

MOLECULAR MECHANISTIC STUDIES OF STEVIOSIDE IN PANCREATIC
CANCER



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MOLECULAR MECHANISTIC STUDIES OF STEVIOSIDE IN PANCREATIC
CANCER

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ABSTRACT

MOLECULAR MECHANISTIC STUDIES OF STEVIOSIDE IN PANCREATIC CANCER

Pancreatic cancer is a highly aggressive malignant tumor that is characterized by poor diagnosis and a 5-year survival rate. In 2020, according to GLOBOCAN pancreatic cancer is the seventh leading cause of cancer-related deaths with which 6 of every 100,000 people died because of pancreatic cancer. Enhanced glycolysis is considered the dominant alteration in tumorigenesis of pancreatic cancer; however, the promotion of metastasis requires enhanced OXPHOS in PDAC. Disruption of the energy balance might inhibit the metastatic progression of pancreatic cancer. Stevioside (ST) is a diterpene glycoside containing 3 glucose molecules and used as a zero-calorie natural sweetener. Studies have shown that ST has an effective anti-cancer activity. However, the mechanism of action for ST at the cellular level remains unclear.

In this study, the molecular mechanism of stevioside in normal and cancer pancreatic ductal cells was examined. Analysis by MTS and Annexin V-PI staining showed ST-related anti-proliferative effect and apoptosis in PDAC cells, and no growth inhibitory effect was induced in normal cells. In non-metastatic Panc 02 cells, Rhodamine 123 and MitoTracker Green FM results revealed an ST-related decrease in the mitochondrial mass and inner membrane potential. ST triggered induction of the metabolic switch to glycolysis, preventing energy crisis by increasing the LDHA expression levels by 1.7-fold. Next, ST has induced energy crisis and overload in mitochondria through elevated inner membrane potential and diminished mitochondrial mass in metastatic PANC-1 cells. Cellular ATP levels decreased by 44% due to ST treatment. In contrast, ST treatment in HPNE cells triggered the elimination of defective mitochondria and increase cellular ATP levels.

In conclusion, ST might be a potential therapeutic agent for the disruption of energy balance and mitochondria in pancreatic cancer progression.

ÖZET

PANKREAS KANSERİNDE STEVİOSİTİN MOLEKÜLER MEKANİSTİK ÇALIŞMASI

Pankreas kanseri, zayıf tanı ve 5 yıllık sağkalım oranı ile karakterize, oldukça agresif bir malign tümördür. 2020'de GLOBOCAN'a göre pankreas kanseri, her 100.000 kişiden 6'sının ölümüyle sonuçlanmış ve kansere bağlı ölümlerin yedinci önde gelen nedenidir. Artan glikoliz aktivitesi, pankreas kanserinin ilerlemesinde baskın değişiklik olarak kabul edilir; bununla birlikte, metastazın tetiklenmesi, PDAC'de OXPHOS gerektirir. Enerji dengesinin bozulması pankreas kanserinde metastatik ilerlemeyi engelleyebilir. Stevioside (ST), 3 glukoz molekülü içeren ve sıfır kalorili doğal tatlandırıcı olarak kullanılan bir diterpen glikozittir. Çalışmalar, ST'nin etkili bir anti-kanser aktivitesine sahip olduğunu göstermiştir. Ancak, hücresel düzeyde ST mekanizmasının etkisi belirsizliğini korumaktadır.

Bu çalışmada, normal ve kanser pankreas duktal hücrelerinde steviosidin moleküler mekanizması incelenmiştir. MTS ve Annexin V-PI boyaması ile yapılan analiz, PDAC hücrelerinde ST ile ilişkili anti-proliferatif etki göstermiş ve apoptozu tetiklemiştir. Ek olarak, normal hücrede hiçbir büyüme inhibe edici etki görülmedi. Metastatik olmayan Panc 02 hücrelerinde, Rhodamine 123 ve MitoTracker Green sonuçları, mitokondriyal kütle ve iç zar potansiyelinde ST ile ilişkili azalmayı ortaya çıkarmıştır. ST, metabolik geçişin glikolize indüksiyonunu tetiklemiş ve LDHA ekspresyon seviyelerini 1,7 kat artırarak enerji krizini önlemiştir. Daha sonra, metastatik PANC-1 hücrelerinde ST, mitokondriyal kütleyle azaltarak ve iç zar potansiyelini artırarak mitokondride aşırı yüklenmeye ve enerji krizine neden olduğu gözlemlenmiştir ek olarak, ST tedavisine bağlı olarak hücresel ATP seviyeleri, %66 azalmıştır. Buna karşılık, HPNE hücrelerinde ST tedavisi, kusurlu mitokondrilerin ortadan kaldırılması tetiklenmiş ve hücresel ATP seviyeleri artmıştır.

Sonuç olarak ST, pankreas kanseri ilerlemesinde enerji dengesinin ve mitokondrinin bozulması için potansiyel bir terapötik ajan olabilir.

TABLE OF CONTENTS

ACKNOWLEDGEMENTS.....	iv
ABSTRACT.....	v
ÖZET	vi
TABLE OF CONTENTS.....	vii
LIST OF FIGURES	x
LIST OF TABLES.....	xii
LIST OF ABBREVIATIONS/ SYMBOLS.....	xiii
1. INTRODUCTION.....	1
1.1 CANCER.....	1
1.2 CANCER METABOLISM	5
1.2.1 Warburg Effect	5
1.2.2 Mitochondrial Metabolism	6
1.3 PANCREATIC CANCER.....	11
1.3.1 Anatomy of Pancreas	11
1.3.2 Pathogenesis of Pancreatic Cancer	12
1.3.3 Pancreatic Cancer Epidemiology.....	14
1.3.4 Risk Factors of Pancreas Cancer	16
1.3.5 Diagnosis and Therapeutic Strategies.....	19
1.4 STEVIOSIDE.....	21
1.5 GLUCOSE METABOLISM.....	23
1.5.1 Glucose Metabolism in Pancreatic Cancer	23
1.6 AIM OF THE STUDY	25
2. MATERIALS	26
2.1 CELL LINES AND CELL CULTURE	26
2.2 CHEMICALS.....	26
2.2.1 Kits.....	27
2.2.2 Antibodies.....	27
2.3 INSTRUMENTS.....	27
2.3.1 Equipment.....	28
3. METHODS.....	29
3.1 MOLECULAR DOCKING OF STEVIOSIDE	29

3.2	CELL CULTURE	29
3.2.1	Cell Culture Conditions	29
3.2.2	Thawing of The Cell	30
3.2.3	Subculturing.....	30
3.2.4	Determination of Total Cell Number.....	30
3.2.5	Cryopreservation.....	30
3.3	PREPARATION OF STEVIOSIDE	31
3.3.1	Preparation of Stevioside	31
3.3.2	Stevioside Treatment	31
3.4	CELL VIABILITY ASSAY.....	31
3.5	MITOTRACKER GREEN FM STAINING	32
3.6	RHODAMINE 123 STAINING.....	32
3.7	REACTIVE OXYGEN SPECIES DETECTION ASSAY	33
3.8	ATP BIOLUMINESCENCE ASSAY	33
3.9	ANNEXIN V + PI STAINING	33
3.10	IMMUNOBLOTTING OF PROTEIN SAMPLES.....	34
3.10.1	Cellular Lysis and Isolation of the Proteins.....	34
3.10.2	BCA (Bicinchionic Acid) Protein Assay	34
3.10.3	Sample Preparation and SDS- PAGE	35
3.10.4	Western Blotting.....	35
3.11	STATISTICAL ANALYSIS.....	36
4.	RESULTS.....	37
4.1	MOLECULAR DOCKING BINDING AFFINITY OF STEVIOSIDE WITH PROTEINS	37
4.2	THE INTERACTION BETWEEN GLUT1 AND STEVIOSIDE.....	38
4.3	THE INTERACTION BETWEEN LDHA AND STEVIOSIDE	39
4.4	THE INTERACTION BETWEEN CASPASE-3 AND STEVIOSIDE	41
4.5	THE INTERACTION BETWEEN LC-3 AND STEVIOSIDE.....	43
4.6	THE INTERACTION BETWEEN VEGFA AND STEVIOSIDE	44
4.7	CELL VIABILITY DETERMINATION OF PDAC CELLS TREATED WITH STEVIOSIDE	46
4.8	DETERMINATION OF CELL DEATH MECHANISM BY ANNEXIN V-FITC + PI STAINING.....	47

4.9	DETERMINATION OF INTRACELLULAR ROS LEVELS OF PDAC CELLS TREATED WITH ST	48
4.10	LIVE CELL IMAGING OF MITOCHONDRIAL MEMBRANE POTENTIAL AND MITOCHONDRIAL MASS OF PDAC CELLS BY CONFOCAL MICROSCOPY.....	49
4.11	QUANTITATIVE ANALYSIS OF MITOCHONDRIAL MEMBRANE POTENTIAL AND MASS BY FLOW CYTOMETRY	51
4.12	MEASUREMENT OF TOTAL ATP LEVELS.....	52
4.13	DETERMINATION OF CHANGES IN OXPHOS COMPLEXES EXPRESSION LEVELS IN ST-TREATED PDAC CELLS	53
4.14	DETERMINATION OF THE CHANGES IN LDHA LEVELS IN PDAC CELLS WITH ST TREATMENT	55
5.	DISCUSSION.....	56
6.	CONCLUSION AND FUTURE ASPECTS.....	61
	REFERENCES	63

LIST OF FIGURES

Figure 1.1. Classification of most common cancer types incidence and mortality rates in the year 2020 by GLOBOCAN for both sexes (A), male (B), and female (C).	2
Figure 1.2. The number of diagnoses of all cancer types, age between 0-85 in both sexes estimated between the years 2020 and 2040.....	3
Figure 1.3 Fourteen hallmarks of cancer	5
Figure 1.4 The functions of mitochondria	8
Figure 1.5 Schematic representation of OXPHOS complexes.	9
Figure 1.6 Schematic representation of intrinsic and extrinsic apoptosis pathways.	11
Figure 1.7 Schematic representation of macroscopic and microscopic pancreas anatomy .	12
Figure 1.8 Visualization of pancreatic cancer types	13
Figure 1.9 Formation of PDAC from normal pancreatic exocrine tissue	14
Figure 1.10 Pancreatic cancer incidence (above) and mortality (below) cases according to GLOBOCAN	15
Figure 1.11 Treatment order of pancreatic cancer according to tumor stage and ECOG score performance status..	21
Figure 1.12 The structure of steviol (1) and stevioside (2).....	22
Figure 1.13 Metabolism of steviol glycosides	22
Figure 1.14 Differences between energy metabolism in normal and cancer cells	24
Figure 4.1 Molecular interaction between GLUT1 and glucose and ST in 2D and 3D configuration analyzed by Discovery studio visualizer.	38
Figure 4.2 Molecular interaction between LDHA and pyruvate, ST and GNE-140 in 2D and 3D configuration analyzed by Discovery studio visualizer	40
Figure 4.3 Molecular interaction between caspase-3 and ST and Ac-DEVD-CHO in 2D and 3D configuration analyzed by Discovery studio visualizer	42
Figure 4.4 Molecular interaction between LC-3 and ST in 2D and 3D configuration analyzed by Discovery studio visualizer.....	43
Figure 4.5 Molecular interaction between VEGFA and ST and SOR in 2D and 3D configuration analyzed by Discovery studio visualizer	45
Figure 4.6 Cell growth inhibition determination of ST treatment for 24, 48, and 72 H on HPNE, Panc 02, and PANC-1 cell lines	47

Figure 4.7 Cell death mechanisms of HPNE, Panc 02, and PANC-1 after ST treatment for 24 and 48 H were determined by Annexin V-FITC + PI staining.....	48
Figure 4.8 Relative changes in intracellular ROS levels after 24 H ST treatment determined by DCFDA/H2DCFDA staining.....	49
Figure 4.9 Mitochondrial membrane potential ($\Delta\Psi_m$) and mass were detected by Rhodamine 123 and MitoTracker Green FM staining by confocal microscope.	50
Figure 4.10 Quantitative mitochondrial membrane potential and mass were analyzed by Rhodamine 123 and MitoTracker Green FM staining by flow cytometry..	51
Figure 4.11 Changes in the cellular ATP levels after 24h ST treatment by chemiluminescence measurement.....	52
Figure 4.12 The changes in the OXPHOS complexes levels were examined by immunoblotting.....	54
Figure 4.13 The changes in the LDHA levels were examined by immunoblotting	55

LIST OF TABLES

Table 1.1 TNM (Tumor node metastasis) grouping of pancreatic cancer stages	17
Table 1.2 Pancreatic stages according to TNM	17
Table 4.1 Molecular docking scores of ligands in their best confirmation.....	37
Table 4.2 Docking site amino acids of GLUT1 and interaction with ST and glucose.	39
Table 4.3 Docking site amino acids of LDHA and interaction with ST, pyruvate, and GNE-140.	41
Table 4.4 Docking site amino acids of caspase-3 and interaction with ST and Ac-DEVD-CHO.....	43
Table 4.5 Docking site amino acids of LC-3 and interaction with ST.	44
Table 4.6 Docking site amino acids of VEGFA and interaction with ST and SOR.....	46

LIST OF ABBREVIATIONS/ SYMBOLS

P53	Tumor protein 53
DNA	Deoxyribonucleic acid
SASP	Senescence associated secretory phenotype
TME	Tumor microenvironment
ETC	Electron transport chain
OXPPOS	Oxidative phosphorylation
ATP	Adenosine triphosphate
mtDNA	Mitochondrial DNA
ROS	Reactive oxygen species
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine di-nucleotide phosphate
ADP	Adenosine diphosphate
FADH ₂	Flavin adenine dinucleotide
TCA	Tricarboxylic acid cycle
APAF1	Apoptotic peptidase activating factor 1
SMAC	Second mitochondria-derived activator of caspase
XIAP	X-linked inhibitor of apoptosis
DR	Death receptor
TNF	Tumor necrosis factor
TRAIL	TNF-related apoptosis-inducing ligand
FADD	Fas-associated death domain
TRADD	TNF receptor-associated death domain
DISC	Death inducing signal complex

PDAC	Pancreatic ductal adenocarcinoma
PanINs	Pancreatic intraepithelial neoplasia
IPMNs	Intraductal papillary mucinous neoplasms
TNM	Tumor node metastasis
BRCA2	Breast cancer gene 2
CDKN2A	Cyclin-dependent kinase inhibitor 2A
CT	Computed tomography
EUS	Endoscopic ultrasonography
MRI	Magnetic resonance imaging
MRCP	Magnetic resonance cholangiopancreatography
ERCP	Endoscopic retrograde cholangiopancreatography
CA19-9	Carbohydrate antigen 19-9
CA12-5	Carbohydrate antigen 12-5
CEA	Carcinoembryonic antigen
5-FU	5-fluorouracil
ECOG	Eastern Cooperative Oncology Group
ST	Stevioside
KRAS	Kirsten Rat Sarcoma viral oncogene homolog
PI3K	Phosphoinositide 3-kinase
LDHA	Lactate dehydrogenase A
FBS	Fetal bovine serum
DPBS	Dulbecco's phosphate buffered saline
DMSO	Dimethyl sulfoxide
TEMED	Tetramethyl ethylenediamine

APS	Ammonium persulfate
BCA	Bicinchoninic acid assay
LC-3	Microtubule-associated protein 1A/1B-light chain 3
SDS	Sodium dodecyl sulfate
PVDF	Polyvinylidene difluoride
VEGFA	Vascular endothelial growth factor A
SOR	Sorafenib
FCCP	Trifluoromethoxyphenylhydrazine
TBHP	Tert-butyl hydroperoxide
DCFDA	2'-7'-Dichlorodihydrofluorescein diacetate
PI	Propidium iodide
RIPA	Radioimmunoprecipitation assay
BSA	Bovine serum albumin
TBS	Tris buffered saline
TBS-T	Tris buffered saline-Tween 20
RT	Room temperature
ECL	Enhanced chemiluminescence

1. INTRODUCTION

1.1 CANCER

Cancer is the first or second leading cause of death [1]. In 2020, nearly 19.3 million people were diagnosed with cancer and 9.9 million people died because of cancer worldwide (Fig 1.1). In Türkiye, approximately 234 thousand people were diagnosed, and 126 thousand of them died of cancer [2]. It is estimated that by the year 2040 the cancer incidence in the world to reach 30.2 M cases compared to 2020, which is almost a 50% increase and can be considered phenomenal (Fig 1.2) [2, 3].

