AMERICAN UNIVERSITY OF BEIRUT

ANTITUMOR EFFECT OF THE ADAMANTYL RETINOID ST1926 IN COMBINATION WITH 5-FLUOROURACIL IN PANCREATIC CANCER

by
WAFAA TOUFIQ BEQAI

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ABSTRACT
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Title: Antitumor Effect of the Adamantyl Retinoid ST1926 in Combination with 5-Fluorouracil in Pancreatic Cancer

Pancreatic cancer is considered as one of the most fatal malignancies, ranking as the seventh leading cause of worldwide cancer-related deaths in industrialized countries and the third most common in the U.S.A. Despite recent advances in surgical techniques, chemotherapy and radiation therapy, the five year's survival rate has not significantly improved, confirming the need for novel therapeutics strategy.

Retinoid related molecules are crucial potential agents for cancer treatment. One of them is ST1926, a synthetic adamantyl retinoid that has shown potent antitumor effect in many models of human cancer.

As the molecular pathogenesis of pancreatic cancer is very complex and the development and metastasis related to the abnormality of various gene mutations and cell signaling pathways, then combining several drugs that target different signaling pathways provide a research hot spot. Therefore, we aimed to investigate the antitumor activities of ST1926 alone and in combination with 5-fluorouracil (5-FU) in pancreatic cancer in *in vitro* models.

We used human pancreatic cancerous cell lines (Panc-1 and Capan-1) that harbor different genetic mutations. We showed that ST1926 in combination with 5-FU were significantly more effective in inhibiting pancreatic cancer cell lines proliferation than either cytotoxic agent alone. Furthermore, ST1926/5-FU induced apoptosis as evidenced by PARP cleavage and mitochondrial membrane potential dissipation. In addition, ST1926/5-FU increased the protein levels of total p53 and γH2AX while decreasing that of DNA polymerase α (POLA-1).

In conclusion, our study supports the possibility that the combined treatment of ST1926 and 5-FU may be potentially effective and a critical strategy for pancreatic cancer treatment.

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ABBREVIATIONS

- ACCs Acinar cell carcinoma
- AJCC American Joint Committee on Cancer
- APAF-1 Apoptotic protease activating factor 1
- APC Antigen-presenting cell
- APL Acute promyelocytic leukemia
- ASR Age-standardized incidence rates
- ATL Adult T-cell leukemia
- ATRA All-trans-retinoic acid
- AUBMC American University of Beirut Medical Center
- Bax Apoptosis regulator BAX, also known as bcl-2-like protein 4
- BRCA1 Breast cancer type 1 susceptibility protein
- BRCA2 Breast cancer type 2 susceptibility protein
- CA19-9 Carbohydrate antigen 19-9
- CA-50 Gastrointestinal tumor antigen 50
- cat D Cathepsin D
- CD133 Cluster of differentiation 133
- CD24 Cluster of differentiation 24
- CD437 6-[3-(1-Adamantyl)-4-hydroxyphenyl]-2-naphthalenecarboxylic Acid
- CD44 Cluster of differentiation 44
- CDKN2A Cyclin-dependent kinase inhibitor 2A
- CH2THF 5,10-Methylenetetrahydrofolate (N5,N10-Methylenetetrahydrofolate; 5,10-CH2-THF)
- CI Combination Index
- CML Chronic myeloid leukemia
- CRABP2 Cellular retinoic acid binding protein 2
- CSC Cancer stem cells
- CT Computed tomography
- CXCL12 C-X-C motif chemokine 12
- DCK Deoxycytidine kinase
- DHFU Dihydrofluorouracil
- DMEM Dulbecco’s Modified Eagle’s Medium
- DMSO Dimethyl sulfoxide
- DNA Deoxyribonucleic acid
- DPD Dihydropyrimidine dehydrogenase
- DR4 Death receptor 4
- DR5 Death receptor 5
- DSB Double strands breaks

- dTMP Deox-ythymidine monophosphate
- dUMP Deoxyuridine monophosphate
- DUPAN-2 Duke Pancreatic Monoclonal Antigen Type 2
- dUTP Deoxyuridine triphosphate
- ECM Extracellular matrix
- EMT Epithelial-mesenchymal transition
- EPIC European Prospective Investigation into cancer
- FABP5 Fatty acid binding protein 5
- FDA Food and Drug Administration
- FdUMP Fluorodeoxyuridine monophosphate
- FdUTP Fluorodeoxyuridine triphosphate
- FGF Fibroblast growth factor
- FOXO1 Forkhead box protein O1
- FUTP Fluorouridine triphosphate
- GAPDH Glyceraldehyde-3-phosphate dehydrogenase
- Gem Gemcitabine
- h Hour
- HDACs Histone deacetylases
- IL-2 Interleukins 2
- IL-6 Interleukins 6
- INF-α Interferon Alfa
- IPMN Intraductal papillary mucinous neoplasms
- JNK C-Jun N-terminal kinase
- MAP kinase Mitogen-activated protein kinase
- MCN Mucinous cystic neoplasms
- MDR Multidrug-resistance protein
- MLH1 Mut L homolog 1
- MRI Magnetic Resonance Imaging
- MSH2 Mut S homolog 2
- MSH6 Mut S homolog 6
- MUC Mucins
- NUR77/TR3 Orphan Nuclear Receptor
- p53 Tumor protein P53
- PAGE Polyacrylamide gel electrophoresis
- PAK1 Phosphorylates p21-activated kinase 1
- PALB2 Partner and localizer of BRCA2
- PanIN Pancreatic intraepithelial neoplasm
- Pan-NET Pancreatic neuroendocrine tumor
- PARP Poly ADP (Adenosine Diphosphate)-Ribose Polymerase
- PAX3 Paired box gene3

- PBs Pancreatoblastomas
- PC Pancreatic Cancer
- PDAC Pancreatic ductal adenocarcinoma
- POLA-1 DNA polymerase α
- PPAR Peroxisome proliferator-activated receptor
- PSCs Pancreatic stellate cells
- RA Retinoic acid
- RAREs Retinoic acid responsive response element
- RARs Retinoic acid receptors
- RNA Ribonucleic acid
- RRMs Retinoid related molecules
- RT Room Temperature
- RXRs Retinoid X receptors
- SDH Succinate dehydrogenase
- SDS Sodium dodecyl sulfate
- SEM Standard Error of Mean
- SPAN-1 Surface presentation of antigens protein
- SPNs Pseudo papillary neoplasms
- ST1926 E)-3-[4-[3-(1-adamantyl)-4-hydroxyphenyl]phenyl]prop-2-enoic acid
- STAT-3 Signal transducer and activator of transcription 3
- STK11 Serine/threonine kinase 11
- Tax Trans-activator x
- TGF-β Transforming growth factor β
- TS Thymidylate synthase
- U.K United kingdom
- U.S.A United states of America
- UDG Uracil-DNA glycosylase
- WHO World Health Organization
- γH2AX H2A histone family member X
- µM Micromolar µM
- 13-cis-RA 13-cis retinoic acid
- 5-FU 5-fluorouracil
- 9-c RA 9-cis retinoic acid

CHAPTER I

INTRODUCTION

A. Pancreatic Cancer

1. Overview

Pancreatic cancer is a fatal malignancy, ranking as the seventh leading cause of worldwide cancer-related deaths in industrialized countries [1] and the third most common in the U.S.A [2]. According to GLOBOCAN estimates in 2018, pancreatic cancer ranked as the eleventh most common cancer all over the world adding up 458,918 new cases and leading to 432,242 deaths (that is 4.5% of all deaths caused by cancer) in 2018. Globally, pancreatic cancer incidence and mortality are associated with increasing age and is a bit more common in men compared to women [1]. In spite of the great efforts in recognizing the potential risk factor of pancreatic cancer and advancement in early diagnostic tools, it is predicted that pancreatic cancer will surpass breast, prostate and colorectal cancers as a leading cause of cancer related deaths in the U.S.A. by the year 2030 [3].

Generally pancreatic cancer is classified into two categories: pancreatic adenocarcinoma that occurs in the exocrine gland of the pancreas, which is the most common (85% of the cases) and pancreatic neuroendocrine tumor (pan-NET) that arises in the endocrine tissue of the pancreas, which is less common (less than 5%) [4].

Pancreatic adenocarcinoma is one of the worse prognosis carcinomas, typically after diagnosis only 24% of people survive for one year and 9% survive for five years [5] leading to pancreatic ductal adenocarcinoma to become as the second leading cause of cancer- related death in 2020 in U.S.A [6] . In Lebanon the percentage of incidence and

mortality was 2.6% and 4.7 % respectively according to GLOBOCAN estimates in 2020. [7]

2. Epidemiology

Pancreatic cancer epidemiology analysis can be a cornerstone of developing an efficient prevention strategy by playing a role in explaining the etiology of pancreatic cancer.

a. Incidence

Pancreatic cancer incidence differs among regions and populations (Fig.1). Globally, 458,918 new cases of pancreatic cancer were registered in 2018 (2.5% of all cancers). The age-standardized incidence rates (ASR) was highest in Europe (7.7 per 100,000 people) followed by North America (7.6 per 100,000 people) and Oceania (6.4 per 100,000 people) and lowest in Africa (2.2 per 100,000 people) showing a significant different geographic distribution in pancreatic cancer incidence (Figure 1) [1].The reason for these differences among countries is insufficiently known. An important thing to be considered is that the diagnostic tools and the variety in the usage of many diagnostic strategies differ between developed and undeveloped geographic areas [8]. In addition, some difference in the evaluated incidence may refer to the quality of registries, as coverage, completeness, and accuracy differ by country [9]. Also, the observed geographic variation in the incidence of pancreatic cancer may be attributed to exposure to certain risk factor. For instance, some findings show that some of this difference may refer to tobacco smoking [10] while others refer it to the dietary style and obesity [11, 12]. On the other hand, a slight difference in the pancreatic cancer incidence was

observed between genders. It is more common in men (5.5 per 100,000, 243,033 cases) than in women (4.0 per 100,000, 215,885 cases) [1]. It is not entirely known the reason for the higher incidence of pancreatic cancer in men. Women are either less exposed to risk factors from the environment or less susceptible to these types of malignant tumors [13, 14] However, in both sexes, the incidence rate increases with age [1]. Pancreatic cancer is described as a disease for elderly populations as the highest incidence is reported in people above 70 years and rarely diagnosed before 55 years of age [15].

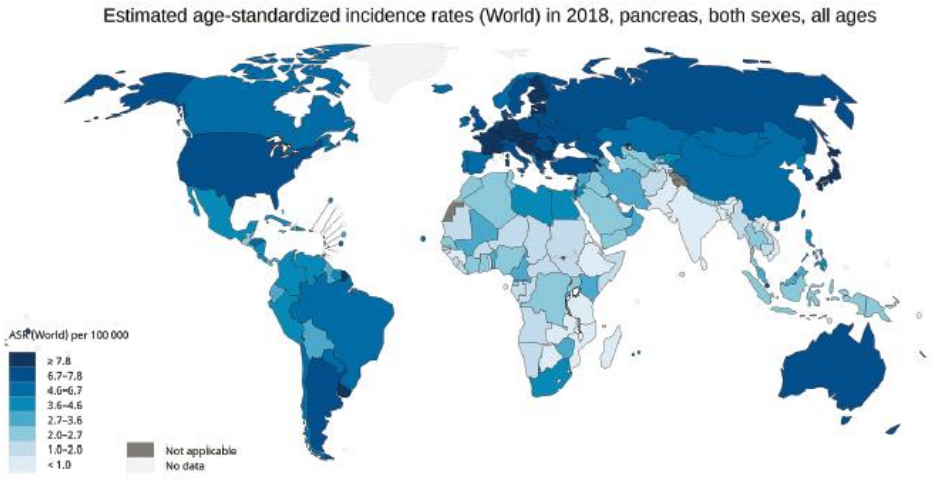

Figure 1. Map shows estimated age-standardized incidence rates (ASR) for pancreatic cancer

b. Mortality

Pancreatic cancer mortality ASR vary considerably among countries. Based on GLOBOCAN 2018 estimates, the highest age-standardized mortality rates were reported in Western Europe (7.6 per 100,000 people) followed by Central and Eastern Europe (7.3), and (Northern Europe and North America equally (6.5)) and the lowest was registered in the countries of Eastern Africa (1.4), followed by South-Eastern

Asia and Western Africa (equally: 2.1) (Figure 2). To sum up, in 2018 more than half of pancreatic cancer deaths were reported in the most developed countries (52.3%, 226,272 of deaths), slightly less than half of the deaths for pancreatic cancer were recorded in Asia (46.4%, 200,681 of deaths) and a bit more than one-third occurred in Europe (29.6%, 128,045 of deaths). In addition, in both males and females, the pancreatic cancer mortality rate increases with age, and approximately 90% of the all deaths occur after the age of 55 years [1].

Pancreatic cancer is the most common malignancy detected at the autopsy studies [8, 16], 80-90% of the patients have unresectable tumors due to the advanced stage at diagnosis because it is hard to diagnose pancreatic cancer due to the lack of early symptoms. Furthermore, the available chemotherapeutics strategies are limited and in many cases ineffective, particularly in adenocarcinoma that is most of the time diagnosed at stage III or IV [17-19].

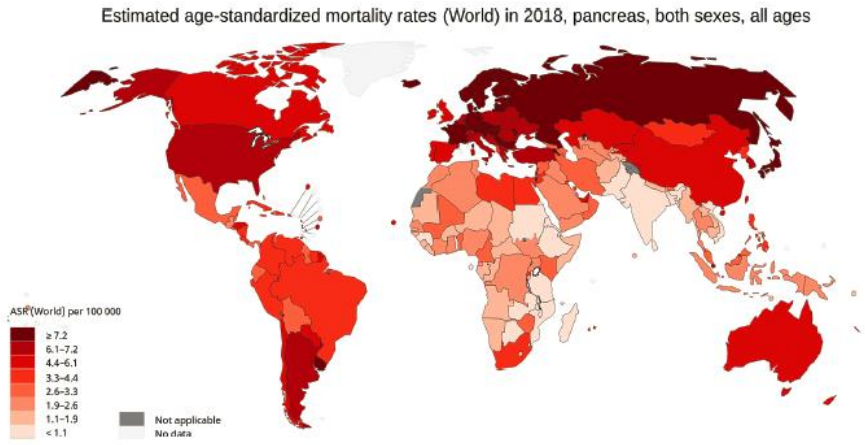

Figure 2. Map shows estimated age-standardized mortality rates (ASR) for pancreatic cancer

3. Risk Factors

The etiology of pancreatic cancer has been widely studied and thus numerous risk factors have been identified and can be divided into two groups: modifiable and non-modifiable risk factors [20].

The modifiable group includes smoking [10], alcohol [21], dietary factors [22] obesity [23], and exposure to toxic substances [24]. The non- modifiable group includes family history of pancreatic cancer [25], gender (higher in males than females), age [1], genetic factors [26], non-O blood group [27], ethnicity [28], diabetes mellitus [29], chronic infections [30, 31], and chronic pancreatitis [32]. Despite that the cause of pancreatic cancer is multifactorial and complex, family history [33] and cigarette smoking [34] are most dominant.

It is reported that about 5-10% of individuals with pancreatic cancer have family history of pancreatic cancer [25]. Prospective analysis of families with pancreatic cancer shows that first-degree relatives of individuals with familial pancreatic cancer have a nine-fold more risk of developing pancreatic cancer over the general population [33]. This risk increases to 18-fold when at least two of the first-degree relatives in the family have pancreatic cancer [35] and to 32-fold when three or more of first degree relatives with pancreatic cancer [36]. Besides, the risk of pancreatic cancer increase with genetic variation or mutation (germ-line mutation) [26]. About 10% of patients with pancreatic cancer have some genetic predisposition like gene variations or alterations to developing this malignant disease [37]. Numerous germ-line mutations such as BRCA1, BRCA2, PALB2, ATM, CDKN2A, APC, MLH1, MSH2, MSH6, PMS2, PRSS1, and STK11 have been involved in hereditary forms of pancreatic cancer [36, 38]. [39]. Moreover, main genes (KRAS,

p53 and SMAD4) in inherited genetic mutations play an important role in increasing risk of pancreatic cancer[40].

Worldwide, more than one thousand millions of people do tobacco smoking and according to the (International Agency for Research on Cancer) smoking is causally related with pancreatic cancer [10, 41]. Duration of smoking and number of cigarettes smoked daily increase the risk of pancreatic cancer. The risk is approximately two times more in smokers than in non-smokers [42]. A meta-analysis of 82 studies showed that the relative risk of pancreatic cancer was 1.74 in current smokers and 1.2 in former smokers and this risk persists for at least 10 years after they stop smoking [43]. In addition, a study for the European Prospective Investigation into cancer (EPIC) in 2012 showed that for every five cigarettes smoked per day the risk of pancreatic cancer increases and that the risk can be increased by 50% from passive smoking [44]. Although smoking is decreasing in developed countries, it is still high in others and increasing in developing countries and among women. A study in 2011 reported that about 26.2 % of pancreatic cancers in men and 31% in women were related to tobacco smoking in the U.K [45].

4. Pancreas

The pancreas is a secondarily retroperitoneal organ located on the posterior wall of the abdominal cavity. An adult human has a pancreas of an average volume of 72 cm^3 (parenchyma -44$cm^{3,}$ fat -28 cm^3) [46]. It measures 12-20 cm in length, 3-5 cm in height, and 1-3 cm in width [47]. The shape of the pancreas is elongated looking like a hook or hammer. It can be divided into four parts the head (surrounded by the duodenum), neck (lies near the superior mesenteric vessels), body (located

behind the posterior wall of the stomach, and the tail (expand to the hilum of the spleen) from which originates the pancreatic duct (duct of Wirsung) that runs across the entire organ and connects with the common bile duct to form the hepatopancreatic ampulla (ampulla of Vater) that found at the major duodenal papilla. Unlike most glands in the human body, pancreas does not have a fibrous capsule [48].

The pancreas consists of two morphologically and functionally distinct components integrated into one anatomical structure. It is a complex gland composed of both endocrine (islets of Langerhans) and exocrine (acinar and ductal cells) parts (Figure 3).

The connective tissue divides the pancreas into lobules that are composed of grape-like clusters of exocrine cells called acini. The enzyme-secreting exocrine cells that form acini have conical shape and organized around a central lumen. The acini are connected to the intralobular ducts through the intercalated ducts, which drain to the interlobular ducts. This duct system is lined with simple squamous epithelium at the proximal part and simple cuboidal epithelium at the distal part. The main ducts and larger interlobular ducts are lined with columnar epithelium and contain mucous-secreting cells [49]. The exocrine pancreas secretes the pancreatic juice (approximately 1200-1500 ml per day) [50] that is made of water, bicarbonate ions (regulating its alkaline reactions) and numerous enzymes including amylase, trypsinogen, chymotrypsinogen, carboxypeptidases, lipase, elastase, phospholipase A, DNAse, and RNAse [51], which are secreted into pancreatic ducts and excreted into the small intestine to breakdown carbohydrates, proteins, and fats for absorption.

Commonly the endocrine part of the pancreas is called the islets of Langerhans. Most of the islets are located in the body and tail of the pancreas. The

shape of the islets is spherical although it may be flat or elongated and range from 50-500 µm in diameter. The human pancreas contains around 1 million islets, which accounts for 1-2% of the pancreas mass [52]. The endocrine pancreas consists of five types of hormone-secreting cells. Alpha cells secrete glucagon, beta cells secrete insulin, delta cells secrete somatostatin, gamma (PP) cells secrete pancreatic polypeptide and, epsilon cells secrete ghrelin. These hormones are transported via bloodstream to target organs and tissues [49]. The main hormones secreted by the endocrine pancreas are insulin and glucagon that are directly released to the blood circulation via a dense intra-islets vascular network, which regulate the level of glucose in the blood, and somatostatin, which prevents the release of insulin and glucagon [53].

