromised, providing a reliable read-out for cell death. The percentage of dead cells was determined for each treatment group, as outlined in Material and Methods. Untreated PANC-1 and MIA PaCa-2 cell lines showed little cell death. However, PANC-1 cells treated with as little as 50 nM YM155 died at a rate of ~30%, while nearly 20% of the MIA PaCa-2 cells died when treated with the same amount of drug. (Fig. 6, see bars labeled 50 nM). Increasing the concentration of YM155 used to treat cells to 100 nM caused between 60-70% of PANC-1 and MIA PaCa-2 cells to die, while 200 nM caused approximately 90% cell death. (Fig. 6, see bars labelled 100 nM and 200 nM). These findings demonstrate that two commonly studied human pancreatic cell lines are highly sensitive to the reduction in Survivin expression caused by YM155 treatment.

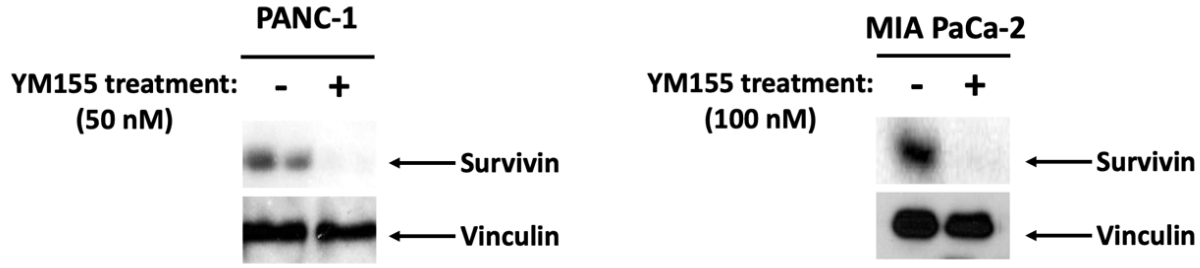

Figure 5. Treatment of pancreatic cancer cell lines with YM155 inhibits Survivin expression. PANC-1 (left) and MIA PaCa-2 (right) cells were treated without (-) or with the indicated concentration of YM155 for 24 hours, before the cells were lysed and analyzed by Western blot using a Survivin antibody (top panels).Vinculin was used as the loading control (bottom panels).

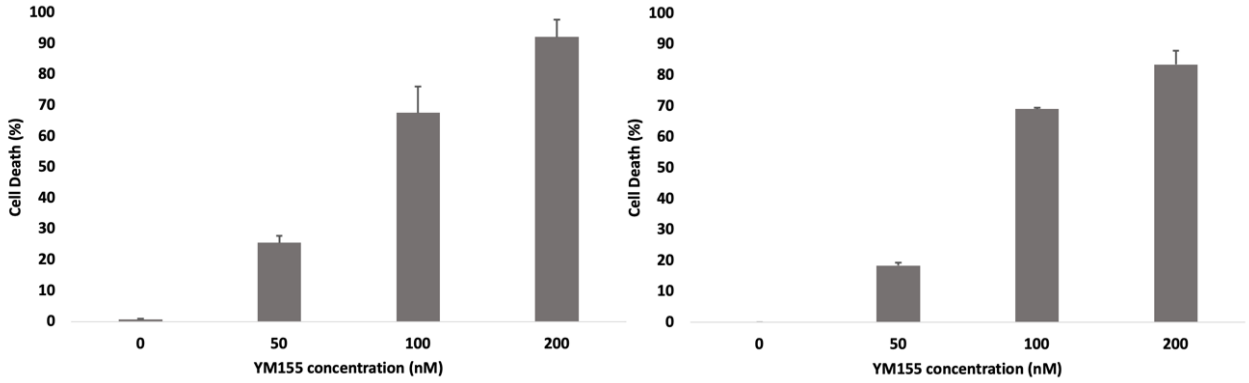

Figure 6. Pancreatic cancer cells treated with YM155 die.

PANC-1 (left) and MIA PaCa-2 (right) cells were cultured in DMEM medium supplemented with 1.5% fetal bovine serum (FBS) and nothing (0), or the indicated concentrations of YM155 (from 50-200 nM). Approximately 40 hours later, the viability of the cells was determined using Trypan Blue. The ratio of dead cells to total cells was used to determine the percentage of cell death for each condition. The data shown in represent the mean ± standard error. All experiments were performed a minimum of three independent times.

