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Targeting Ribosome Biogenesis for Pancreatic Cancer Treatment

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The University of Texas Rio Grande Valley

TARGETING RIBOSOME BIOGENESIS FOR PANCREATIC CANCER TREATMENT

A Thesis
by
CARLOS PEREZ III

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ABSTRACT

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Pancreatic cancer (PanCa) is one of the leading causes of cancer-related death in the United States. Currently, PanCa is one of the recalcitrant cancers that has very limited therapeutic options available for its treatment. The current standard care of PanCa is gemcitabine (GEM) alone or in combination with FOLFIRINOX, nab-paclitaxel, erlotinib, or 5-FU PanCa, which often show poor response. Therefore, the discovery of new molecular targets and new treatment strategies are required for the prevention and treatment of PanCa. Ribosome biogenesis process is dysregulated in most of the cancer types of results in production of more ribosomes and synthesis of oncoproteins which lead to the induction, progression, and metastasis of cancer. This process in the cancer cells can be controlled by pharmacological inhibitors targeting various components of ribosome biogenesis. BMH-21 is a non-toxic selective inhibitor of RNA Polymerase I that inhibits ribosome biogenesis via binding to GC rich ribosomal DNA (rDNA) resulting inhibition of RNA polymerase I activity. BMH-21 has been shown for its potent anti-cancer effects against solid tumors in preclinical mouse models, but no study exists in the literature to explore its therapeutic efficacy against PanCa.

DEDICATION

The completion of my masters' studies would not have been possible without the love and support of my family. My mother, Beatriz Perez, my father, Carlos Perez Jr, my wife, Melissa Guevara Perez, and my children, Carlos IV, and Cruz, who have wholeheartedly inspired, motivated, and supported me by all means to accomplish this degree. Thank you all for giving me all your love, motivation, and patience as I pursue and complete my educational goals. I also would like to thank my siblings Christina Perez, Rene Perez, and David Perez as they are always pushing me to accomplish my goals and expand my career.

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CHAPTER I

INTRODUCTION

1.1 Statement of Problem

Pancreatic cancer (PanCa) is the third leading causes of cancer-related deaths in the United States (Siegel, Miller, and Jemal 2020) with limited therapeutic options. Gemcitabine (GEM), a deoxycytidine nucleoside analog, is currently considered the most effective therapy for PanCa; however, it shows only a marginal survival benefit of 6 months (Burris et al. 1997). Moreover, combinations of GEM with other therapeutic regimens show only marginal improvements in a patient's overall survival (Chapman et al. 2018; Frese et al. 2012). This poor response is mainly due to desmoplastic pancreatic tumor microenvironment (TME) which includes excessive fibrosis, infiltration of immune cells, extracellular matrix deposition and occurrence of various oncogenic events including dysregulation of ribosome biogenesis (Merika, Syrigos, and Saif 2012; Whatcott et al. 2012; El Hassouni et al. 2018; Ruggero 2012; Ruggero and Pandolfi 2003; Derenzini et al. 1998; Sheng et al., 2005).

About 95 % of the patients that suffer from PanCa has a mutation in *KRAS* gene. In most of the cases individuals that are diagnosed with PanCa have no symptoms and disease has already been metastasize in distant organs. Therefore, understanding the PanCa biology and discovery of new molecular targets and their inhibitors are urgently required for the management of this disease. In this context, Dr. Hafeez laboratory at Department of Immunology and Microbiology, SOM, UTRGV working on to understand the dysregulation of ribosome

biogenesis process in PanCa and how targeting various ribosome biogenesis components could be a novel strategy for the prevention and treatment of PanCa. In this thesis dissertation, we identified some interesting ribosome biogenesis molecules of ribosome biogenesis which are overexpressed in PanCa cells and tumor tissues when compared with normal pancreatic ductal epithelial cells or normal adjacent pancreatic tissue. Moreover, we also evaluated therapeutic potential of an RNA polymerase inhibitor BMH-21 against PanCa cells. The rational for using BMH-21 is 1) for its potential anti-cancer activity against cancer but no toxicity in normal epithelial cells and 2) currently there is no study listed on PubMed to define the anti-cancer activity of BMH-21 against cancer.

1.2 Statement of the Purpose

Dysregulation of ribosome biogenesis occurs in PanCa. We observed overexpression of ribosome biogenesis components in PanCa cells and tissues when compared to normal pancreatic ductal epithelial cells and normal adjacent tissues. However, no study has been listed on PubMed if targeting ribosome biogenesis process can lead to the tumor growth inhibition of PanCa. We hypothesize that targeting ribosome biogenesis process by small molecule inhibitor of RNA polymerase I inhibit the growth and metastasis of PanCa .

The main objective of this thesis proposal is to identify the expression profile of various ribosome biogenesis components and if targeting this process by RNA polymerase inhibitor (BMH-21) suppresses the growth of PanCa. This study for the first time will develop BMH-21 as a new therapeutic drug against PanCa which has significant translational impact in the clinic. To accomplish the objectives of our thesis, we proposed following two specific Aims:

AIM 1: To investigate the expression patterns of various components of ribosome biogenesis in pancreatic cancer cells and tissues.

AIM 2: To investigate the therapeutic efficacy of RNA Polymerase I inhibitor (BMH-21) against pancreatic cancer.

CHAPTER II

REVIEW OF LITERATURE

2.1 Pancreatic Ductal Adenocarcinoma

Pancreatic cancer (PanCa) is the most significant malignancy and third leading cause of cancer related deaths in the United States. The generic term PanCa includes a class of malignancies originating from the exocrine or endocrine tissue of the pancreas. Pancreatic adenocarcinoma is the most common form, representing approximately 85% of cases. In the United States there is a 5-year survival rate expectancy with the ranges of 5% to 15% and the survival rate of 6% (Puckett & Garfield, 2022). In addition, there are other less frequent types of exocrine cancers such as squamous cell carcinomas, adenosquamous carcinomas, signet ring cell carcinomas, undifferentiated carcinomas, and undifferentiated carcinomas with giant cells. Gemcitabine (GEM), a deoxycytidine nucleoside analog, is currently considered the most effective therapy for PanCa; however, it shows only a marginal survival benefit of 6 months (Burris et al. 1997). Moreover, combinations of GEM with other therapeutic regimens show only marginal improvements in a patient's overall survival (Chapman et al. 2018; Frese et al. 2012).

2.2 Molecular Pathology of Pancreatic Cancer

PanCa represents various driver mutations in KRAS, TP53, SMAD4 and CDKN2A, which occurs as alone or in combination of in more than 50% cases, whereas recurrent mutations in a number of other genes, including *ARIDIA, KDM6A, MLL3, TGFBR2, RBM10, and BCORL1,* are found in 10 % of tumors (Waddell et al. 2015; Bailey et al. 2016). However, amplification of *ERBB2,* fusion/mutation of BRAF gene and mutation in DNA damage repair

genes *BRCA1, BRACA2 or PALB2* are found in response to targeted therapy (Witkiewicz et al. 2015; Biankin et al. 2012). Additionally, “bulk” transcriptomic profiling has identified two broad PDAC subtypes with distinct biology, namely Classical and Basal-like, with Basal-like tumors associated with significantly poorer outcome (Collisson et al. 2011; O'Kane et al. 2020; Chan-Seng-Yue et al. 2020). These subtypes are characterized by the differential expression of pancreatic specific transcription factors, such as *GATA6, PDX1, and HNF1A,* which are maintained in Classical tumors and are lost in Basal-like PDACs. In addition to these molecular genetic events, formation of desmoplastic tumor microenvironment (TME) plays an important role in induction, progression, and therapy resistance of PanCa.(Candido et al. 2018; Steele et al. 2020; Hutton et al. 2021). The pancreatic TME is generally considered as immunologically cold tumor, exhibiting low numbers of CD8+ cytotoxic T cells and high numbers of immunosuppressive immune cell populations specifically tumor associated macrophages and myeloid derived suppressive cells, rendering most PanCa patients poor candidates for immunotherapy (Karamitopoulou 2019; Ho, Jaffee, and Zheng 2020).