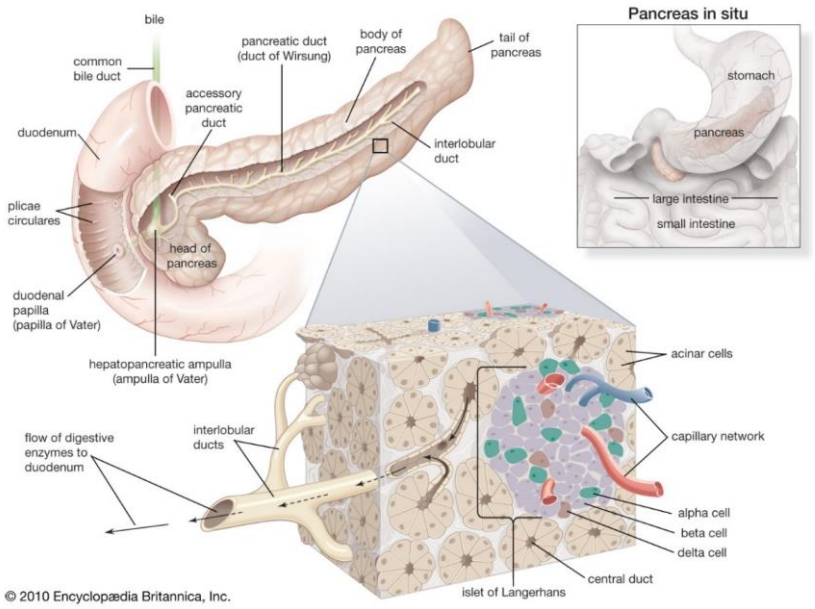

Figure 3. Structures of the human pancreas. Acinar cells produce digestive enzymes, which are secreted into tiny ducts that feed into the pancreatic duct. Islets of Langerhans are clusters of cells that secrete hormones such as insulin and glucagon directly into a capillary network, which also joins the pancreatic duct. Encyclopædia Britannica, Inc. (https://www.britannica.com/science/pancreas)

5. Pancreatic cancer

Depending on the type of cells they start in, pancreatic tumors are either exocrine or neuroendocrine (endocrine). Most of the pancreatic cancers are exocrine and minimal are neuroendocrine tumors (pancreatic NETs or PNETs). Adenocarcinoma is an exocrine tumor that starts in the cells lining the pancreatic duct and is the most common type of pancreatic cancer.

a. Exocrine Tumors

i. Pancreatic Ductal Adenocarcinoma

Approximately 90% of pancreatic carcinomas are pancreatic adenocarcinoma and its variants [54]. About 60-70% of pancreatic adenocarcinomas arise in the head of the pancreas with the remaining (15% in the body and 15% in the tail). Unfortunately, most of the pancreatic adenocarcinomas are being already spread beyond the pancreas through nodal metastases at the time of diagnosis [55].

World Health Organization (WHO) classification of pancreatic tumors recognized morphological variants of pancreatic adenocarcinoma which have distinct histological characteristics compared to the conventional pancreatic adenocarcinoma. A summary of main variants of pancreatic adenocarcinoma are showed in Table 1 [56].

Table 1.Summary of the different subtypes of pancreatic ductal

Morphological Variant	Characteristics
Adenosquamous carcinoma	Significant components of ductal/glandular and squamous differentiation (at least 30%). Considered to have a worse prognosis than pancreatic adenocarcinoma
Colloid/mucinous carcinoma	Production of copious amounts of extracellular stromal mucin. Most arise in association with intraductal papillary mucinous neoplasms; thought to have more favorable prognosis than pancreatic adenocarcinoma
Undifferentiated /anaplastic carcinoma	Minimal or no differentiation; highly atypical cells which may appear spindle shaped or sarcomatoid, often admixed with osteoclast-like giant cells. One of the most aggressive forms of pancreatic cancer with extremely poor survival rates
Signet ring cell carcinoma	Discohesive, singly invasive cells with intracytoplasmic mucin that may displace the nucleus. Similar tumors throughout the gastrointestinal tract. Very rare form of pancreatic cancer with prognosis similar to that of pancreatic adenocarcinoma
Medullary carcinoma	Syncytial arrangement of pleomorphic epithelial cells with associated intratumoral lymphoid infiltrate. Prognosis is slightly better than pancreatic adenocarcinoma
Hepatoid carcinoma	Morphological similarity to hepatocellular carcinoma. May produce bile. Very rare tumor with a poor prognosis similar to that of pancreatic adenocarcinoma

Pancreatic adenocarcinoma evolves through a series of step-wise mutations (hereditary germline or somatic acquired mutations in cancer related genes such as oncogenes, tumor suppressor genes, cell cycle genes, apoptosis and genome maintenance genes from normal mucosa to specific precursor lesions and finally to invasive malignancy. Furthermore, cell turnover, shortened telomerase, and genomic instability play an important role in the development of pancreatic epithelial cells to pancreatic cancer [57]. Figure 4 represents the progression of normal pancreatic duct epithelium to pancreatic adenocarcinoma in early (telomerase shortened, KRAS mutation, p16 loss) and late (p53 loss, SMAD4/DPC loss) stage [58]. Abnormalities in sonic hedgehog pathways and notch signaling have as well been implicated in the development of pancreatic adenocarcinoma where 80% of these mutations appear to be sporadic [36].

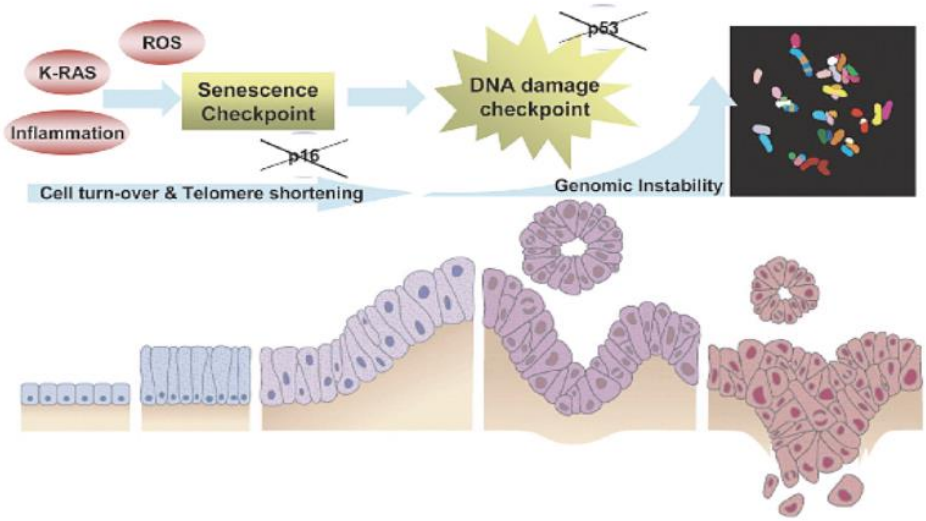

Figure 4. Progression to pancreatic ductal Adenocarcinoma and Genetic

There are some precursor lesions to pancreatic cancer, each of these has a unique molecular, clinical and, pathological traits. The three best characterized precursors to this malignancy are pancreatic intraepithelial neoplasm (PanIN), intraductal papillary mucinous neoplasms (IPMN), and mucinous cystic neoplasms (MCN) that is believed to originate from pancreatic cancer stem cells (CSC) [59].

1. PanIN: PanINs are microscopic non-invasive epithelial neoplasm that are usually located at the small pancreatic duct in the head of the pancreas. By epithelial atypia they are divided into three subgroups. PanIN-1 (minimal atypia which is also divided to two subgroups PanIN-1A “flat type” and PanIN-1B “papillary type”), PanIN-2, and PanIN-3 (limited atypia). PanIN is correlated with chronic pancreatitis and invasive carcinomas. Figure 6 illustrates detected mutations in PanIN [58, 59]. Molecular studies showed that PanIN is the most common precursor to pancreatic adenocarcinoma as these lesions have common genetic abnormalities with the pancreatic adenocarcinoma and the histological progression of them parallels the accumulation of molecular abnormalities.[54]

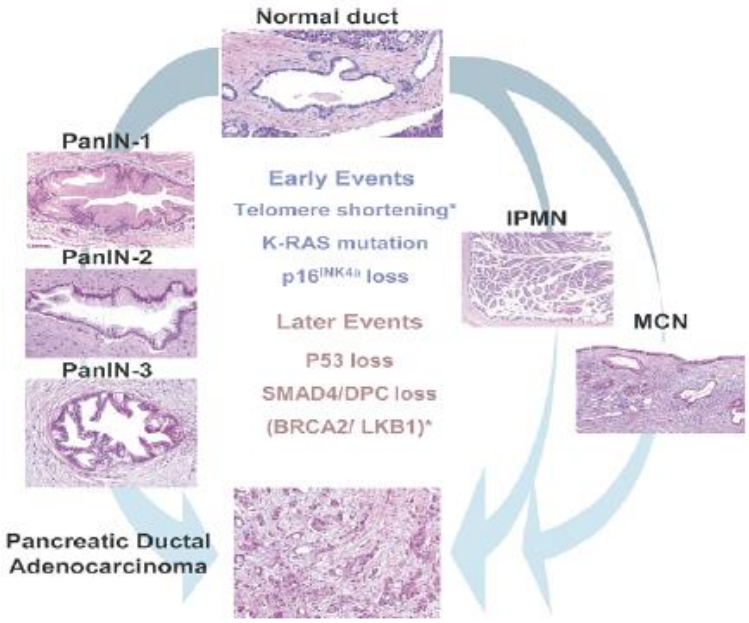

Figure 5. Events during Progression from Normal Pancreatic Ducts Structure to Pancreatic Adenocarcinoma [58]

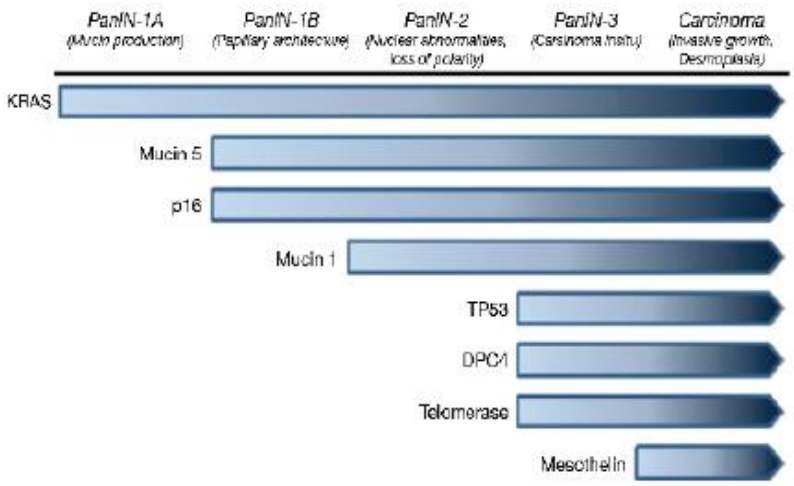

Figure 6. Genetic Mutations Detected in the PanIN

2. **IPMN**: IPMN actual incidence is unknown as it is small and asymptomatic. It is divided into two subtypes (IPMN-MD “main duct type” and IPMN-BD “branch duct

type"). In 2010, based on the malignant transformation properties, WHO classified IPMN as low, intermediate and high-grade dysplasia with invasive cancer properties. Genes involved in IPMN include KRAS (80%), RNF43 (75%), GNAS (60%), P16/CDKN2A, TP53, SMAD4 (variable by histological degree), and PIK3CA (10%). [59].

3. **MCN:** Generally it is solitary, has thick fibrotic wall, and may contain (mucin, hemorrhagic fluids or necrotic material. It is rare and asymptomatic, so its diagnosis is usually incidental with a median age of onset around 40-50 years Genes involved in MCN include KRAS (75%), RNF43 (40%), P16/CDKN2A, TP53, and SMAD4 (variable by histological degree).[59]

ii. Non-Ductal Pancreatic Neoplasms

Non-ductal pancreatic tumors account for less than 5% of pancreatic tumors [60]. It includes solid pseudo papillary neoplasms (SPNs), acinar cell carcinoma (ACCs), and pancreatoblastomas (PBs). These types share overlaying gross, microscopic, and immunohistochemical characteristics such as well-delineated neoplasms, monotonous cellular tumor cells with a bit intervening stroma and abnormal beta-catenin expression. Non- ductal pancreatic neoplasms differentiation is based on the identification of histologic findings such as pseudopapillae, acinar cell features, and squamoid corpuscles [61].

b. Pancreatic Neuroendocrine Tumors (PNETs)

PNETs are rare neoplasms that account for 1-2% of all pancreatic tumors and have an incidence of approximately one per 100,000 individuals per year [62]. PNETs

may appear at any age, but they most frequently happen in the fourth and sixth decades of life. In general, there is no predominance, however the multiple subtypes may show a slight predominance for men or women [63].

Broadly PNETs may be classified as functional and nonfunctional. Commonly functional tumors show a wide range of presenting symptoms from inappropriate secretion of hormones, and are able of manifesting fascinating clinical syndromes. Insulinomas are most common, followed by gastrinomas, glucogonomas, VIPomas, somatostatinomas, and other rare types in decreasing order. Nonfunctional PNETs are either incidentally found or clinically present late with symptoms such as weight loss, nausea, vomiting, anorexia, obstructive jaundice, invasion into an adjacent structure, from mass effect of tumor, or metastasis. In addition nonfunctional PNETs are much more common than functional PNETs [64, 65].

i. Functional Pancreatic Neuroendocrine Tumors

Functional PNET patients may be misdiagnosed for years before an accurate diagnosis is made where the clinical sequelae from hormone hypersecretion can be debilitating. Insulinomas, gastrinomas, glucogonomas, VIPomas, and somatostatinomas have a well-described clinical syndrome. Hormone elevation needs to be detected in the serum and commercial assays are accessible for each [66].

ii. Nonfunctional Pancreatic Neuroendocrine Tumors

Approximately70% of PNETs are classified as nonfunctional because they do not manifest symptoms of hormone excess. Although, most of these nonfunctional tumors do produce peptides that can be detected in the serum. Both functional and

nonfunctional endocrine tumors of the pancreas frequently secrete a number of other substances including chromogranins (particularly chromogranin A), pancreatic polypeptide, neuron-specific enolase, subunits of human chorionic gonadotropin or ghrelin, but these substances do not cause a specific hormonal syndrome. Serum chromogranin A is a neuroendocrine secretory protein that may be used as a marker of disease activity for both functional and nonfunctional PNETs [67]. Additionally, it may be useful in assessment of response to treatment and monitoring disease progression. Although it is not a universal biomarker for PNETs, it is the most sensitive and most well studied serum tumor marker available at this time [68].

6. Pancreatic Cancer Staging

After a patient is diagnosed with pancreatic cancer, doctors will try to evaluate if it has spread, and if so, how far. This is called Staging of a cancer. It outlines how much cancer is in the body, assists determine how serious the cancer is and how best to treat it. The earliest stage of pancreatic cancer is stage "0" (carcinoma in situ), followed in series by stage "I" to stage "IV". The pancreatic cancer staging system mostly used is the **AJCC** (American Joint Committee on Cancer) **TNM** system, which is based on three principal parts of information. The extent of **tumor (T),** the spread to nearby lymph **nodes (N),** and the spread **(metastasized)** to distant sites **(M).** The most recent AJCC system (effective January 2018) is described below. This staging system is used to stage most pancreatic cancers (Table 2) excluding the well-differentiated pancreatic neuroendocrine tumors (NETs), which possess their own staging system (Tables 3 and 4) [69, 70].

Table 2. Summary of Pancreatic Cancer Staging System according to AJCC

AJCC Stage	Stage Grouping	Stage Description
0	Tis N0 M0	The cancer is confined to the top layers of pancreatic duct cells and has not invaded deeper tissues. It has not spread outside of the pancreas. These tumors are sometimes referred to as carcinoma *in situ* (Tis) It has not spread to nearby lymph nodes (N0) or to distant sites (M0)
IA	T1 N0 M0	The cancer is confined to the pancreas and is no bigger than 2 cm (0.8 inch) across (T1) It has not spread to nearby lymph nodes (N0) or to distant sites (M0)
IB	T2 N0 M0	The cancer is confined to the pancreas and is larger than 2 cm (0.8 inch) but no more than 4cm (1.6 inches) across (T2) It has not spread to nearby lymph nodes (N0) or to distant sites (M0)
IIA	T3 N0 M0	The cancer is confined to the pancreas and is bigger than 4 cm (1.6 inches) across (T3) It has not spread to nearby lymph nodes (N0) or to distant sites (M0)
IIB	T1 N1 M0	The cancer is confined to the pancreas and is no bigger than 2 cm (0.8 inch) across (T1) AND it has spread to no more than 3 nearby lymph nodes (N1) It has not spread to distant sites (M0)
	T2 N1 M0	The cancer is confined to the pancreas and is larger than 2 cm (0.8 inch) but no more than 4 cm (1.6 inches) across (T2) AND it has spread to no more than 3 nearby lymph nodes (N1) It has not spread to distant sites (M0)
	T3 N1 M0	The cancer is confined to the pancreas and is bigger than 4 cm (1.6 inches) across (T3) AND it has spread to no more than 3 nearby lymph nodes (N1) It has not spread to distant sites (M0)
III	T1 N2 M0	The cancer is confined to the pancreas and is no bigger than 2 cm (0.8 inch) across (T1) AND it has spread to 4 or more nearby lymph nodes (N2) It has not spread to distant sites (M0)
	OR	
	T2 N2 M0	The cancer is confined to the pancreas and is larger than 2cm (0.8 inch) but no more than 4 cm (1.6 inches) across (T2) AND it has spread to 4 or more nearby lymph nodes (N2) It has not spread to distant sites (M0)
	OR	

	T3 N2 M0	The cancer is confined to the pancreas and is bigger than 4 cm (1.6 inches)across (T3) AND it has spread to 4 or more nearby lymph nodes (N2) It has not spread to distant sites (M0)
	OR	
	T4 Any N M0	The cancer is growing outside the pancreas and into nearby major blood vessels (T4). The cancer may or may not have spread to nearby lymph nodes (Any N) It has not spread to distant sites (M0)
IV	Any T Any N M1	The cancer has spread to distant sites such as the liver, peritoneum (the lining of the abdominal cavity), lungs or bones (M1). It can be any size (Any T) and might or might not have spread to nearby lymph nodes (Any N)

The following additional categories are not listed in the table above:

- T0: No evidence of a primary tumor
- TX: Main tumor cannot be assessed due to the lack of information
- NX: Regional lymph nodes cannot be assessed due to the lack of information

Table 3. WHO Classification of Pancreatic Neuroendocrine

Tumor	**Size (cm)**	**Mitotic count (per 10 hpf)**	**KI-67 Index**	**Angioinvasive**	**Metastasis**
Well differentiated endocrine tumor					
Benign	<2	<2	<2%	Absent	Absent
Uncertain	>2	2-10	>2%	Present	Absent
Well differentiated endocrine carcinoma	>2	<10	>2%	Present	Present
Poorly differentiated endocrine carcinoma	-	>10	>20%	Present	Present

Table 4. TNM staging system for pancreatic neuroendocrine

Primary Tumor	**Size (cm)**
Tx	Primary tumor cannot be assessed
T1	<2, limited to pancreas
T2	2-4, limited to pancreas
T3	Beyond pancreas, no invasion of celiac axis or superior mesenteric artery
T4	Beyond pancreas, invasion of celiac axis or superior mesenteric artery
Regional Lymph nodes	
Nx	Regional lymph nodes cannot be assessed
N0	No regional lymph node metastases
N1	Regional lymph node metastases
Distant metastases	
Mx	Distant metastases cannot be assessed
M0	No distant metastases
M1	Distant metastases
Staging	
Stage Ia	T1N0
Stage Ib	T2N0
Stage IIa	T3N0
Stage IIb	T1-3N1
Stage III	T4 N any M0
Stage IV	T any N any M1

7. Screening and Prevention

Early detection can be the clue to decrease mortality of pancreatic cancer and it can be supported by patient's screening and prevention. Generally, screening of large groups in the general population is not considered effective to detect early stage disease, however more recent techniques, and the screening of firmly targeted groups (especially those of family history), are being evaluated [71], including blood markers for pancreatic cancer CA19-9, CA-50, SPAN-1, DUPAN-2, cell surface-associated mucins (MUC), carcinoembryonic antigen, and heat shock proteins. Although, these tests have not been well studied yet. And the focus up to now in screening has been to detect preinvasive lesions, in preference to early pancreatic cancer, as the resection of preinvasive lesions can restrict development of an invasive pancreatic cancer, while

once an invasive pancreatic cancer develops, its spread beyond the pancreas is likely rapid, restricting use of markers of invasive pancreatic cancer. Therefore, primary prevention is of utmost importance [72, 73].