Cancer is the general name for a group of diseases that appear in different organs with more than a hundred types and subtypes, which differentiate at the cellular level both genotypically and phenotypically from normal cells within tissues. Cancer has abnormalities in regulatory circuits controlling homeostasis and cell proliferation, causing tumor development. Cancer demonstrates fourteen biological capabilities and/or hallmarks to proliferate, survive, and disseminate. These are cell senescence, genome instability, collection of mutations, deregulation of cellular energy metabolism, unlocking phenotypic plasticity, non-mutational epigenetic reprogramming, avoiding immune destruction, tumor-promoting inflammation, polymorphic microbiomes, accessing or inducing vasculature, enabling replicative immortality, activating metastasis and invasion, resisting cell death, sustaining growth signals, and evading growth suppressors (Fig 1.3) [4–6].

Cancer cells can induce and sustain positive-growth signals; however, growth suppressors or tumor suppressors are powerful molecules within cells that negatively regulate cell growth and proliferation by multiple pathways. Therefore, cancer cells can overcome these tumor suppressors by loss-of-function or gain-of-function to evade them. P53 is a well-known tumor suppressor which generally found as mutated and plays a central role in the life and death decisions of the cells such as promoting proliferation or activating cell death mechanisms or senescence [5, 7]. P53 decision-making is a result of abnormalities in the genome and stress levels in the cells. If the damage in DNA or stress within the cell is high, p53 triggers the apoptosis or stops the cell cycle until cellular conditions become optimum; however, if DNA is repaired and stress levels are low with high nutrient conditions p53

promotes proliferation and continues the cell cycle. Therefore, cancer cells circumvent tumor suppressors to promote continuous growth.

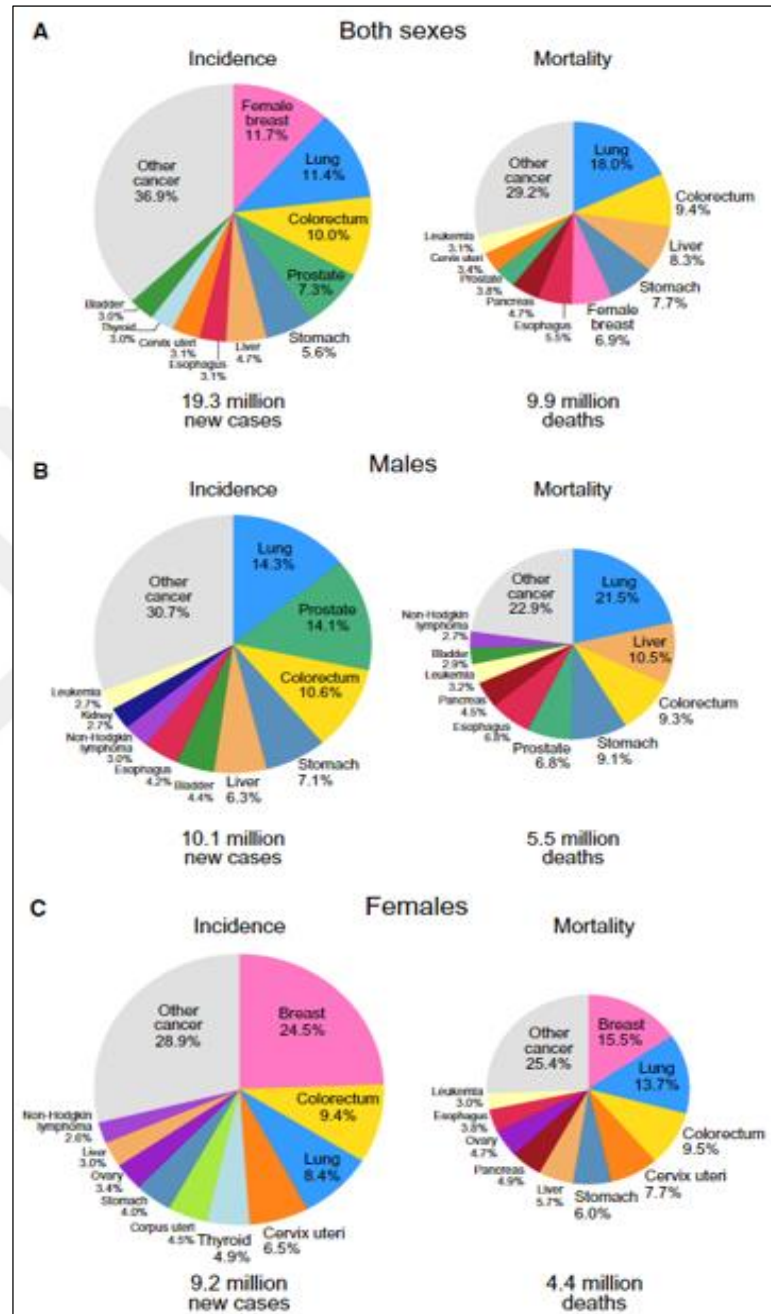


Figure 1.1. Classification of most common cancer types incidence and mortality rates in the year 2020 by GLOBOCAN for both sexes (A), male (B), and female (C)[2].

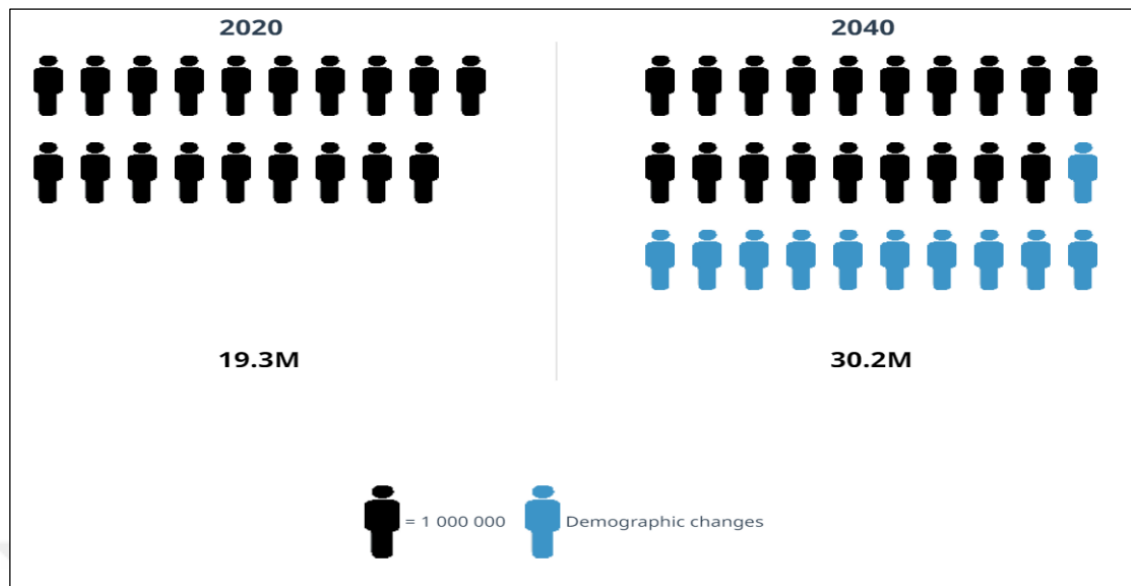


Figure 1.2. The number of diagnoses of all cancer types, age between 0-85 in both sexes estimated between the years 2020 and 2040[8].

Other than evading growth suppressors, cancer cells also resist cell death. Programmed cell deaths, commonly apoptosis, work as a barricade to tumor growth. Imbalanced signaling is caused by increased levels of oncogenes and hyperproliferation causing impaired DNA replication. Apoptosis in cells can be controlled by anti-apoptotic (Bcl-2, Bcl-w, Bcl-xL) and pro-apoptotic (Bax, Bak) Bcl-2 family members, anti-apoptotic Bcl-2 family members can inhibit the apoptosis by binding pro-apoptotic Bcl-2 members when Bax and Bak separated from Bcl-2 they trigger apoptosis by disrupting the mitochondrial outer membrane integrity [7]. Loss-of-function tumor suppressors such as p53 with hyperactivated oncoproteins, or downregulating pro-apoptotic proteins, and also, overexpression of anti-apoptotic proteins are the main strategies of cancer cells to resist apoptosis regardless of cellular stress conditions[4, 5, 7].

Senescence is a mechanism that protects cells to preserve homeostasis by irreversibly causing cell cycle arrest to block proliferation. Therefore, senescence is thought to support programmed cell death and removes the aberrant cells from the tissue by aging and changing its metabolism and morphology. Cancer cells favor to block cell death and proliferate uncontrollably, however, recent studies showed that cancer also uses cellular senescence to protect the cells from genomic damage either caused by radiotherapy or chemotherapy, increased stress conditions (nutrient deprivation, etc.), and oncogene-stimulated hyperproliferation. Tumor development and progression of malignancy are promoted in

various ways by senescent cells which are using paracrine-like transmission by senescence-associated secretory phenotype (SASP) [6]. Therefore, different SASP systems by senescent cancer cells can promote angiogenesis, proliferation, and invasion and avoid programmed cell death in the tumor microenvironment (TME). Also, senescent cancer cells have a distinguishing characteristic that allows them to reverse the senescence to go under a proliferative state when the microenvironmental stress levels are decreased. This also means after the cancer therapy senescent cancer cells can escape non-proliferative mechanisms and cause resistance to cancer therapy [6].

Cancer neoplasticity is generally associated with uncontrolled cell proliferation which requires cellular reprogramming of the energy metabolism besides deregulation of cell growth and death pathways. Normal cells in aerobic conditions process glucose to pyruvate in the cytoplasm and then carbon dioxide in mitochondria via the Krebs cycle and electron transport chain (ETC). Glycolysis is preferred under anaerobic conditions and a bit of pyruvate is transported to the mitochondria to consume oxygen and metabolize carbon dioxide. Cancer cells have aberrant glucose metabolism whether oxygen is present or not and cells favor glycolysis over oxidative phosphorylation named aerobic glycolysis [5].

In tumor tissues, cells need to uptake oxygen and nutrients and clear the metabolic wastes and CO₂, like normal tissues. Neovascularization in the tumor tissue is generated by the process called angiogenesis which is the sprouting of new vessels from existing vessels and the proliferation of endothelial cells. In normal tissues, the angiogenesis process is transiently active while in cancer tissues it is activated at all times, to provide sustenance to expanding tumor tissue [5].

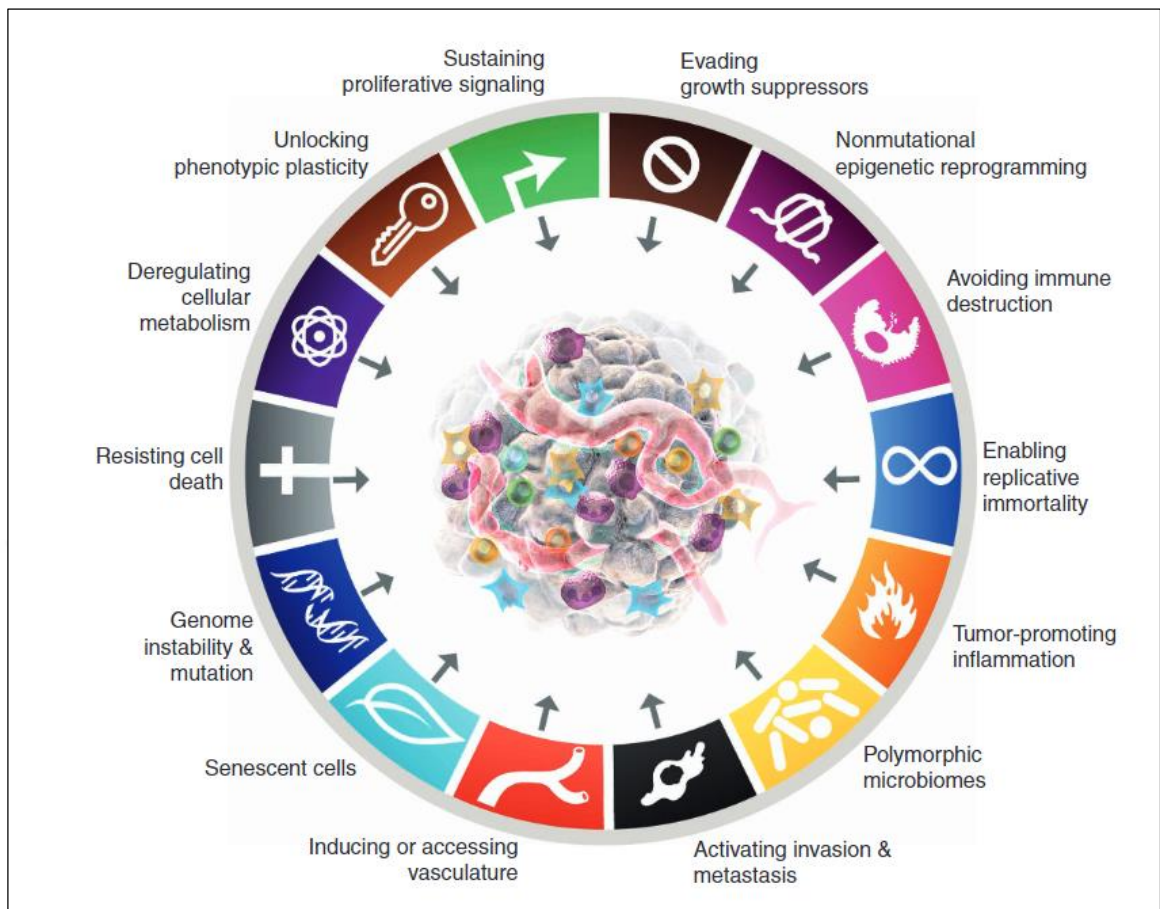


Figure 1.3 Fourteen hallmarks of cancer [6].

1.2 CANCER METABOLISM

Deregulated cancer metabolism like enhanced anabolic pathways and aerobic glycolysis plays a critical role in metastasis, tumorigenesis, cancer stemness, and drug resistance. Aberrant metabolic pathways partake in signal transduction pathways in the cell that supply reactive redox species, energy, and cellular building blocks for the uncontrolled growth of cancer cells. The phenotypes of cancer metabolism are heterogeneous which is a result of changes in the genome, proteome, and TME [9, 10].

1.2.1 Warburg Effect

Reprogramming of the metabolism is an important factor for cancer cell survival in adverse conditions such as nutrient limitations, immune surveillance, and hypoxia other than

upregulated cell proliferation [10]. In the year 1924, Otto Warburg was the first one to describe that for survival and proliferation cancer cells switched metabolism, which was even under normoxic conditions glucose metabolized anaerobically with increased production of lactate. Cancer cells count on glycolysis as a replacement for oxidative phosphorylation even though the mitochondria are intact in tumor cells and can use OXPHOS for ATP [9, 11].

The Warburg effect is characterized by excessive lactate production with increased aerobic glycolysis. According to Warburg's study, in tumor cells glucose uptake was approximately 46-70% while normal cells were 3-18%. Furthermore, 66% of glucose is converted to lactate in tumor cells [11].

1.2.2 Mitochondrial Metabolism

Mitochondrial metabolism is designed to meet cells' metabolic and energy demands. Mitochondria consists of an inner membrane and an outer membrane within between mitochondrial intermembrane space and a matrix. Nevertheless, the efficiency of work changes widely resulting from polymorphism in genetic information both in nuclear (nDNA) and mitochondrial DNA (mtDNA). Problematic environmental conditions and pathologies interrupt the mitochondria function (such as deletion mutations or impaired replication of mtDNA) and cause mitochondrial dysfunction [12]. There are up to ten thousand copies of mtDNA in mitochondria, while mtDNA depletion is still documented as causing mitochondrial dysfunction in a few cancer types [13]. This disruption results in concentrated toxic metabolites coming closer to mtDNA open for mutations and damage considering the inefficient mitochondrial repair mechanism.

Besides being invaluable in energy metabolism, mitochondria carry out different roles such as generating metabolites, reactive oxygen species (ROS), and redox molecules, organizing biosynthetic metabolism, cell death, and cellular signaling (Fig 1.4). These functions confirm mitochondria as critical sensors of stress and adaptors of changing environments. In cancer, mitochondria grant flexibility for tumorigenesis such as the production of oncometabolites, oncoproteins, and higher ROS levels, during nutrient deprivation and hypoxia. In addition to tumor growth, mitochondria provide endurance to the cell exposed to unfavorable

environments such as cancer therapy. For these reasons, mitochondria are critical players in tumorigenesis and cancer treatment [10, 13, 14].

The major ROS source of the cell is the mitochondria, where oxygen consumed during OXPHOS can be converted to radicals such as hydrogen peroxide or superoxide [15, 16]. Mitochondrial ROS level is tightly controlled in normal cells, which acts as a signaling molecule in different pathways in the cell like metabolic adaptation, and differentiation [16, 17]. Deregulation resulting in elevated ROS levels ends up in cell death for normal cells. However, for cancer cells, mitochondrial ROS plays an important role and affects tumorigenesis starting from initiation to metastasis which is resulting from the loss of tumor suppressors and activation of oncogenes to cause the production of aberrant levels of mitochondrial ROS [17]. Which later activates oncogenic pathways that are related to proliferation in the cancer cells. The balance between mitochondrial ROS production and scavenging enables tumorigenesis in cancer cells without cell death caused by high ROS accumulation. Therefore, high expression levels of antioxidants and generation of NADPH in both mitochondria and cytoplasm are also correlated with cancer cells to eliminate the possibility of cell death [10, 18, 19].