Treatment of pancreatic cancer cell lines with YM155 and other drugs.

Given the sensitivity of human pancreatic cell lines to YM155, it is important to determine whether combining it with other drugs have additive or synergic effects on cell death, which would potentially minimizing drug doses and off-target effects while still effectively targeting the cancer cells.[53] Although predicting which drugs combine in a beneficial manner with YM155 is difficult,[53] a good place to start is to target proteins have not only been implicated in cancer progression, but have also been shown to interact with Survivin or mediate its effect. In this regard, I focused on using the Heat shock protein 90 (HSP90) protein inhibitor, geldanamycin, and the Focal adhesion kinase (FAK) inhibitor, FAK inhibitor III. HSP90 is believed to bind and promote the stability of Survivin.[50] This same protein has also been shown to function as a chaperone that helps mediate several different cellular processes that promote aggressive phenotypes. On the other hand, Survivin-mediated cancer cell invasion has been shown to require the activation of FAK.[52] Therefore, Geldanamycin and FAK inhibitor III were combined with YM155 to test whether these combinations of inhibitors achieved a better killing efficacy, compared to using either of these drugs alone.

The viability of PANC-1 and MIA PACA-2 cells treated with nothing (untreated), or various combination of 50 nM YM155, 75 nM Geldanamycin and 7.0 μM FAK inhibitor III for 40 hours was determined using Trypan Blue. The concentration of YM155 was set at 50 nM, which is approximately the amount of drugs that I determined to cause 25% cell death after 40 hours of treatment (the I.C. 25). Fig. 7A shows that treating PANC1 and MIA PaCa-2 with 75 nM

Geldanamycin caused only a small amount of the cells to die. However, combining Geldanamycin and YM155 killed a larger proportion of PANC-1 cells, with 50.22% of the cells dying, compared to 33.40% cell death caused by YM155 treatment alone. However, treating MIA PaCa-2 with the combination on YM155 and Geldanamycin showed almost no advantage compared to the cell death achieved with YM155 treatment alone.

The viability of the PANC-1 and MIA PaCa-2 cell lines were similarly determined for the YM155 and FAK inhibitor III treatment. The results of this experiment showed that this drug combination did not increase cell death over what is achieved with YM155 alone (Fig. 7B).