Understanding the etiology, clarifying the risk factors, and identifying high-risk individuals are principle to the primary prevention of this frequently rapidly deadly disease. Risk reduction is the best preventive strategy against pancreatic cancer by acting on the modifiable risk factors (tobacco smoking, overweight and alcohol use, reducing red meat consumption and increasing fruit and vegetable intake, having regular exercise) and through regular control of health issue [74].

Tobacco smoking is strongly correlated with pancreatic cancer risk, and it has been shown that about 30% of pancreatic cancers could be prevented by the cessation of smoking [45]. Interestingly, after 10 years of smoking cessation, the risk is reduced to the levels of a non-smoker [44].

Dietary modification is major in preventing pancreatic cancer for several reasons. First, high consumption of red and processed meat is associated with greater risk of pancreatic cancer [75], whereas high fruit and vegetable intake [76], as well as nut consumption, is found protective [77]. As a result, a well-balanced diet enriched in fruits, vegetables, and vitamins is highly recommended. In addition, lower intake of saturated fat, together with increased physical activity, is highly suggested to help to reduce the risk of overweight or obesity, which are also associated with risk of pancreatic cancer.

Approximately 60-70% of patients who have cystic neoplasms of the pancreas develop pancreatic cancer [78]. The complete extirpation of cystic neoplasms is now carried out as a cancer preventive strategy [79]. If a non-suspicious cyst is found,

surveillance should be repeated every 6-12 months. Solid lesions, not meeting the criteria for immediate resection, and main pancreatic duct strictures should have repeat imaging after three months [117]. Moreover, alcohol use limitation is considered necessary to reduce the risk of pancreatic cancer through the development of pancreatitis [80].

Non-modifiable risk factors cannot be controlled. Although, patients with family history and genetic susceptibility may do screening tests for early detection of pancreatic cancer. Unfortunately, widely applied screening tests are not yet available, and researchers are working on developing effective screening tests. For high risk people of pancreatic cancer (patients with hereditary pancreatitis or with a family history of pancreatic cancer), some screening techniques are promising, such as endoscopic ultrasound and spiral computerized tomography, but have not been totally evaluated [81]. In hereditary pancreatitis patients with PRSS1 germ-line mutation who are at a higher risk of early onset of pancreatic cancer, screening can start at the age of 40 years even though, there is no consensus about when to begin the screening [82].

8. Diagnosis

Pancreatic cancer detection and diagnoses is hard because of the location of the pancreas. About 80-90% of patients with pancreatic cancer have unresectable tumors at the moment of diagnoses as most pancreatic cancer is diagnosed at an advanced stage and there are many reasons for this.

To start with, most people who have symptoms attributable to pancreatic cancer have advanced disease as early-stage pancreatic cancer is usually clinically silent. Symptoms are non-specific and include abdominal pain, pruritus, dark urine, jaundice,

and acholic stools, which may be symptoms resulting from an obstruction within the biliary tree [83]. Because of the wide range of non-specific symptoms, a broad number of diseases need to be differentiated [84], which include but are not restricted to: cholangitis, choledocholithiasis, choledochal cysts and primary or secondary cancers of the biliary tree, liver, stomach, intestine or pancreas. Therefore, pancreatic cancer is considered as the most common tumor detected at the autopsy studies as diagnosis can be delayed or missed [8].

Up to date, there are numerous diagnostic tools available, for example magnetic resonance imaging (MRI) [84], abdominal ultrasonography, tri-phasic pancreatic-protocol CT (the standard for diagnosis and staging [85, 86], and endoscopic ultrasound-guided fine-needle aspiration for cytological diagnosis (reported with 80% sensitivity) [87]. In the high risk population outlined by the International Cancer of the Pancreas Screening Consortium group, consensus was that a combination of EUS and MRI/MRCP are the recommended imaging modalities for screening in these individuals [88].

Furthermore, in symptomatic patients, measurement of blood levels of cancer antigen 19-9 can help to confirm the diagnosis and predict prognosis and recurrence after resection [89]; however, it cannot stand as an individual screening tool for asymptomatic patients because it is not tumor-specific [90].

Of note, the use of various diagnostic modalities and diagnostic tools differs between developed and undeveloped countries, which may explain the noted vast differences in incidence and mortality rates. As an example, in 2012, Europe recorded one-third of the total incidence, which can result from the more accurate diagnosis despite etiology [13].

The relative rarity of pancreatic cancer also means that many primary care physicians will only see a case every few years on average. It is therefore imperative to maintain awareness among these professionals in order that those with relevant symptoms are investigated in a timely and appropriate fashion. A retrospective case-control study in primary care found that patients sought medical attention 18 times on average in the period preceding their pancreatic cancer diagnosis [91].

9. Treatment

Pancreatic cancer patient's survival rates are extremely low as the majority of tumors being at an advanced stage at diagnosis. In fact, only 10% of cases are resectable at the presentation and more than 90% of patients who go through potentially curative resection still die of the disease due to local recurrence and/or distant metastases in the absence of adjuvant therapy (Figure 7) [92]. Up to date, surgical resection is the only treatment that provides a potential cure of pancreatic cancer and the addition of chemotherapy in the adjuvant setting has been shown to improve survival rates. In addition, some optimistic results show a further improvement in survival with the administration of chemo-radiotherapy in the neo-adjuvant setting but further work is needed to identify which group of patients will benefit the most [93].

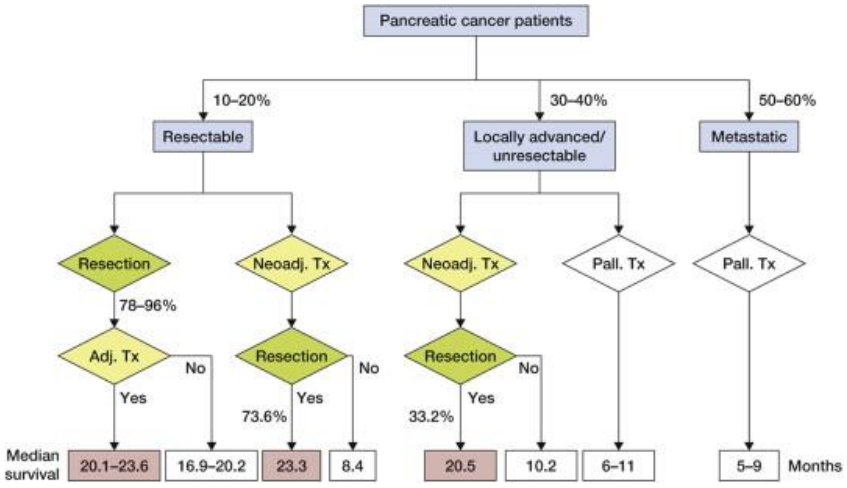

Figure 7. Summary of survival and resection percentages for patients with pancreatic cancer. Reproduced with permission from Gillen et al., 2010. Neoadj: neoadjuvant; Tx: treatment; Pall: palliative; Adj.: adjuvant.

a. Medical Management

i. Adjuvant Treatment

The use of adjuvant chemotherapy was supported by the landmark randomized CONKO-001 study which compared adjuvant gemcitabine after complete surgical resection against surgery alone. This study demonstrated a significantly improved median disease-free survival (13.4 months vs 6.7 months) and overall survival with a five-year survival of 20.7% vs 10.4% and a ten-year survival of 12.2% vs 7.7%. However, despite these promising results, the median overall survival only improved from 20 to 23 months (P = 0.01)[94]

Further studies have sought to identify the best chemotherapy regimen. The ESPAC-3 trial demonstrated that gemcitabine was the chemotherapy agent of choice when compared to 5-fluorouracil. Although survival outcomes were comparable in both

groups, the latter was less well tolerated. Due to the success of dual therapy of capecitabine and gemcitabine in both advanced and metastatic disease, Neoptolemos et al performed the ESPAC-4 trial in patients with resected disease and found that the median overall survival was 28 months (95%CI: 23.5-31.5) in dual therapy compared to 25.5 months (22.7-27.9) in gemcitabine alone (HR: 0.82, 95%CI: 0.68-0.98; P = 0.032)[95].

Other chemotherapy regime have been studied, for example, in the PRODIGE24/CCTG randomized clinical trial which compared the outcomes of gemcitabine or mFOLFIRONOX (a combination of oxaliplatin, irinotecan, and leucovorin) in patients with an R1 or R0 resection of pancreatic adenocarcinoma[96]. The results at a median follow up time of 33.6 months have shown that administration of mFOLFIRONOX was associated with a significantly improved disease-free survival (21.6 months vs 12.8 months), and overall survival (54.4 months vs 35 months) compared to gemcitabine. Administration of mFOLFIRONOX was associated with a significantly increased risk of complications although the only death that occurred was within the gemcitabine treatment group. The current standard of care is guided by post-operative fitness and mFOLFIRONOX is used for very fit patients with tumors of the head, body and tail of the pancreas whereas in less fit patients dual therapy with gemcitabine and capecitabine is given [97].

ii. Neo-adjuvant Treatment

Although there has been shown to be a survival benefit with adjuvant treatment, between 71% and 76% percent of patients still relapse within two years up. Furthermore, due to complications associated with surgery up to 40% of patients are not

suitable for progression to adjuvant therapy [97]. Such figures coupled with the success seen with neo-adjuvant treatment in several other cancers including rectal, esophageal, and gastric cancer have led to the exploration of the impact of neo-adjuvant treatment in pancreatic cancer [98]. The theoretical advantage of neo-adjuvant therapy includes eliminating micro-metastases and shrinkage of the primary tumor and both these factors are associated with a decreased incidence of tumor recurrence [99]. However, patients receiving neo-adjuvant treatment may develop complications which can delay or prevent the progression to surgery and tumors may be unresponsive to the chemo radiotherapy leading to disease progression and previously resectable disease becoming unresectable. Furthermore, the administration of chemo radiotherapy induces fibrosis within the pancreas which can increase the complication rate associated with pancreatectomy [100]. Studies looking at the impact of neo-adjuvant treatment have been performed in patients with resectable or borderline resectable disease. Multiple meta-analyses have been performed studying the impact of neoadjuvant treatment on survival in pancreatic adenocarcinoma. A recent one was by Versteijne et al. [101] which included 38 studies with a combination of 3 randomized controlled trials, 9 phase one or phase two trials, 12 prospective cohort studies and 14 retrospective cohort studies. In intention-to-treat analysis there was a median overall survival of 18.8 months in the neo-adjuvant group compared to 14.8 months in the surgery first group [102].

iii. Treatment in Metastatic Patients

The management of metastatic pancreatic cancer involves symptom control, management of jaundice and palliative chemotherapy with the preferred chemotherapy regime FOLFIRONOX (mFOLFIRONOX with 5-fluorouracil). Conroy et al. [103]

performed a multicenter, randomized trial in 48 French centers with patients receiving either gemcitabine or FOLFIRINOX within a week of enrolment. There were 171 patients within each group and intention to treat analysis was performed. The median overall survival in the FOLFIRONOX group was 11.1 months (95%CI: 9.0-13.2) compared to 6.8 months (95%CI: 5.5-7.6) in the gemcitabine group (HR: 0.57 95%CI: 0.45-0.73; $P < 0.001$). There was an increased incidence of adverse effects within the group receiving FOLFIRONOX, however, this group concluded that FOLFIRONOX should be the treatment of choice in patients with metastatic disease [104].

The limitations of current treatment strategies in pancreatic cancer reinforces the need for new avenues of research to be explored, in order to achieve potential breakthroughs.

B. Chemotherapy

As mentioned earlier, PDAC patients are usually treated with gemcitabine (gem) and/or 5-fluorouracil (5-FU) in case of inoperability [105]. The pyrimidine analog gemcitabine is currently a principal drug for the treatment of metastatic, or unresectable pancreatic tumors, either alone or in combination with other medications [106]. 5-FU, a member of fluoropyrimidines group, is used for the palliative therapy of PDAC as a monotherapy or in combination with other chemotherapies such as in the FOLFIRINOX [107]. The combination of chemotherapeutics agents has earned increasing attention. Intriguingly, upon the combination of gem with 5-FU a considerable antagonistic effect was observed. Despite that the use of combination therapy is an accepted standard for most human malignancies more attention needed to be paid to drug interactions [108].

1. 5-Fluorouracil

5-FU is one of the most important drugs for human pancreatic cancer. Although recent studies have questioned the effectiveness of 5-FU against pancreatic cancer, it remains a good choice for pancreatic cancer. In 1957, 5-FU, a widely accepted anti-cancer drug, was first introduced [109].The mechanism of cytotoxicity of 5-FU, as a pyrimidine analog, has been described through the inhibition of thymidylate synthase (TS) and the misincorporation of its metabolites into RNA and DNA [110]. Despite initial doubts concerning the efficacy of 5-FU, numerous studies have since demonstrated a valuable role for 5-FU in combined treatment protocols compared with single gemcitabine chemotherapy [94]. However, 5-FU chemoresistance, which may result from deficient drug uptake, activation of DNA repair pathways, alterations of targets, resistance to apoptosis and the tumor microenvironment, and other serious problems have been noticed [111]. Thus understanding the mechanisms by which 5-FU leads to cell death and by which tumors become resistant to 5-FU is a crucial step towards predicting or overcoming that resistance.

2. Mechanism of Action of 5-FU

5-FU is a uracil analogue with a fluorine instead of hydrogen atom at the C-5 position. It uses the same facilitated transport mechanism as uracil to enters the cell rapidly [112]. Intracellularly 5-FU is converted to many active metabolites: fluorodeoxyuridine monophosphate (FdUMP), fluorodeoxyuridine triphosphate (FdUTP), and fluorouridine triphosphate (FUTP) (Figure 8) [113] .These active metabolites disrupt the action of TS and RNA synthesis. Dihydropyrimidine

dehydrogenase (DPD) is the rate-limiting enzyme in 5-FU catabolism which converts 5-FU to dihydrofluorouracil (DHFU). Normally, more than 80% of administered 5-FU is catabolized primarily in the liver, where DPD is abundantly expressed [114]

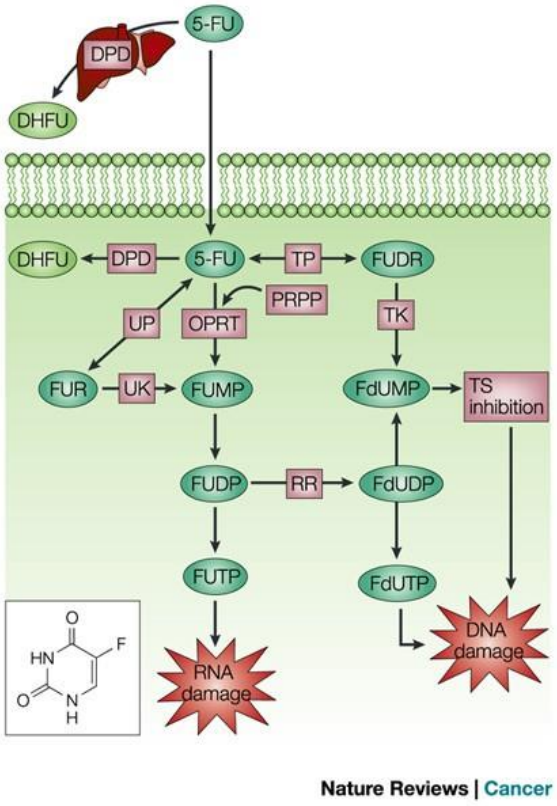

Figure 8. 5-Fluorouracil metabolism. 5-Fluorouracil (5-FU; see structure) is converted to three main active metabolites: fluorodeoxyuridine monophosphate (FdUMP), fluorodeoxyuridine triphosphate (FdUTP) and fluorouridine triphosphate (FUTP). The main mechanism of 5-FU activation is conversion to fluorouridine monophosphate (FUMP), either directly by orotate phosphoribosyltransferase (OPRT) with phosphoribosyl pyrophosphate (PRPP) as the cofactor, or indirectly via fluorouridine (FUR) through the sequential action of uridine phosphorylase (UP) and uridine kinase (UK). FUMP is then phosphorylated to fluorouridine diphosphate (FUDP), which can be either further phosphorylated to the active metabolite fluorouridine triphosphate (FUTP), or converted to fluorodeoxyuridine diphosphate (FdUDP) by ribonucleotide reductase (RR). In turn, FdUDP can either be phosphorylated or dephosphorylated to generate the active metabolites FdUTP and FdUMP, respectively. An alternative activation pathway involves the thymidine phosphorylase catalyzed conversion of 5-FU to fluorodeoxyuridine (FUDR), which is then phosphorylated by thymidine kinase (TK) to FdUMP. Dihydropyrimidine dehydrogenase (DPD)-mediated conversion of 5-FU to dihydrofluorouracil (DHFU) is the rate-limiting step of 5-FU catabolism in normal and tumor cells. Up to 80% of administered 5-FU is broken down by DPD in the liver [113]

To explain more, TS catalyzes the reductive methylation of deoxyuridine monophosphate (dUMP) to deox-ythymidine monophosphate (dTMP), with the methyl donor the reduced FOLATE 5, 10-methylenetetrahydrofolate (CH2THF). This reaction provides the sole de novo source of thymidylate, which is necessary for DNA replication and repair. The TS protein functions as a dimer, both subunits of which contain a nucleotide-binding site and a binding site for CH2THF. The 5-FU metabolite FdUMP binds to the nucleotide-binding site of TS, forming a stable ternary complex with the enzyme and CH2THF, thereby blocking binding of the normal substrate dUMP and inhibiting dTMP synthesis [115]. Even though, the exact molecular mechanisms that mediate events downstream of TS inhibition have not been fully elucidated. The latter results in deoxynucleotide (dNTP) pool imbalances and increased levels of deoxyuridine triphosphate (dUTP), and both of which cause DNA damage [116, 117]. The DNA damage limit caused by dUTP is dependent on both the levels of the pyrophosphatase dUTPase (which limits intracellular accumulation of dUTP) and nucleotide excision repair enzyme uracil-DNA glycosylase (UDG) [118, 119]. Furthermore, thymidine kinase can recover thymidylate from thymidine, thereby relieving the effects of TS deficiency. This pathway can be considered as a potential mechanism of resistance to 5-FU [120] (Figure 9).