2.3 Statistic and Risk Factors

PanCa is estimated to cause the death of over 331,0000 people every year and there is also a 5-year survival rate for individuals that get this type of cancer (Puckett & Garfield, 2022). In the areas of North America males and females have the highest incidences of PanCa if compared to African or Asia countries. However, when males or females are older in age such as 70-years old the incidence rates for this type of disease increases. It has been identified that about 90% of individuals with PanCa are over the age of 55 years old (Puckett & Garfield, 2022). Over 90% of pancreatic adenocarcinomas occur in the ductal cell while the rest occur in

cystadenocarcinoma and acinar cell carcinoma. Pancreatic adenocarcinomas occur in 2/3 of the pancreatic head; 1/3 occurs at the pancreases body or tail. There is ongoing research to study the genetic information to the various types of pancreatic adenocarcinomas to develop better therapeutic strategies.

Pancreatic adenocarcinoma becomes more susceptible to individuals that consume increased amounts of alcohol, obesity, diabetes, smoking, African Americans, and genetic history. As individuals smoke cigarettes it increases their risk of PanCa if they are smoking 35 cigarettes or more (Klein, 2021). However, individuals that decide to quit smoking will reduce the odds that they get PanCa if compared to smokers and non-smokers. Patients that suffered from adenocarcinoma and had ceased smoking for 10 to 20 years they have a greater risk of the cancer returning (Klein, 2021). Diabetes that is over three years increases the risk factor towards PanCa formation in the pancreas. Individuals that have a higher risk facto to PanCa are those that suffer from fasting glucose, insulin, or insulin resistance levels (Klein, 2021). Another key factor for individuals that are susceptible to PanCa are individuals with elevated $HbA_{1c.}$ Elevated levels of HbA_{1c} are signs of excessively high sugar levels and are a sign that an individual has diabetes.

<u>Figure 1:</u> Worldwide burden of risk factors and trends in pancreatic cancer: A. Statistics of Males and Females That Participate in Consumption and Smoking: -B. New cases, deaths, and 5-year relative survival of PanCa in US. C. PanCa death rate per 100,000 persons by race/ethnicity. D. Percent PanCa death by age group. Picture was taken from *Gastroenterology 2021; 160;744-754.*

2.4 Ribosomes

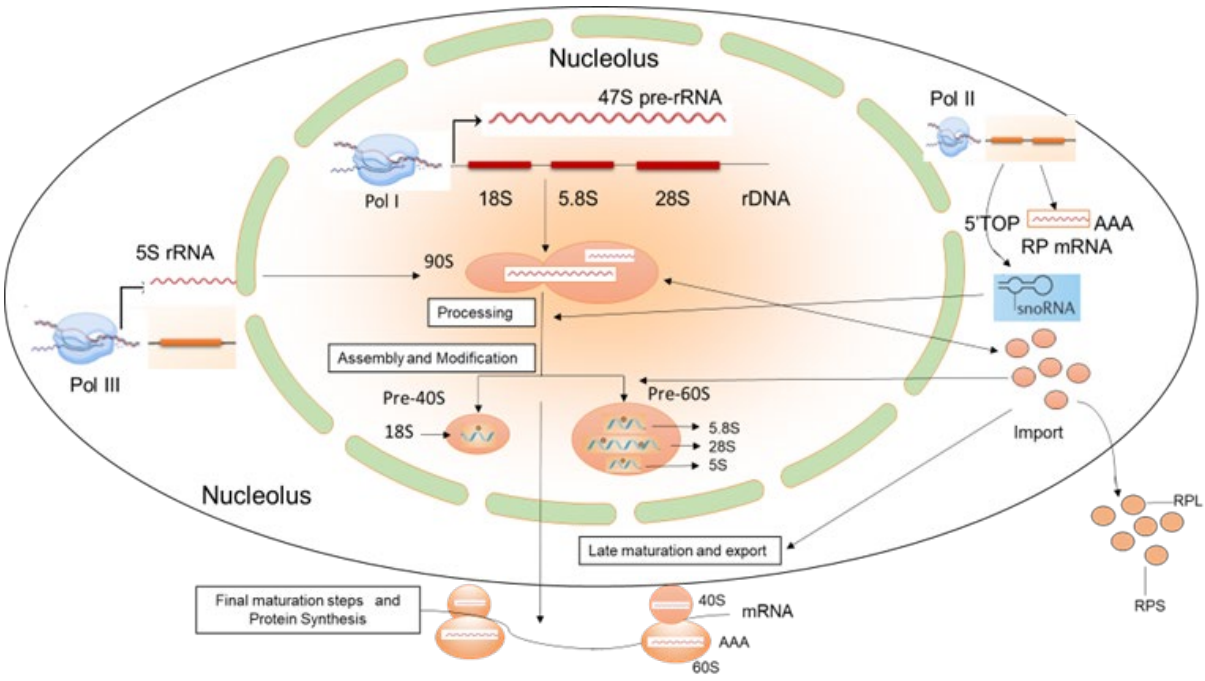
Ribosomes were first discovered by George Palade in 1955. They are also called as Palade granules after their discoverer. Ribosomes are macro-molecular machines made up of ribonucleoproteins that play an indispensable role in central dogma. As an important component of central dogma chain, ribosomes decode the instructions stored in mRNA transcripts and help in the synthesis of polypeptides and proteins (Lafontaine and Tollervey 2001). The synthesis and functioning of ribosomes are essential for maintenance of normal gene expression and cellular homeostasis. Moreover, biogenesis of ribosomes is a very high energy consuming process accounting for half of energy needs of the cells at some stages(Pelletier and Sonenberg 2019). Thus, ribosome biogenesis and function has been subjected to very stringent controls (Bianco

and Mohr 2019; Gościńska and Topf 2020). Ribosomes are classified on the basis of rate of sedimentation. Smaller ribosomes are 70S and are found in prokaryotes and some organelles of eukaryotes such as mitochondria. They are composed of 30S and 50S subunits. Large ribosomes are 80S and occur in eukaryotes. They are composed of 40S and 60S subunits (Wilson and Doudna Cate 2012; Pelletier, Thomas, and Volarević 2018). Each ribosomal unit has its own function. The 40S subunit of human ribosomes is made up of 18S rRNA and 33 different ribosomal proteins. It deals with binding, unwinding, and scanning of mRNAs received from genes. 60S subunit of human ribosomes in involved in the synthesis of peptide bonds and maintenance of quality of newly synthesized nascent peptides. 60S subunit is made up of three rRNA strands (5S, 5.8S and 28S) and 47 different ribosomal proteins. Two decades ago the three dimensional structure of intact ribosome and ribosomal units was solved using X-ray crystallography with atomic level resolution (Schluenzen et al. 2000). The new structural details strengthen the idea that rRNAs are the main functional element of ribosomes. Also, as per the X-ray crystallography data the shape of ribosomes is to a large extent determined by rRNAs. Earlier it was believed that cells produce a single type of ribosomes. However, it has been shown that cells produce a heterogenous populations of ribosomes that differ in composition and structure (Genuth and Barna 2018). These findings could lead to paradigm shift in our understanding of processes of translation and protein synthesis in normal and pathological states.