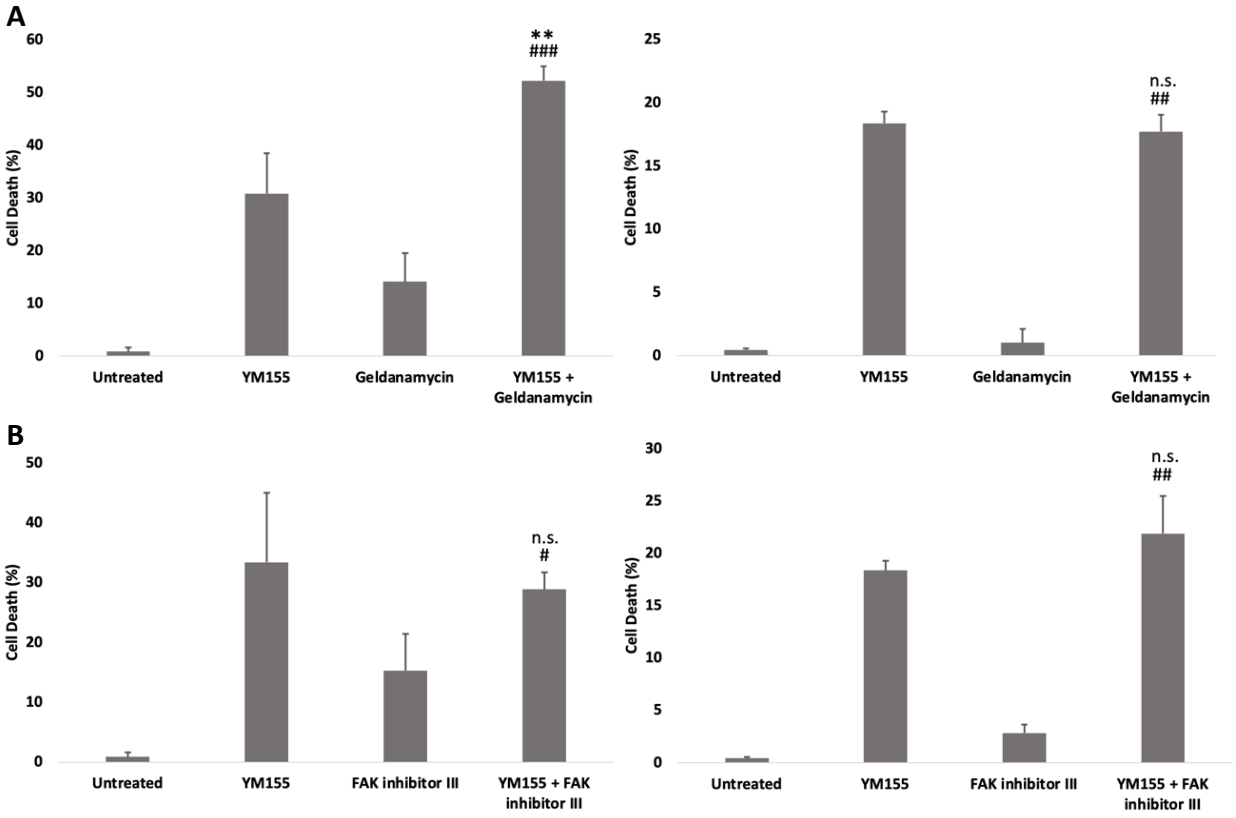

Figure 7. Drug combination of YM155 + Geldanamycin and YM155 + FAK inhibitor III in Pancreatic cancer cells.

(A) PANC-1 (left) and MIA PaCa-2 (right) cells were cultured in DMEM medium supplemented with 1.5% fetal bovine serum (FBS) with nothing (0), 50 nM YM155, 75 nM Geldanamycin or a combination of 50 nM YM155 + 75nM Geldanamycin.

(B) PANC-1 (left) and MIA PaCa-2 (right) cells were cultured in DMEM medium supplemented with 1.5% fetal bovine serum (FBS) with nothing (0), 50 nM YM155, 7.0 μM FAK inhibitor III or a combination of 50 nM YM155 + 7.0 μM FAK inhibitor III.

The viability of the cells was determined using Trypan Blue approximately 40 hours later. The ratio of dead cells to total cells was used to determine the percentage of cell death for each condition. The data shown represent the mean ± standard error. All experiments were performed a minimum of three independent times, and statistical significance was determined using Student's t-tests; ** $p < 0.01$, n.s. not significant, compared to YM155 treatment group. ### $p < 0.001$, ## $p < 0.01$, compared to Geldanamycin treatment or FAK inhibitor III treatment group.

Generation of a YM155 resistant pancreatic cancer cell line.

As mentioned earlier, drug resistance is one of the biggest challenges confronted by oncologists when treating pancreatic cancer patients. These patients are either inherently resistant to therapies, or quickly develop resistance to them[1,2,3]. As YM155 treatment can efficiently kill both PANC-1 and MIA PaCa-2 cells, it is interesting to consider the possibility that YM155 resistant cell lines could be developed, as they would be important tools that could be used to help understanding the mechanisms that underly drug resistance. To attempt to generate YM155-resistant cell lines, PANC-1 and MIA PaCa-2 pancreatic cells were treated weekly with increasing concentrations of YM155, starting with 5 nM and ending at 120 nM in their conventional growth medium (DMEM containing 10% FBS) for 2 months. Several attempts to generate a YM155 resistant PANC-1 cell line proved to be unsuccessful. In fact, 5 nM YM155 was sufficient to kill virtually all of the cells within 5 days. On the other hand, a YM155-resistant MIA PaCa-2 pancreatic cancer cell line, referred to as MIA PaCa-2/R, was successfully generated and is currently being maintained in growth medium supplemented every third day with 120 nM YM155. The morphology of the MIA PaCa-2/R cells tends to be more rounded, compared to their parental counterparts (MIA PaCa-2 Wildtype cells) (Fig. 8). Despite the morphology difference, the growth rate of the YM155 resistant and the wildtype (i.e. parental) MIA PaCa-2 cell lines are similar.