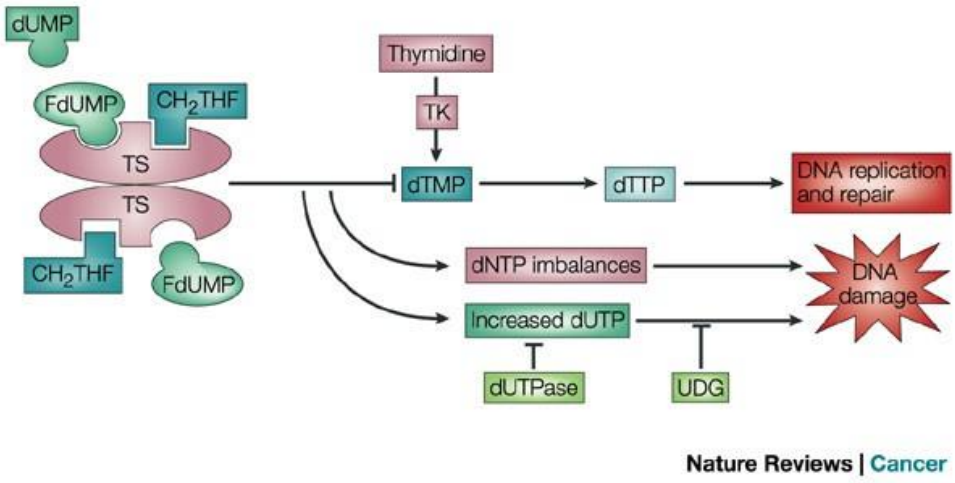

Figure 9: Mechanism of thymidylate synthase inhibition by 5-fluorouracil [113]

In addition, the 5-FU metabolite FUTP is considerably incorporated into RNA, disrupting normal RNA processing and function. 5-FU misincorporation can lead to toxicity to RNA at many levels. It inhibits the processing of pre-rRNA into mature rRNA [121], disrupts post-transcriptional modification of tRNAs [122], and the assembly and activity of snRNA/protein complexes, thereby inhibiting splicing of pre-mRNA [123]. In addition, post-transcriptional conversion of uridine to pseudouridine has been inhibited by 5-FU in rRNA, tRNA and snRNA that all contain the modified base pseudouridine [124]. These studies indicate that 5-FU misincorporation can potentially disrupt many aspects of RNA processing, leading to profound effects on cellular metabolism and viability.

3. 5-FU Resistance in Pancreatic Cancer

Resistance to 5-FU is a serious challenge in the treatment of pancreatic cancer. Equilibrated nucleoside transporter 1 and multidrug-resistance protein (MRP) 5 and MRP8, rather than P-glycoprotein, play important roles in 5-FU transport.

Thymidylate synthase, dihydropyrimidine dehydrogenase, methylenetetrahydrofolate reductase and thymidine phosphorylase are four key enzymes involved in 5-FU metabolism. Other metabolic enzymes, including uridine monophosphate synthetase, also contribute to chemoresistance. Intracellular signaling pathways are an integrated network. The nuclear factor kappa-light-chain-enhancer of activated B cells, AKT and extracellular signal-regulated kinases are signaling pathways that are particularly relevant to 5-FU resistance. In addition, recent reports indicate that STAT-3 is a crucial survival protein. Furthermore, a powerful way for identifying target proteins and understanding the role of microRNAs and stromal factors is provided by the proteomic assays in order to facilitate the development of strategies to combat 5-FU resistance. To describe the main molecular mechanisms of 5-FU chemoresistance, an illustration based on many related studies was created; further studies are necessary to elucidate the roles of these networks (Figure 10) [125]

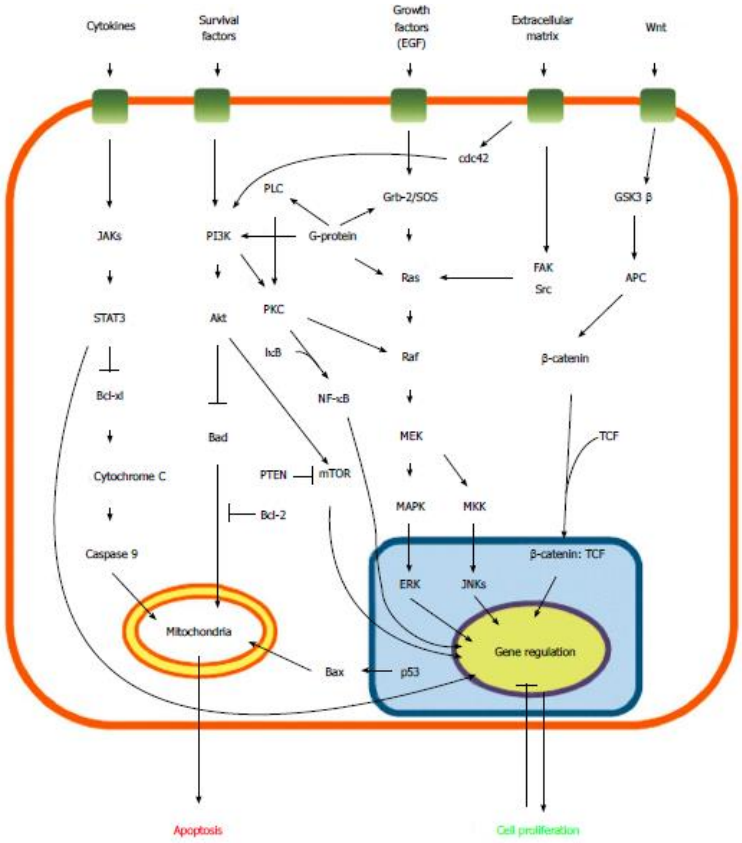

Figure 10: Pancreatic cancer cell survival pathways in 5-fluorouracil resistance. DNA and/or RNA damage caused by 5-fluorouracil (5-FU) leads to the activation of DNA repair systems or the apoptosis cascade. Several cell survival pathways, including the epidermal growth factor receptor (EGFR)/mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) pathway, Akt/mechanistic target of rapamycin (mTOR) pathway, STAT3 dependent pathway, phosphatidyl inositol 3-kinase (PI3K)/nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway and Wnt/GSK3β/β-catenin pathway, are involved in 5-FU resistance in pancreatic cancer [125].

C. Retinoids

Conventional treatment strategies such as surgery, chemotherapy, and/or radiation have had little influence on the course of this aggressive cancer regardless the efforts over the past several years. Even though metastatic pancreatic cancer patients have shown better outcomes with combinations of cytotoxic chemotherapy agents, not all patients can encounter these regimens because of severe intolerable toxicities [103]. Therefore, gentler alternative approaches are highly sought, and the strategy of

investigating the less toxic cancer bioactive vitamins to augment cytotoxic therapy is very attractive.

Retinoids (natural and synthetic vitamin A derivatives) have been studied for decades in clinical trials due to their established role in regulating cell growth, differentiation, proliferation, and apoptosis [126]. This fat-soluble vitamin is stored in lipid vesicles in interstitial cells in the pancreas called stellate cells (PSCs) [155]. All-trans-retinoic acid (ATRA) and 9-cis retinoic acid (9-c RA) are two biologically active stereoisomers of RA that binds to retinoic acid receptors (RARs) with high affinity [127]. The intra-cellular transportation of ATRA to the nuclear receptors is performed by either fatty acid binding protein 5 (FABP5) or cellular retinoic acid binding protein 2 (CRABP2), dictating the transcription of downstream genes and, thus, eventual cell phenotype. [128]. Several biologically active vitamin A derivatives such as ATRA, have been tested for potential use in cancer therapy and chemoprevention. The most effective clinical use of ATRA was demonstrated in acute promyelocytic leukemia (APL) treatment [129]. Retinoids have been studied as chemopreventive agents in clinical trials, with many endpoints focusing on safety and tolerability and antitumor activity these supplements may have alone or in combination with other agents.

1. Mechanisms of Action of Retinoids in Pancreatic cancer

RA has been demonstrated to block cell proliferation and induce cell differentiation in many malignant tissues. RA exerts its pleiotropic effects on cellular growth and differentiation through nuclear receptors, RARs and retinoid X receptors (RXRs). There are three RARs: RAR-α, RAR-β, and RAR-γ, encoded by the *RARA, RARB, and RARG* genes, respectively, as well as 3 RXRs: RXR-α, RXR-β, and RXR-γ,

encoded by the *RXRA*, *RXRB*, and *RXRG* genes, respectively. RAR forms a heterodimer with RXR, increasing its affinity for binding to the retinoic acid responsive response element (RAREs) located on the promoter region of retinoids target genes. In the absence of a ligand, it becomes complexed with a corepressor protein, causing transcriptional repression through histone deacetylation with histone deacetylases (HDACs). When an agonist ligand such as RA binds RAR on the RAR/RXR heterodimer, it results in dissociation of the corepressor and recruitment of a coactivator protein, promoting histone acetylation with histone acetyltransferases and activation of mRNA transcription [130]. In addition, RA was also shown to function independently of the classical mechanisms of nuclear receptor action via non-genomic pathways where it can efficiently adjust the activities of proteins involved in signal transduction in a way that is highly cell type specific [131]

Several studies have established an effect of retinoids on many types of cancer cells including leukemia, lymphomas, breast, ovarian, colon, skin, lung, prostate and most notably pancreatic cancer [130, 132-134]. One main focus is on the stimulation of RAR, specifically the RAR-β subtype, in which decreased expression of RAR-β plays a key role in the maintenance of a malignant phenotype in human pancreatic adenocarcinoma, representing a novel target for treatment [135]. In animal studies, stimulation of the RAR-α subtype by retinoids resulted in a time- and dose-dependent inhibition of cell growth [136]. Retinoids also play a role in other intracellular signaling pathways. They have been shown to be involved in the differential regulation of PKC-α, which plays a central role in pancreatic carcinoma cell growth [137]. They have also been implicated in decreasing pancreatic carcinoma cell adhesion to laminin, a component of the basement membrane, during infiltrative growth and metastasis [138].

Retinoids also inhibit pancreatic carcinoma cell migration and epithelial-mesenchymal transition (EMT) of tumor cells through downregulation of IL-6 in cancer-associated fibroblast cells (CAFs) [139]. Furthermore, ATRA inhibitory effect on cell proliferation, colony formation, and migration/invasion was associated with downregulation of p21-activated kinases (PAKs) and depletion of PAK1. Plus the combination of ATRA and gemcitabine synergistically reduced cell growth in both wild-type and gemcitabine-resistant pancreatic ductal adenocarcinoma cells where it enhances gemcitabine cytotoxicity by increasing deoxycytidine kinase (dCK) expression [140, 141]. To add more, the effect of the combination of gemcitabine and ATRA on pancreatic stellate cells (PSCs) showed that PSC activity (as measured by deposition of extracellular matrix proteins such as collagen and fibronectin) and PSC invasive ability were declined in response to combination therapy. These effects were taking place through a range of signaling cascades in the tumor-stroma cross-talk such as reduction of Wnt signaling in the tumor compartment, disrupted fibroblast growth factor (FGF) signaling in the stromal compartment or targeting of other signaling cascades such as hedgehog, IL6, and CXCL12 in cancer as well as stellate cells, affecting epithelial cellular functions such as epithelial-mesenchymal transition, cellular polarity, and lumen formation [142]. Another example for the cross-talk is that healthy PSCs are quiescent, but upon activation during disease progression, they adopt a myofibroblast-contractile phenotype and secrete and concomitantly reorganize the stiff extracellular matrix (ECM). Transforming growth factor β (TGF-β) is a potent activator of PSCs. ATRA inhibits the ability of PSCs to mechanically release active TGF-β [143] and also suppresses its pro-angiogenic activity [144]. They have also been demonstrated to reduce the expression of pancreatic stem cell markers CD24, CD44, CD133, and

aldehyde dehydrogenase 1. However gemcitabine treatment increases the expression of some of these markers especially CD44 when it is combined with RA, a notable reduction in all of them is observed [145]. Moreover, retinoids have been shown to regulate expression of some miRNA, for example, it upregulated expression of miR-10a [146] and increased expression of miR-375 that in most cases interferes with myotrophin (Mtpn) expression and decreases its expression at both the mRNA and protein levels [147]. Collectively, these data provided biologically plausible evidence that RA could be used as a promising agent in pancreatic cancer.

In fact, many studies have searched for a link between the administration of RA and the prevention of metastasis and recurrence in pancreatic cancer. Although no objective responses were observed, prolonged stable disease occurred in pancreatic cancer patients in a phase I study of the HDAC inhibitor entinostat in combination with 13-cis-RA [148]. Another very interesting study used the concept of maintenance immunotherapy consisting of IL-2 and 13-cis-RA after treatment for stage III pancreatic cancer. The study subjects were progression-free stage III patients who had received cisplatin and gemcitabine therapy consolidated with radiotherapy with concurrent capcitabine. Patients treated with the maintenance immunotherapy had a median progression-free survival of 16.2 months, with overall survival still pending after an average of >24 months. These outcomes were superior over historical controls of standard cytotoxic chemotherapy with chemoradiation therapy for stage III pancreatic cancer [149]. A phase II trial of 13-cis-RA combined with INF-α in locally advanced pancreatic cancer was noted to be well tolerated but with no improvement in the response rate [150]. A phase II pilot trial of 13-cis-RA and INF-α in patients with advanced cancer also noted tolerance of the therapy but no substantial response rates

[151]. A most recent study on Phase I clinical trial for pancreatic cancer patients with advanced, unresectable PDAC showed that ATRA re-purposed as a stromal-targeting agent in combination with gemcitabine-nab-paclitaxel chemotherapy using a two-step adaptive continual re-assessment method trial design is safe and tolerable [152]. So, many studies have reported the promising role of retinoids in attaining better outcomes for several types of cancer. However, even this promising therapy has some limitations: retinoid toxicity and intrinsic or acquired resistance have been observed in many patients

2. Mechanisms of retinoid resistance

Even though pharmacological retinoid doses have been approved by the Food and Drug Administration (FDA) and other regulatory bodies for the treatment of some hematologic malignancies and high-risk neuroblastoma NBL, the chemopreventive and therapeutic effects of retinoids in other solid tumors are still unclear. The therapeutic response of retinoids in some tumors is found to be limited to a small proportion of the treated patients [153]. Retinoid resistance is thought to cause this limited effect. Moreover, after retinoid treatment, some carcinomas not only fail to exert growth inhibition but instead respond with enhanced proliferation. A clue to this paradoxical behavior was suggested by the finding that retinoic acid and its natural receptor also activate peroxisome proliferator-activated receptor (PPAR) β and δ (PPARβ/δ), which are involved in mitogenic and anti-apoptotic activities [154].

Many potential mechanisms have been suggested for retinoid resistance (Figure 11) including decreased retinoid uptake [155], increased retinoid catabolism by cytochrome P450 [156], active drug efflux by membrane transporters, the altered

expression of coactivator or downstream target genes, the downregulated expression of various RAR genes (promoter methylation) and changes in the activities of other signaling pathways [157].

Despite that retinoid resistance remains problematic in the area of biological anticancer therapy, the discovery of biomarkers that indicate retinoid resistance or sensitivity in each individual patient seems to be important for the recent personalized therapy strategy, which is aimed at identifying of the most effective therapy for individual patients.

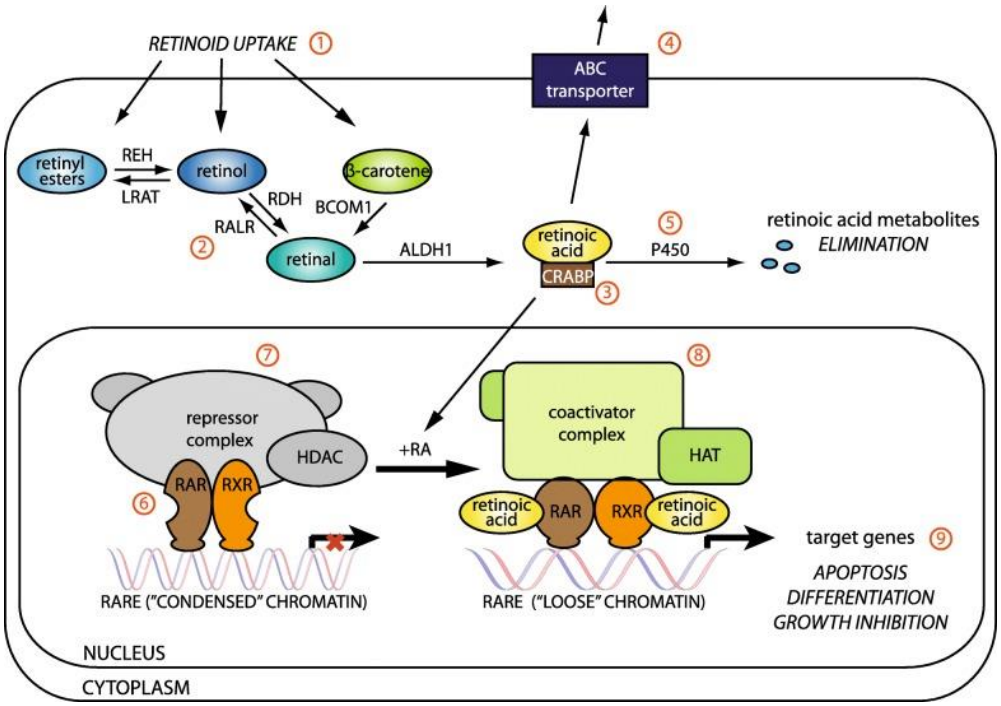

Figure 11. **Possible mechanisms of retinoid resistance**. Cancer cell retinoid resistance may be caused by several independent mechanisms including (1) decreased retinoid uptake; (2) intracellular retinoid metabolism; (3) altered intracellular retinoid availability due to CRAB protein binding; (4) increased retinoid efflux by ABC transporters; (5) increased retinoid catabolism catalyzed by cytochrome P450; (6) decreased RAR and/or RXR expression; (7) inhibited retinoid-induced transcription by the repressor complex, (8) altered coactivator structure, expression, or activity; (9) altered downstream target gene expression

3. Predictive biomarkers of retinoid resistance in pancreatic ductal adenocarcinoma

The disturbances of vitamin A metabolism that result in a decreased intracellular ATRA concentrations were originally described in pancreatic ductal adenocarcinoma (PDAC) [158] and later, in other human malignancies [159]. Previous studies in PDAC cell lines have indicated the ability of ATRA to induce cell cycle arrest and differentiation, although these data revealed highly variable retinoid sensitivity among the PDAC cell lines [160, 161]. Based on the receptor-dependent retinoid mechanism, the potential patient benefit from this treatment is highly dependent on the retinoid receptor expression level in tumor tissue. Among others, RARβ expression is downregulated in PDAC [162] which may explain the negative outcomes of clinical trials focused on retinoid treatments.

Typically, ATRA showed to induce cell differentiation and growth arrest in most epithelial cell types. However, experiments in the pancreatic cancer Capan-1 cell line have shown that in addition to an antiproliferative effect, retinoids increase cell migration, resulting in an invasive phenotype [163]. This effect is believed to be happened by the existence of the nuclear receptors PPARβ/δ (activated by retinoids and form heterodimers with RXR). So, in the time where RAR-dependent gene expression causes growth arrest, PPARβ/δ activation leads to proliferation, cell survival, and tumor growth in mouse model [164]. The levels of two key intracellular ligand-binding proteins: fatty acid-binding protein 5 (FABP5) and cellular retinoic acid-binding protein 2 (CRABP2) regulate the distribution of available ATRA between PPARβ/δ and RAR receptors. According to the relative abundance of FABP5 and CRABP2 within the cell, they transport exogenous retinoids from the cell cytoplasm into the nucleus, to either PPARβ/δ or RARs [154]. A study on 14 PDAC cell lines demonstrated that it might be

possible to predict PDAC cell sensitivity to ATRA on the basis of the relative expression levels of these two retinoid-binding proteins. According to this study, 10 out of 14 cell lines expressed the one or the other binding protein confirming the pattern of reciprocal differential expression of both transcripts in PDAC cells. PDAC cell lines with FABP5 (high) CRABP2 (null) were resistant to ATRA-mediated growth inhibition and apoptosis and exhibited an increased migration and invasion phenotype. On the other hand, cell lines with FABP5 (null) CRABP2 (high) retained ATRA sensitivity. These results were also confirmed in vivo using xenograft models. Immunohistochemical detection of FABP5 in PDAC samples revealed that about 20% of them were completely negative for FABP5 indicating these patients as suitable candidates for retinoid therapy [165]. These drawbacks emphasize the interest in the identification of novel strategies to facilities cancerous cells to undergo cell deaths with increased specificity and decreased toxicity.