2.5 Ribosome Biogenesis Process

The biogenesis of ribosomes occurs in the nucleolar region of nucleus during interphase of cell cycle (Penzo et al. 2019). The portion of DNA that codes for ribosomal genes is called as

ribosomal DNA (rDNA). In humans, rDNA is transcribed by RNA pol I. rDNA is transcribed as 47S rRNA which undergoes several modifications and maturation processing to generate 18S, 5.8S and 28S rRNA. 5S rRNA is transcribed in nucleoplasm by RNA pol III and then translocated to nucleolus (Catez et al. 2019). During the process of biogenesis, ribosomal proteins are assembled with rRNA to produce ribosomal subunits which later combine at the time of protein synthesis to produce functional ribosomes (Pelletier, Thomas, and Volarević 2018). Ribosome biogenesis is one of the most complex processes that occurs in the cell. During this process 84 core components (4 rRNAs and 80 ribosomal proteins) are orchestrated to produce ribosomes with the help of around 200 factors. It occurs in several compartments of cell viz nucleolus, nucleoplasm and cytoplasm. Due to its complexity, the process of ribosome biogenesis poses a constant threat to protein homeostasis of cells (Albert et al. 2019). The unregulated synthesis of ribosomal proteins can lead to cellular stress and consequent cell death. To avoid loss of resources and prevent proteotoxic stress, cells have evolved quality control to regulate ribosomal biogenesis. The key protein involved in quality control of ribosome biogenesis is Heat shock factor 1(Hsf 1) (Li, Labbadia, and Morimoto 2017). Hsf 1 prevents aggregation of ribosomal proteins by several mechanisms inducing expression of chaperones (HSP 70 and HSP 90), aggregates (nuclear and cytoplasmic) and components of proteasomal machinery (Solís et al. 2016; Mahat et al. 2016). In pathological conditions such as cancer, ribosomal biogenesis is dysregulated (Bianco and Mohr 2019). The extent of ribosomal dysregulation and its role in origin, development and metastasis of cancer has not been understood yet. So far, several inhibitors of various aspects of ribosome biogenesis have been developed and some of these molecules are various stages of clinical trials. Further progress in this field of cancer biology may lead to the development of promising anticancer strategy.



<u>Figure 2:</u> Schematic diagram showing the process of ribosome biogenesis: The nucleolus is where the process of ribosome biogenesis occurs it is in this area where the 47S repeats and 47S pre-rRNA initiate the repeated transcribing processes. The SL-1 complex initiates RNA Pol I by using regulatory subunits to cause an interaction with UBF and RRN3 communicate with RNA polymerase I. After RNA Pol I is activated the 47S pre-rRNA modifies so that mature forms of rRNA (28S, 18S, and 5.8S). The matured forms of 28S, 18S, and 5.8S rRNAs will together affect the 5S rRNA from the nucleolus to bind to ribosomal proteins while using RNA Pol III. RNA Pol I will develop the ribosomal proteins in the nucleolus and Polymerase III will cause the 40S and 60S subunits to be created and become exported into the nucleus where they will be transported to the cytoplasm and the mature form of the 80S ribosomes will become developed.

2.6 Ribosome Biogenesis and Cancer

Cancer cells are addicted to the biogenesis of ribosomes (Catez et al. 2019). In cancer cells, increased activation of ribosome biogenesis is reflected by changes in morphology, size and number of nucleoli. Traditionally, these changes have been used by pathologists to establish

tumorigenicity of biopsy samples using silver staining (Trerè 2000). Cancer tissues with high malignancy have more black spots localized through nucleolar region than less malignant tissues. Recently, algorithms have been developed to analyze silver staining quantitatively and qualitatively. The results of silver staining are now presented as indexes which reflects the extent of nuclear disruption. Moreover, use of algorithms allows pathologists to address the issues related to reproducibility and quantifications of silver staining. It has been observed that cancer cells use up-regulation of ribosome biogenesis as mechanism for transformation as well as tumorigenesis(Pelletier, Thomas, and Volarević 2018; Barna et al. 2008). However, various aspects of mechanisms by which ribosome biogenesis leads to transformation and tumorigenesis are not clear. It has also not been understood that why some cancer cells are more dependent on ribosome biogenesis than others (Catez et al. 2019). The activation of various oncogenes like c MYC, mTOR, PI3K and Akt and inactivation of tumor suppressor genes such as TP53, PTEN and RB lead to dysregulation of RNA polymerases which leads to increased ribosome biogenesis(Catez et al. 2019; Pelletier, Thomas, and Volarević 2018; Nait Slimane et al. 2020; Drygin, Rice, and Grummt 2010).

2.7 Ribosome Biogenesis and Pancreatic Cancer

We did not find any study on PubMed which show the role of ribosome biogenesis components in pancreatic carcinogenesis. However, a recent study demonstrated therapeutic effect of RNA polymerase inhibitor (CX-5461) against pancreatic ductal adenocarcinoma (El Hassouni et al. 2019). In our thesis aim I, we investigated the expression profile of ribosome biogenesis components. However, further studies are required to investigate the function impact of targeting ribosome biogenesis components in PanCa. These studies will help in understanding of the

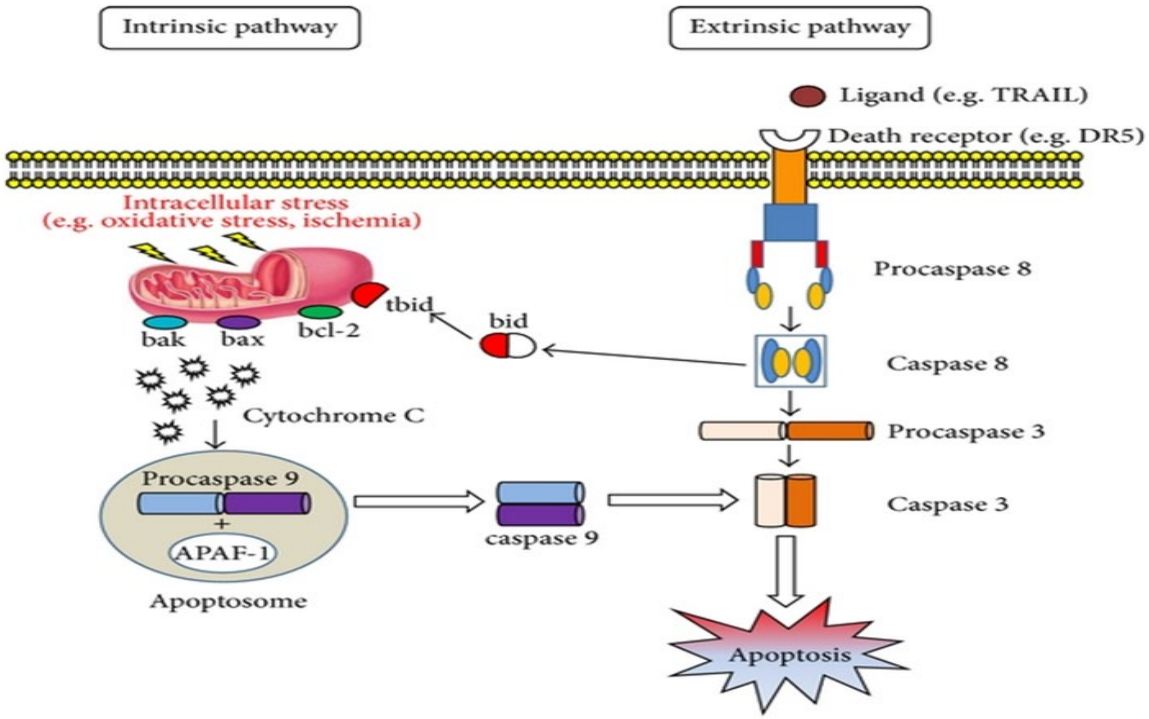
molecular pathobiology of PanCa and determine to identify the potential molecular targets for future drug discoveries against this lethal malignancy.

2.8 Apoptosis and Cancer

Apoptosis normally occurs in a cell to help with maintenance and the removal of various tissues that are no longer needed. In addition, apoptosis is a controlled cell death that results in the bubbling of cellular material that will be consumed by macrophages. The extrinsic pathway contains a death ligand receptor that is found at the outer part of a cell for the mechanism to become activated. This pathway allows various types of caspases to become released one after the other which occurs within the cell so that apoptosis can take place. Death receptors are made up of Fas receptors, tumor necrosis factor receptors, and TNF-related apoptosis-inducing ligand receptors (Krakstad, & Chekenya, 2010). The mechanism works as TNF-R1 employs a tumor necrosis factor which will initiate the accumulation of Fas proteins that comprise FADD. Next TNF-R1 connects with TRADD which is considered to be a death protein and will start a recruitment of caspase-8. The accumulation of caspase-8 and -10 will ultimately lead to the cells going through the controlled aspects of apoptosis. As caspases -6, -3, and -7 are activated in a downstream fashion causing the release of the BID protein to initiate apoptosis.