As the powerhouse of the cells, one of the essential roles of mitochondria is to generate ATP through oxidative phosphorylation. ATP is produced by converting chemical energy within nutrients to an electrochemical gradient in the mitochondrial inner membrane. Inner membrane complexes (I to IV) of the electron transport chain are responsible for the formation of this gradient to act as energy storage which is later converted to ATP from ADP with complex V. The inner membrane of the mitochondria has an electrochemical potential generated by the H^+ continuous flow from the matrix and contains five complexes of the electron transport chain (ETC) which transfers electrons supplied by $FADH_2$ and NADH. As a result of potential and electron transfer between complexes I to IV, the final electron acceptor complex V (ATP synthase) produces ATP with oxygen (Fig 1.5) [18, 20–22].

Under unfavorable conditions such as damage in DNA, high ROS levels, or other stress conditions cells halt the proliferation and activate the cell death mechanisms. Apoptosis is a type of programmed cell death without any spillage of any cellular content to TME. Induction of apoptosis depends on the cysteine-aspartic protease family called caspases activation. There are two groups of caspases according to their function which are initiators and executors.

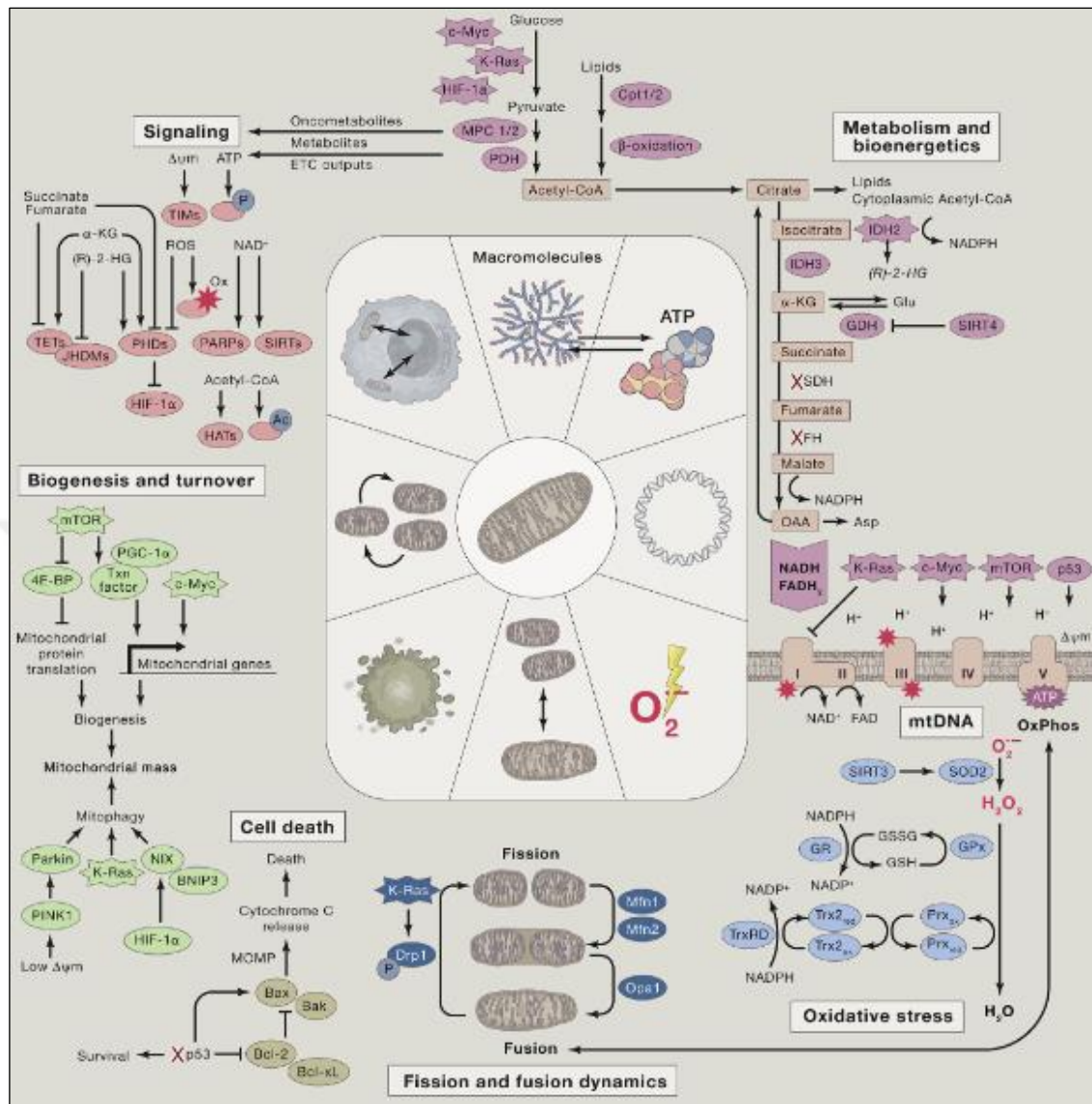


Figure 1.4 The functions of mitochondria. The mitochondria are the center of ATP and metabolite production, regulation of ROS, cell signaling, and death [13].

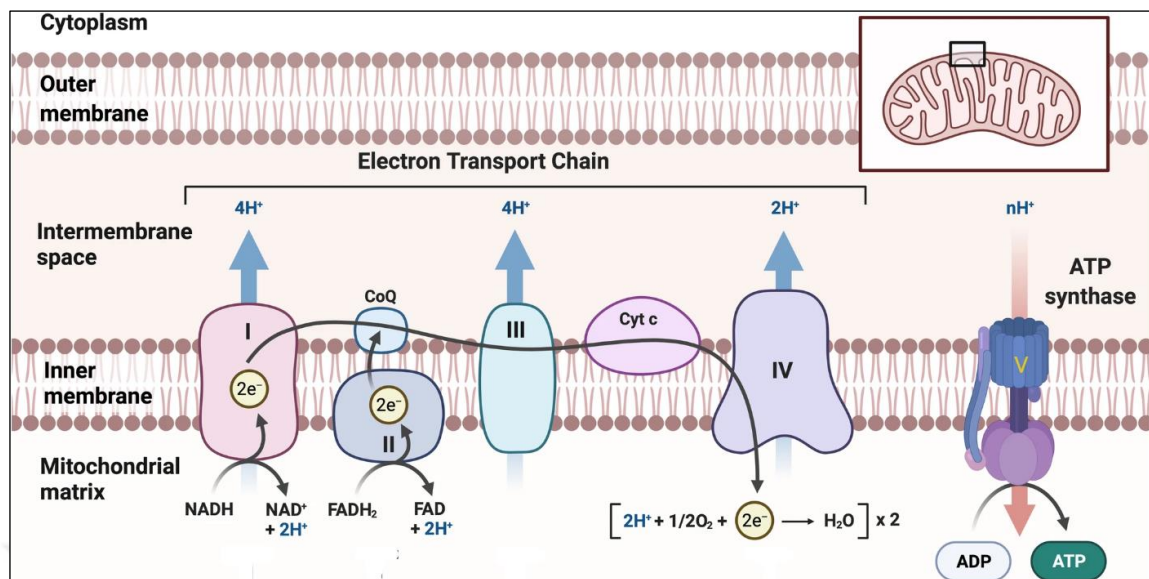


Figure 1.5 Schematic representation of OXPHOS complexes. NADH and FADH₂ electron donors are supplied by the TCA cycle (Created by Biorender).

When damage in the cell is discovered, inactive procaspases are activated to initiator caspases which are caspases 8 and 9. Initiator caspase then activates executioner caspase 6, 7 and 3. Once the executioner caspases are activated caspase cascade is initiated resulting in endonuclease activation for DNA fragmentation, cytoskeleton and nuclear protein degradation, and apoptotic body generation. The formation of apoptotic bodies provides apoptosis to reduce damaging risk to surrounding tissue. There are two types of apoptosis according to source apoptotic sensors called intrinsic and extrinsic pathways. Both pathways synergistically work together to eliminate defective cells and ensure a multicellular organism with healthy cells. Deficiency in apoptosis regulation such as the decrease in apoptosis levels is associated with uncontrolled proliferation and growth.

In the intrinsic pathway (mitochondrial pathway), apoptosis is induced by the cell itself by the detection of cellular signals. Apoptosis signals are induced when pro-survival and growth signals are absent such as growth factors and hormones. Regulation of apoptosis is controlled by a protein family named Bcl-2 which has two categories as pro- and anti-apoptotic factors. BH3-only proteins Bax, bak, bad, and bid are pro-apoptotic and inhibited when pro-survival signals are present in the cell however, anti-apoptotic or pro-survival Bcl-2 proteins such as bcl-2, bcl-w, and bcl-xl are activated other than the induction of apoptosis. A well-known

tumor suppressor and cell cycle controller p53 influences the Bcl-2 family. When p53 detects unrepairable damage, it can directly activate Bax and inhibit bcl-2 to induce apoptosis.

In the absence of survival signals, pro-apoptotic proteins like noxa, puma, and bax are activated and apoptosis become initiated. When the initiation stimuli are present and Bax and Bak are activated, mitochondria become disrupted and the outer membrane of the mitochondria permeabilized with the formation of transition pores for channeling apoptotic factors. This permeabilization causes cytochrome c to be released to the cytoplasm to bind with an apoptotic protease activating factor called APAF1. Then recruits the caspase recruitment domain (CARD) and activates several initiators of caspase-9 of the intrinsic pathway and APAF1 forms an apoptosome. Mitochondrial permeabilization transient pores also release other pro-apoptotic factors like SMAC/DIABLO to activate executioner caspases by binding and inhibiting XIAP (X-linked inhibitor of apoptosis) which is an apoptosis inhibitor. Activated procaspase-9 then activates executioner caspase such as caspase-3 to induce apoptosis in all means and start DNA fragmentation and condensation of the chromatin (Fig 1.6) [7, 23–25].

Another type of apoptosis is the extrinsic pathway which is also known as the death receptor (DR) pathway apoptosis is induced by interaction with macrophages, natural killer, or damaged cells producing death ligands. The extrinsic pathway is induced when death signals bind with DRs called death signal binding to tumor necrosis factor (TNF) family members such as TNF, TRAIL (TNF-related apoptosis-inducing ligand), and FAS receptors anchored via transmembrane domain on the cell membrane [7]. When an extracellular death ligand binds with the TNF receptor, the death signal is sent to cytoplasmic death domains which are FADD (Fas-associated death domain) and TRADD (TNF receptor-associated death domain). Death receptors along with procaspase-8 and the death domain cause the formation of a death-inducing signal complex (DISC) which recruits procaspase-8 to dimerize for activation. Caspase-8 and 10 then activated executioner caspase-3 and 7 to trigger proteolytic cleavage of cellular proteins and apoptosis (Fig 1.6) [23–25].

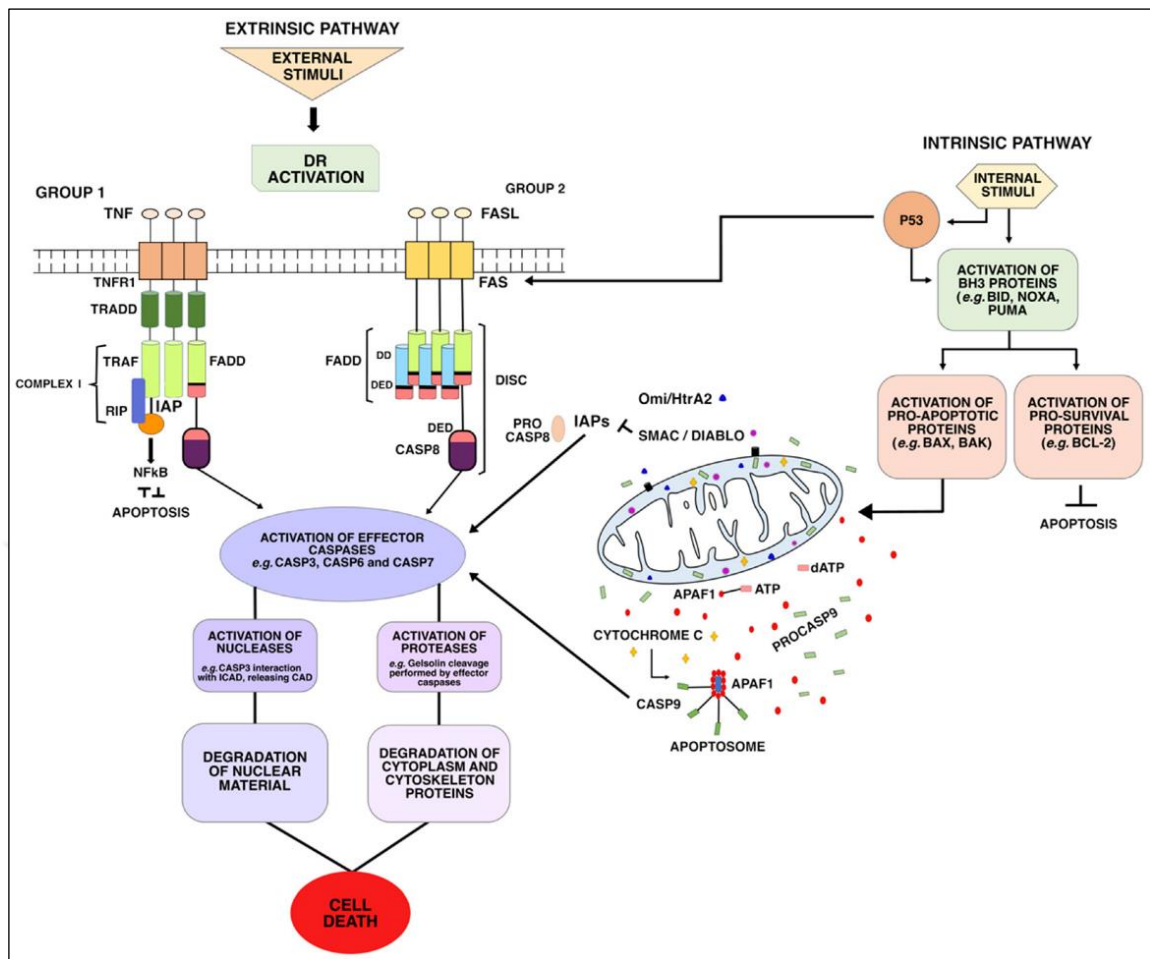


Figure 1.6 Schematic representation of intrinsic and extrinsic apoptosis pathways [26].

1.3 PANCREATIC CANCER

1.3.1 Anatomy of Pancreas

Pancreas is a solitary organ located in the abdomen, behind the stomach that plays an essential role in nutrient metabolism. The pancreas functions as both endocrine and exocrine [27]. 80% mass of the pancreas functions as exocrine and generates a branching network containing acinar and ductal cells regulating the digestion of nutrients. These two cell types produce and transport digestive zymogens to the gastrointestinal tract. Pancreatic zymogens used protein, lipid, and carbohydrate breakdown secreted by the acinar cells into the ductal lumen for transportation to the duodenum (Fig 1.6). The endocrine cells of the pancreas mostly function independently from the exocrine cells. The endocrine part of the pancreas

consists of five different cell types assembled in clusters named islets of Langerhans and responsible for glucose metabolism and homeostasis by releasing hormones into the bloodstream [27, 28]. These five types of cells secrete polypeptide hormones and 50 to 70% of these endocrine cells are beta cells secreting insulin. The second most common is the alpha cells secreting glucagon (glucose elevating hormone in the bloodstream) found in the islets 10-20%. The other three types in the islets are delta, PP (or F-cells) and epsilon secreting somatostatin (growth hormone- inhibiting hormone and inhibits insulin, glucagon, and pancreatic polypeptide release), pancreatic polypeptide (intra-islet secretion regulator), and ghrelin (hunger hormone), respectively, least recurring cells found less than 10 percent [29, 30].