D. Retinoids Related Molecules

There has been a remarkable effort aimed at the identification and characterization of retinoids endowed with apoptogenic properties stronger than those of ATRA and its natural derivatives 9-cis and 13-cis retinoic acid. The availability of new retinoids which accelerate the process of apoptosis and/or render it differentiation-independent would be advantageous in the setting of the first and second-line clinical treatment of various types of leukemia and solid tumors. Retinoid related molecules (RRMs) are an emerging class of apoptotic compounds whose chemical structure is derived from that of ATRA. The prototype of this family of molecules is the

conformationally restricted retinoid CD437 (Figure 9) which was originally developed as a selective RARγ agonist [166].

CD437 undergoes its effects through retinoid receptor dependent and independent pathways leading to apoptosis and tumor growth arrest by binding selectively with RARγ and minimally with RARα or RARβ [166, 167]. RRMs appeared to induce apoptosis in both retinoic acid- and multi-drug-resistant cancerous cells. The apoptotic process triggered by RRMs is independent of p53 activation and proceeds through a novel pathway in which the mitochondrion seems to play a pivotal role [168]. In addition, CD437 was found to be a direct inhibitor of DNA polymerase α, the enzyme responsible for initiating DNA synthesis during the S phase of the cell cycle [169]. Figure 12 summarizes some of the biochemical pathways activated by RRMs in the neoplastic cell. In certain cell types, RRMs activate (red arrow pointing upward) or repress (red arrow pointing downward) the expression of multiple genes. Activation may be the result of transcriptional effects, as indicated in the figure, or post-transcriptional events (not shown). Genes A-B are activated or repressed by RRMs via RARγ-independent pathways, whereas the expression of genes C and D is modulated by ligand-dependent activation of the nuclear retinoic acid receptor. The expression of genes E and F is up-regulated or down-regulated as a result of the potential DNA damage caused by RRMs. In this scheme, the two genes are instrumental in inducing a genotoxic response that causes activation of the MAP kinase cascade, with increased phosphorylation of p38 and JNK. Genotoxic responses can also be triggered through DNA insults that do not require gene activation as indicated by the rightmost line in the scheme. It is also possible that the MAP kinase cascade is activated by as yet unrecognized pathways that are independent of

genotoxicity[168]. For instance p38 activation may result from caspase activation in certain cellular contexts (not shown). RRMs induce the membrane expression of the two death receptors DR4 and DR5 through a p53-dependent pathway. P53 controls the activity of the genes encoding DR4 and DR5. Some of the non-genomic effects triggered by RRMs, are also summarized in the figure. CD437 and congeners induce the translocation of the orphan nuclear receptor NUR77/TR3 from the nucleus to mitochondrial membrane. The translocation of the protein is purported to induce the release of cytochrome c and other factors (not shown) from the mitochondrion to the cytosol. Cytochrome c leakage results in the assembly of the apoptosome, a functional multi-protein complex which includes the death factor APAF-1. Assembly of the apoptosome induces the activation of the caspase cascade (only the upstream caspase-9 and the effector caspases, caspase-3 and caspase-7 are shown in the figure). In many cell types, one of the earliest biochemical events activated by RRMs is an increase in the cytosolic concentration of calcium (Ca++). This is likely to be the result of an inactivation of the process of calcium uptake inside the mitochondrion. CD437 and congeners may also have a direct action on the mitochondria that may result in a depolarization of the corresponding membrane (Ψ). In certain cells, mitochondrial dysfunction leads to an increased production of toxic oxygen radicals that cause the release of cathepsin D (cat D) from the lysosome into the cytosol. All these biochemical effects may have a role in the process of apoptosis triggered by RRMs in the neoplastic cell. Despite that, RRMs manifest synergistic interactions with many classical cytotoxic agents, they also manifest very limited cross-resistance with other classes of chemotherapeutic agents [168]

To add more, this compound has raised remarkable enthusiasm, as it is active in vivo on preclinical models of such diverse types of neoplasia as melanoma [170], lung carcinoma [171] and acute myeloid leukemia [172]. So, CD437 is likely to have a mechanism of action that is rather, if not entirely, different from that of classical cyto differentiating retinoids and many known chemotherapeutic agents. However, despite the anti-neoplastic properties that CD437 held in different tumor types, it showed to have a low pharmacokinetic profile. Several RRM congeners, such as ST1926, were shown to have a more potent apoptotic potential, a lower level of toxicity, and a better pharmacokinetic profile than CD437.

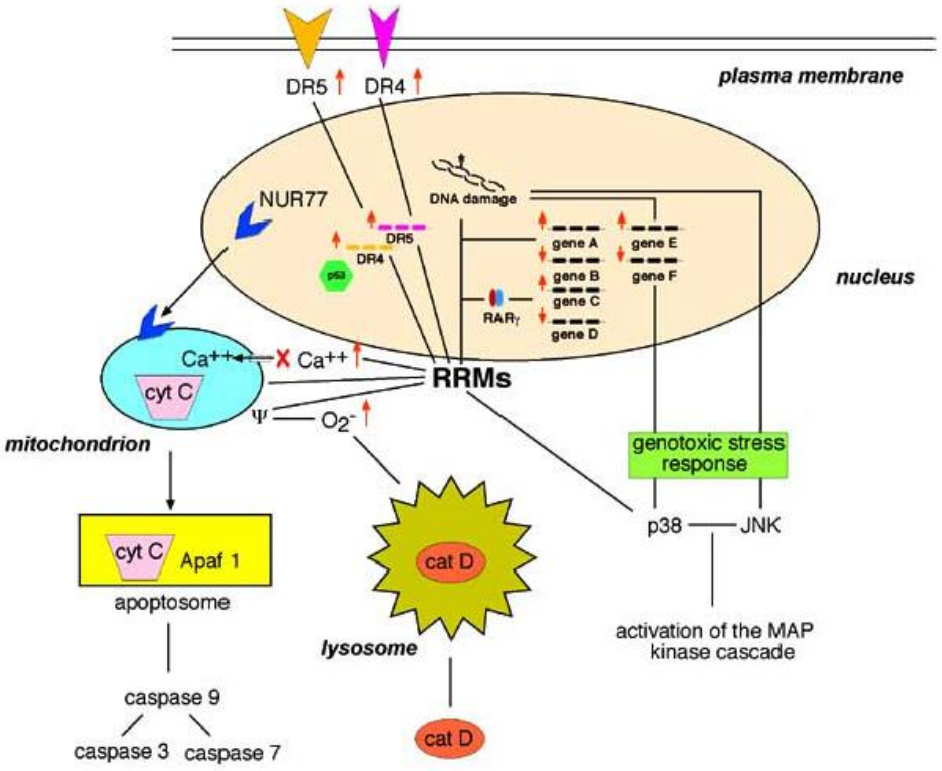

Figure 12. Biochemical pathways activated by RRMs in the neoplastic cell during the process of induced apoptosis [168]

1. The adamantyl Retinoid ST1926

ST1926 an orally available synthetic atypical retinoid is a more specific, stable, and less toxic analogue of CD437 (Figure 13) [168, 173]. ST1926 was shown to be effective against several solid and hematological tumor cells that are resistant to ATRA [174-176]. ST1926 bio-distribution in mouse was found to be favorable as micromolar (μM) concentrations of the compound were achievable in the plasma of mice, with significant extravascular distribution of the compound [176] where many in vivo experiments confirmed the role of ST1926 in suppressing tumor growth with few toxicity [174-177].

Compared to CD437, ST1926 was found to be able to trans-activate RARγ less potentially but with greater apoptotic effective. Thus, indicating its ability to mediate its mechanisms of action independently of the retinoid receptor pathway.

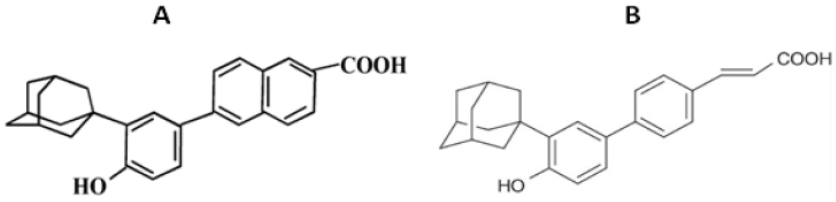

Figure 13. Chemical Structures of Retinoids Related Molecules: (A) CD437 (B) ST1926

ST1926 induced apoptosis, DNA damage, and cell cycle arrest by several pathways. it was shown to down regulate expression of many targets necessary for tumor progression (such as Tax in adult T-cell leukemia (ATL) [174], BCR-ABL in chronic myeloid leukemia (CML) [175], Wnt/β-catenin pathway in breast cancer cells in 2D and 3D cell culture models [178]), to decrease PAX3-FOXO1 fusion oncoprotein levels at a post-transcriptional level, and reduce protein levels of the cell cycle kinase

CDK1 in rhabdomyosarcoma [176], to cleavage of PARP a hallmark of apoptosis [179] and poly (ADP-ribose) polymerase, to significantly impair complex II (CII) function which was associated with the inhibition of succinate dehydrogenase (SDH) activity in Glioma [177]. In contrast, to up regulate expression of (p53, p21, γH2AX, Bax, c-Fos, to activate caspases such as (3, 8, 9), to induce phosphorylation of p38 and JNK and to cause an immediate increase in the cytosolic calcium level that is directly related to apoptosis [173, 175, 176, 180]. Also, it induces specific phase DNA double strands breaks (DSB) and cell death through the mitochondrial pathways of apoptosis [181].

Mechanistically, histone H2A.Z protein was found to specifically bind ST1926 clarifying the link between perturbations of histone pathway and DNA damage and apoptotic responses but this was achieved at suprapharmacological levels. To add more, pharmacological inhibition of proteasome blocked apoptotic response afforded by ST1926, whereas inhibition c-AMP dependent protein kinase A partially reverted resistance to ST1926 [182]. Similarly to ST1926 parent molecules CD437, ST1926 inhibited DNA polymerase α (POLA-1) activity and reduced its protein expression levels [183]

Besides, ST1926 demonstrated synergistic interactions with a number of classical cytotoxic agents such as the epidermal growth factor receptor inhibitor ZD1839[184], cisplatin [185], ATRA [186], and sensitization to ST1926 by histone deacetylases inhibitor RC307 [187]

CHAPTER II

AIM

Previous studies have demonstrated the anticancer effects of ST1926 on several cancer models, including acute myeloid leukemia, T-cell leukemia, chronic myeloid leukemia, breast, ovarian, melanoma, neuroblastoma, lung, colorectal, prostate, and glioma cancers [173, 174, 177-179, 183]. In addition, they have shown that ST1926 in combination with a number of classical cytotoxic agents such as the epidermal growth factor receptor inhibitor ZD1839 [184], cisplatin [185], and ATRA [186] showed synergistic interactions and thus suggesting that studies of synergistic effect is a promising path for more efficient anti-cancer therapies.

In this study, we examined the anti-tumor effect of the synthetic retinoid ST1926 on an *in vitro* pancreatic cancer model. Also, we examined whether combination therapy with ST1926 and

5-FU had enhanced anti-tumor efficacy in pancreatic cancer. The effect of ST1926 alone or in combination with 5-FU was investigated on pancreatic cancerous cell proliferation, viability, and cell death mechanisms. The synergistic activity of the combination of ST1926 with 5-FU was evaluated using the CompuSyn analysis method. Protein expression for some targets was measured by western blot.

CHAPTER III

MATERIALS AND METHODS

A. Cell Culture

Human pancreatic cancer cell lines, Panc-1 and Capan-1, were maintained in Dulbecco's Modified Eagle's Medium (DMEM) high glucose (Sigma Aldrich) with 10% Fetal Bovine Serum (FBS) (Sigma Aldrich) for Panc-1 and 20% for Capan-1, 1 mM sodium pyruvate (Sigma Aldrich), 1% penicillin/streptomycin (Sigma Aldrich), and 0.5% kanamycin (Sigma Aldrich). Cells were maintained at standard incubator conditions (humidified atmosphere, 21% O_2, 5% CO_2, 37 °C). The cells were passaged and maintained every two to three days depending on their growth status. Regularly, when the confluence of the cells reached 70-80%, they were collected and centrifuged for 3 min at 900 rpm and the pellet was resuspended in fresh media and transferred into new cell culture flasks for maintenance.

B. ST1926 and 5-FU Preparation

ST1926 was kindly provided by Biogem SCARL (Ariano Irpino-Martiri, Italy), prepared as stock solution, dissolved in dimethyl sulfoxide (DMSO) at $1x\ 10^{-2}$ M, and stored in amber tubes at -80 °C. For experiments, ST1926 was applied on the cells after a serial dilutions in DMEM to obtain concentrations ranging from 0.01 µM to 10 µM. 5-FU is a commercially available drug and was purchased from AUBMC pharmacy at 384 mM concentrations and stored at room temperature (RT). For experiments, 5-FU was diluted in DMEM to obtain concentrations of 5 µM and 10 µM. ST1926 and 5-FU were applied to the cells while the visible light inside the cell culture cabinet was turned off.

C. Cell Growth Assay

To test for the anti-proliferative effects of ST1926, 5-FU or ST1926 in combination with 5-FU, pancreatic cancer (PC) cells were treated with varying concentrations of ST1926 (0.01 µM, 0.05 µM, 0.1 µM, 0.5 µM, 1 µM, 5 µM, and 10 µM) or/and 5-FU (5 µM and 10 µM). PC cells seeded in 96-well plates at a concentration of 5000 cells/well. Cell growth at 24, 48, and 72 hours post-treatment was assayed using MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) non-radioactive cell proliferation assay. Such assay quantifies the metabolic activity in the mitochondria of the cells that convert tetrazolium salt into a blue formazan crystals, 3 to 4 hours post-addition of the dye. The formazan dye is then solubilized in an SDS-based stop solution and the absorbance of the blue color is assessed in triplicate wells by measuring the optical density (OD) at 595 nm using an ELISA microplate reader. The results were expressed as a percentage of control and they represent an average of up to three independent experiments ± standard error (SE).

D. Determination of Combination Index

The combined effect of ST1926 and 5-FU was evaluated using the CompuSyn analysis method. The combination index (CI) value is interpreted as follow CI 0.1-0.3 (Strong Synergism), CI 0.3-0.7 (Synergism), CI 0.7-0.85 (Moderate Synergism), CI 0.85-0.9 (Slightly Synergism), CI 0.9-1.1 (Nearly Additive), CI 1.2-1.45 (Slight Antagonism) CI 1.45-3.3 (Antagonism).

E. Cell Viability Assay

Cell growth was confirmed with trypan blue exclusion assay. Panc-1 cells were seeded in 6-well plate at a concentration of $15x10^4$ cells/well and treated with 1 µM ST1926, 10 µM 5-FU and 1 µM ST1926 in combination with10 µM 5-FU. Upon the treatment addition (24 and 48 hours post-treatment), trypan blue dye enters the disrupted membranes of the non-viable cells and causes the cells to turn blue in color which can be distinguished from the colorless viable ones under the light microscope. Cells were counted on the four corner chambers of a hemocytometer according to the formula: cells/ml = average number of cells x dilution factor x volume of suspension x 10^4. The results were expressed as a percentage of control and they represent an average of up to three independent experiments ± standard error (SE).

F. Microscopic Imaging

Panc-1 cells were seeded in 6-well plates at a concentration of $15x10^4$ cells/well and treated with 1 µM ST1926, 10 µM 5-FU and 1 µM ST1926 in combination with 10 µM 5-FU for 24 and 48 hours. Representative bright field images were acquired using Zeiss axiovert light microscope and compared to their respective controls.

G. Mitochondrial Membrane Dissipation Assay

The mitochondrial membrane potential for Panc-1 after treatment with ST1926, 5-FU, and ST1926 in combination with 5-FU was monitored using Rhodamine-123 fluorescent dye (Ex/Em = 488/534nm; Sigma), a cell permeable cationic green-fluorescent dye, which preferentially enters into mitochondria of living cells due to the highly negative mitochondrial membrane potential ($\Delta\Psi m$) and sequestered by active

mitochondria without cytotoxic effects. Depolarization of ΔΨm results in the loss of Rhodamine from the mitochondria and a decrease in intracellular fluorescence.

On the day of the experiment, $15x10^4$ cells/well were seeded in 6-well plate and treated with 1 µM ST1926, 10 µM 5-FU, and 1 µM ST1926 in combination with10 µM 5-FU. Cells were then collected 24 or 48 hours post-treatment, washed and resuspended in Rhodamine washing buffer. Then cells were incubated for 1 hour at 37°C in dark with Rhodamine-123 dye at a final concentration of 5 µM. Cells were then washed and resuspended with Rhodamine washing buffer, transferred into polystyrene round bottom tubes (Falcon) and the fluorescence was measured through FACScan flow cytometer.

H. Western Blotting

Panc-1 cells were seeded at a concentration of $15x10^4$ cells/well in 6-well plate and treated with 1 µM ST1926, 10 µM 5-FU, and 1 µM ST1926 in combination with10 µM 5-FU. Cells were then collected 72 hours post-treatment, and washed with 1x PBS, followed by their lysis with 100 µl of 2X Laemmli Lysis Buffer (Bio-Rad) to extract the proteins. Proteins were then quantified using the NanoDrop-1000 (Thermo Scientific) through the protein A280 method. Then, 5% β-mercaptoethanol was added to each sample to reduce the disulfide bonds in the proteins. Using Bio-Rad electrophoresis cell, a gel casting system was set according to the manufacturer's instructions. Subsequently, equal amount of the protein extracts was loaded into each well of the 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), which was run first at 80v for around 25 minutes followed by 100v for around 2 hours using the Mini-PROTEN II electrophoresis cell unit (Bio-Rad). After the run, the proteins were transferred into nitrocellulose membrane using the Electrophoretic

Transfer Cell (Bio-Rad) at 30v overnight. Following the transfer, the membranes were incubated with the blocking solution (10 mm tris-HCl pH 8.0, 150 mm NaCl, 0.05 % Tween 20 with 5% dry milk (fat free) for 1 hour while shaking at room temperature. Then, the membranes were incubated in primary antibodies diluted in a blocking solution at cold room +4°C overnight. The next morning, the membranes were washed briefly with wash buffer and incubated with a secondary antibody (horseradish peroxidase-conjugated anti-rabbit or rabbit anti-mouse; Santa Cruz Biotechnology) for 2 hours at room temperature while shaking.

Finally, membranes were washed and the proteins were detected by enhanced chemiluminescence using ECL system (Bio-Rad). The membranes were then exposed to chemiluminescence detection by the ChemiDoc MP Imaging System (Bio-Rad). The following antibodies were used: PARP (1:1000, Cell Signaling), P53 (1:500, Santa Cruz Biotechnology), POLA-1 (1:1000, Abcam) and γH2AX (1:1000, Cell Signaling). The membranes were also probed with anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1: 25000, Abnova) for assessment of equal protein loading. The Data were analyzed using the ImageJ software.