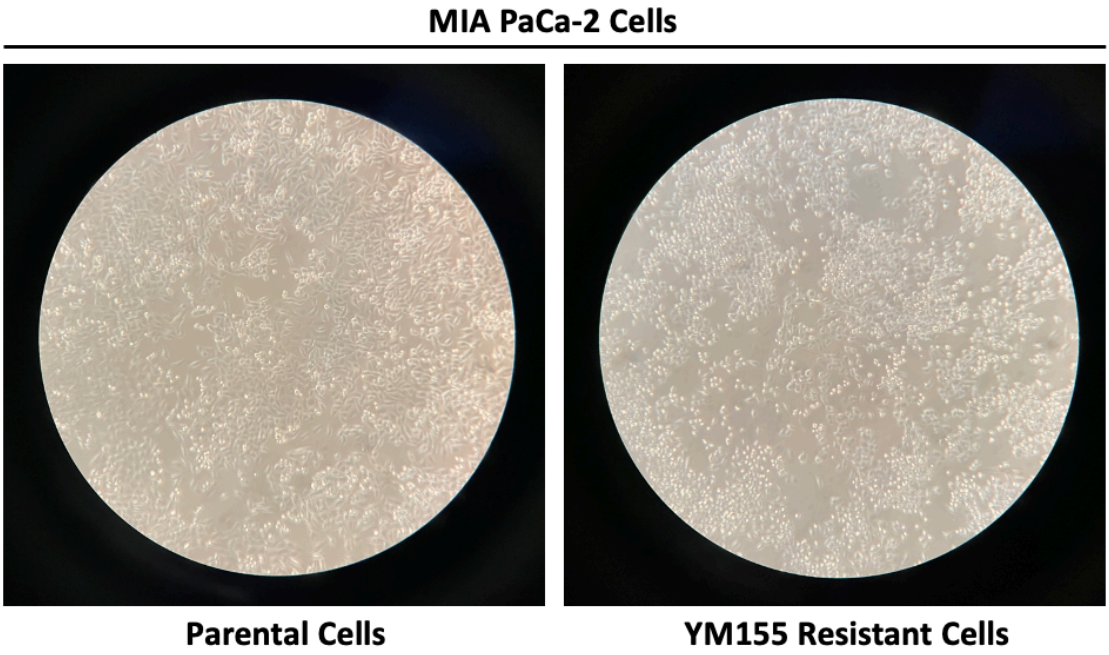

Figure 8. A YM155 resistant MIA PaCa-2 cell line has been generated.

Images of parental (YM155 sensitive), and YM155 resistant, MIA PaCa-2 cells. The resistant cell line was cultured for approximately three months in increasing concentrations of YM155, starting with 5 nM. The resistant line is currently being maintained in 120 nM of YM155. No obvious changes in cell growth has been observed.

To begin to investigate the mechanism underlying YM155 resistance in the MIA PaCa-2/R cell line, I first determined the levels of Survivin expression in these cells. The wildtype MIA PaCa-2 cells and the MIA PaCa-2/R cells were treated without (-), or with 50 nM or 100 nM YM155 for 24 hours, at which point the cells were lysed and analyzed by Western blot analysis using a Survivin antibody. The findings in Fig. 9A (top panel) show again that the Survivin expressed in untreated wildtype MIA PaCa-2 cells can be reduced upon their treatment with YM155. However, the levels of Survivin detected in the YM155 resistant MIA PaCa-2 line (MIA PaCa-2/R) were comparable to those seen in the YM155-treated wildtype MIA PaCa-2 cells. This suggests that the resistance of the MIA PaCa-2/R cell line to YM155 could not be explained by the cells finding an alternative mechanism to upregulate Survivin expression levels.

As shown in Fig 4A, MEFs expressing an activated form of KRAS (specifically $KRAS^{G12D}$) can increase Survivin expression. This led me to consider whether the RAS-RAF-MEK-ERK pathway is upregulated, for example might ERK activity be enhanced in MIA PaCa-2/R cells. I discovered that compared to the level of ERK activity detected in the wildtype MIA PaCa-2 cells, it was higher in the YM155 resistant MIA PaCa-2/R cell line, as determined by Western blot analysis using an antibody that recognize the activated, or phosphorylated form of ERK (i.e. P-ERK, Fig. 9B).