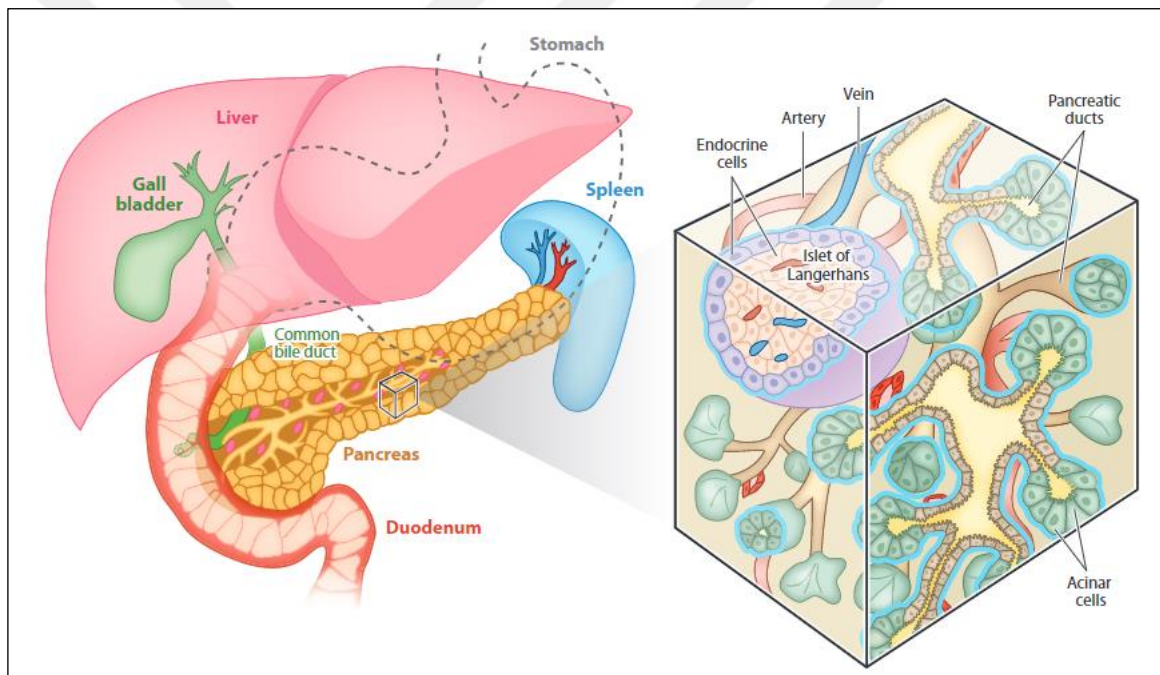


Figure 1.7 Schematic representation of macroscopic and microscopic anatomy of the pancreas located behind the stomach (shown with a dashed line) [27].

1.3.2 Pathogenesis of Pancreatic Cancer

Pancreatic cancer can be grouped as solid or cystic according to gross appearance or classified as acinar, ductal, or endocrine according to differentiation pattern. Pancreatic ductal adenocarcinoma, acinar cell carcinoma, neuroendocrine neoplasms, and pancreatoblastoma are solid pancreatic cancer. Mucinous cystic neoplasm, solid-

pseudopapillary neoplasm, and intraductal papillary mucinous neoplasm are the less aggressive and cystic types of pancreatic cancer (Fig 1.8).

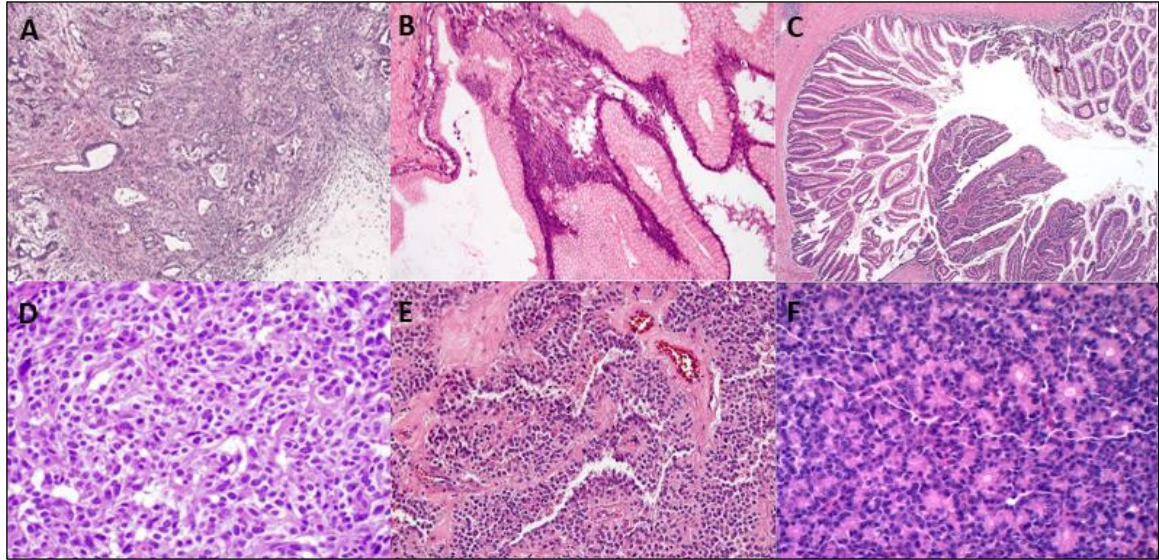


Figure 1.8 Visualization of pancreatic cancer types. Pancreatic ductal adenocarcinoma (A), mucinous cystic neoplasm (B), Intraductal papillary mucinous neoplasm (C), pancreatic neuroendocrine neoplasm (D), solid pseudopapillary neoplasm (E), acinar cell carcinoma (F) [31]

Conventional exocrine pancreatic ductal adenocarcinomas (PDAC) are among the mostly diagnosed pancreatic cancer with 90 percent and endocrine-originating neoplasms are the 5% of all cases [32, 33]. Pancreatic neuroendocrine tumors are the 2nd most frequent neoplasm in the pancreas, while PDAC displays a different molecular profile compared to neuroendocrine tumors. Early histological molecular profiling showed that intraductal precursors undergo low to high-grade lesion progression by gaining genetic anomaly and cytological atypia causing the formation of invasive adenocarcinomas. Two pancreatic cancer precursor lesions have been detected, pancreatic intraepithelial neoplasia (PanINs) and cystic intraductal papillary mucinous neoplasms (IPMNs). PanINs are the most common microscopic precursor lesions that cannot be visualized by abdomen scanning. It contains three grades named PanIN-1, PanIN-2, and PanIN-3 depending on the stages of progression. On the other hand, IPMNs are macroscopic precursors and arise inside pancreatic duct branches or the duct itself (Fig 1.9). Almost 90% of all PDAC cases originate from PanINs and the remaining 10% are from cystic precursors [32–35].

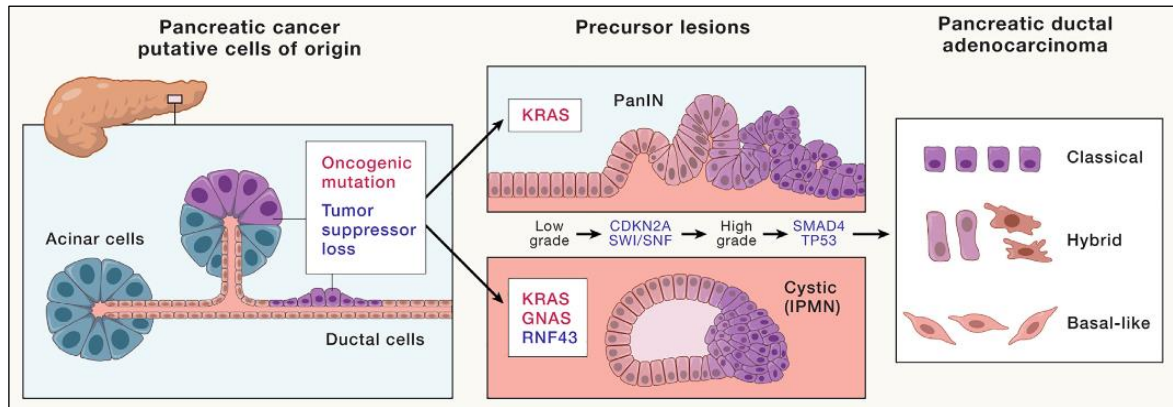


Figure 1.9 Formation of PDAC from normal pancreatic exocrine tissue. Following mutations in oncogenes and loss of tumor suppressors, the development of cancer proceeds through PanINs or IPMNs. Precursor lesion mutation causes a higher-grade neoplasm with a formation of PDAC subtypes [32].

1.3.3 Pancreatic Cancer Epidemiology

Pancreatic cancer is a malignant tumor that is characterized by a poor prognosis and a 5-year survival rate. The last twenty years show that pancreas cancer diagnosis doubled compared with 1990 [36]. In 2020, according to GLOBOCAN pancreas cancer incidence rate was 2.5% of total cancer cases worldwide with approximately 496,000 new cases [2]. However, pancreas cancer is highly aggressive and hard to diagnose, causing this cancer to be one of the deadliest cancers with a $6.0/10^5$ mortality rate, as a result, pancreas cancer is the 7th cause of cancer-related deaths in both sexes worldwide according to GLOBOCAN 2020 statistics (Fig 1.1A) [37]. Also, the incidence rate is higher in men compared to women (Fig 1.1 B) [36]. Especially, Turkey is the 11th country in new pancreas cancer cases and cancer-related deaths with men having a higher number in both incidence and mortality compared to women [2].

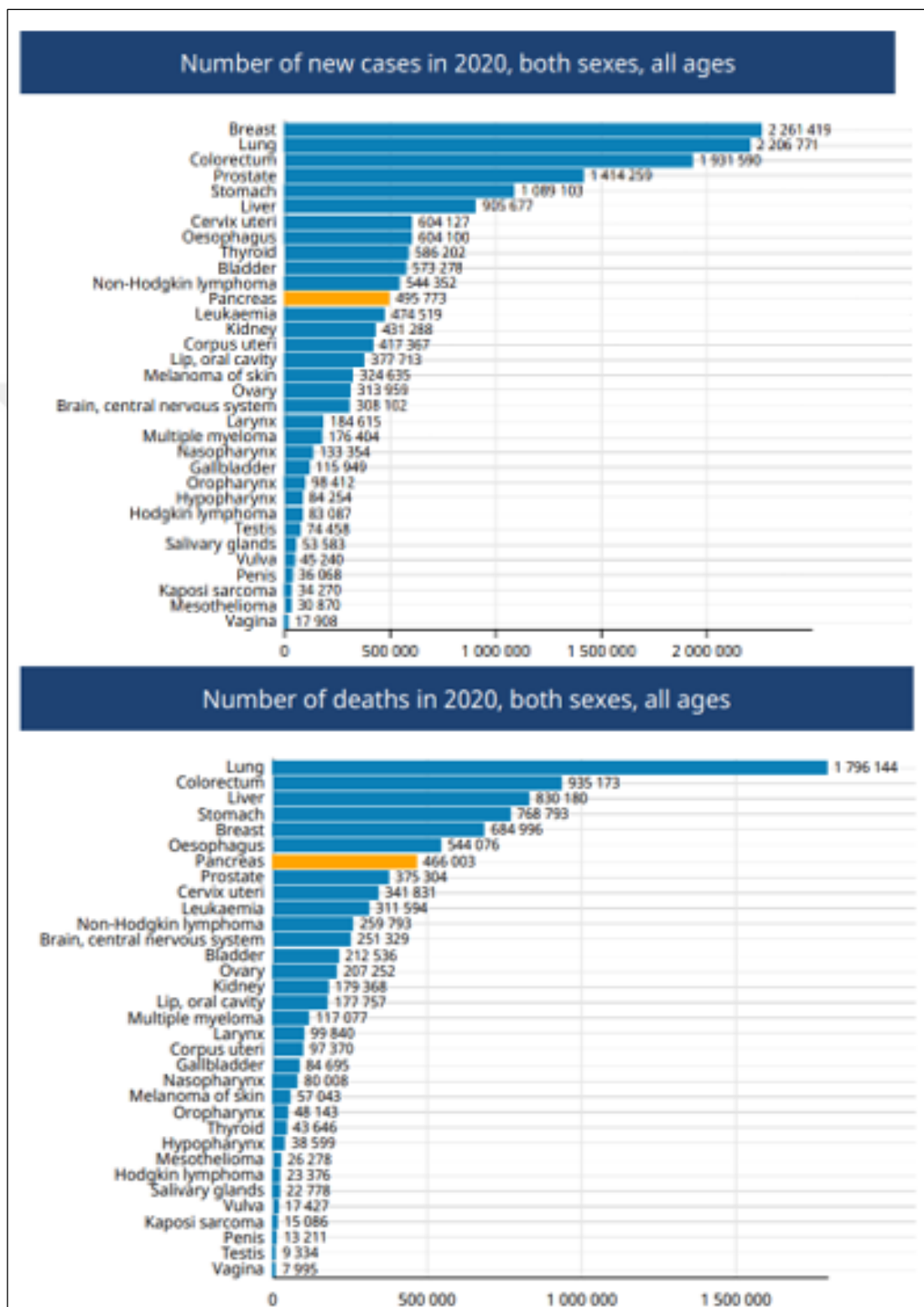


Figure 1.10 Pancreatic cancer incidence (above) and mortality (below) cases according to GLOBOCAN [38]

More than 85% of pancreatic cancer cases occur as PDAC with a low 5-year survival rate of 5%. This survival ratio is related to the diagnosis at advanced stages because of the no obvious symptoms, only 20 percent of the diagnosis occurs in early stages having operatable cancer. Surgical resection increases the 5-year survival rate to 20 percent and if the diagnosis stage is 1A (Table 1.1 & 1.2) survival rate increases up to 80%. Early-phase surgical operation and diagnosis are the only effective treatment for pancreas cancer way to improve patient outcomes. Therefore, it is critical to determine the risk factors and populations related to higher pancreatic cancer risk other than improved early diagnosis such as biomarker selection or clinical examination [36].

1.3.4 Risk Factors of Pancreas Cancer

Pancreatic cancer development in a human in a lifetime is 1.49% which means in every sixty-seven cases there is one pancreatic cancer[39]. Risk factors can be grouped as modifiable (smoking, obesity, infection, chronic pancreatitis, intestinal microflora) and non-modifiable (blood group, age, diabetes, family history, gender, and area) risk factors [40].

One of the non-modifiable risk factors is the age at which pancreatic cancer can develop. However, ages between 60 to 80 are the peak point for approximately 90% of the incidence rate [36, 37, 40]. As mentioned above, the incidence rate of pancreatic cancer depends on the sex of the patient which incidence rate is higher in men, while no significant change in mortality based on sex. The difference between incidence rates in both sexes has a relation with steroid levels which is higher in women than men [40]. Estrogen can reduce the possibility of developing pancreatic cancer[40]. Ethnicity is another non-modifiable risk factor that highest incidence rates in males observed in African Americans followed by non-Hispanic and white populations with the lowest occurrence in Asians. In women again highest incidence rates were observed in African American, following non-Hispanics, and white with the lowest incidence rate by American Indian/Alaska natives. This increasing risk factor with ethnicity might be caused by changing lifestyle, metabolism, diet, and income levels. In addition, the mortality rate of the Asian population is again less aggressive than non-Asian populations and the survival rate is higher. Blood groups other than 0 which are

A, B, and AB have a higher risk of developing pancreatic cancer. Blood group AB is associated with a higher risk of pancreatic cancer development [40].

Table 1.1 Tumor node metastasis (TNM) grouping of pancreatic cancer stages [41].

Classification	Explanation
Primary Tumor (T)	
TX	Primary tumors cannot be assessed
T0	No evidence of a primary tumor
Tis	Carcinoma in situ (includes PanIN-3 classification)
T1	Tumor restricted to the pancreas, ≤ 2 cm greatest dimension
T2	Tumor restricted to the pancreas, ≥ 2 cm greatest dimension
T3	The tumor extends beyond the pancreas, with no involvement of the coeliac axis or superior mesenteric artery, resectable
T4	The tumor affects the coeliac axis or superior mesenteric artery, an unresectable primary tumor
Regional Lymph Nodes (N)	
NX	Regional lymph nodes cannot be assessed
N0	No regional lymph node metastasis
N1	Regional lymph node metastasis
Distant Metastasis (M)	
M0	No distant metastasis
M1	Distant metastasis

Table 1.2 Pancreatic stages according to TNM [39].

Stages	Primary Tumor (T)	Regional Lymph Node (N)	Distant Metastasis (M)	Diagnosis Rate (%)	Median Survival	Explanation
0	Tis	N0	M0	10%	17- 23 months	Local, resectable
IA	T1	N0	M0			
IB	T2	N0	M0			
IIA	T3	N0	M0			
IIB	T1	N1	M0	10%	Up to 20 months	Borderline resectable
	T2	N1	M0			
	T3	N1	M0			
III	T4	Any N	M0	30%	8-14 months	Locally advanced, unresectable
IV	Any T	Any N	M1	60%	4-6 months	Metastatic

The other two non-modifiable risk factors are genetic factors and diabetes. Five to ten people within 100 were diagnosed with familial pancreatic cancer. More than 80% of the new cases have sporadic genetic mutations including somatic and germ cells, also genetic syndromes related to pancreatic cancer. Hereditary pancreatitis (HP), hereditary breast and ovarian cancer syndrome (HBOC), Lynch syndrome (LS), and familial adenomatous polyposis (FAP) hereditary syndromes can cause the development of pancreatic cancer. Genetic alterations in BRCA2, ATM, and CDKN2A/p16 are the most common genetic changes in pancreatic cancer, in addition to the hereditary syndrome, the development of pancreatic cancer significantly increases with the first-stage relatives having pancreatic cancer [32, 37, 40]. Diabetes mellitus can be both a factor of risk and a result of pancreatic cancer. In many newly diagnosed cases, beginning diabetes and worsening disease among diabetic patients are reported. Also, patients having diabetes over 3 years have an elevated risk of developing cancer. Other than diagnosis, diabetes biomarkers are associated with pancreatic cancer incidence risk including insulin resistance, fasting glucose, and insulin levels [36, 37, 40].