I. Statistical Analysis

The i*n vitro* studies data presented are the means (± SE) of three independent experiments. Statistical analysis and significance were performed and analyzed using GraphPad Prism 6 software. Statistical significance was reported when the P value was ($P<0.05$, *, $P<0.01$, **, $P<0.001$, ***).

CHAPTER IV

RESULTS

A. Inhibitory effect of ST1926, 5-FU, and their combination on the proliferation of pancreatic cancer cell lines as evident by MTT proliferation assay

First, we started characterizing the anti-proliferative effect of ST1926 on the two pancreatic cancer cell lines (Panc-1 and Capan-1) that harbor different genetic mutations. Cell growth was assayed using non-radioactive MTT proliferation assay. The tested ST1926 concentrations ranged from 0.01 to 10 µM, concentrations of ST1926 that can be achieved physiologically [176]. We have shown that, using concentrations up to 10 µM, ST1926 inhibits the proliferation of the tested PC cell lines Panc-1 and Capan-1 in a dose- and time- dependent manner and by 33% and 58%, respectively 3 days post-treatment (Figure 14). This indicates that both PC cell lines show moderate sensitivity to ST1926 by using concentrations up to 10 µM with Capan-1 being more sensitive compared to Panc-1.

In addition, we have tested several combinations of ST1926 with 5-FU. The combined effect of ST1926 and 5-FU on cell proliferation was measured by incubation of the PC cells with (1 µM and 5 µM) ST1926 and (5 µM and 10 µM) 5-FU alone or in combination. As mentioned earlier ST1926 alone slightly inhibits cell proliferation in both PC cells. Concerning 5-FU, both PC cells also showed some also moderate sensitivity that is time- and dose-dependent, to 5-FU where using concentrations up to 10 µM of 5-FU until 3 days inhibit the proliferation of Panc-1 and Capan-1 by 49% and 50%, respectively.

Interestingly, further reduction of cell proliferation was observed in both cell lines when cells were incubated with combination of ST1926 and 5-FU (Figure 15).

Cell proliferation in Panc-1 cells treated with ST1926 plus 5-FU showed a further decrease by 16 to34% and 10 to 20%compared with cells treated with ST1925 and 5-FU alone, respectively 3 days post-treatment. Similar effect was observed with Capan-1, where compared with ST1926 and 5-FU alone, cell proliferation in capan-1 cells treated with ST1926 plus 5-FU further decreased by 13 to 28 % and (8 to 23%), respectively.

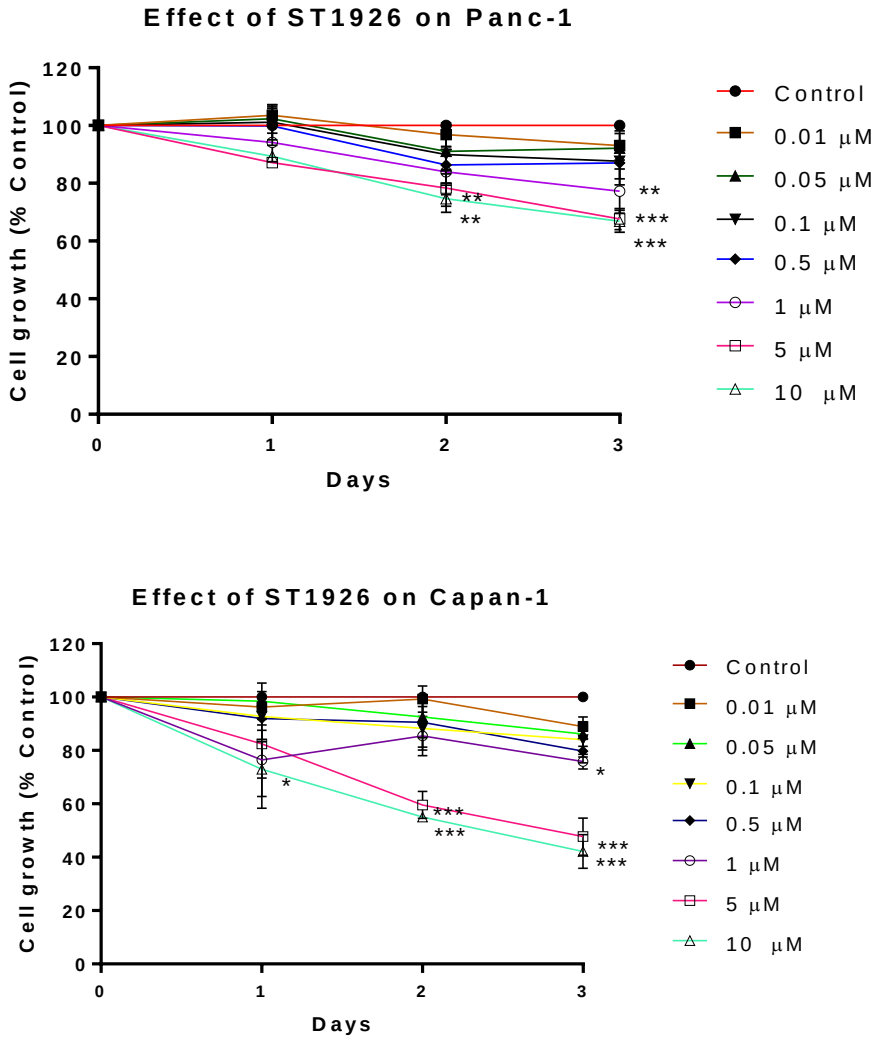

Figure 14. Effect of ST1926 on the proliferation of PC cells using MTT assay. Panc-1 and Capan-1 were seeded at a concentration of $5x10^3$ cells/well in a 96 well plate and treated with the indicated concentrations of ST1926 up to three days. Cell growth was assayed in triplicate wells using non-radioactive cell MTT proliferation assay. Results are expressed as a percentage of control and they represent an average of three independent experiments (± SE) (*, P<0.05;**, P<0.01; ***P<0.001)

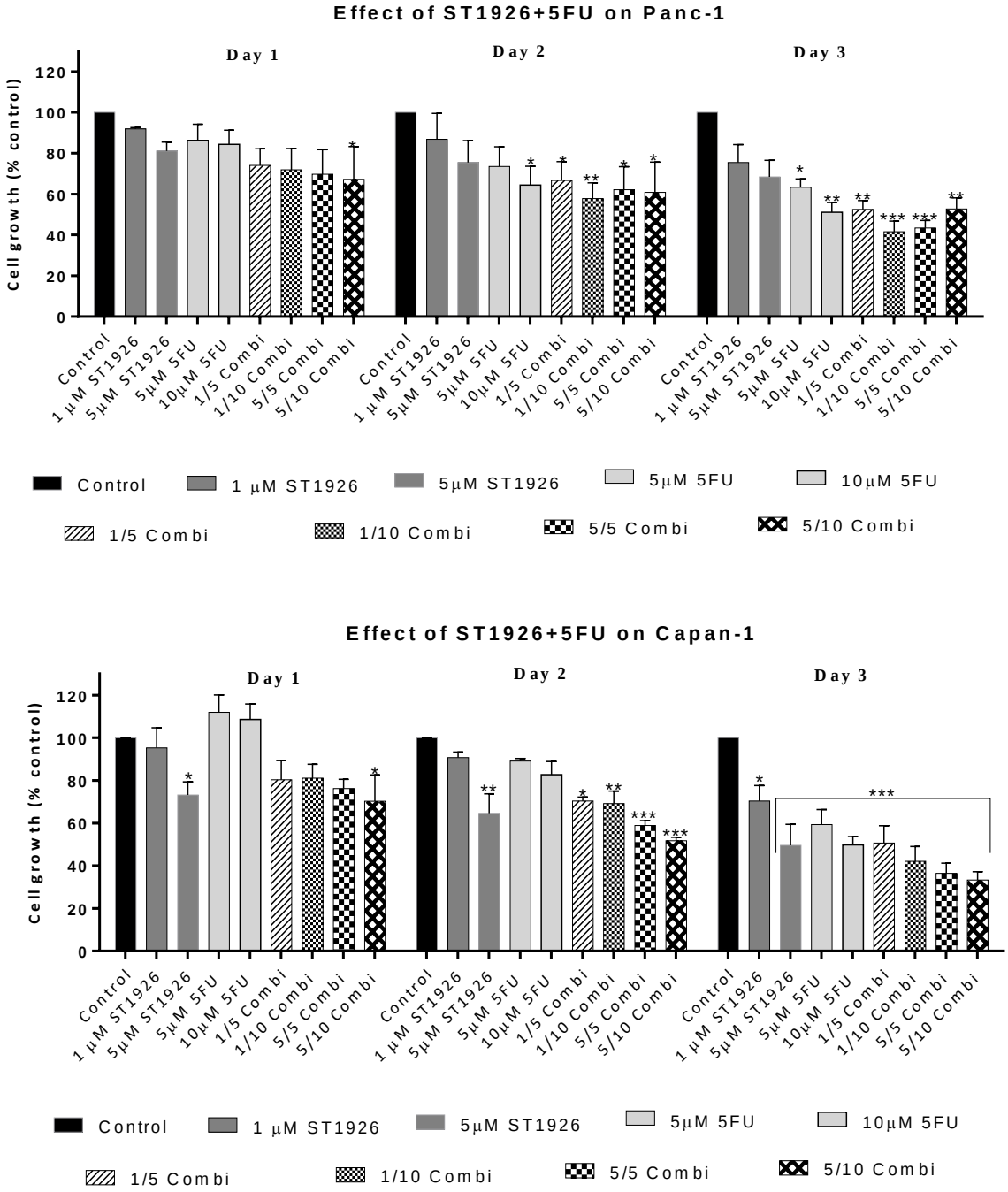

Figure 15. Effect of ST1926 in combination with 5-FU on the proliferation of PC cells using MTT assay. Panc-1 and Capan-1 were seeded at a concentration of $5x10^3$ cells/well in a 96 well plate and treated with the indicated concentrations of ST1926 and 5-FU up to three days. Cell growth was assayed in triplicate wells using non-radioactive cell MTT proliferation assay. Results are expressed as a percentage of control and they represent an average of three independent experiments (± SE) (*, P<0.05; **, P<0.01; ***P<0.001)

B. Combined treatment with ST1926 and 5-FU synergistically inhibited both pancreatic cancer cell lines, as evident by CompuSyn Analysis

The combined effect of ST1926 plus 5-FU was shown to be mostly synergistic, with a CI <1 when analyzed by the CompuSyn software (Table 5). In Capan-1, some antagonistic values were noticed at 24 hours potentially explained by the proliferation induced by the 5-FU upon its addition on the cells at this time point.

Based on the results of our MTT assay and CompuSyn analysis, 1 µM ST1926, 10 µM 5-FU, and 1/10 combination were selected to be the working concentration to further decipher the mechanisms of growth suppression in treated cells. Also, we will continue to work with only Panc-1 as Capan-1 need collagen type-1 to be seeded in larger well plates which is unfortunately was not available. Figure 16 represents the CI of 1/10 combination at 24, 48, and 72 hours in both cell lines.

Table 5. Combined effects of ST1926 and 5-FU on pancreatic cancerous cells using CompuSyn Analysis

Drug Concentration (µM)	**ST 1926**		**1**	**1**	**5**	**5**
	5FU		**5**	**10**	**5**	**10**
Combination Index (CI)	**Panc-1**	**24 h**	0.13673	0.12870	0.37630	0.31715
		48 h	0.66555	0.66719	0.68572	1.00894
		72 h	0.54972	0.58665	0.33342	1.13467
	Capan-1	**24 h**	0.67313	1.08521	1.52402	1.63324
		48 h	0.45742	0.61571	0.89524	0.74228
		72 h	0.74689	0.68428	0.55766	0.57621

Combination Index (CI): CI 0.1-0.3 (Strong Synergism), CI 0.3-0.7 (Synergism), CI 0.7-0.85 (Moderate Synergism), CI 0.85-0.9 (Slightly Synergism), CI 0.9-1.1 (Nearly Additive), CI 1.2-1.45 (Slight Antagonism) CI 1.45-3.3 (Antagonism)

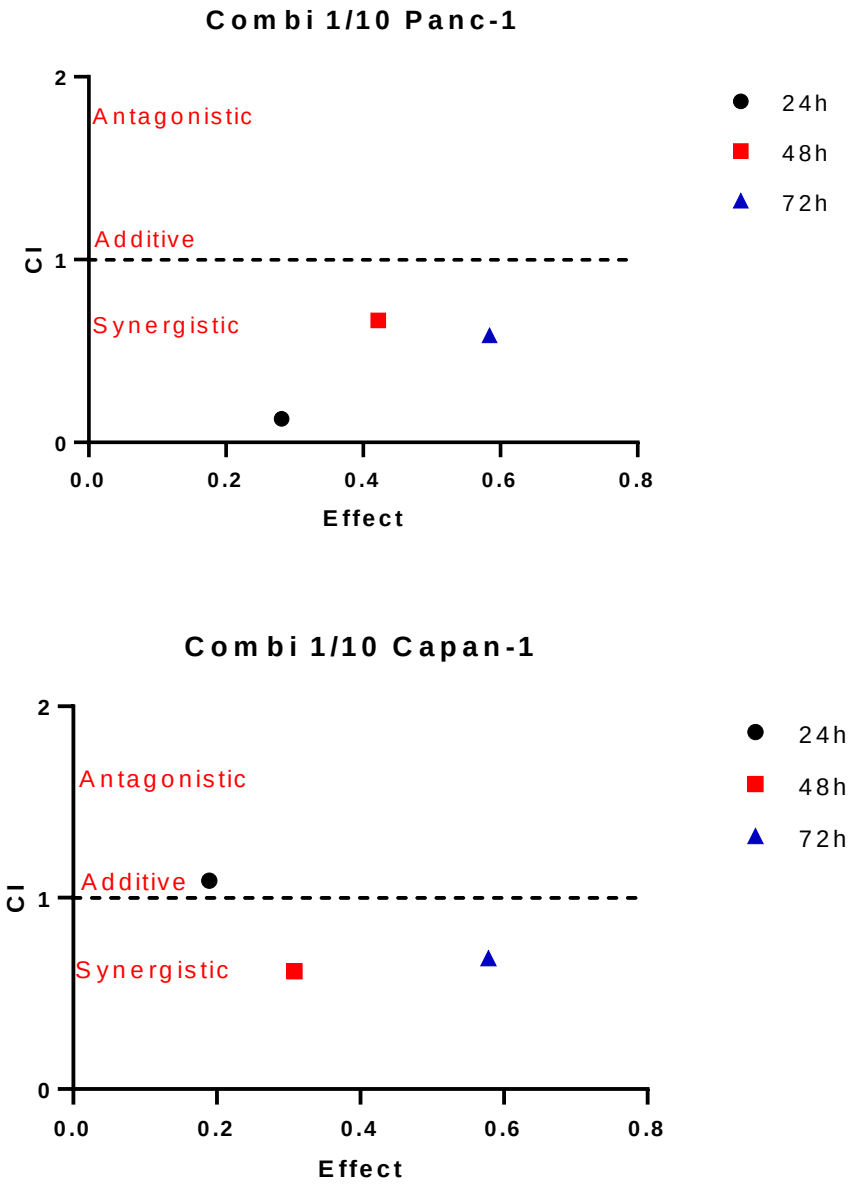

Figure 16. The combinations of ST1926 and 5-FU synergistically reduce cell proliferation. The synergistic effect of the combination treatment was evaluated by the calculation of the combination index (CI) using the CompuSyn software. A CI <1 indicates synergistic inhibition. Data represent an average of three independent experiments (± SE).

C. Inhibitory effect of ST1926 in combination with 5-FU on the viability of Panc-1 cells as evident by trypan blue exclusion assay

We also tested the effect of 1 µM ST1926, 10 µM 5-FU, and 1/10 combination on the viability of Panc-1 cells using trypan blue exclusion assay. Similar to MTT results, we have shown that 1 µM ST1926 and 10 µM 5-FU alone inhibit the viability of panc-1 cells by 8 % one day and 18% two days post-treatment and by 9 % one day and 15 % two days post-treatment, respectively, compared to control untreated cells. However, 1/10 combination significantly further inhibits the viability of Panc-1 by (13% at 24h -16% at 48h) and (15% at 24h -17% at 48h) compared to ST1926 and 5-FU alone respectively (Figure 17), consistently with our MTT results.

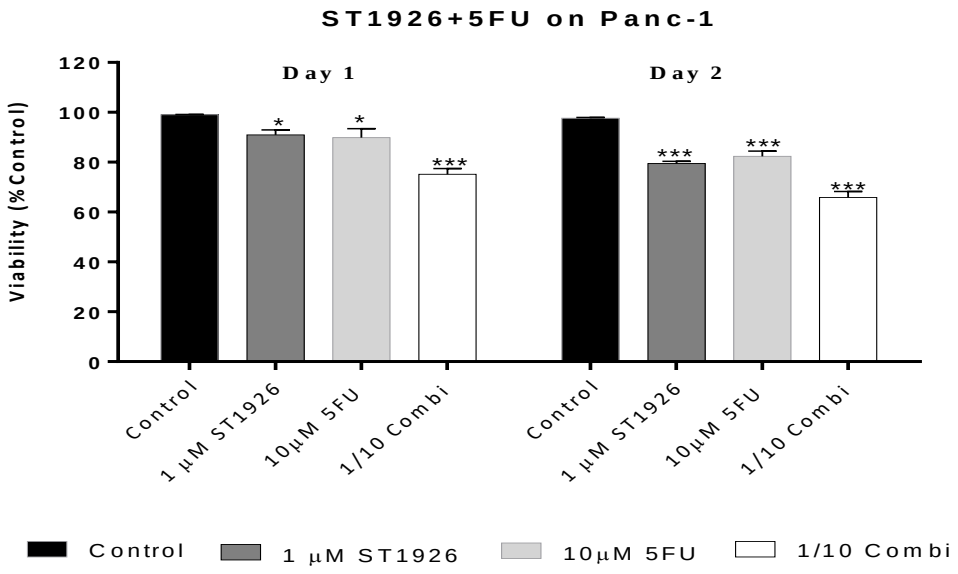

Figure 17. Effect of ST1926 in combination with 5-FU on the viability of Panc-1 cells using trypan blue exclusion assay. Panc-1 cells were seeded at a concentration of $15x10^4$ cells/well in 6-well plate and treated with indicated concentration for 24h and 48h.Cell viability was assessed by trypan blue exclusion assay. Results are expressed as a percentage of control and they represent an average of three independent experiments (± SE) (*, P<0.05; **, P<0.01; ***P<0.001)

D. Effect of ST1926 in combination with 5-FU on Panc-1 cells confluency and morphology

To test the effect of ST1926 in combination with 5-FU on Panc-1 cells confluency and morphology, bright field images were acquired using Zeiss axiovert light microscope. We can barely notice a decrease in confluency when comparing 1 µM ST1926 and 10 µM 5-FU alone to untreated control cells at both time points. However, a substantial decrease in confluency in 1/10 combination treated cells was observed especially at 48 hours, in addition to cell contraction (Figure 18).