Another pathway that a cell can reach the apoptosis phase of controlled cell death is through the use of an intrinsic pathway that occurs with the use of the cell's mitochondria. Proteins Bax and Bak open the pores of the mitochondria causing cytochrome c to become released from the mitochondria. However, when Bcl-2 and Bcl-xl is active in the mitochondria it results in the cytochrome c protein to stay within the mitochondria. With the inactivation of cytochrome c, it will cause and interruption in cell death and results in the accumulation of cells. The release of cytochrome c into the cytoplasm will cause the creation of a complex referred to as apoptosome

which is needed to activate the caspases needed for apoptosis. The apoptosome contains an apoptotic protease activating factor 1(APAF-1) which will the inactive caspase-9 to become initiated with the hydrolyzation of adenosine triphosphate. As adenosine triphosphate becomes hydrolyzed it will cause the activation of caspases -9 which is used to activate caspases – 3, -6, and -7 and result in cells activating apoptosis.



<u>Figure 3.</u> Schematic diagram showing intrinsic and extrinsic pathways involved in the induction of apoptosis

CHAPTER III

MATERIALS AND METHODS

3.1 *In Silico* Analysis

Gene Expression Profiling Interactive Analysis (GEPIA) is a web server to determine the gene expression profiling and interactive analyses in human cancer (Williamson et al. 2006). We used this web server to investigate the expression profile of RPA194 and RPA135 in pancreatic tumor tissues, tumor tissue grade and compared with normal pancreatic tissues. We also investigated how their expression correlate with the overall survival of pancreatic cancer patients. Experimental Atlas: This web server contains the RNA Seq-mRNA data base of various human cancer cell lines. With the help of this web server, we investigated the expression profile of various ribosome biogenesis components (TAF-1/SL-1, UBTF, POLR1 and POLR1B) in 15 human PanCa cells.

3.2 Cell lines, Reagents, and Treatments

MiAPaCa-2 (CRM-CRL-1420), Panc-1(CRL-1469), HPAF-II (CRL-1997), Capan-2 (HTB-80), BxPC-3 (CRL-1687), are specific types of PanCa cell lines of human origin. HPNE and HPDE are human normal transformed cell lines. All cell lines were obtained from American Type Culture Collection (ATCC, TIB-71, Manassas, VA). Panc-1 were cultured in complete Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% heat inactivated FBS (Life Technologies, Grand Island, NY), 1x Antibiotic-Antimycotic and incubated at 37°C in a

humidified environment of 5% CO2. BMH-21 was purchased from Cayman chemical (Ann Arbor, MI) and dissolved in Dimethyl Sulfoxide (DMSO, Sigma, St. Louis M.3 MTT Assay

Percent viability of normal and cancer cells upon treatment with RNA pol I inhibitors was done by MTT (3-(4,5-dimethylthiazol-2-yl)- 2,5-diphenyltetrazoliumbromide) as per established protocols with minor modifications (Heo et al. 1990; Hafeez et al. 2017). Briefly, cells were seeded at a density of 2 to 5 × 103 cells/well in 96-well plates and cultured until cells were 75-80% confluent. Confluent cells were then treated with either 0.01 to 0.1 % DMSO (control) or with different concentrations of RNA pol I inhibitors for 24 to 48 hr. After the completion of incubation, 100 µl of 0.5 mg/mL of MTT dye was added and plates were incubated for 2.5 to 5 hours at 37°C. After this, media was removed. The formazan crystals formed from MTT dye were dissolved in DMSO (150µL per well). Absorbance was then measured immediately measured at 570nm.

3.3 Western Blot Analysis

Western blotting was performed as per established methodology(Hafeez et al. 2017).Briefly, cells were 1 to 2 x10^6 cells were seeded in 100 mm plates and allowed to grow to in incubator at 37°C in a humidified environment of 5% CO2. After achieving the proper confluency (60 to 75 %) cell were treated with 0.01 to 0.1 % DMSO or BMH-21 for 4 to 48 hr. After treatment duration, cells were lysed using RIPA lysis Buffer System (Santacruz Biotech., Dallas, TX), passed through syringe (10 to 15 times), centrifuged (14000g x 15 min). The supernatant was collected, and protein concentration estimated by bicinchoninic acid (BCA) assay (23225, Thermo Fisher Scientific). 20 to 40 µg protein was denatured by heating for 5 to 7 min after adding 4X Lammeli buffer. The denatured protein was loaded in wells and separated

using 4 to 10% precast TGX gels at voltage of 100 V. Tris-Glycine-SDS buffer (28362, Thermo Fisher Scientific) was used as running buffer. The proteins were then transferred to PVDF membrane using cold Tris Glycine buffer. Proteins were then blocked in 5% fat free milk in TBST (0.1 % Tween 20-Tris buffered saline) at room temperature for 1-2 hr. after. After completion of blocking membranes were incubated primary antibodies overnight at 4 degrees Celsius with gentle shaking (20 rpm). After this, membranes were washed three times TBST buffer (10 min each) and later incubated with HRP-conjugated secondary antibodies for an hour at room temperature. Membranes were again washed with TBST buffer three times (15 min each) After membranes were incubated sufficient amount of HRP substrate 30 to 60 seconds and images were taken using ChemiDocTM MP (Bio-rad, Hercules, CA).

3.4 RNA Isolation and Quantitative Real time PCR

RNA was extracted from cells using TRIzol reagent (Life Technologies, Carlsbad CA)(Rio et al. 2010). Cells were cultured and treated with inhibitors as discussed in western blotting methodology. The purity and concentration of isolated RNA was determined using Nanodrop OneC (Thermo Scientific, Madison, WI). 2000ng RNA was used to synthesized RNA using High-Capacity cDNA Reverse Transcription Kit (Thermofisher, Graiciuno, LT). RT-PCR was ran using CFX 96 Real Time SystemTM (Bio-Rad Inc., Singapore) to determine the expression level of various genes normalized to the fold change of GAPDH.

3.5 Immunofluorescence Assay

Immunofluorescence was done as per established protocols (Peltonen, Colis, Liu, Trivedi, et al. 2014). 0.005 to 0.01 $x10^6$ cells/well were seeded in Lab-Tek IICC2 4-well chamber slide system (Thermo Fisher Scientific, Rochester, NY). Slide chambers were placed in incubator at 37°C in a humidified environment of 5% CO2. After achieving 60 % confluency, cells were

treated with 0.01 to 0.1 % DMSO or BMH-21 for 4 to 48 hr. After this, cells were fixed with 4% Paraformaldehyde for 15 minutes at 4°C and permeabilized with 0.2% triton X-100 for 5 minutes, blocked with 10% donkey serum at room temperature for 1 hour and then incubated with respective primary antibody overnight at 4°C. Control wells were set up to check autofluorescence of primary and secondary antibodies. Cells were then washed with TBST(1x tris-buffered saline, 0.1% tween 20) and incubated in the secondary antibody (Alexa Fluor 488-conjugated antibody, Jackson Immuno Research) for one hour at room temperature, washed and mounted in antifade mounting medium with DAPI (Vectashield, Burlingame, CA). Images were taken and analyzed using the Nikon AX confocal microscope system.