The first example of a modifiable risk factor for pancreatic cancer is smoking which is a well-established factor that increases the incidence rate more than 1.5-fold compared to non-smokers. Studies showed that the diet of the patient showed a relation between pancreas cancer occurrences [37]. Consumption of sugar-sweetened products, red meat, and saturated fat increases the development of pancreatic cancer. In contrast, consuming vegetables, fruits, and whole-grain and nuts decreases the development possibility [37]. Also, people who drank more than 30 grams of alcohol are associated with higher pancreatic cancer risk [37]. However, there is no association between pancreatic cancer and low or moderate drinking showing alcohol affects risk in dose-dependent. In addition to diet, Body mass index is an important determinative of pancreatic cancer risk higher than 30 kg/m² increases the risk 1.7-fold compared to 25 kg/m². With the increasing BMI globally, obesity will lead to an increasing number of pancreatic cancer patients.

Pancreatitis is the progressive inflammatory phase causing islet cell loss and pancreatic fibrosis. The damage and inflammation resulting from pancreatitis leads to pancreatic endocrine or exocrine insufficiency causing abnormal pancreatic enzymes. In addition to the macroscopic level, chronic pancreatitis disrupts lysosomal autophagy, and mitochondria systems of pancreas cells leading to increased DNA damage, mutation, and activation of oncogenes. For these reasons, pancreatitis is also a risk factor, and patients with pancreatitis

have a significantly higher risk of developing pancreatic cancer with a 13.3-fold ratio [36, 40].

1.3.5 Diagnosis and Therapeutic Strategies

Pancreatic cancer patients often demonstrate unspecified symptoms causing delayed diagnosis. Most of the patients present weight loss, anorexia, asthenia more than 80%, jaundice, diarrhea, vomiting, dark urine, and pain in the back and abdomen are the other signs of pancreatic cancer [33]. Most patients are diagnosed with pancreatic cancer when pain levels become agonizing. As a result of lacking typical clinical manifestations and efficient diagnostics, patients diagnosed with pancreas cancer are often in the advanced stage with a low possibility of surgical operation and effective treatment. Hence, early-phase diagnosis and screening are critical for patients' life quality and earlier treatments with increased opportunities for surgery and other cancer therapies.

For diagnosis, computed tomography (CT), endoscopic ultrasonography (EUS), magnetic resonance imaging (MRI), magnetic resonance cholangiopancreatography (MRCP), and endoscopic retrograde cholangiopancreatography (ERCP) are used in clinical, however, these visualization methods are not precise enough to identify early phase pancreatic cancer and often hard to differentiate from pancreatitis and normal pancreas. Therefore, proteomic, biomarkers named CA19-9 (carbohydrate antigen 19-9), CA12-5, and CEA (carcinoembryonic antigen) are used for early detection and screening but still, the sensitivity of biomarkers in symptomatic patients reach 80% and can be found in benign tumors [33]. Therefore, the accuracy of the diagnosis is not adequate [37].

Pancreatic cancer treatment covers chemotherapy, radiotherapy, surgery, and palliative care. In addition to these treatments, brachytherapy, radiopharmaceutical therapy, immunotherapy, and microbial therapy also started to be used as targeted therapy and combined with known traditional methods in pancreatic cancer. The method of treatment is determined according to the stage of the disease (Fig 1.11). Surgery is accepted as the only cure for pancreatic cancer because of its significant effect on prolonged survival. Resectable pancreatic cancer is determined by the absence of a relation between the tumor and major arteries and veins plus the absence of metastasis [39]. If the tumor is unresectable, two options can be preferred: adjuvant and neoadjuvant. Neoadjuvant therapies are preferred to

improve the survival and life quality of the patient by achieving better control of local tumors. Neoadjuvant therapies showed an increased potential in surgical resection after chemotherapy [40]. Studies showed that pre-surgical radiation or chemotherapy can clear metastatic lesions and are more efficient compared to post-surgical radiation or chemotherapy. The reason behind this efficiency change is possibly owing to poor sensitivity, and drug delivery after removal of the tumor. Also, pancreatic cancer with arterial metastasis is unsuitable for a second resection therefore, neoadjuvant therapy is a better option in pancreatic cancer to improve survival [40, 42].

Chemotherapy is fundamental for pancreatic cancer treatment and studies showed that adjuvant chemotherapy after surgical resection can increase overall disease-free survival significantly. Gemcitabine and capecitabine or modified 5-fluorouracil (5-FU), and oxaliplatin (MFOLFIRINOX) are often used as adjuvant chemotherapy for 6 months to eliminate recurrence of the cancer. In the metastatic phase, the FOLFIRINOX regimen (combination of leucovorin calcium, fluorouracil, oxaliplatin, and irinotecan hydrochloride), gemcitabine, and NAB-paclitaxel combinations are the preferred therapy however, if the combination therapy is not appropriate for the patient gemcitabine is the first line therapy [43]. In locally advanced tumor therapy, a combination of radiation and chemotherapy is preferred as the first line using gemcitabine with 54 Gy[33, 40, 44]. A newly developed brachytherapy device named OncoSil™ delivers ^{32}P to the targeted intratumoral area and a combination of OncoSil™ and gemcitabine or FOLFIRINOX treatment showed progression-free survival in the locally advanced PDAC patients [45, 46].

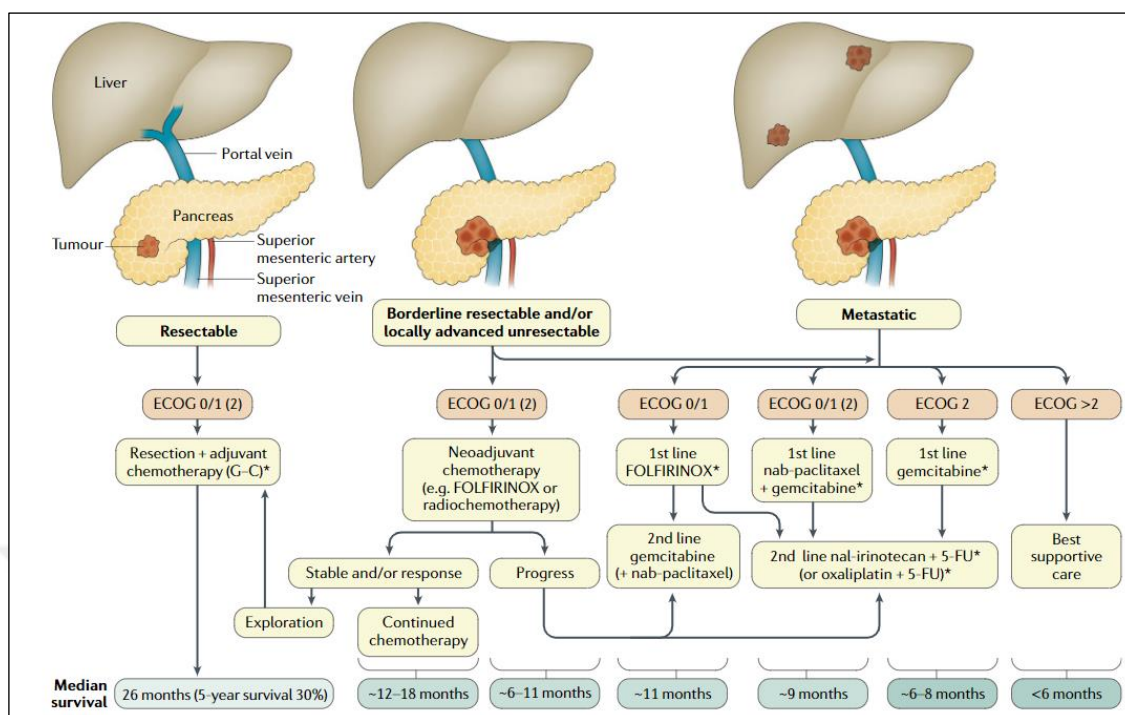


Figure 1.11 Treatment order of pancreatic cancer according to tumor stage (metastatic, resectable, locally advanced unresectable, and borderline resectable) and ECOG (Eastern Cooperative Oncology Group) score performance status. (0: active, all responsibilities can be carried out like in pre-diseased performance, 1: physical restriction in activity able to perform light office or housework, 2: ambulatory, can perform self-care and unable to perform 50% of house and office work, >2: limited self-care or disabled, bounded to a chair or a bed) [44].

1.4 STEVIOSIDE

Stevioside is a diterpene glycoside containing three molecules of glucose and an aglycone named steviol (Fig 1.12) that is extracted from the leaves of a perennial herb called *Stevia rebaudiana* (Bertoni). Stevia extracts are dietary supplements used as sweeteners that contain several other sweet compounds other than stevioside such as steviobioside, ducoside A, and rebaudioside A, B, C, D, and E which all have the same steviol backbone structure with distinct carbohydrate positions [47, 48]. Stevioside is a natural sweetener that is known to be 300-to-400-fold sweeter than sucrose. This increased sweetener capacity and the taste quality of the stevioside are related to the glucose units' ratio on the 13-hydroxyl and 19-

carboxyl groups (Fig 1.12) [49, 50]. Steviol glycosides cannot be digested by pancreatic fluids and stomach enzymes therefore, they are called zero-calorie sweeteners [51, 52].

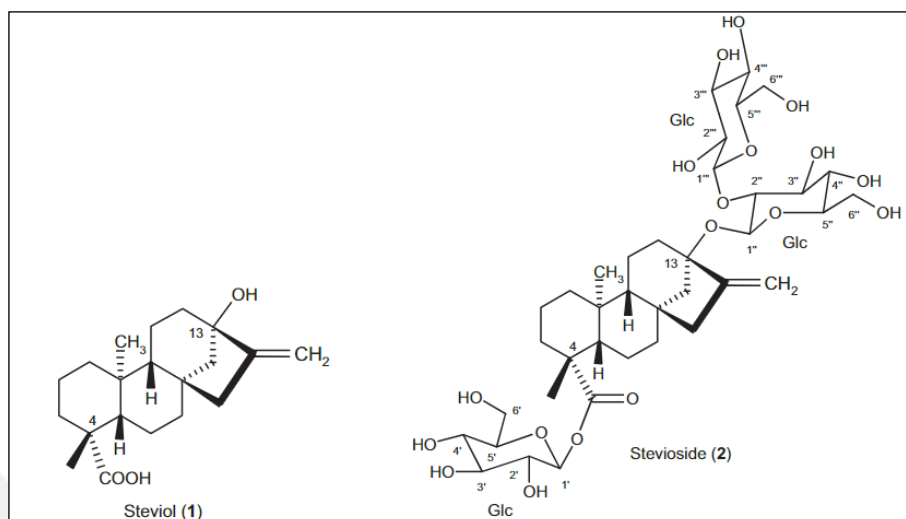


Figure 1.12 The structure of steviol (1) and stevioside (2) [49].

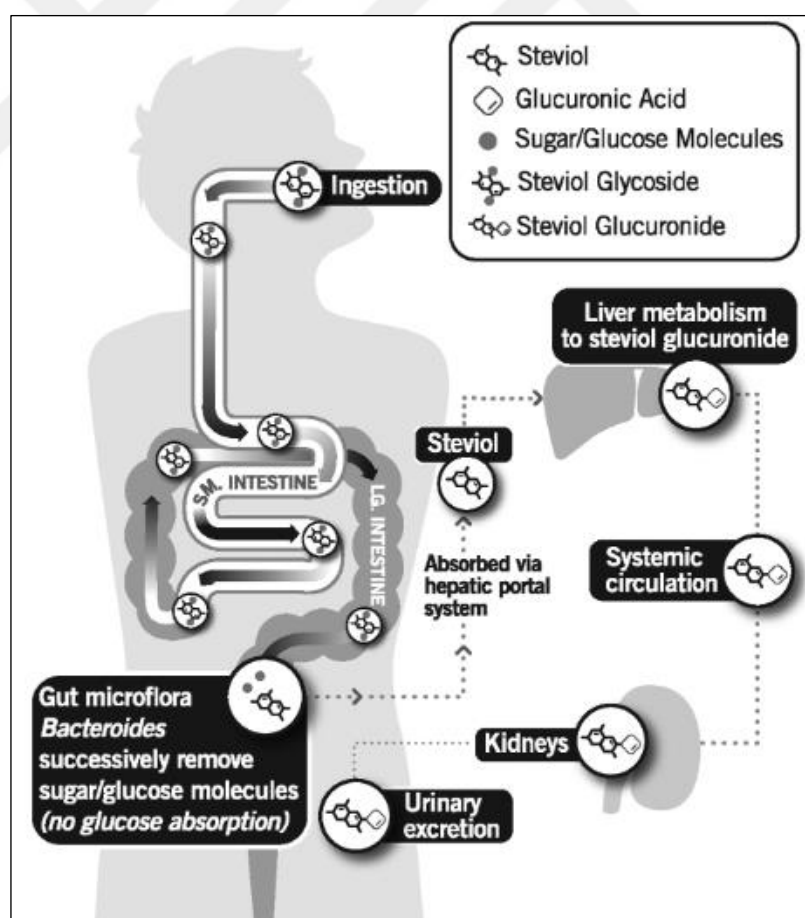


Figure 1.13 Metabolism of steviol glycosides [53]

In both humans and rats, oral administration of the stevioside can be absorbed in two ways as degradation via intestinal flora to steviol and three glucose molecules or direct stevioside absorption [47, 50]. Digestion of the stevioside is performed by a group of bacteria named *Bacteriodes* sp. which takes three glucose molecules for energy and releases steviol for intestinal absorption [51].

After the absorption, steviol and stevioside are transported to the liver via the hepatic portal system and glucuronidated. In the final step, steviol and stevioside were excreted via urine and feces in humans and rats (Fig 1.13). However, the detailed metabolism of steviol glycosides is still unknown [53]. Wheeler *et al.* showed that stevioside was digested to steviol before absorption in the gastrointestinal tract and most of the steviol circulating in the form of steviol glucuronide which is excreted via the urinary system in 72 H. In addition to steviol conjugate small amount of stevioside is also found in the urine [54].

In addition to being a food additive and zero-calorie, stevioside also displays various health effects such as anti-cancer [55], anti-diabetic [56], and anti-hypertensive [57]. Studies of breast cancer showed that stevioside causes the cell cycle to shift G0/G1 phase and decrease the mitochondrial potential with an increase in intracellular ROS levels in the MCF-7 cell line [58]. In addition, a combination of 5-FU with stevioside (ST) increases the treatment efficiency compared to 5-FU and sensitizes to apoptosis by decreasing the Bcl-2 and increasing the expression levels of Bax and caspase-9 the MDA-MB-231 breast cancer cells [59]. In bladder cancer, stevioside induce intrinsic pathway by upregulating caspase-9 and treatment cause mitochondrial depolarization and mitochondrial stress. The ST treatment cause increased levels of ROS accumulation similar to breast cancer studies [60]. Finally, colon cancer studies of steviol showed cell cycle arrest at the G1 phase and induce apoptosis by upregulating expression levels of p21 and p53 and Bax/Bcl-2 ratio whereas downregulating the expression of Cyclin D1 [61].

1.5 GLUCOSE METABOLISM

1.5.1 Glucose Metabolism in Pancreatic Cancer

Pancreatic cancer progression requires adequate energy and biosynthetic precursors to supply cell division, migration, and invasion. However, in PDAC hypovascularization causes nutrient deprivation for biosynthesis and a crisis of energy.