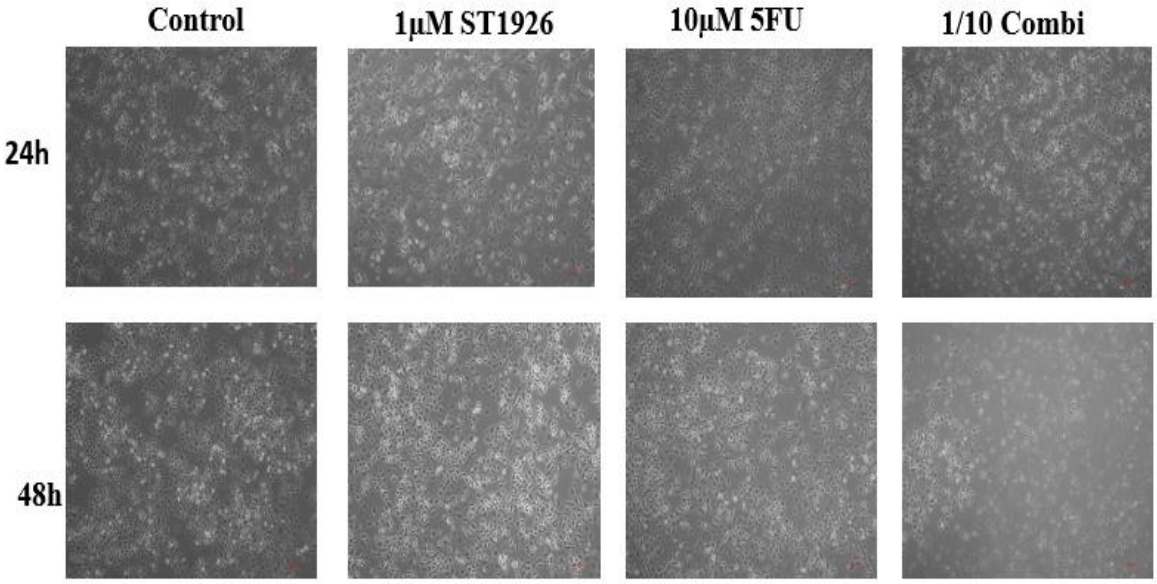

Figure 18. Effect of ST1926 in combination with 5-FU on the confluency and morphology of Panc-1 cells in culture. Panc-1 cells were seeded at a concentration of $15x10^4$ cells/well in 6-well plate and treated with1 µM ST1926, 10 µM 5-FU, and 1/10 combination for 24 h and 48 h. Representative bright field images were acquired using Zeiss axiovert light microscope (x4). Scale bar represents 1000 µM

E. Effect of ST1926 in combination with 5-FU on mitochondrial membrane potential of Panc-1 cells as assessed by Rhodamine-123 efflux

We aimed to further investigate the effect of the combined treatment on the mitochondrial membrane potential. Using Rhodamine-123 efflux assay, the mitochondrial membrane potential was monitored in Panc-1 cell line. Rhodamine-123 is a cell permeable cationic dye that enters the mitochondria and settles there, and any depolarization of the mitochondrial potential results in the loss of Rhodamine from the mitochondria leading to a drop in the intracellular fluorescence.

Panc-1 cells were treated with 1 µM ST1926, 10 µM 5-FU and 1/10 combination for up to 48 hours and then incubated with Rhodamine-123 dye. Using flow cytometry based analysis, our results show that the intracellular fluorescence of Rhodamine dye decreased by 13% -14% at 24 hours and 48 hours, respectively post-treatment with ST1926 compared to control. Concerning 5-FU, no difference can be noticed in the intracellular fluorescence of Rhodamine relative to control. Interestingly, a further notable reduction in the intracellular fluorescence by 10% at day one and 33% at day two post-treatment with 1/10 combination were observed compared to ST1926 alone and by 26% at day one and 50% at day two post-treatment with 1/10 combination compared to 5-FU alone (Figure 19). Figure 20 shows representative results from three independent experiments after flow cytometric analysis , where we display an overlay of the mitochondrial membrane dissipation of Rhodamine dye in the 1 µM ST1926, 10 µM 5-FU, and 1/10 combination Panc-1 treated cells as compared to their respective controls. These results show the loss of mitochondrial potential upon treatment of Panc-1 cells with ST1926/5-FU.

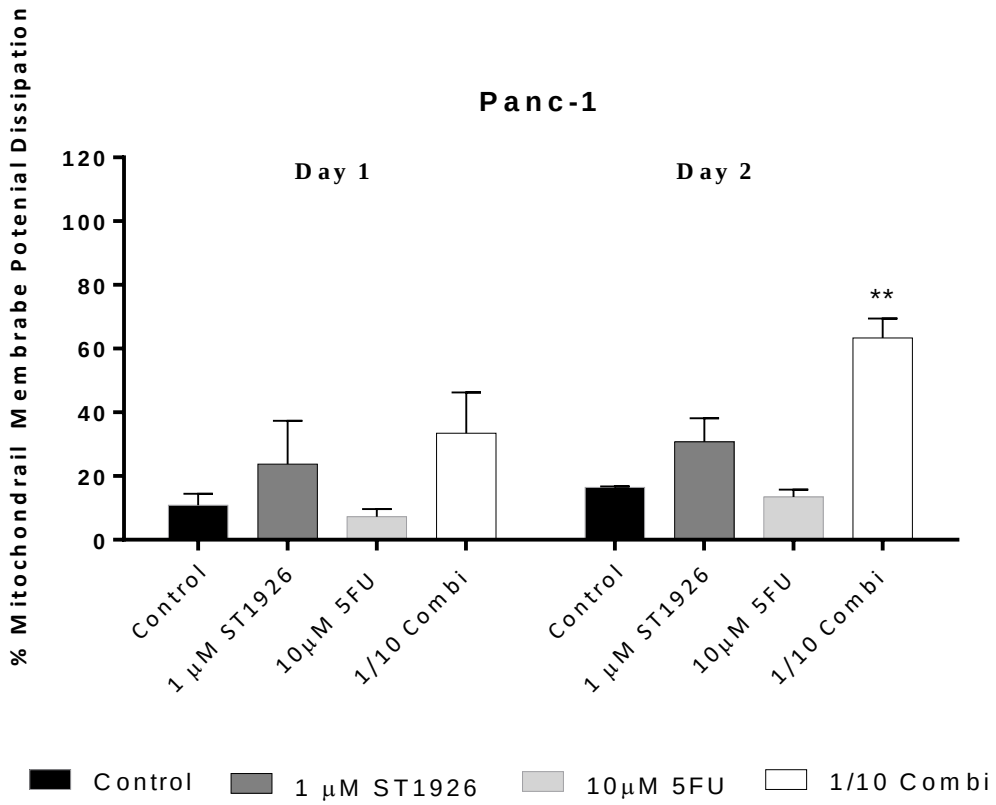

Figure 19. The percentage change in the mitochondrial membrane potential dissipation in ST1926 in combination with 5-FU treated panc-1 cells. Panc-1 cells were seeded at a concentration of $15x10^4$ cells/well in 6-well plate and treated with1 µM ST1926, 10 µM 5-FU, and 1/10 combination for up to 2 days. Cells were then exposed to 5µM of Rhodamine-123 for 1 hour at 37°C. Then, the intracellular accumulation of Rhodamine dye was measured through FACScan flow cytometer. The change in the mitochondrial Rhodamine dye in the control to treated cells was calculated and plotted. Results represent an average of three independent experiments (± SE) (*, P<0.05;**, P<0.01; ***P<0.001)

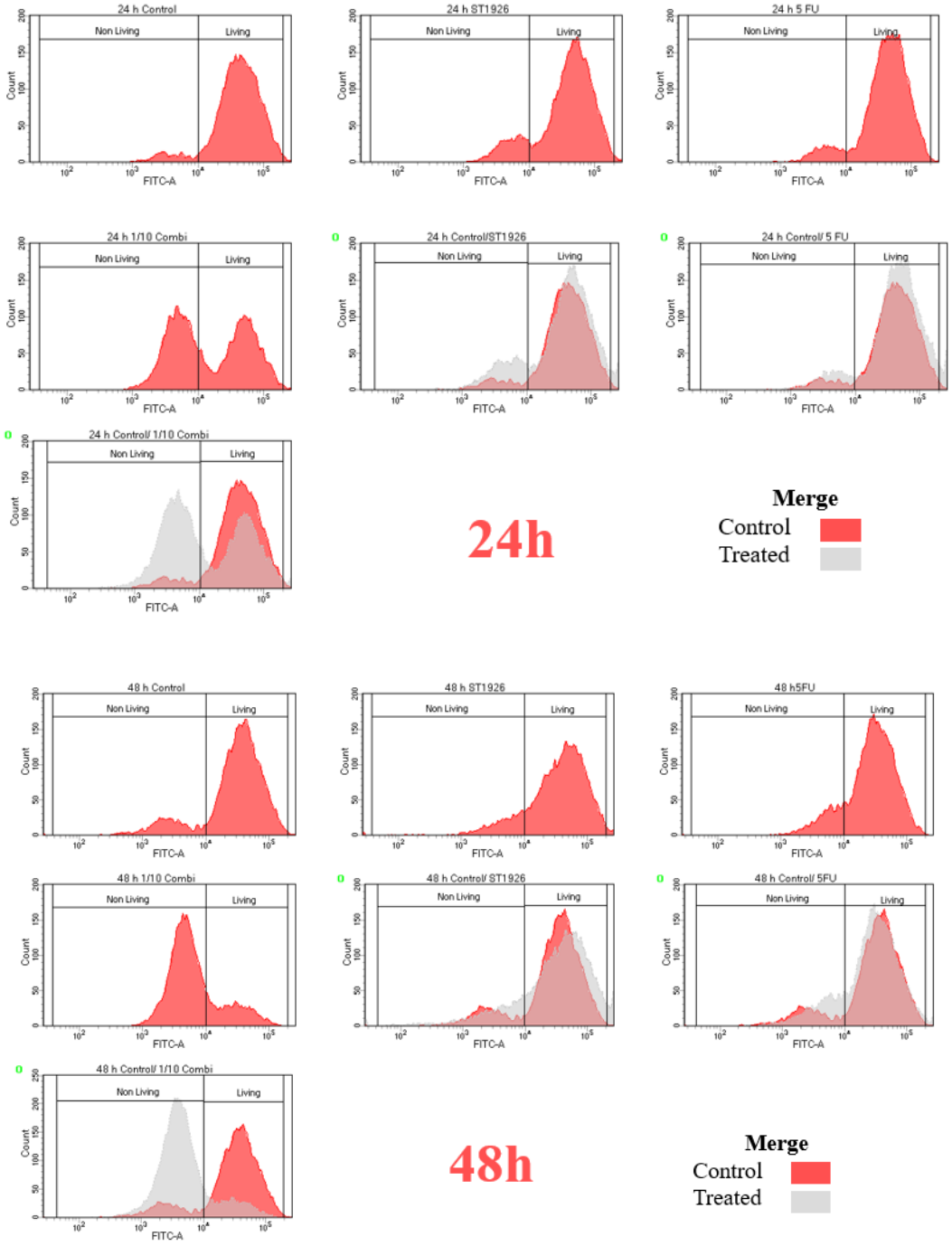

Figure 20. Loss of the mitochondrial membrane potential after ST1926 in combination with 5-FU treatment of Panc-1 cells. Panc-1 cells were seeded at a concentration of $15x10^4$ cells/well in 6-well plate and treated with1 µM ST1926, 10 µM 5-FU, and 1/10 combination for up to 2 days. Cells were then exposed to 5 µM of Rhodamine-123 for 1 hour at 37°C. Histograms represent flow cytometry-based analysis of Rhodamine-123 fluorescence (x-axis) over cell counts (y-axis) and the panels represent an overlay of 1 µM ST1926, 10 µM 5-FU, and 1/10 combination treated cells (grey) over control (red). The results shown are representative of at least three independent experiments.

F. Effect of ST1926 in combination with 5-FU on PARP cleavage in Panc-1 treated cells

PARP is a nuclear protein involved in DNA repair and apoptosis. During apoptosis several downstream signals are activated including caspases; leading to the cleavage of 116 kDa PARP stress response protein to an 89 kDa death associated fragment. Cleavage of PARP is considered to be a hallmark of apoptosis [188]

In our experiments, PARP antibody was probed on the membranes of whole cell lysates of Panc-1 cells treated with 1 µM ST1926, 10 µM 5-FU, and 1/10 combination for 72 hours. Our results show that PARP cleavage was more pronounced in 1/10 combination treated cells compared to 1 µM ST1926 and 10 µM 5-FU panc-1 treated cells (Figure 21)

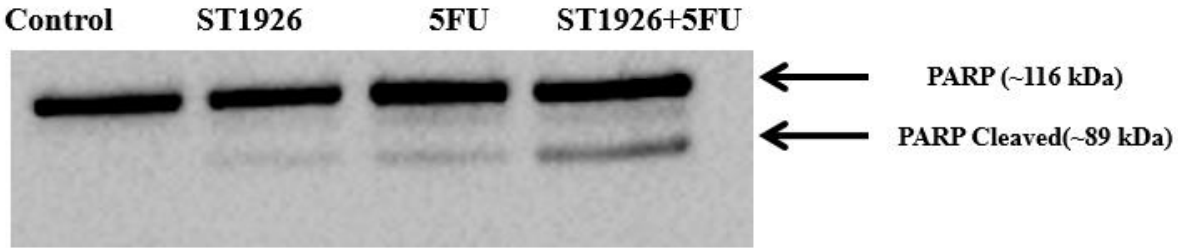

Figure 21. Induction of PARP cleavage after ST1926 in combination of 5-FU treatment in Panc-1 cells. Panc-1 cells were seeded at a concentration of $15x10^4$ cells/well in 6-well plate and treated with1 µM ST1926, 10 µM 5-FU, and 1/10 combination. Cells were then collected 3 days post-treatment and the protein lysates were prepared and immunoblotted against PARP antibody. Results shown are representative of three independent experiments.

G. Effect of ST1926 in combination with 5-FU on P53 total protein level in Panc-1 treated cells

Under normal and unstressed conditions, p53 remains undetected intracellularly due to its short half-life. However, upon DNA damage caused by apoptosis activation,

upregulation of the tumor suppressor protein p53 is observed. Therefore, overexpression of p53 can be an early detector of apoptosis [189]. Previous studies have already demonstrated that ST1926 induces its antitumor effects on several cancerous cells through p53 independent mechanisms [173]. Nevertheless, we wanted to investigate the effect of ST1926 in combination with 5-FU on total p53 protein levels in Panc-1 treated cells. Therefore, total p53 antibodies were probed on the membranes of whole cell lysates of Panc-1 cells treated with 1 µM ST1926, 10 µM 5-FU, and 1/10 combination for 72 hours. Our results show that total p53 increased by about 2 folds in ST1926 treated cells and by approximately 4 folds in both 5-FU and ST1926 plus 5-FU Panc-1 treated cells compared to control. (Figure 22)

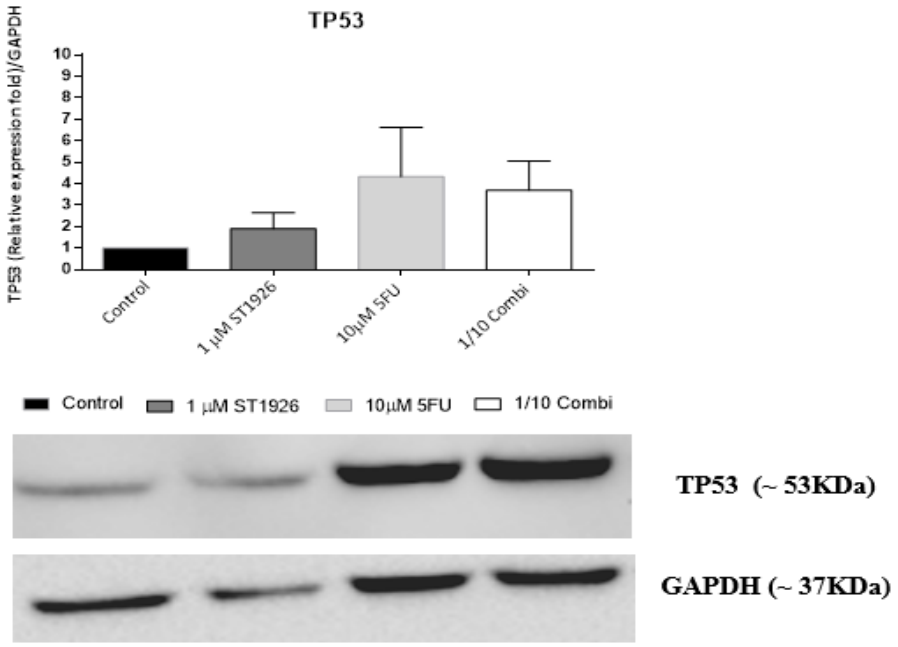

Figure 22. Effect of ST1926 in combination with 5-FU on total P53 protein levels in Panc-1 treated cells. Panc-1 cells were seeded at a concentration of $15x10^4$ cells/well in 6-well plate and treated with1 µM ST1926, 10 µM 5-FU, and 1/10 combination. Cells were then collected 3 days post-treatment and the protein lysates were prepared and immunoblotted against phosphorylated and total p53 antibodies. Blots were also probed with GAPDH antibody to ensure equal protein loading. Results shown are representative of at least three independent experiments.

H. Effect of ST1926 in combination with 5-FU on γH2AX protein levels in Panc-1 treated cells

Phosphorylation of the histone H2AX to γH2AX is considered as a sensitive marker that can be used to inspect cellular DNA damage and its subsequent repair Correspondingly, γH2AX antibody was probed on the membranes of whole cell lysates of Panc-1 cells treated with 1 µM ST1926, 10 µM 5-FU, and 1/10 combination for 72 hours. Our results show a more pronounced accumulation of γH2AX, an indication of DNA damage, in both ST1926 (410 folds) and ST1926 plus 5-FU (350 folds) compared to 5-FU (40 folds) Panc-1 treated cells as compared to control (Figure 23)

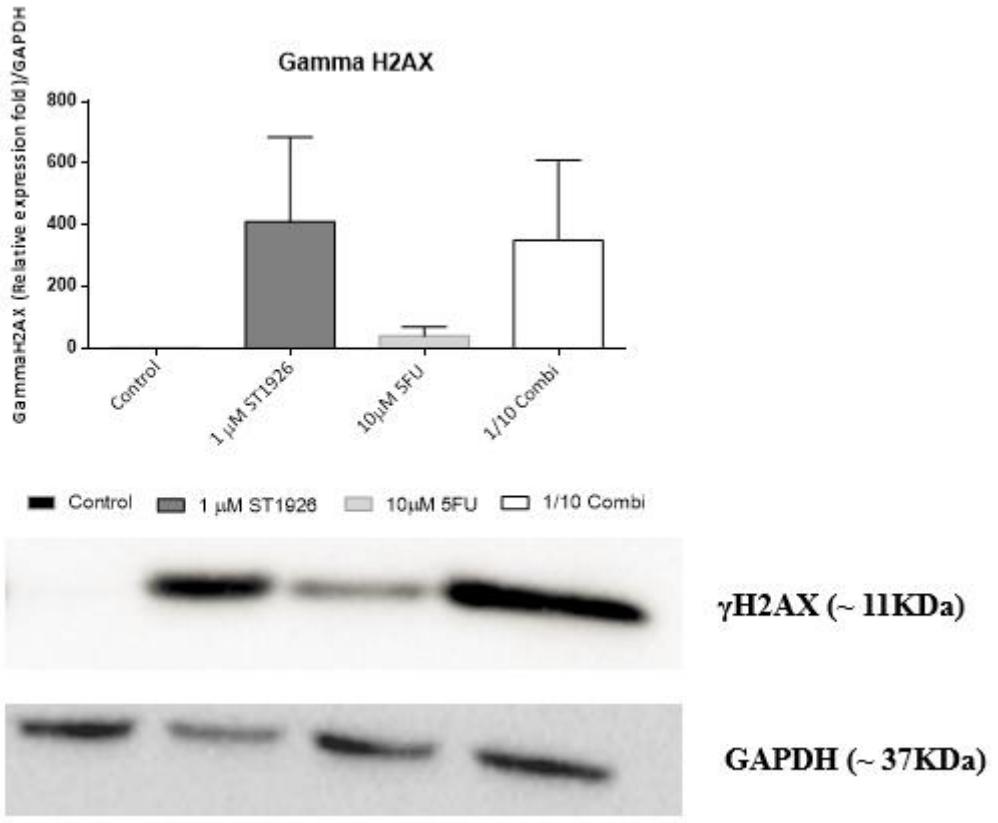

Figure 23. Effect of ST1926 in combination with 5-FU on γH2AX protein levels in Panc-1 treated cells. Panc-1 cells were seeded at a concentration of $15x10^4$ cells/well in 6-well plate and treated with1 µM ST1926, 10 µM 5-FU, and 1/10 combination. Cells were then collected 3 days post-treatment and the protein lysates were prepared and immunoblotted against γH2AX antibody. Blots were also probed with GAPDH antibody to ensure equal protein loading. Results shown are representative of at least three independent experiments

I. Effect of ST1926 in combination with 5-FU on POLA-1 protein levels in Panc-1 treated cells

Interestingly, ST1926 recently proved to inhibit POLA-1 activity in colorectal cancers proving that POLA-1 is involved in the mechanism of action of ST1926 in colorectal cancer [183]. Accordingly POLA-1 antibody was probed on the membranes of whole cell lysates of Panc-1 cells treated with 1 µM ST1926, 10 µM 5-FU, and 1/10 combination for 72 hours. Our results show that POLA-1 protein levels in ST1926 Panc-1 treated cells increased by about 2 folds as compared to control. However, in both 5-FU and ST1926 plus 5-FU Panc-1 treated cells, POLA-1 expression interestingly decreased by about two-thirds as compared to control (Figure 24).