I then determined whether the MIA PaCa-2/R cells were sensitive to treatment with Geldanamycin, FAK inhibitor III, and also the ERK inhibitor, SCH772984, after seeing ERK activity was upregulated in these cells. MIA PaCa-2/R cells were treated with 100nM YM155

alone (as a control) or together with 75nM Geldanamycin, 7.0 μM FAK inhibitor III or 2.0 μM SCH772984 for 40 hours, at which the viability of the cells were determined using Trypan Blue.

Fig.10 shows that combining YM155 with Geldanamycin, FAK inhibitor III, and SCH772984 all showed increased killing efficacies of MIA PaCa-2/R cells, with 28.70%, 38.14%, and 10.43% cell death caused by Geldanamycin, FAK inhibitor III, and SCH772984 respectively, compared to 1.09% caused by YM155 treatment alone. Moreover, these findings also suggests that HSP90, FAK and ERK might play important role to compensate for the loss of Survivin in MIA PaCa-2/R cells.

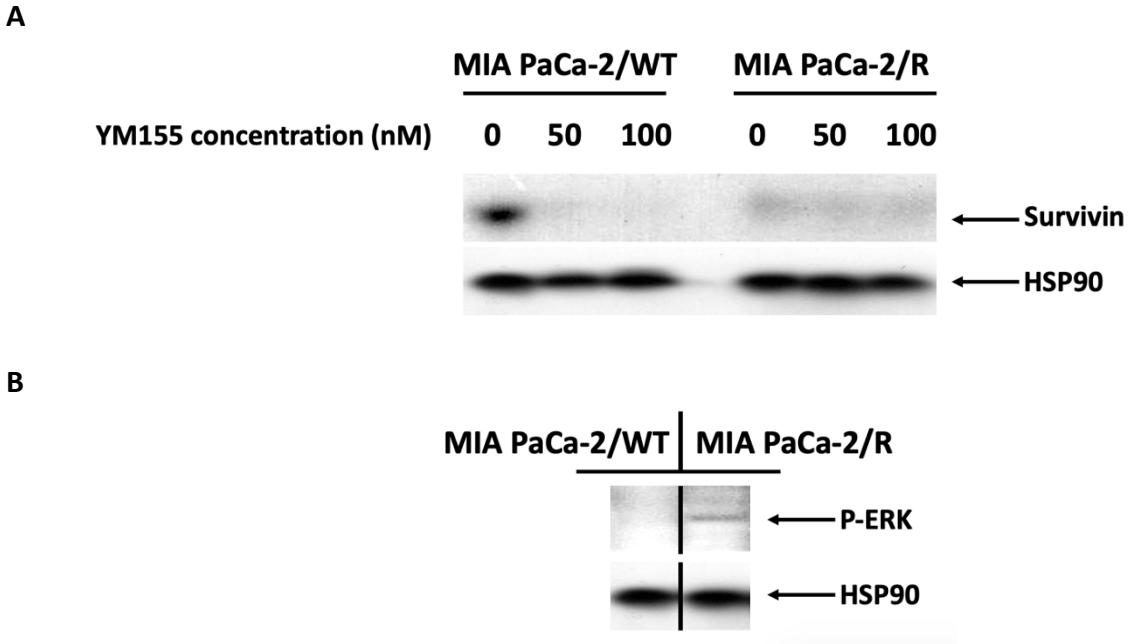

Figure 9. Survivin expression is still inhibited in MIA PaCa-2/R cells.

(A) Wildtype MIA PaCa-2 cells(left), and MIA PaCa-2/R cells (right) were treated without (-) or with the indicated concentration of YM155 for 24 hours, before the cells were lysed and analyzed by Western blot using a Survivin antibody (top panel). HSP-90 was used as the loading control (bottom panel).

(B) Wildtype MIA PaCa-2 parental (left) and MIA PaCa-2/R (right) cells were lysed and analyzed by Western blot using an antibody that recognizes the activation (i.e. phosphorylated) form of ERK (top panel). HSP-90 was used as the loading control (bottom panel).The line in the blots indicates a portion of the blot was deleted.