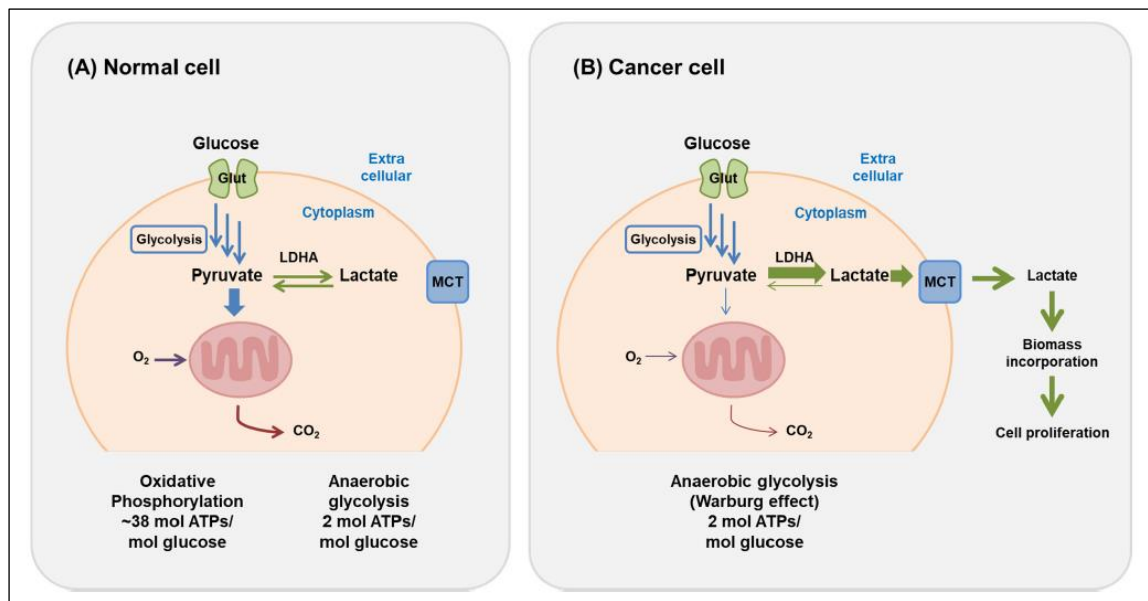


Figure 1.14 Differences between energy metabolism in normal and cancer cells [62]

Therefore, cancer cells reprogram their energy metabolism to survive under unfavorable conditions leading to enhanced glycolysis by elevated glucose uptake and lactate production. Enhanced glycolysis is driven by certain genomic alterations such as Kras and P53 [63]. Both molecules are critical for PDAC development and progression. Kras is responsible for uncontrolled proliferation through activating PI3K/AKT/mTOR and MAPK pathways[63]. Oncogenic Kras can elevate glycolysis levels by regulating glucose uptake and rate-limiting glycolysis enzymes such as LDHA, HK2, and GLUT1. P53 on the other hand, mutated as a gain-of-function version and modulates genes for cell growth by enhancing glycolysis [63].

Macromolecular precursor and reducing equivalent supply and energy provided by the Warburg effect can benefit pancreatic cancer via apoptosis inhibition and cancer cell growth. In addition, upregulated glucose anabolism and glycolytic enzymes enhance metastasis in PDAC cells. For example, increased lactate production by glycolysis leads to lactate deposition in the TME which can be fuel for oxidative PDAC cells and signal for invasion and metastasis [63]. Although increased glycolysis levels are taken as a dominant alteration in metabolism, OXPHOS activity is also essential in some cancers with metabolic heterogeneity. PDAC cells showed that enhanced OXPHOS-like glycolysis also promotes metastasis and high OXPHOS levels can stabilize the functionality of the cancer stem cells [64–66].

1.6 AIM OF THE STUDY

Stevioside is a glucose-mimicking zero-calorie sweetener with an anti-cancer effect and our recent study (unpublished data) showed a decrease in the cell viability with stevioside treatment. This study aimed to determine the molecular mechanism and effects in the energy metabolism of stevioside in pancreatic cancer by examining glycolytic profile, cellular, and mitochondrial metabolism. *This is the first molecular mechanistic study of the effect of stevioside on pancreatic cancer by utilizing a cell model.*



2. MATERIALS

2.1 CELL LINES AND CELL CULTURE

Human pancreatic cancer cell lines PANC-1 (ATCC®, CRL-1469™), hTERT-HPNE, human normal pancreatic cell line (ATCC®, CRL-4023™), and Panc 02.03 (ATCC®, CRL-2553™), were obtained from the cell/tissue biobank at the department of Genetics and Bioengineering, Yeditepe University.

2.2 CHEMICALS

RPMI-1640 (w/L-glutamine) (Gibco™ #11875093, USA), DMEM (w/ 4.5 g/L D-glucose, L-glutamine) (Gibco™ #11965092, USA) and FBS (Fetal Bovine Serum) (Gibco™ #10500-064, USA) were purchased from Sigma Aldrich. Trypsin/EDTA (2.21 mM EDTA, 0.25% trypsin) (Multicell #325-043-EL, Canada), and Antibiotic-antimycotic solution (100x) (Multicell #450-115-EL, Canada) were purchased from Wisent Bioproducts. DPBS (w/o Mg and Ca) (#P04-53500, Germany) was purchased from Pan Biotech. DMSO 99.9% (dimethyl sulfoxide) (Fluka #41641-250ML, USA) was purchased from Thomas Scientific. Stevioside hydrate (#B1242, USA) was purchased from BioVision Incorporated. Sodium dodecyl sulfate (SDS) (Invitrogen #171055309, USA) was purchased from Thermo Fisher. Glycine (#A610235-0500, Canada) was purchased from Bio Basic and Tris-base (#P2183C20, China) from Sangon Biotech. Tween-20 (Sigma-Aldrich #P1379-250ML, USA), TEMED (BioFroxx #8027ML100, Germany), Sodium Chloride (Isolab #9690331000-1KG, Germany), 2- propanol (Sigma-Aldrich #24137-2.5L-R, USA), Acetic Acid (Sigma-Aldrich #27225, USA), Methanol 99.9% (Sigma-Aldrich #34885, USA), β -mercaptoethanol (Merck #8057400250, Germany), Bromophenol Blue (Sigma-Aldrich #1143915G, USA), APS (ammonium persulfate) (Sigma-Aldrich #A3678100G), Coomassie Brilliant Blue R250 (Sigma-Aldrich #2781625G, USA), Tris hydrochloride (Sangon Biotech #A6101030250, China), Glycerol 99% (Sigma-Aldrich #G2025, USA), MitoTracker™ Green FM (Life Technologies #M7514, USA), Acrylamide/Bis Solution 29:1 (30%) (Bio-Rad #1610158), Precision Plus Protein Dual Color Standards (Bio-Rad #1610374),

Rhodamine 123 (Sigma-Aldrich #R8004, USA), 2-NBDG (Invitrogen #N13195, USA) were purchased from different companies.

2.2.1 Kits

Pierce™ BCA Protein Assay Kit (Thermo Fisher #23225, USA), DCFDA/H2DCFDA - Cellular ROS Detection Assay Kit (Abcam #ab113851, USA), eBioscience™ Annexin V Apoptosis Detection Kit (Invitrogen #88800574, USA), CellTiter 96® Aqueous One Solution Cell Proliferation (MTS) Assay Kit (Promega #G3582, USA), Clarity Western ECL Substrate (Bio-Rad #1705061, USA), ATP Bioluminescence Assay Kit HS II (Roche #11699709001, USA), Apoptosis Sampler Kit (Cell Signaling Technology #9915, USA) were obtained for the experiments.

2.2.2 Antibodies

Anti – LDHA Rabbit (Sigma-Aldrich #SAB2101329, USA), Mouse Total Anti – OXPHOS WB Ab Cocktail (Mitosciences #MS604, USA), Anti -LC-3 mouse (Abcam #ab51520, UK) were applied during the experiments.

2.3 INSTRUMENTS

SpectraMax® Paradigm® Multi-Mode Detection Microplate reader (Molecular Devices #PARADIGM, USA), Chemidoc™ XRS+ System (Bio-Rad, #1708265, USA), FACSCalibur Flow cytometry (BD Biosciences #342973, USA), Mini-PROTEAN Electrophoresis System (Bio-Rad #1658001, USA), Air jacketed CO₂ incubator (Heal Force #HF151UV, China), Laminar flow hood (ESCO Life Sciences #AC2-4E8, USA), Pierce™ G-2 Fast-Blotter (Thermo Scientific, #62287, USA), Bead bath (Lab Armor® #74309-714, USA), Scientific Balance (Shimadzu, #AUX320, China), Rocker (Daihan Scientific, #RK2D, China), Inverted light microscope (Zeiss Primo vert #491206-0005-000, Germany), pH meter (Hanna Instruments #HI2020, USA), Vortex (Dragon Lan #MX-S, Spain), Confocal microscope (Zeiss #LSM800, Germany) were employed during the experiments.

2.3.1 Equipment

Cryovials (Corning #430659, USA), Tissue Culture flasks; T25, T75, and T175 (TPP, Switzerland), 6-well plates (SPL #30006, Korea), 96-well plates (Greiner Bio-One #655001, Austria), Hydrophobic PVDF (Polyvinylidene Fluoride) Blotting Membrane (Bio-Rad #IPVH00010, Germany), Serological pipettes; 25ml (NEST #328001, China), 5 ml (NEST #327001, China) 10 ml (CAPP #SP10C, Denmark), Micropipette tips; 10 μ l, 200 μ l, 1000 μ l (CAPP, Denmark), Minisart® NML plus 0.45 μ m syringe filter (Sartorius #17829, Germany), 50ml syringe (Set Inject, Turkey), Parafilm® (Sigma-Aldrich #P7793, USA), Hemocytometer (Naubauer- improved) (Marienfeld, # 0610030, Germany), Whatmann Paper (3mm)(GE Healthcare Life Sciences #3030917, USA), Mini-PROTEAN® short glass plates (Bio-Rad #1653308, USA), Mini-PROTEAN® Spacer glass plates (1.0 mm) (Bio-Rad #1653311, USA), Conical Centrifuge Tubes; 15 ml, 50 ml (Falcon®, USA), 1.5 ml, 2.0 ml (Eppendorf, USA) were the materials and pieces of equipment for the experiments.

3. METHODS

3.1 MOLECULAR DOCKING OF STEVIOSIDE

Molecular docking was performed for stevioside and substrates or inhibitors of GLUT1 (glucose transporter 1, PDB ID: 6THA), VEGFA (Vascular endothelial growth factor A, PDB ID: 6ZFL), caspase-3 (PDB ID: 7RNC p12), LC-3 (PDB ID: 5MS6) and LDHA (Lactate dehydrogenase A, PDB ID: 4ZVV). The substrates of GLUT1, caspase-3, and LDHA were chosen as glucose, Ac-DEVD-CHO [67], and pyruvate [68], respectively. For the inhibitors of LDHA and VEGFA, GNE140 [69] and sorafenib (SOR) [70] were chosen.

The chemical structures of stevioside, inhibitors, and substrates were drawn using the ChemSketch tool (ACD Labs 1.2). Structure optimization of the ligands, energy minimization, and proper 2D orientation arranged by Avogadro ORCA (Avogadro 4.2.1). Protein crystal structures were downloaded from the RCSB protein data bank (PDB) and by using AutoDock 4.2 (MGL tools 1.5.7) water and ligand molecules were removed. With the addition of polar hydrogens, proteins became prepared. Ligands and proteins were uploaded to an online docking algorithm, SwissDock. The best docking conformation was chosen and the interaction between ligand and protein was visualized by USCF Chimera 1.16 and the receptor-ligand interaction tool Discovery Studio Visualizer (Biovia).

3.2 CELL CULTURE

3.2.1 Cell Culture Conditions

PANC-1, and HPNE were cultured and preserved with Dulbecco's Modified Essential Medium (DMEM) containing high glucose (4.5 g/L) L-glutamine and sodium bicarbonate. Panc 02.03 was preserved with RPMI-1640 containing L-glutamine. Both FBS and antibiotic and antimycotic were added with final concentrations of 10% and 1% (v/v) respectively to RPMI-1640 and DMEM to complete the mediums. All cells were incubated in an air-jacketed CO₂ incubator conditioned to 37°C with 5% CO₂.

3.2.2 Thawing of The Cell

Cells in cryovials were taken from the nitrogen tank and quickly heated to 37°C using a water bath. Thawed cells were transferred to a falcon tube and 9ml of fresh medium was slowly added and mixed. Cells were centrifuged at 300 x *g* for 5 minutes. The supernatant containing freezing medium was discarded and cells dissolved in fresh complete medium and seeded in a T25 tissue culture flask. Flask was placed into an air-jacketed CO₂ incubator conditioned to 37°C with 5% CO₂ for incubation.

3.2.3 Subculturing

When overall confluency in the culture plate become 80%, the cells were rinsed with dPBS and treated with 0.25% trypsin/EDTA solution for five minutes at 37°C. Cell-cell and cell-surface dissociation was observed under the inverted microscope and the culture flask was centrifuged at 300 x *g* for 5 minutes. The supernatant was discarded and fresh medium was seeded to the appropriate culture flask after calculating and determining the cell number and required volumes. Flask was placed back into an air-jacketed CO₂ incubator conditioned to 37°C with 5% CO₂ for incubation.

3.2.4 Determination of Total Cell Number

Following the dissociation of the cells by trypsin and centrifugation, cells were resuspended in the fresh medium and loaded in between the coverslip and hemocytometer for cell counting. Four squares of the mirror-grid hemocytometer were counted with the inverted microscope at 10X objective lens magnification. To calculate the cell number the equation 3.1 given below was used.

$$\text{total number of cells} = \frac{\text{average of cells in four squares} \times \text{dilution factor}}{10^{-4}} \quad (3.1)$$

3.2.5 Cryopreservation

Cells were harvested with trypsin and pellets were isolated with centrifugation when tissue flask surface confluency reach 80%. Cell number was counted and the appropriate number

of cells was dissolved in 1ml freezing medium which contains 10% dimethyl sulfoxide(DMSO), and 50% FBS within the complete medium. The cell suspension was transferred into a cryovial which was immediately placed at -80°C overnight within an isopropanol box and later into a nitrogen tank from the department biobank.

3.3 PREPARATION OF STEVIOSIDE

3.3.1 Preparation of Stevioside

Stevioside (ST) dissolved in 10% DMSO within a specific medium for the cell type described in 3.1.1. 1 mg ST was weighed and dissolved 1 ml of 10% DMSO in a medium to prepare the main stock of 1.28 mM.

3.3.2 Stevioside Treatment

PANC-1, PANC 02.03, and HPNE cells were treated with 100 μ M ST and placed into air-jacketed CO₂ incubator conditioned to 37°C with 5% CO₂ for 24 H incubation. For Annexin V+PI, treatment was performed also for 48 H, and for MTS treatment time was performed for 24, 48, and 72 H.

3.4 CELL VIABILITY ASSAY

MTS is a colorimetric-based biochemical assay that is used for cell viability determination. MTS is based on tetrazolium salt which is reduced to formazan by the living cells by the reduction of NADH and NADPH done by a group of enzymes called dehydrogenases.

For the colorimetric assay, 10,000 cells/well were counted for PANC-1, and HPNE, and 7,500 cells/well were counted for Panc 02.03. Cells were seeded into 96-well plates with final volumes of 100 μ l and incubated overnight at 37°C. The old medium was discarded and cells were starved by serum-free medium for 2 H incubation. After the starvation, different groups of treatments were applied and placed into an air-jacketed CO₂ incubator conditioned to 37°C with 5% CO₂ for 24, 48, and 72H incubation. After incubation, the treatment medium was discarded and 10% MTS in a specific medium was added to the cells

and incubated for 30, 60, and 90 minutes. The absorbance of treatments was measured at 490 nm by the plate reader.

3.5 MITOTRACKER GREEN FM STAINING

Coverslips were washed with 70% ethanol and placed into a 6-well plate. The lid of the flask was opened and sterilized under UV light. Wells were washed with 1X PBS, then coated with poly-L-Lysine. HPNE, PANC-1, and Panc 02 were trypsinized and counted 200,000 cells/well. Cells were incubated overnight at 37 °C with 5% CO₂. The next day, stevioside treatment was applied as in section 3.2.2. Old media was discarded, and cells were rinsed twice with PBS. 1 ml of 200 nM MitoTracker Green FM was added in serum-free medium (SFM), and incubated for 30 minutes at 37 °C. Wells were rinsed with PBS three times for 5 minutes. Coverslips were mounted to microscope slide and cells were observed under confocal microscope.

For the Flow cytometry, cells were seeded as 300,000 cells/ well and incubated overnight at 37 °C with 5% CO₂. Stevioside treatment was applied following the instructions from section 3.2.2. The next day, cells were collected and rinsed with 100 µl PBS. Cells were resuspended in 100µl of 200 nM MitoTracker Green FM and incubated for 30 minutes in the same incubator conditions. Lastly, samples were analyzed with the flow cytometer.

3.6 RHODAMINE 123 STAINING

Coverslips were washed with 70% ethanol and placed into a 6-well plate. The lid of the flask was opened and sterilized under UV light. Wells were washed with 1X PBS, then coated with poly-L-Lysine. HPNE, PANC-1, and Panc 02 were trypsinized and counted 200,000 cells/well. Cells were incubated overnight at 37 °C with 5% CO₂. The next day, stevioside treatment was applied as in section 3.2.2. Before staining, 600nM of FCCP dissolved in serum-free media was added and incubated for 2 minutes at 37 °C as a positive control. Old media was discarded, and cells were rinsed 2 times with PBS. 1 ml of 5µg/ml Rhodamine 123 was added in serum-free medium (SFM), and incubated for 30 minutes at 37 °C. Wells were rinsed with PBS three times for 5 minutes. Coverslips were mounted to microscope slide and cells were observed under confocal microscope.