3.6 Statistical Methods

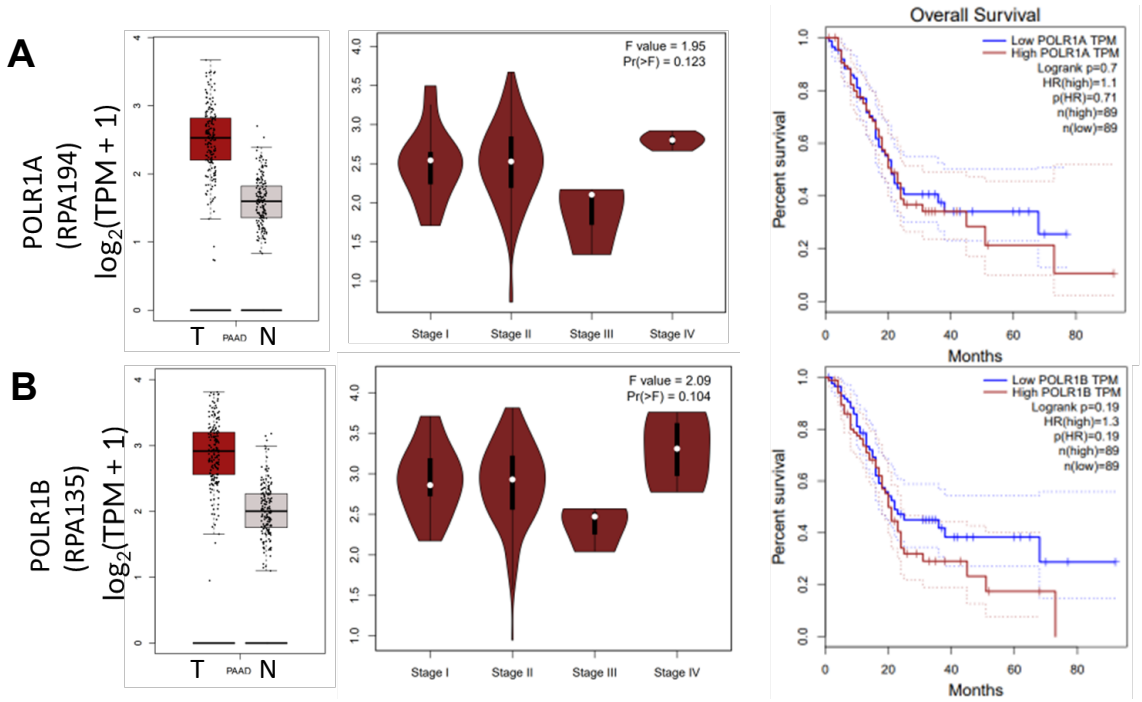
All data obtained during this study were presented as Mean ±Standard error of mean (SEM). Statistical analyses were done using unpaired, two-tailed student's *t*-test and one-way analysis of variance (ANOVA) with the level of significance set at *p*-values < 0.05 with the levels of significance represented respectively as follows *p< 0.05, **p<0.01, ***p<0.001. All graphs were generated using GraphPad Prism (5.03, GraphPad Software, Inc., La Jolla, CA, USA).

CHAPTER IV

RESULTS

Dysregulation of ribosome biogenesis occurs in cancer cells as they require more protein synthesis compared to normal cells. Ribosomes show heterogeneity in their protein and rRNA composition [36]. In cancers, ribosomes have been found that have different types of rRNA modifications than ribosomes of healthy persons. The most prevalent difference in the modification profile of rRNAs between ribosomes of healthy and cancer patients is 2'-O-methylation of nucleotides [37]. Alterations in pattern of 2'-O-methylation of nucleotides of rRNA affects the composition of ribosomes which modulates their activity[38]. These differences in function and composition of ribosomes provides opportunity for specific targeting of cancer ribosomes. Therefore, comprehensive study is required to determine the expression profile of various components of ribosome biogenesis. In our first experimental approach, we performed *in silico* analysis to investigate the expression pattern of POLR1A and POLR1B in in normal and pancreatic tumor tissues using GEPIA web server. We observed overexpression of both RNA Pol I catalytic subunits (POLR1A and POLR1B) in pancreatic tumor tissues (n=170) compared to normal pancreatic tissue tissues (n=70) (Fig. 2A-B). Next, we determined POLR1A and POLR1B expression pattern in various stages of PanCa. Results revealed almost similar pattern of POLR1A but the expression pattern of POLR1B was relatively higher in high grade tumors. We also determined if POLR1A and POLR1B linked to the poor prognosis in PanCa patients. Data suggest higher expression of both POLR1A and POLR1B linked to the overall

poor survival in PanCa patients (Fig. 2A-B). Higher expression of POLR1B correlates poorer prognosis of PanCa as compared to POLR1A (Fig. 2A-B). The expression of POLR1A, POLR1B varies in different stages of PanCa as indicated in stage plot graphs (Fig 2A-B). We next determine the expression pattern of mRNA expression of both POLR1A (Fig. A iii) and POLR1B (Fig. A iv) in various PanCa cells through RNA seq. data base analysis. Results indicated differential expression of POLR1A and POLR1B in various human pancreatic cancer cells. Relatively expression pattern of POLR1A was relatively higher in all the PanCa cells compared to POLR1B. The highest expression of POLR1A (~55 TPM) was observed in Panc 08.1.3 cells, even though highest expression of POLR1B was observed in SUIT-2 cells. Western blot data analysis results also showed relatively higher protein levels of RPA-194 in all the PanCa cells compared to normal pancreatic epithelial cells as determined by Western blot analysis (Fig. 3B).



<u>Figure 4 :</u> POLR1A and POLR1B are upregulated in pancreatic cancer tissues. A**.** POLR1A (A) and POLR1B (B) were upregulated in PanCa samples compared to normal tissues, their expression varies in different stages of PanCa as indicated in stage plot graphs, and higher expression of POLR1A correlates with poor survival. The expression pattern of POLR1A was analyzed using gene expression profiling interactive analysis (GEPIA) datasets. T=Tumor (n=170) and N=Normal (n=70).

4.1 Pancreatic Cancer Cells Showed Increase Pubtf Protein Levels in PanCa Cells.

Upstream binding transcription factor (UBTF) is a multiple HMGbox architectural protein and has double function in the modulation of Pol I and Pol II mediated transcription. UBTF is not only considered to play a role in RNA Pol I-specific transcription of the ribosomal genes, but also enrich at Pol II-transcribed genes throughout human genomes. UBTF can regulate differentiation, proliferation, and cell growth through different signaling pathways. A

recent study has demonstrated that UBTF is linked to the melanoma progression via modulating MEK-1/2 and ERK1/2 signaling pathways (Zhang et al. 2021) suggesting a potential molecular target for the management of skin cancer. However, no study exists in the literature to elucidate the role of UBTF in PanCa. Here, we observed a marked increase of UBTF phosphorylation in PanCa. The phosphorylation of UBTF was relatively higher in HPAF-II cells compared to other PanCa cells or with HPDE and HPNE cells (Fig.5B). Interestingly, highest expression of pUBTF was observed in mouse PanCa cells (KPC) which are derived from genetically engineered mouse model of PanCa. This mouse model was developed in introducing mutant Kras^{G12D} and mutant p53 in pancreatic ductal epithelial cells by using Cre-Lox technology. We observed very little protein levels of pUBTF1 and total UBTF-II isoform in WT-*KRAS* expressing BxPC3 PanCa cells (Fig. 5B). In confocal microscopy results demonstrated higher expression of UBTF in mutant *KRAS* expressing AsPC1 cells compared to HPNE cells but expression of UBTF in BxPC3 cells was almost similar to HPNE cells (Fig. 5C).