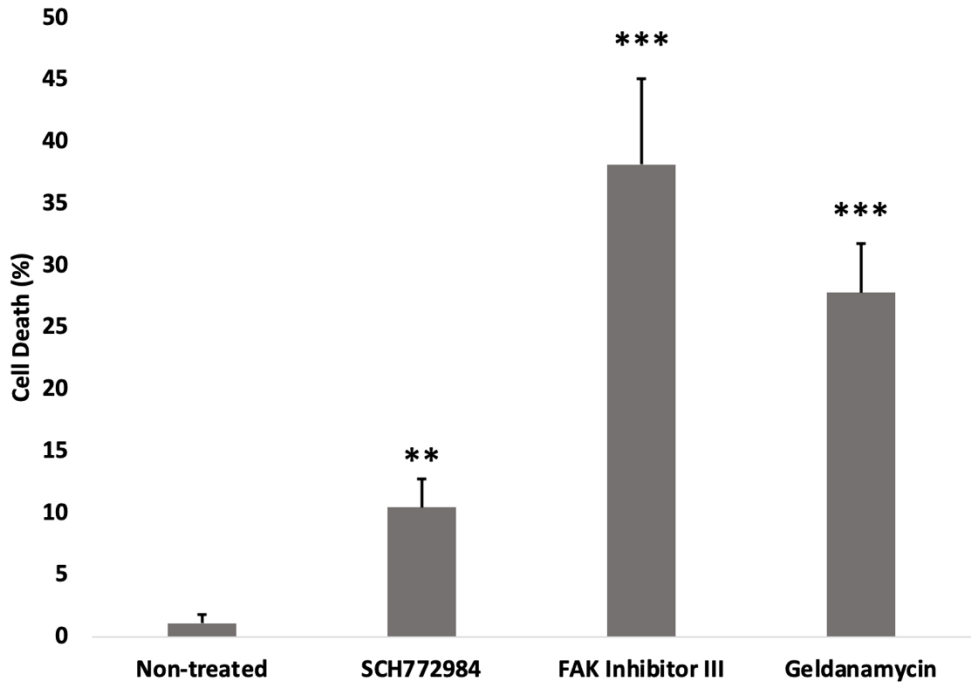

Figure 10. MIA PaCa-2/R cells are sensitive to ERK, FAK, and HSP90 inhibition.

The MIA PaCa-2 cells resistant to 120 nM YM155 were maintained in DMEM supplemented with 1.5% FBS, and either nothing (non-treated) or 2.0 μM SCH772984, 75 nM Geldanamycin or 7.0 μM FAK inhibitor III. Approximately 40 hours later, , the viability of the cells was determined for each condition using Trypan Blue. The ratio of dead cells to total cells was used to determine the percentage of cell death for each condition. The data shown in represent the mean ± standard error. All experiments were performed a minimum of three independent times and statistical significance was determined using Student's t-tests; ** $p < 0.01$, *** $p < 0.01$, compared to YM155 treatment group.

4. Conclusion and Discussion

The major objective of this thesis was to determine important mechanisms that underlie pancreatic cancer progression and to identify possible novel drug combinations that could be used to treat this disease. This is especially important given that pancreatic cancer is quite common and highly deadly.[1-3] Experiments showed that MEFs that were induced to express a mutant form of KRAS ($KRAS^{G12D}$), or human pancreatic cancer cell lines that express a mutant form of KRAS, strongly upregulated the expression of a protein called Survivin (Fig. 4). Blocking Survivin expression using YM155 in two different pancreatic cancer cell lines, PANC-1 and MIA PaCa-2, effectively killed them (Fig. 5-6), suggesting that these cells are dependent on Survivin to maintain their viability. The ability of Survivin to promote cell survival might be important in many stages of PDAC. For example, Survivin likely plays an important role in the early phase of pancreatic cancer, when the cells acquire a mutation in KRAS. During this process, cells transition from a non-transformed state to a transformed, cancer-like state. This transition is often accompanied by intense cell stress and cell death.[54] However, since KRAS can increase Survivin expression, a protein that is known to promote cell survival by binding and inhibiting the apoptotic machinery, the cells gain survival advantages. Increased Survivin expression may also be important during tumor expansion, when a tumor gets to a certain size that oxygen and nutrients are become limited.[55,56] Under these stressful conditions, Survivin may help the cancer cells survive. One last context where Survivin may potentially play a role in pancreatic cancer is in response to therapies. One of the major challenges confronted by oncologists when treating pancreatic cancer patients is

the fact that the patients often respond poorly to treatments, accounting for the high mortality rate associated with this disease.[1-3] Survivin has been showed to promote chemotherapy resistance and thus could be a major contributor to tumor recurrence following treatments.[35-39]