For the flow cytometry, cells were seeded as 300,000 cells/ well and incubated overnight at 37 °C with 5% CO₂. Stevioside treatment was applied according to section 3.2.2. Then, cells were collected and rinsed with 100 µl PBS. Cells were resuspended in 100µl of 5µg/ml Rhodamine 123 and incubated for 30 minutes in the same incubator conditions. Lastly, samples were analyzed with the flow cytometer.

3.7 REACTIVE OXYGEN SPECIES DETECTION ASSAY

HPNE, Panc 02, and PANC-1 (200,000 cells/well) were seeded into a 6-well plate and incubated overnight at 37 °C with 5% CO₂. Cells were collected into Eppendorf tubes and rinsed with 1X buffer included in the DCFDA kit. After the centrifugation, cells were resuspended in DCFDA (100 µL 20µM in 1X Buffer) and incubated for 30 mins at 37 °C. Results were analyzed using the flow cytometer.

3.8 ATP BIOLUMINESCENCE ASSAY

ATP bioluminescence assay was used to measure the ATP levels in the cell. The assay is based on luciferase and luciferin with the help of a luminometer to measure the light that is emitted. Cells were seeded to the T25 flask (HPNE and Panc 02 500,000 cells, PANC1 1,000,000 cells). After the 24h treatment according to section 3.2.2, cells were trypsinized and transferred to 96 well plates. ATP standards provided by the kit were added to the plate at the concentrations of 10, 1,0.1, 0.01, 0.001, and 0.0001 µM. The detergent (50 µl) was added to every well and centrifuged for 5 minutes at 650 rpm. Then, substrate buffer was added to the wells as 50µl and again centrifuged at 650 rpm for 5 minutes. The plate was incubated at room temperature for 10 minutes and necessary measurements and calculations were performed. Both ATP standards and substrate solution are light sensitive therefore, the experiment was performed in the dark.

3.9 ANNEXIN V + PI STAINING

Annexin V and PI (propidium iodide) staining were used for the determination of the death mechanism caused by the ST treatment, annexin V determines apoptosis while PI is a nuclear

stain that is related to necrotic cell deaths. The combination of annexin V with PI also enables the detection of diverse apoptotic stages. Cells (200,000 cells per well) were seeded and then collected after stevioside treatment following the parameters in section 3.2.2. Samples were transferred to Eppendorf tubes and washed with PBS. Cells were resuspended in 200 μ L of 1X Binding Buffer and centrifuged at 300 *g*. Annexin (3 μ L) was mixed with 200 μ L of binding buffer and cells were incubated in the dark for 15 minutes at room temperature. Cells were washed with 200 μ L binding buffer and centrifuged. PI (1 μ L) was added to 200 μ L of binding buffer and analysis was performed by flow cytometry.

3.10 IMMUNOBLOTTING OF PROTEIN SAMPLES

3.10.1 Cellular Lysis and Isolation of the Proteins

After HPNE, PANC-1, and Panc 02.03 cells were treated with ST for 24H, cells were trypsinized and pellets were washed with PBS and centrifuged at 4°C 14,000 rpm for ten minutes, the supernatant was discarded carefully. RIPA (Radioimmunoprecipitation assay) buffer was added to the pellet including a 4% (v/v) protease inhibitor cocktail (PMSF, DIC, SQ, PI each 1%), and 1% Na₃VO₄ (Sodium Orthovanadate) and incubated at 4°C for 30 minutes. After the incubation, pellets were centrifuged for 10 minutes at 14,000 rpm.

3.10.2 BCA (Bicinchionic Acid) Protein Assay

To determine the protein concentration colorimetric assay called BCA assay can be used, before determination of the protein samples, bovine serum albumin (BSA) protein standard of 2.0 mg/ml was diluted serially to 1.0, 0.75, 0.50, and 0.25 mg/ml. After the preparation of the BSA standards, the working solution was prepared and the reagent A to reagent B ratio to each other was taken as 50:1 according to the kit. BSA standards and working solution were transferred to the 96 well plates as 10 and 90 μ l respectively to finalize the volume of 100 μ l per well. The protein samples were diluted 1:5 in RIPA buffer, and samples and working solution were added to the wells as 5 and 95 μ l respectively to finalize the volume 100 μ l per well. Both BSA and protein samples were added as 3 replicates.

Finally, the 96-well plate was incubated at 37°C for 30 minutes and absorbance values were measured at 562 nm by the plate reader.

3.10.3 Sample Preparation and SDS- PAGE

According to the BCA protein quantification results 20 µg protein was mixed with 2X Laemmli buffer and incubated at 37°C for 30 minutes. An electrophoresis tank was prepared and 1X running buffer was poured into the tank. Samples were loaded to the 4-20% gradient gel slowly and run at 150V 40mA for 70 minutes.

3.10.4 Western Blotting

After the SDS-PAGE, the gel was separated from glass plates and conditioned with semi-dry transfer buffer for 5 minutes while the PVDF membrane was washed with 99% methanol and conditioned with transfer buffer for 5 minutes. Whatman papers were soaked in a transfer buffer. The gel and the membrane were put in the Thermo G2 Fast Blotter platform and the transfer was done at 2.5 A 25V for 24 mins. PVDF membrane was transferred into a blocking solution (5% skim milk powder in TBS-T) and incubated for 1 H at RT. The membrane was washed with 1X TBS-T three times. After the rinsing, the primary antibody was prepared in TBS-T (OXPHOS 1:000, LDH-A 1:1000, beta-actin 1:5000, and HSP60 1:5000) and was poured to the membrane and incubated overnight at 4°C then 1 H at RT. The membrane was rinsed with TBS-T three times and the secondary antibody was prepared in 1X TBS-T (1:5000). The membrane was incubated with the secondary antibody at RT for 120 minutes. After that, the membrane was washed with TBST two times and TBS one time. Lastly, antibodies were visualized by a reagent named western Clarity ECL Substrate with Chemidoc™ XRS+ and necessary analysis was performed with ImageLab software.

3.11 STATISTICAL ANALYSIS

The statistical analysis of the data was conducted by using Microsoft Excel. Analysis of all the experimental data was done by Student's *t*-test with a *p*-value set to 0.05 to determine the statistical significance of the samples with variations. All data are displayed as mean and $\pm$ standard deviation from the mean.



4. RESULTS

4.1 MOLECULAR DOCKING BINDING AFFINITY OF STEVIOSIDE WITH PROTEINS

Molecular docking studies were performed by SwissDock before *in vitro* experiments to check possible targets. Five targets GLUT1, LDHA, Caspase-3, LC-3, and VEGFA were selected with their known substrates and/or inhibitors. Calculated binding free energies (ΔG) and full fitness showed that in all proteins stevioside showed lower ΔG with a higher full fitness score (Table 4.1). Stevioside showed the highest binding affinities which almost two times lowered the ΔG in both GLUT1 and LDHA compared to their substrates glucose and pyruvate respectively. Also, caspase-3 and VEGFA stevioside showed a small decrease in ΔG however, caspase-3 full fitness score decreased by 30% compared to its substrate Ac-DEVD-CHO and shows unfavorable docking compared with other fitness scores.

Table 4.1 Molecular docking scores of ligands in their best confirmation.

Name of the Protein (PDB ID)	Stevioside		Substrate		Inhibitor	
	ΔG (kcal/mol)	Full Fitness (kcal/mol)	ΔG (kcal/mol)	Full Fitness (kcal/mol)	ΔG (kcal/mol)	Full Fitness (kcal/mol)
GLUT1 (6THA)	-12.29	-1049.17	-7.19	-1245.00	-	-
LDHA (4ZVV)	-11.12	1470.90	-6.67	-1694.44	-8.74	-1649.73
Caspase-3 (7RNC p12)	-9.08	-720.38	-8.00	-1036.89	-	-
LC-3 (5MS6)	-9.67	-1028.54	-	-	-	-
VEGFA (6ZFL)	-9.93	-1027.08	-	-	-8.59	-1302.96

GLUT1 Substrate: glucose, LDHA substrate: pyruvate, LDHA inhibitor: GNE-140, Caspase-3 substrate: Ac-DEVD-CHO, VEGFA inhibitor: SOR

4.2 THE INTERACTION BETWEEN GLUT1 AND STEVIOSIDE

Interaction and similar amino acids between GLUT1 and ST or glucose (substrate) examined by BIOVIA Discovery Studio. Confirmations were chosen according to the lowest ΔG and full fitness scores. Results showed that both glucose and ST bind to GLUT1 neither hydrophobic nor hydrophilic (Fig 4.1 a&b). In addition to hydrophobicity analysis, receptor-ligand interaction of glucose and ST showed that both ligands use the same docking pocket (Fig 4.1 c&d). According to Table 4.2, glucose interacts with 5 different amino acids in the docking site which also partake in stevioside docking.

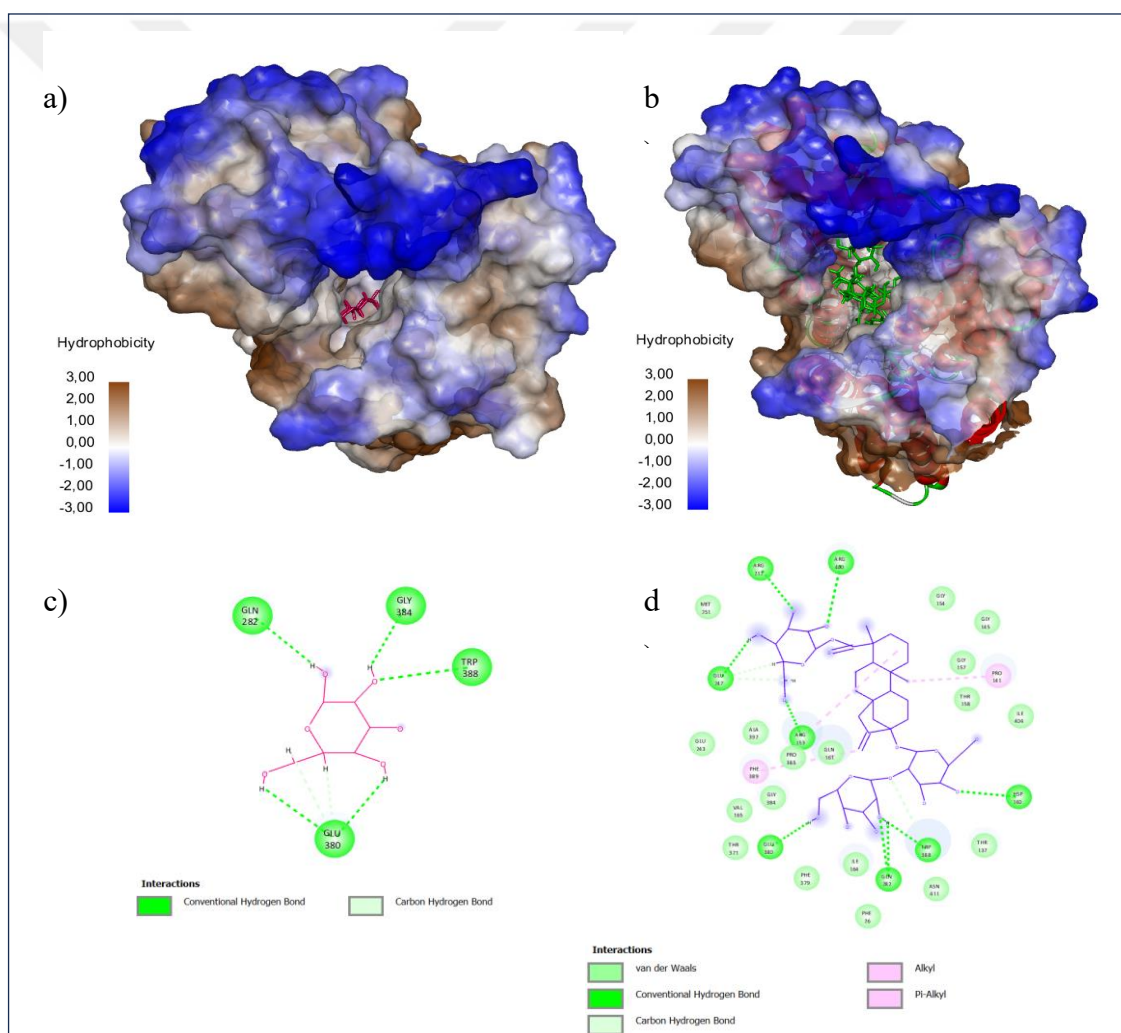


Figure 4.1 Molecular interaction between GLUT1 and glucose and ST in 2D and 3D configuration analyzed by Discovery studio visualizer (BIOVIA 2021). Docking hydrophobicity between A) glucose (red) and GLUT1, B) ST (green) and GLUT1, receptor-ligand interaction between C) glucose and GLUT1, D) ST and GLUT1.

Table 4.2 Docking site amino acids of GLUT1 and interaction with ST and glucose.

Name of the Ligand	H- Bond	Van der Waals	Hydrophobic interactions
Glucose	GLN 282, GLY 384, TRP 388, GLU 380	-	-
ST	ARG 153, GLU 247, ARG 212, ARG 400, HSP 160, TRP 388, GLN 282, GLU 380	GLU 243, ALA 392, PRO 385, GLN161, GLY 384, VAL 165, THR 321, PHE 379, ILE 164, ASN 411, THR 137, ILE 404, THR 158, GLY 157, GLY 145, GLY 154, MET 251	PHE 389, PRO 141

Amino acids written in blue are identical and involved in docking for both glucose and ST.

4.3 THE INTERACTION BETWEEN LDHA AND STEVIOSIDE

Interaction and similar amino acids between LDHA and pyruvate (substrate), ST, or GNE-140 (inhibitor) were examined. Confirmations were chosen according to the lowest ΔG and full fitness scores. Results showed that the docking site of the pyruvate and GNE-140 is more hydrophobic than ST (Fig 4.2 a, b&c). In addition to hydrophobicity analysis, receptor-ligand analysis showed 50% of the docking site amino acids of pyruvate are identical to the ST. Also, ST shared 61% of the GNE-140 docking site amino acids in the docking site with similar chemical interaction showing a high possibility of using the same docking pocket (Table 4.3).

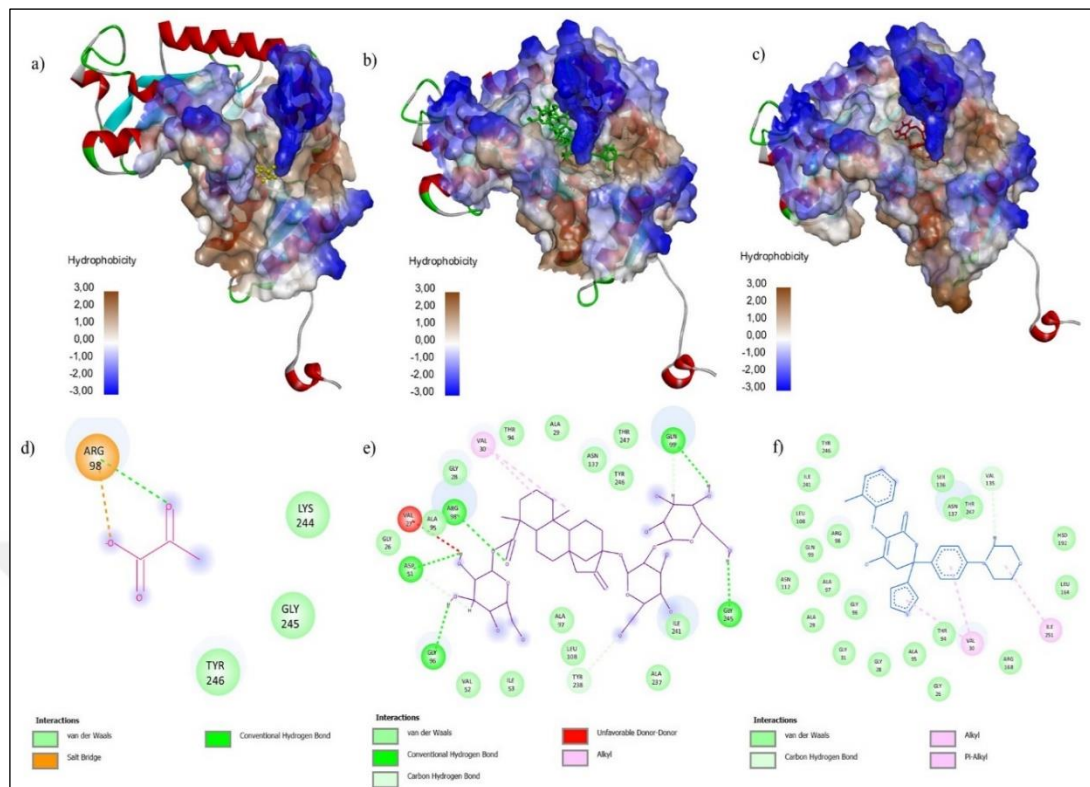


Figure 4.2 Molecular interaction between LDHA and pyruvate, ST and GNE-140 in 2D and 3D configuration analyzed by Discovery studio visualizer (BIOVIA 2021). Docking hydrophobicity between A) pyruvate (yellow) and LDHA, B) ST (green) and LDHA, C) GNE-140 (red) and LDHA, receptor-ligand interaction between D) pyruvate and LDHA, E) ST and LDHA, F) GNE-140 and LDHA.