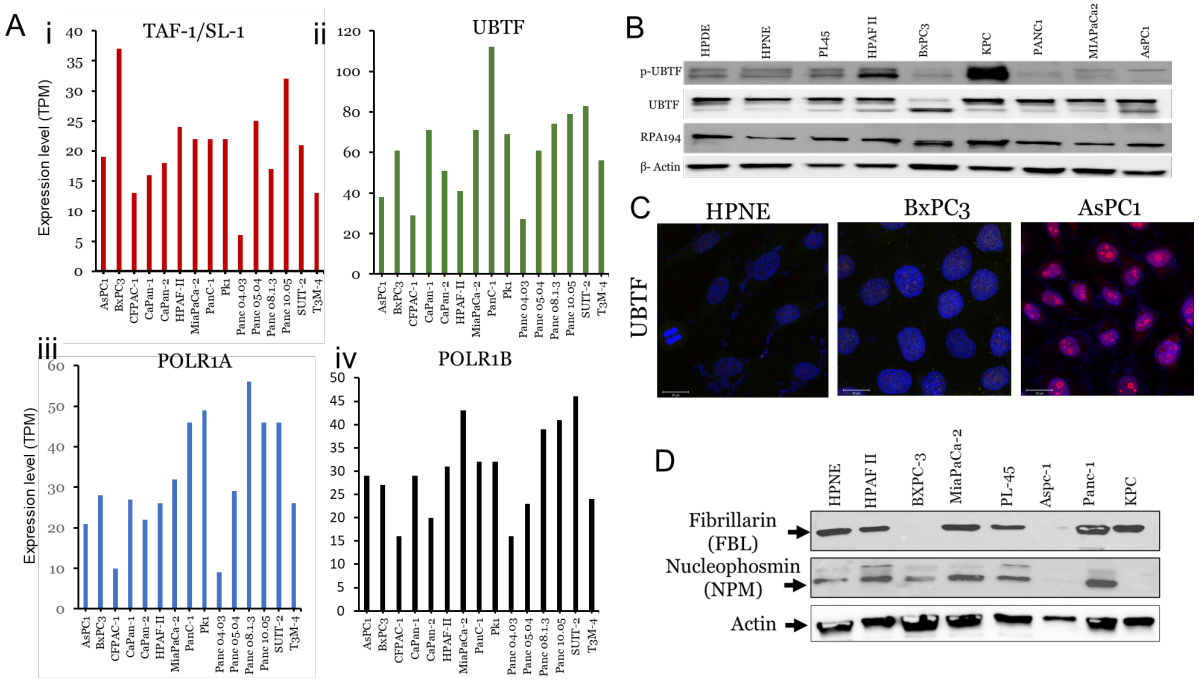


Figure 5 : ***In silico*** **and biochemical analyses in the expression of various ribosome biogenesis components in pancreatic cancer cells vs normal pancreatic epithelial cells.**

A. Expression pattern of TAF-1/SL-1, UBTF, POLR1A, and POLR1B I in various human pancreatic cancer cells. Values in bar graph represent in transcript per million (TPM) as determined by RNA-Seq data base. **B.** Expression pattern of pUBTF, total UBTF transcription factor and RPA194 in transformed normal pancreatic epithelial cells (HPNE), normal pancreatic ductal epithelial cells, and in various indicated human pancreatic cancer cell lines and mouse pancreatic cancer cells (KPC) as determined by Western blot analysis. **C.** Expression pattern of nucleolar proteins (FBL and NPM) in transformed normal pancreatic epithelial cells (HPNE) and in various indicated human pancreatic cancer cell lines and mouse pancreatic cancer cells (KPC) as determined by Western blot analysis. Actin antibody was used as a loading control.

4.2 BMH-21 Treatment Preferentially Induces Cytotoxicity in Human PanCa Cells.

BMH-21 is a specific RNA polymerase I inhibitor which has been shown its anti-cancer effects in various cancer types. However, its role as a therapeutic agent against PanCa was not explored. MTT Assay results demonstrated that BMH-21 preferentially induces cytotoxic effects in PanCa cells but not in human normal pancreatic ductal epithelial (NPNE) cells. BMH-21 has differential cytotoxic action in PanCa cells as we observed a more pronounced effect of BMH-21 in MIA PaCa-2 with an IC50 value of 0.5 μM (Fig. 6A-B). However, the IC50 of BMH-21 in Panc-1, BxPC3, AsPC1, and PL-45 was 3.0, 2.3, 3.0, and 1.8 μM respectively (Fig. 6A-B). BMH-21 did not show any cytotoxic effect against HPNE till 5 μM (Fig. 6C). We observed 60 μM IC50 of BMH-21 in HPNE cells, which was 8-fold higher than PanCa cells (Fig. 6C).

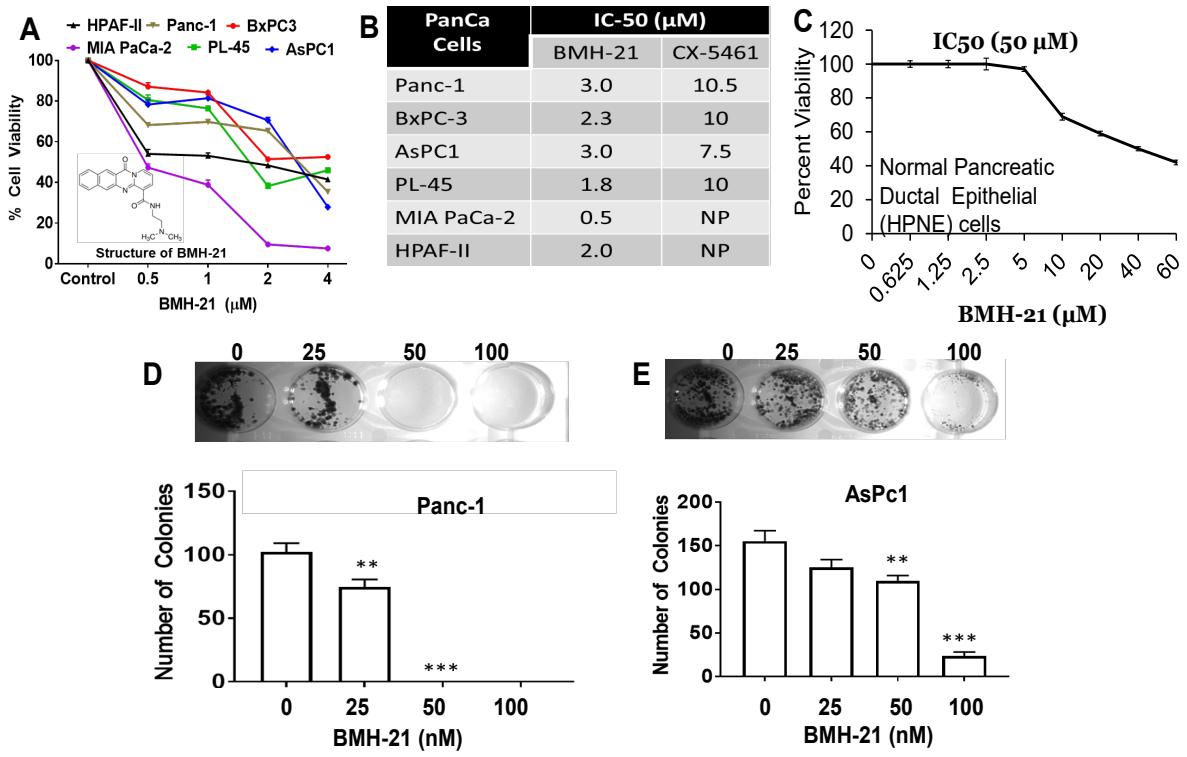


Figure 6: BMH-21 Inhibits the Growth of PanCa Cells and Induces Apoptosis. **A.** Effect of BMH-21 and CX-5461 (RNA polymerase I inhibitors) on cell viability of various PanCa cells (Panc-1, BxPC-3, AsPC1, PL-45, HPAF-II, and MIA PaCa-2) as determined by MTT assay. **B.** Inserted table is showing the IC50 values of BMH-21 and CX-5461 in various cancer cells. NP denotes not performed. **C.** Effect of BMH-21 on the cell viability of normal pancreatic ductal epithelial cells as performed by MTT assay at 24 hr. **D-E.** Effect of BMH-21 on the colony formation potential of Panc-1 and AsPC-1 cells. Representative images are showing colony formation in control and BMH-21 treatment group. Values are in bar graphs represent Mean±SEM of triplicate wells. **P<0.01 and ***P<0.001 are considered statistically significant.

4.3 BMH-21 Treatment Inhibits the Colony Formation Ability of Human PanCa Cells.

Clonogenic assay is a routine test to determine the effect of test drugs on cell proliferation. In this assay, we assume that the number of colonies formed reflect the proliferation of a cell. In this experiment 200 cells were seeded in the 6 well plates and allowed them for 7 days to form the colonies. Media was replaced with fresh media at alternate day. After 7 days, cells were treated with vehicle and approximately 1/10 of IC50 concentration of BMH-21 (25, 50 and 100 nM). BMH-21 treatment effectively inhibited colony forming potential of Panc1 (Fig. 6D) and AsPC-1 (Fig. 6E) cells.