Therefore, Survivin may represent a unique vulnerability in pancreatic cancer that could be taken advantage of as a treatment option. In addition to the promising efficacy of YM155 treatment alone, its combination with the HSP90 inhibitor, Geldanamycin showed an increased ability to cause PANC-1 cells to die (Fig. 7A), a cell type which has been reported to be resistant to several different chemotherapy drugs.[57,58] Moreover, even after multiple attempts, a YM155 resistant cell line could not be generated from PANC-1. This result suggests that PANC-1 cells are exceptionally dependent on Survivin and thus YM155 treatment could be an effective treatment option for pancreatic cancer patients that failed to respond to other therapies. Although MIA PaCa-2 cells are also sensitive to YM155, which caused a considered amount of cell death, a YM155 resistant cell line could be generated overtime (referred as MIA PaCa-2/R). Despite MIA PaCa-2/R cells being resistant to YM155, they are still sensitive to treatment with other drugs including Geldanamycin, a HSP90 inhibitor, FAK inhibitor III, a FAK inhibitor, and SCH772984, an ERK inhibitor (Fig. 10). Also, as discussed in the Introduction, a recent study has shown that blocking ERK activation using SCH772984, as well as autophagy using hydroxychloroquine, could strongly inhibited the growth of pancreatic cancer cells.[28] Therefore, it would be interesting to see if combining YM155 and hydroxychloroquine would have additive or synergetic effect in pancreatic cancer cells.

These findings might offer novel strategies for the treatment human pancreatic cancer that

would increase PDAC patient survival. They also lay the ground work for researchers to better understand how Survivin mediates its effects. YM155 has shown manageable toxicity in phase I clinical trials, although its phase II clinical trials in lung and prostate cancers were less successful.[48,49] Thus, after obtaining the promising *in vitro* results in this thesis, it would now be important to expand this line of study and examine whether YM155 alone, or in combination with Geldanamycin, would be able to inhibit tumor growth and invasive activity in mouse models of pancreatic cancer. If successful, these findings could then potentially lead to clinical trials.

The MIA PaCa-2/R cell line developed that is resistant to YM155 is also an important tool that can give crucial insights into drug resistance. Here, MIA PaCa-2/R cells can be compared to the wild type MIA PaCa-2 cells to search for differences that could explain the drug resistant phenotype. As Survivin expression of MIA PaCa-2/R was still inhibited under YM155 treatment (Fig. 9A), this resistance cannot easily be explained by an alternative mechanism to upregulate Survivin expression levels. Thus, I would be especially interested in performing proteomic analysis, or RNA seq on each cell line and look for proteins that are involved in promoting cell survival that are differentially expression in these two cell lines.

Although a good deal of work still needs to be done to fully understand the mechanism of YM155 resistance in these cells, ERK activation is elevated in YM155 treatment (Fig. 9B). ERK is an effector of KRAS and is important for promoting cell growth and survival. Also, treating the MIA PaCa-2/R cells with ERK inhibitor SCH772984 was able to cause them to die (Fig. 10). Therefore, an important question is raised: is KRAS or one of its effectors (i.e. ERK) is up-

regulated in the MIA PaCa-2/R cells as a way to compensate for the loss of Survivin expression.

Another mechanism of drug resistance involves the altered mechanism observed in cancer cells.[59] Unlike normal cells, which rely primarily on mitochondrial oxidative phosphorylation to generate energy, cancer cells depend on aerobic glycolysis for rapid energy production, which can be fueled not only by glucose, but also amino acid glutamine (Gln).[60,61] Mutant forms of KRAS, have been suggested to reprogram Gln metabolism in PDAC cells.[62] Moreover, Survivin has also been reported to enhance aerobic glycolysis by regulating mitochondrial fusion and fission.[63] These findings raised an interesting possibility that the glutamine dependent aerobic glycolysis of the MIA PaCa-2/R cells was up regulated. This possibility might be further tested by metabolism assays, for example lactate assay.

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