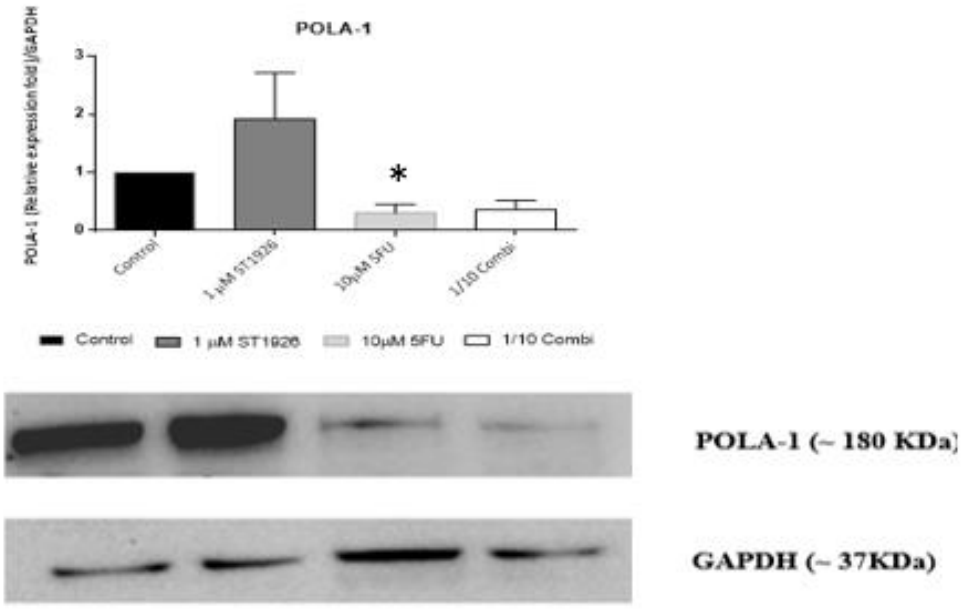

Figure 24. Effect of ST1926 in combination of 5-FU on POLA-1 protein levels in panc-1 treated cells. Panc-1 cells were seeded at a concentration of $15x10^4$ cells/well in 6-well plate and treated with1 µM ST1926, 10 µM 5-FU, and 1/10 combination. Cells were then collected 3 days post-treatment and the protein lysates were prepared and immunoblotted against POLA-1 antibody. Blots were also probed with GAPDH antibody to ensure equal protein loading. Results shown are representative of at least three independent experiments.

CHAPTER V

DISCUSSION

Pancreatic cancer is one of the most aggressive and deadliest cancer types, ranking as the seventh leading cause of worldwide cancer-related deaths in industrialized countries[1] and the third most common in the U.S.A [2]. About 80-90% of patients with pancreatic cancer have unresectable tumors and diagnosed at an advanced stage. In spite of the great efforts in recognizing the potential risk factor of pancreatic cancer and advancement in early diagnostic tools, it is predicted that pancreatic cancer will surpass breast, prostate, and colorectal cancers as a leading cause of cancer related deaths in the U.S.A by the year 2030 [3]. Conventional treatment strategies such as surgery, chemotherapy, and/or radiation have had little influence on the course of this aggressive cancer regardless the efforts over the past several years. The limitations of current treatment strategies in pancreatic cancer reinforce the need for new avenues of research to be explored.

Retinoids (natural and synthetic vitamin A derivatives) have been studied for decades in clinical trials due to their established role in regulating cell growth, differentiation, proliferation and apoptosis [126]. All-trans-retinoic (ATRA) acid and 9-cis retinoic acid (9-cRA) are two biologically active stereoisomers of RA that bind to retinoic acid receptors (RARs) with high affinity [127]. The most effective clinical use of ATRA was demonstrated in acute promyelocytic leukemia (APL) treatment[129]. Even though pharmacological RA doses have been approved by the Food and Drug Administration (FDA) and other regulatory bodies for the treatment of some hematological malignancies and high-risk neuroblastoma NBL, the chemopreventive and therapeutic effects of retinoids in other solid tumors are still unclear. The therapeutic

response of retinoids in some tumors is found to be limited to a small proportion of the treated patients [153]. Thus, despite that many studies have reported the promising role of retinoids in attaining better outcomes for several types of cancer, this promising therapy has some limitations: retinoid toxicity and intrinsic or acquired resistance that have been observed in many patients.

Therefore, there has been a remarkable effort aimed at the identification and characterization of retinoids endowed with apoptogenic properties stronger than those of ATRA and its natural derivatives 9-cis and 13-cis RA. Retinoid related molecules (RRMs) are an emerging class of apoptotic compounds whose chemical structure is derived from that of ATRA. The prototype of this family of molecules is the conformationally restricted retinoid CD437 which was originally developed as a selective RARγ agonist [166]. This compound has raised remarkable enthusiasm, as it is active *in vivo* on preclinical models of such diverse types of neoplasia as melanoma [170], lung carcinoma [171], and acute myeloid leukemia [172]. However, despite the anti-neoplastic properties that CD437 held in different tumor types, it showed to have a low pharmacokinetic profile. Several RRM congeners, such as ST1926, were shown to have a more potent apoptotic potential, a lower level of toxicity, and a better pharmacokinetic profile than CD437.

ST1926 an orally available synthetic atypical retinoid is a more specific, stable and less toxic analogue of CD437 (Figure 9) [168, 173]. ST1926 was shown to be effective against several solid and hematological tumor cells that are resistant to ATRA [174-176]. ST1926 bio distribution in mouse was found to be favorable as micromolar (μM) concentrations of the compound were achievable in the plasma of mice, with significant extravascular distribution of the compound [176] where many *in vivo*

experiments confirmed the role of ST1926 in suppressing tumor growth with limited toxicity [174-177]. Compared to CD437, ST1926 was found to be able to trans-activate RARγ less potentially but with greater apoptotic activity. Thus indicating its ability to mediate its mechanisms of action independently of the retinoid receptor pathway.

ST1926 exhibited a potent antiproliferative activity on a large panel of human tumor cells such as acute myeloid leukemia, chronic myeloid leukemia, T-cell leukemia, breast, ovarian, melanoma, neuroblastoma , lung, colorectal, prostate, and glioma cancer cell lines [173, 174, 177-179, 183]. However, its anticancer activity has not been investigated against pancreatic cancer cell lines yet.

To the best of our knowledge, no reports have been published to date about ST1926 effect in pancreatic cancer and this is the first study to elucidate the anti-neoplastic properties and mechanism of action of ST1926 in pancreatic cancer. Panc-1 and Capan-1 are two epithelial adherent pancreatic cancer cell lines that derived from pancreatic duct and metastatic site (liver), respectively, and harbor different genetic mutations.

In our study, we found that using up to 10 µM of ST1926 for up to three days barely reached 50 % inhibition of cell proliferation of our tested pancreatic cancer cell lines. On the other hand, previous studies proved that the concentrations of ST1926 required for 50% growth inhibition in various types of human cancer cell lines ranged from 0.1 to 0.5 µM which are pharmacologically relevant, where using up to 10 µM can lead to 100% inhibition of cell proliferation except in the case of glioma cells where using 20 µM of ST1926 for 24 hours decreased cell viability to around (20-70%) depending on the cells type and more interestingly is that it significantly reduced tumor volume and weight in ST1926 treated mice with few toxicity [173, 174, 177-179, 183].

Therefore, combining several drugs that target different signaling pathways can be a way to increase drug sensitivity.

So, the present study is the first one to determine the combinatory effects of ST1926 plus the clinically used anticancer agent thymidylate synthase inhibitor 5-FU. We noted that, ST1926 in combination with 5-FU was significantly more effective in inhibiting pancreatic cancer cell lines proliferation than either cytotoxic agent alone.

Moreover, the synergistic activity we noticed upon the combination of ST1926 with 5-FU was evaluated using the CompuSyn analysis method. Many combinations showed strong synergistic activity with (CI < 0.3) and some showed an antagonistic activity (CI >1.2) that are explained by the pro-proliferative effect of 5-FU upon its addition on the Capan-1 cancer cells at 24 hours post-treatment. The combination of ST1926 with 5-FU in a ratio of 1:10 showed the best synergistic activity on both cell lines. Therefore, 1 µM ST1926, 10 µM 5-FU, and 1/10 combination were selected to be the working concentrations to further decipher the mechanisms of action of ST1926 in pancreatic cancer. Similar synergistic interactions were observed upon the combination of ST1926 with a number of classical cytotoxic agents such as the epidermal growth factor receptor inhibitor ZD1839 [184], cisplatin [185], and ATRA [186] on several types of cancer cells proving that studies of synergistic effect is a promising path for more efficient anticancer therapies.

Furthermore, in many systems apoptosis is associated with the loss of the mitochondrial membrane potential and signals through intrinsic apoptotic pathways. Previous reports demonstrated that ST1926 induced loss of mitochondrial membrane potential in various cancer cells [175] [179]. Our study found that ST1926 alone induced minimal loss of mitochondrial membrane potential and this loss was

significantly noticed two days post-treatment with ST1926 in combination with 5-FU in our tested pancreatic cancer cells as assessed by the Rhodamine efflux assay, thus, suggesting mitochondrial- mediated apoptosis. In addition, our preliminary data showed that ST1926 might induce S-phase arrest while ST1926+5FU showed a significant accumulation of cells in the pre-G1 region of cell cycle.

Upon mitochondrial membrane dissipation, several downstream signals are activated including caspases, which are associated with the cleavage of caspase substrate (PARP) to its death associated fragment. PARP cleavage is considered to be a hallmark of apoptosis [188]. It has been previously shown that ST1926 treatment induced PARP cleavage in various cancer cells such as ovarian, breast, lung, prostate, ATL, CML, and AML cells and thus demonstrating that ST1926-induced apoptosis is partially caspase-dependent [173, 174, 178, 179]. In our study, both ST1926 and 5FU alone weakly induced PARP cleavage and a pronounced PARP cleavage was observed upon their combination, suggesting caspase-dependent mechanism of apoptosis. Future experiments using caspase inhibitors will test the effects of ST1926/5-FU on apoptosis induction.

Another fundamental marker of apoptosis is the activation and upregulation of the tumor suppressor p53 [189]. Previous studies have demonstrated that ST1926 induces its anti-tumor effect in several cancerous cells through p53-independent mechanism and that ST1926 manifests a potent apoptotic effect regardless of p53 status. Interestingly, ST1926 was shown to increase the expression of total p53 in cancerous cells with wild type *p53* status. Therefore, the importance of ST1926 lies in its anti-tumor efficacy regardless of the p53 status, which is important in the treatment of tumors where p53 signaling pathway may be compromised [173, 174, 179]. Nevertheless, when

investigated, we found that ST1926 slightly increased the protein levels of the total p53 in our tested pancreatic cancer cells. Whereas 5-FU alone or in combination with ST1926 showed a pronounced increase in total p53.Many *in vitro* studies have reported that the loss of p53 function reduces cellular sensitivity to 5-FU. Previous studies showed that 5-FU chemosensitivity is related to p53 status in pancreatic cancer cell lines where a significant inhibitory effect on tumor cell growth was achieved in cells with wild-type p53[190, 191]. Additional studies are needed to test for the levels of phosphorylated p53.

Besides, ST1926 has been previously characterized as a genotoxic drug that causes DNA damage in various types of tumor cells [174, 179, 181, 182]. H2AX phosphorylation to γH2AX is considered as a sensitive marker that can be used to examine cellular DNA damage produced and its subsequent repair [192]. Our results show a pronounced accumulation of γH2AX in both ST1926 and ST1926 plus 5-FU treated pancreatic cancer cells indicating DNA damage. Whereas 5FU alone induced fewer accumulation of γH2AX that may be resulted from the upregulation of p53 that we noticed that will ultimately leads to cellular apoptosis. For ST1926 we noticed a marked accumulation of γH2AX in p53- independent manner. This triggers us to further decipher the mechanism of action of ST1926 in pancreatic cancer.

Recently CD437 the parent molecule of ST1926 was reported as a direct and irreversible inhibitor of the DNA polymerase α, POLA-1 [169]. Suspecting that these two adamantyl retinoids may have a comparable targets, we have verified that POLA-1 is involved in the mechanism of action of ST1926 in colorectal cancer and that mutation in POLA-1 specifically reduce the antiproliferative activity of ST1926 but not another DNA damaging agents such as 5-FU [183]. The expression of POLA-1 was then

analyzed in publically available cohort of solid tumors obtained from eleven different tissue sites including the colon. It was found that POLA-1 expression levels was significantly higher in colon adenocarcinoma relative to other carcinomas such as pancreatic adenocarcinoma when compared to their normal counterpart suggesting that POLA-1 is an attractive target in colorectal cancer. Nevertheless, we tested the effect of our drugs on the expression of POLA-1 in pancreatic cancer cells. Surprisingly we found that ST1926 increased the protein levels of POLA-1 rather than decreasing them. In contrast, the ST1926/5-FU combination and 5-FU remarkably reduced the expression of POLA-1 as compared to control. It is important to mention that our study is the first one to study the effect of 5-FU on POLA-1 expression.

These results summarized in figure 25 insure the importance to further decipher the mechanism of action of ST1926 to understand how it alone or in combination with 5-FU work to inhibit pancreatic cancer cells growth while proposing that the synergistic effect of combination we noticed is due to the activation of different signaling pathways by each drug that need to be further studied. Moreover, ST1926 exposure at the concentrations used in our study should be tested on normal pancreatic cells to evaluate toxicity. Further *in vivo* studies are needed before drawing any conclusion as *in vitro* chemosensitivity are not always predictive of *in vivo* activity.

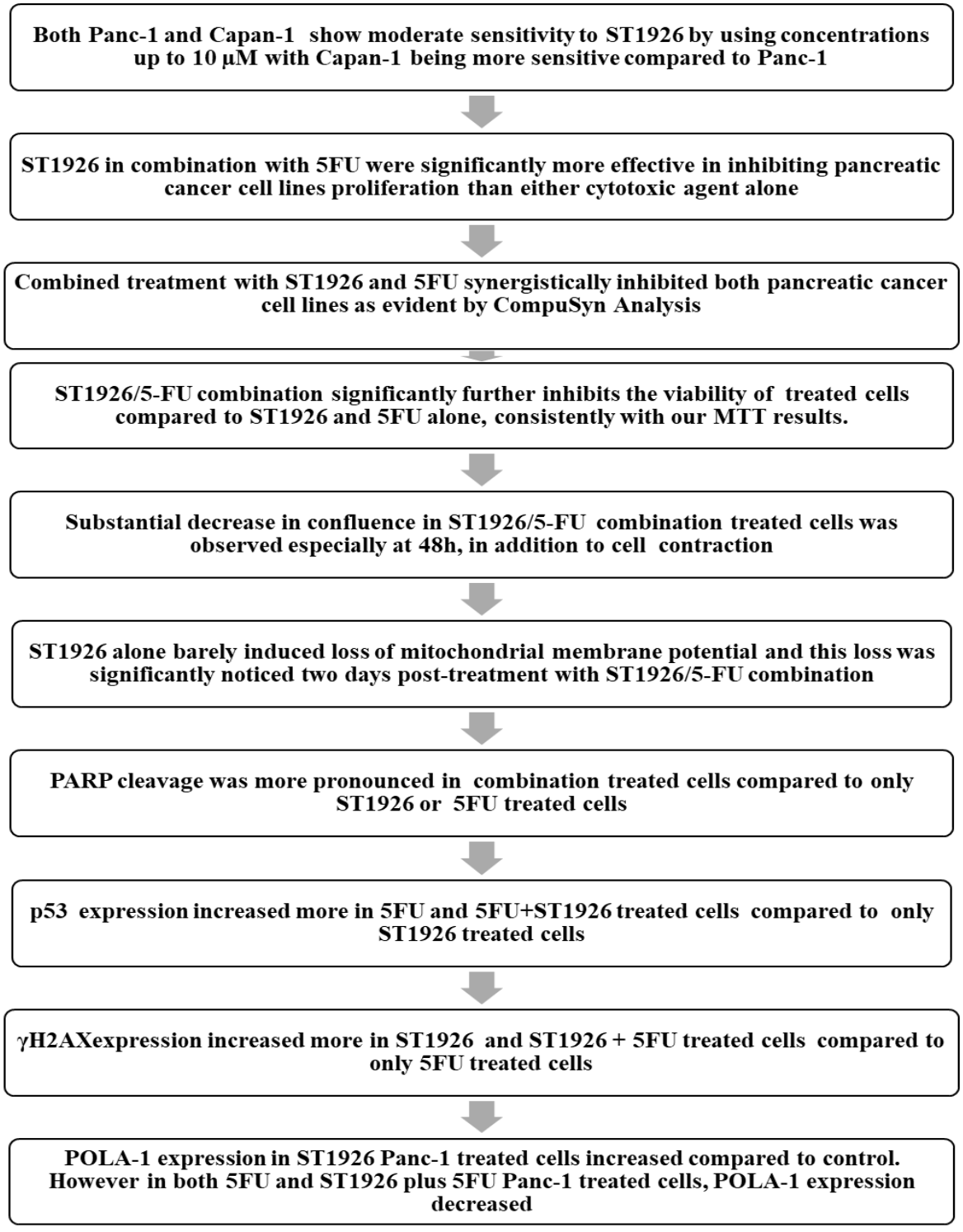

Figure 25. Summary of the Results

CHAPTER VI

CONCLUSION

In conclusion, our study is the first one that determined the effect of ST1926 on pancreatic cancer cell lines and showed that the combinatory effects of ST1926 plus the clinically used anticancer agent thymidylate synthase inhibitor 5-FU were significantly more effective in inhibiting pancreatic cancer cell lines proliferation than either cytotoxic agent alone. It proved that combination therapy is superior to any single agent and is a way to overcome drug resistance through targeting different signaling pathways. In addition, our data provides the basis for further research to elucidate the mechanisms of action causing the observed synergistic interaction. However *in vivo* studies and clinical trials are needed before drawing any conclusion as in vitro chemosensitivity are not always predictive of *in vivo* activity. In addition drug exposure at the concentrations used in our study should be tested on normal pancreatic cells to evaluate toxicity.

To end up, the results of this study support the possibility that the combined treatment of ST1926 and 5-FU may be potentially effective and critical strategy for pancreatic cancer treatment.

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