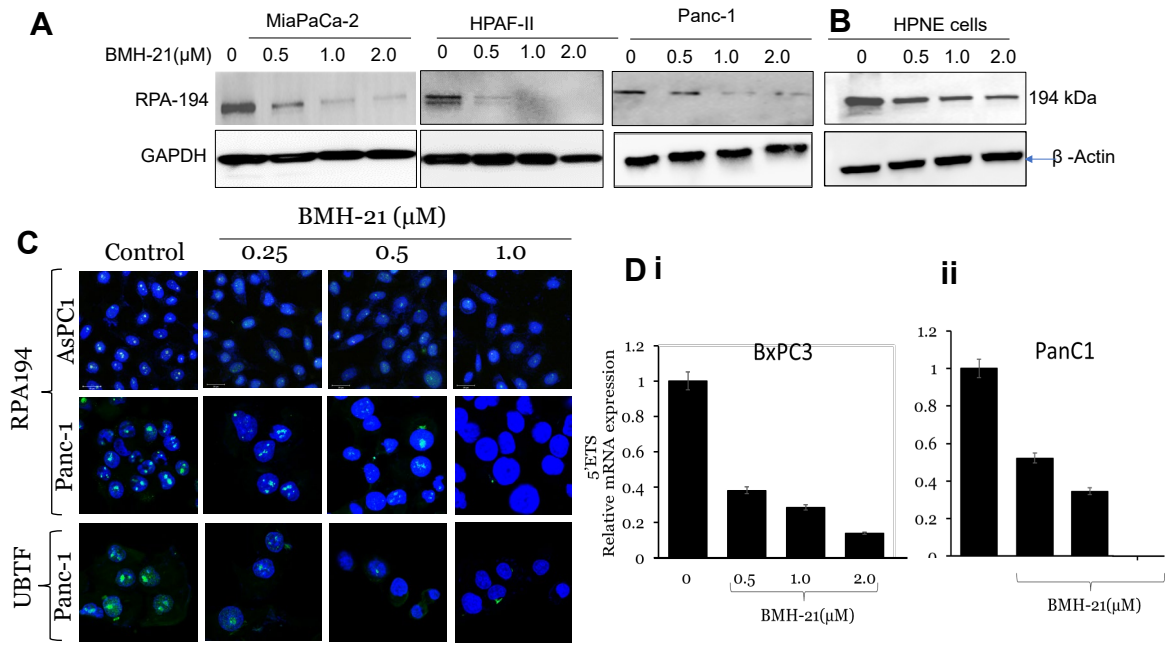


Figure 7: BMH-21 inhibits ribosome biogenesis in PanCa cells. In this experiment, cells were treated with indicated concentrations of BMH-21 for 24 hours and cell lysates and RNA were prepared for respective experiments. **A.** Effect of BMH-2 on the protein level of RPA194 in indicated PanCa cells as determined by Western blot analysis. GAPDH was used as a loading control. **B.** Effect of BMH-21 on the protein levels of RPA194 in human normal pancreatic

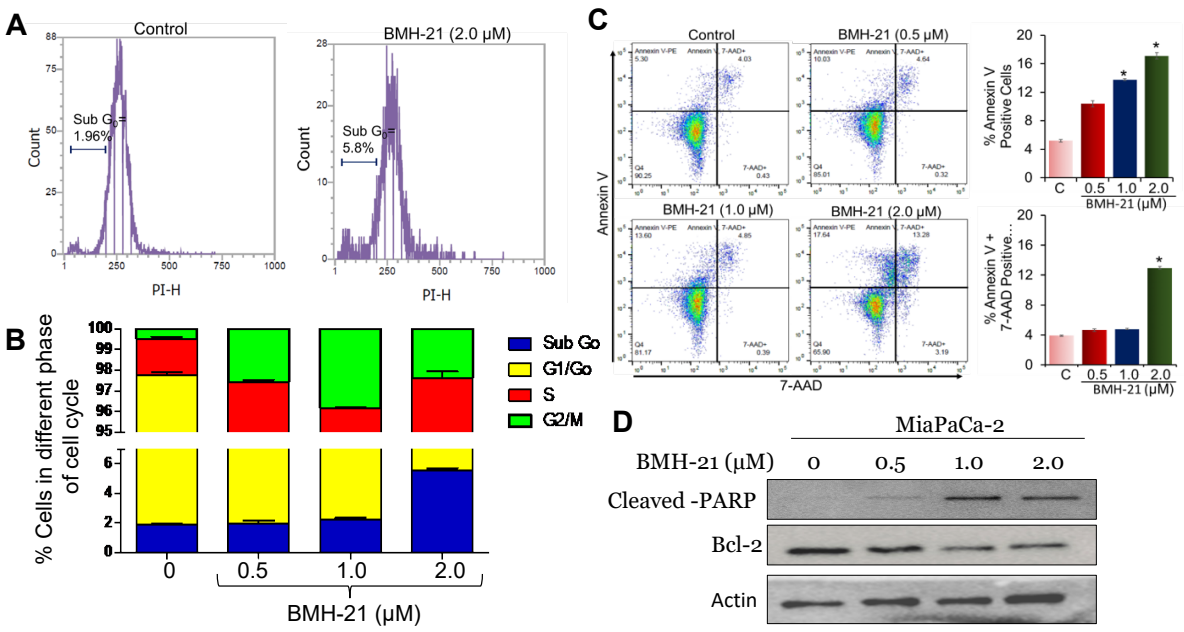
ductal epithelial cells as determined by Western blot analysis. β-actin was used as a protein loading control. **C.** Representative confocal microscopy images showing the effect of BMH-21 on the localization of RPA194 and UBTF in AsPC1 and Panc-1 cells. **D.** Effect of BMH-21 on the mRNA expression of 5ETS as determined by qPCR analysis.

4.4 BMH-21 Inhibits the Protein Levels of RPA194 and 5ETS Expression in PanCa Cells.

Inhibition of rDNA transcription is made possible because BMH-21 causes an interruption of the Polymerase I complex as it degrades RPA194 subunit (Ferreira, et al., 2020). Interruption of the RPA194 subunit within the RNA Pol 1 complex with BMH-21 will cause rDNA transcription to shut down and inhibit ribosome biogenesis in cancer cells. Our results demonstrated that BMH-21 dose-dependently inhibits the constitutive expression of RPA194 protein in all the tested PanCa cells (Fig. 7A). Interestingly, we also observed the similar results in normal pancreatic ductal epithelial cells. We next performed confocal microscopy experiments after BMH-21 treatment in various PanCa cells. We observed the similar observation as BMH-21 inhibited expression of RPA194 (Fig. 7B). Transcription factor UBTF regulates the RNA Pol activity via its recruitment on rDNA helping in rDNA transcription. Therefore, we also determined the effect of BMH-21 on the expression pattern of UBTF by confocal microscopy. Our results revealed a dose-dependent inhibition of BMH-21 on UBTF localization in PanCa cells. BMH-21 also inhibited the mRNA expression of 5ETS in PanCa cells (Fig. 7C). These results clearly suggest that BMH-21 inhibits the ribosome biogenesis process in PanCa cells.

4.4 BMH-21 Arrests Cell Cycle in Sub G0-1 Phase of Cell Cycle And Induces Apoptosis in PanCa Cells

Various chemotherapeutic drugs arrests cell cycle in different phases of cell cycle via targeting essential enzymes required for cell cycle progression of targeting tubulin polymerization and depolarization. Because we observed cytotoxic and inhibition of colony formation potential of BMH-21 in PanCa cells. Therefore, we were interested to know if BMH-21 causes cell cycle arrest of PanCa cells. Our flow cytometry analysis results of cell cycle analysis demonstrated that BMH-21 treatment arrests the cell cycle in sub G0-1 phase of cell cycle (Fig. 8A-B). It is well documented that arresting the cell cycle leads to the induction of cell apoptosis. We next performed apoptosis analysis by flow cytometry in control and BNH-21 treated cells. Results shows a dose-dependent increase of cell apoptosis in PanCa cells as measured by increase Annexin V positive cells (Fig.8C). Late apoptosis was observed at 2 μM dose of BMH-21 as determined by 7-AAD positive cells (Fig.8C). BMH-21 also dose-dependently inhibited the protein levels of mitochondrial anti-apoptotic protein Bcl2 and induces cleavage in PARP protein in MiaPaCa-2 cells (Fig. 8D).



<u>Figure 8:</u> BMH-21 induces cell cycle arrest in sub-G0 phase and induces apoptosis in PanCa cells. A. Effect of BMH-21 on cell cycle analysis in Panc-1 cells**.** Cells were treated with indicated concentrations of BMH-21 for 24 hours, washed with 1X PBS and fixed in 70% ethanol. Cell cycle analysis was performed using FL-2 channel of Attune NxT Acoustic Focusing Cytometer from Invitrogen. **(A)** Histogram is showing distribution of cells in different phases of cell cycle. **B.** Bar graph is showing % distribution of cells in various phases of cell cycle. **C.** Effect of BMH-21 on apoptosis in MiaPaCa-2 cells as determined by flow cytometry using commercially available kit. Bar graph clearly indicating that BMH-21 treatment induces apoptosis as observed by significant increase of Annexin V positive and Annexin V 7-AAD positive cells. **D.** Effect of BMH-21 on the protein levels of anti-apoptotic protein Bcl-2 and cleaved PARP protein as analyzed by Western blot analysis.

CHAPTER 5

DISCUSSION AND CONCLUSION

Pancreatic cancer is one of the deadliest type of cancers has no specific biomarker for early diagnosis and show very poor response to available therapeutic regimens. Various molecular genetic changes such as mutation in KRAS, p53 and SMAD-4 occur which are link to pancreatic carcinogenesis. In addition to these genetic events desmoplastic TME containing various tumor immune cells population (TAM, MDSC, regulatory T-cells), cancer stem cells and tumor associated fibroblasts play significant role in disease progression, metastasis and chemoresistance of PanCa. Therefore, it is an empirical need to identify new molecular targets and therapy for the management of this disease.

Cancer cells require more energy and protein to survive. Therefore, ribosome biogenesis process is dysregulated in cancer cells. Recent efforts have been made to understand the role of ribosome biogenesis in induction and progression of cancer. However, no study exists to investigate the role of ribosome biogenesis in PanCa. Here, we have explored the expression pattern of various ribosome biogenesis components in PanCa cells and tissues and evaluated the therapeutic efficacy of RNA Pol I inhibitor (BMH-21) against PanCa. Our data suggest the overexpression of catalytic subunits of RNA Pol I (RPA194 and RPA135) in PanCa cells and tissues compared to normal cells and tissues. It is well known that RPA194 helps in recruitment of RNA polymerase I on rDNA. These results indicate possible increase of rDNA transcription

in PanCa cells and tissues. Transcription factor UBTF is known to regulate the RNA polymerase I and II which suggest its role in both ribosome biogenesis as well as in transcription of other proteins. UBTF expression was found to be overexpressed in prostate, colon and liver cancer and targeted disruption of UBTF inhibits the growth of tumor cells and metastasis. We for the first time demonstrated that phosphorylation of UBTF is increased in PanCa cells compared to normal cells however, total UBTF expression was almost similar to normal cells. Further studies are required to explore the functional impact of UBTF in pancreatic cancer. In addition to these findings, our results also indicate overexpression of nucleolar proteins (Nucleophosmin) and differential expression of fibrillarin. Overall, these data provide strong indication of increase ribosome biogenesis process in PanCa cells.

Recent studies have shown that targeting RNA Polymerase I (POL I) inhibits the growth of various cancers in pre-clinical models (Bywater et al. 2013; Bywater et al. 2012; Drygin et al. 2011; Devlin et al. 2016; Rebello et al. 2016; Lawrence et al. 2018). A recent phase I human clinical trial of RNA polymerase I inhibitor (CX-5461) against hematologic malignancy showed promising results with no to manageable photo-toxicity (Khot et al. 2019). Overall, these studies including our preliminary data further prove that targeting ribosome biogenesis via RNA polymerase I inhibitor will serve as a novel effective therapeutic approach for the inhibition of advanced PanCa and overcome chemoresistance. BMH-21 (Fig. 7A) is a non-toxic selective inhibitor of RNA Pol I was first developed in the laboratory of Prof. Laiho, Johns Hopkins Institute. BMH-21 inhibits ribosome biogenesis via binding to GC rich ribosomal DNA (rDNA) resulting inhibition of RNA polymerase I activity (Peltonen, Colis, Liu, Jaamaa, et al. 2014). It induces the destabilization and the following degradation of the largest subunit of RNA Pol I,

RPA194 (Peltonen, Colis, Liu, Jaamaa, et al. 2014; Colis et al. 2014; Peltonen, Colis, Liu, Trivedi, et al. 2014). The rapid degradation of RPA194 results in cancer cell death and is not revealed in normal cells (Wei et al. 2018). This activity is specific for polymerase I in fact, and BMH-21 does not cause alteration of RNA Pol II-mediated transcription. *In vitro* and genetic data from yeast mutants suggest that BMH-21 treatment is associated to inhibit RNA Pol I elongation step that activates the degradation of RPA194 (Wei et al. 2018). BMH-21 has also been shown to induce apoptosis in ovarian cancer cell lines via targeting p53-mediated nucleolar stress pathway (Fu et al. 2017). BMH-21 has been shown for its potent anti-cancer effects against solid tumors in preclinical mouse models (Peltonen, Colis, Liu, Jaamaa, et al. 2014; Peltonen, Colis, Liu, Trivedi, et al. 2014; Guner et al. 2017). However, no study has been performed for its anti-cancer effect against PanCa. Our results clearly indicate that BMH-21 has preferential cytotoxic effects in PanCa cells without affecting normal pancreatic ductal epithelial cells which make it enhance therapeutic window for developing as a new anti-cancer drug against PanCa. However, we observed BMH-21 inhibits RPA194 protein level at the similar fashion in normal pancreatic ductal epithelial cells as well as in PanCa cells. These results suggest that apart from RPA194, BMH-21 has other molecular targets to show its cytotoxic action which is not present in normal pancreatic ductal epithelial cells. Decrease expression of 5ETS transcription suggests the inhibition of ribosome machinery in PanCa cells by BMH-21. Our Flow cytometry results indicate that BMH-21 is a potential agent in arresting the cell cycle in G0/G1 phase and inducing apoptosis in PanCa cells.

Taken together, our study provides strong clue of dysregulation of ribosome biogenesis process in PanCa cells. BMH-21 is a non-toxic potential anti-cancer agent which inhibits the

ribosome biogenesis process in PanCa cells. Furthermore the use of BMH-21could be explored for its therapeutic efficacy in relevant clinical mouse models of pancreatic cancer.

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BIOGRAPHICAL SKETCH

Carlos Perez III has attended Texas State Technical College from August 2014 to May 2016 and has graduated with an Associates of Science in Biology. Prior to attending college, he enlisted in the United States Marine Corps and reached the rank of PFC in the year 1995. After graduating from TSTC he attended The University of Texas Rio Grande Valley and graduated in 2019 with a Bachelor of Science in Biology with a minor in Psychology. In October of 2019 he enrolled into the Master of Science in Biology at Grand Canyon University and completed the master's degree in the spring of 2021with a 4.0 grade point average. While at Grand Canyon University he became a member of Alpha Phi in 2020 because of the 4.0 grade point average. He also enrolled into The University of Texas Rio Grande Valley Master of Science Biochemistry and Molecular biology in the fall of 2020. While attending UTRGV he gave his time as a graduate research assistant for UTRGV's Biomedical Facility to research cancer. He has also participated as a teaching assistant for UTRGV's Biology department teaching freshmen Biology. Carlos may be reached at cperez9375@gmail.com.