Table 4.3 Docking site amino acids of LDHA and interaction with ST, pyruvate, and GNE-140.

Name of the Ligand	H- Bond	Van der Waals	Hydrophobic interactions
Pyruvate	-	LYS 244, <u>GLY 245</u> , TYR 246	<u>ARG 98</u>
ST	<u>GLY 96</u> , ASP 51, <u>ARG 98</u> , <u>GLN 99</u> , <u>GLY 245</u>	<u>GLY 26</u> , <u>ALA 95</u> , <u>GLY 28</u> , <u>THR 94</u> , <u>ALA 29</u> , <u>ASN 137</u> , <u>TYR 246</u> , <u>THR 247</u> , <u>ILE 241</u> , ALA 237, <u>ALA 97</u> , <u>LEU 108</u> , TYR 238, ILE 53, VAL 52	VAL 27, <u>VAL 30</u>
GNE 140-	-	<u>TYR 246</u> , <u>ILE 241</u> , <u>LEU 108</u> , <u>ARG 98</u> , <u>GLN 99</u> , <u>ASN 112</u> , <u>ALA 97</u> , <u>GLY 96</u> , <u>ALA 29</u> , <u>GLY 31</u> , <u>GLY 28</u> , <u>ALA 95</u> , <u>GLY 26</u> , <u>THR 94</u> , ARG 168, LEU 164, HSD 192, VAL 135, <u>THR 247</u> , <u>ASN 137</u> , SER 136	<u>VAL 30</u> , ILE 251

Amino acids written in blue are identical and involved in docking for both pyruvate and ST. Underlined amino acids are identical and involved in docking for both GNE-140 and ST.

4.4 THE INTERACTION BETWEEN CASPASE-3 AND STEVIOSIDE

Interaction and similar amino acids between caspase and ST or Ac-DEVD-CHO (substrate) were examined. Confirmations were chosen according to the lowest ΔG and full fitness scores. Results showed that the docking site of the ST is more hydrophilic than Ac-DEVD-CHO (Fig 4.3 A&B). In addition to hydrophobicity analysis, receptor-ligand analysis showed there are no common amino acids that partake in the docking of the ST and Ac-DEVD-CHO (Table 4.4). As a result, the best conformational scores of the ST and Ac-DEVD-CHO bind caspase-3 using different docking sites.

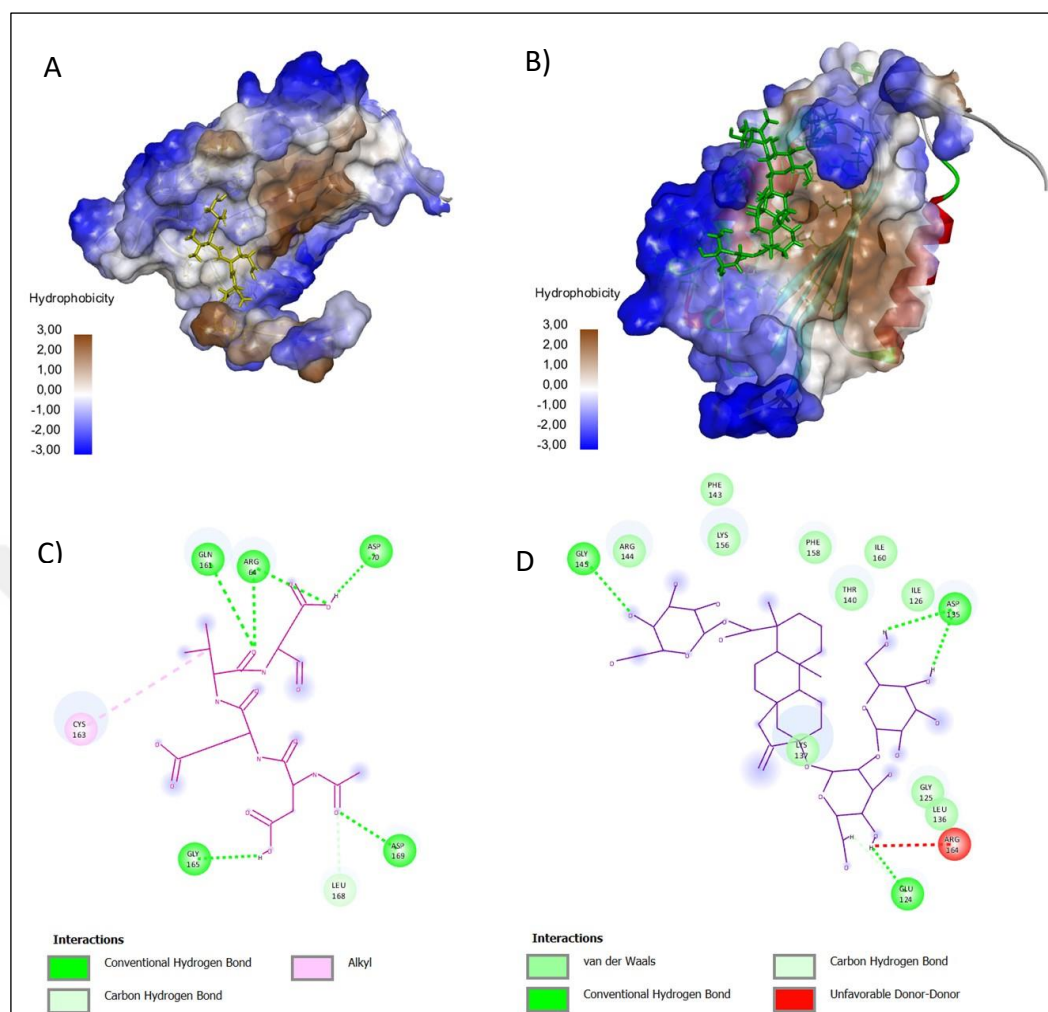


Figure 4.3 Molecular interaction between caspase-3 and ST and Ac-DEVD-CHO in 2D and 3D configuration analyzed by Discovery studio visualizer (BIOVIA 2021). Docking hydrophobicity between A) Ac-DEVD-CHO (yellow) and caspase-3, B) ST (green) and caspase-3, receptor-ligand interaction between C) Ac-DEVD-CHO and caspase-3, D) ST and caspase-3.

Table 4.4 Docking site amino acids of caspase-3 and interaction with ST and Ac-DEVD-CHO.

Name of the Ligand	H- Bond	Van der Waals	Hydrophobic interactions
Ac-DEVD-CHO	GLN 161, ARG 64, ASP 70, ASP 169, GLY 165	LEU 168	CYS 163
ST	GLY 145, GLU 124, ASP 135	ARG 144, PHE 143, LYS 156, PHE 158, THR 140, ILE 160, ILE 126, GLY 125, LEU 136, LYS 137	ARG 164

Amino acids written in blue are identical and involved in docking for both Ac-DEVD-CHO and ST.

4.5 THE INTERACTION BETWEEN LC-3 AND STEVIOSIDE

Interaction and similar amino acids between LC-3 and ST were examined. Confirmations were chosen according to the lowest ΔG and full fitness scores. Results showed that the docking site of the ST is highly hydrophilic (Fig 4.2 a) and receptor-ligand analysis showed more than 50% of the docking site amino acids bind to ST by van der Waals interactions (Table 4.5).

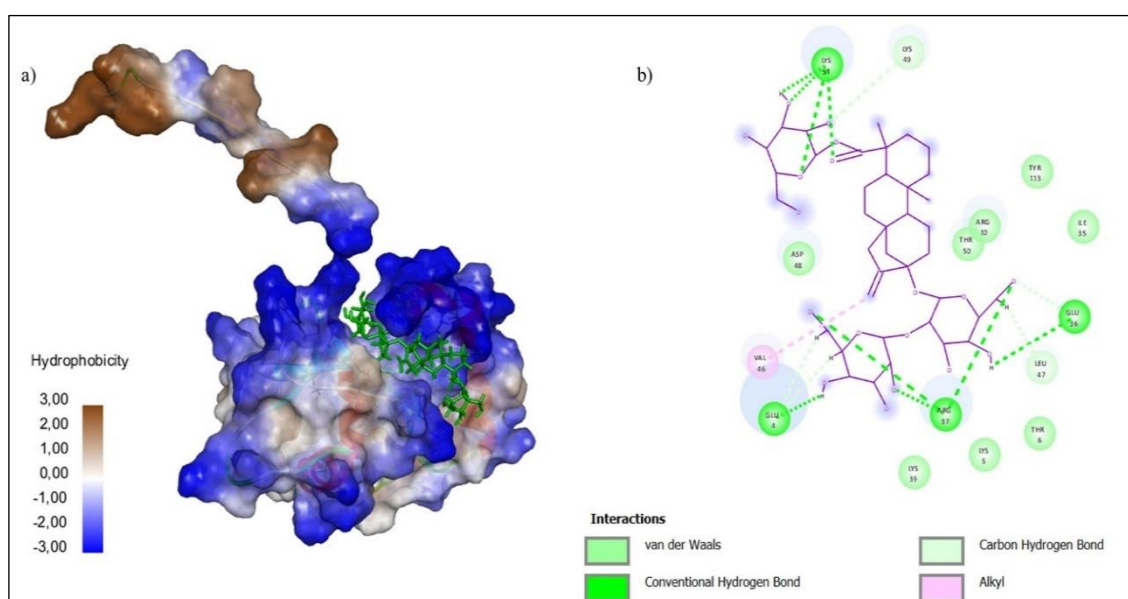


Figure 4.4 Molecular interaction between LC-3 and ST in 2D and 3D configuration analyzed by Discovery studio visualizer (BIOVIA 2021). Docking hydrophobicity between A) ST (green) and LC-3, receptor-ligand interaction between B) ST and LC-3.

Table 4.5 Docking site amino acids of LC-3 and interaction with ST.

Name of the Ligand	H- Bond	Van der Waals	Hydrophobic interactions
ST	LYS 51, GLU 4, ARG 37, GLU36	LYS 49, ASP 48, LYS 39, LYS 5, THR 6, LEU 47, THR 50, ARG 10, ILE 35, TYR 113	VAL 49

4.6 THE INTERACTION BETWEEN VEGFA AND STEVIOSIDE

Interaction and similar amino acids between VEGFA and ST or SOR (inhibitor) were examined. Confirmations were chosen according to the lowest ΔG and full fitness scores. Results showed that the docking site of both SOR and ST is neither hydrophilic nor hydrophobic (Fig 4.4 a&b). Also, receptor-ligand analysis showed almost 60% of the docking amino acids are common in both ST and SOR binding pockets with similar chemical interaction which shows in their best confirmation ST and SOR using the same binding pocket (Table 4.6).

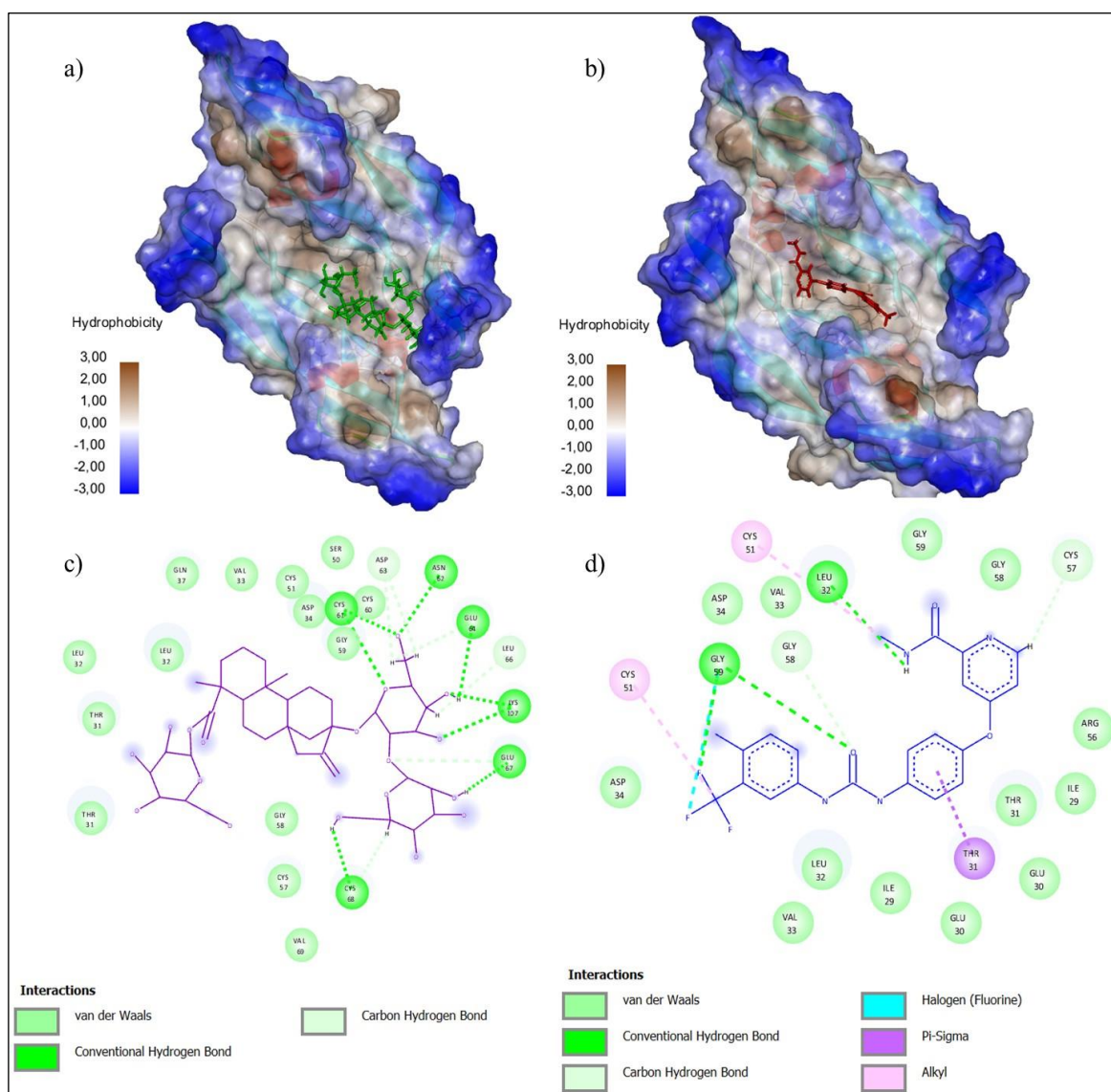


Figure 4.5 Molecular interaction between VEGFA and ST and SOR in 2D and 3D configuration analyzed by Discovery studio visualizer (BIOVIA 2021). Docking hydrophobicity between A) ST (green) and VEGFA, B) SOR and VEGFA receptor-ligand interaction between C) ST and VEGFA, and D) SOR and VEGFA.

Table 4.6 Docking site amino acids of VEGFA and interaction with ST and SOR.

Name of the Ligand	H- Bond	Van der Waals	Hydrophobic interactions
ST	CYS 68, GLU 67, LYS 107, GLU 64, ASN 62, CYS 61	LEU 32, THR 31, GLY 58, CYS 57, VAL 69, LEU 66, ASP 63, CYS 60, GLY 59, ASP34, SER 50, CYS 51, VAL 33, GLN 37	-
SOR	GLY 59, LEU 32	ASP 34, VAL 33, LEU 32, ILE 29, GLU 30, THR 31, ARG 56, CYS 57, GLY 58	CYS 51, GLY59, THR 31

Amino acids written in blue are identical and involved in docking for both SOR and ST.

4.7 CELL VIABILITY DETERMINATION OF PDAC CELLS TREATED WITH STEVIOSIDE

The effect of 100 μ M ST on cell viability was determined by MTS assay on HPNE, Panc 02, and PANC-1 cell lines. Time-dependent cell viability of the ST treatment showed that, on normal pancreatic cell line HPNE, no anti-proliferative effect was observed in all 24, 48, and 72 h treatments. In contrast, in PDAC cell lines Panc 02 and PANC-1 stevioside showed an antiproliferative effect. ST decrease the cell viability to 93 and 86 percent in 24 H, 84 and 85 percent in 48 H treatment, and 66 and 73 percent in 72 H in Panc 02 and PANC-1 cells, respectively (Fig 4.6).

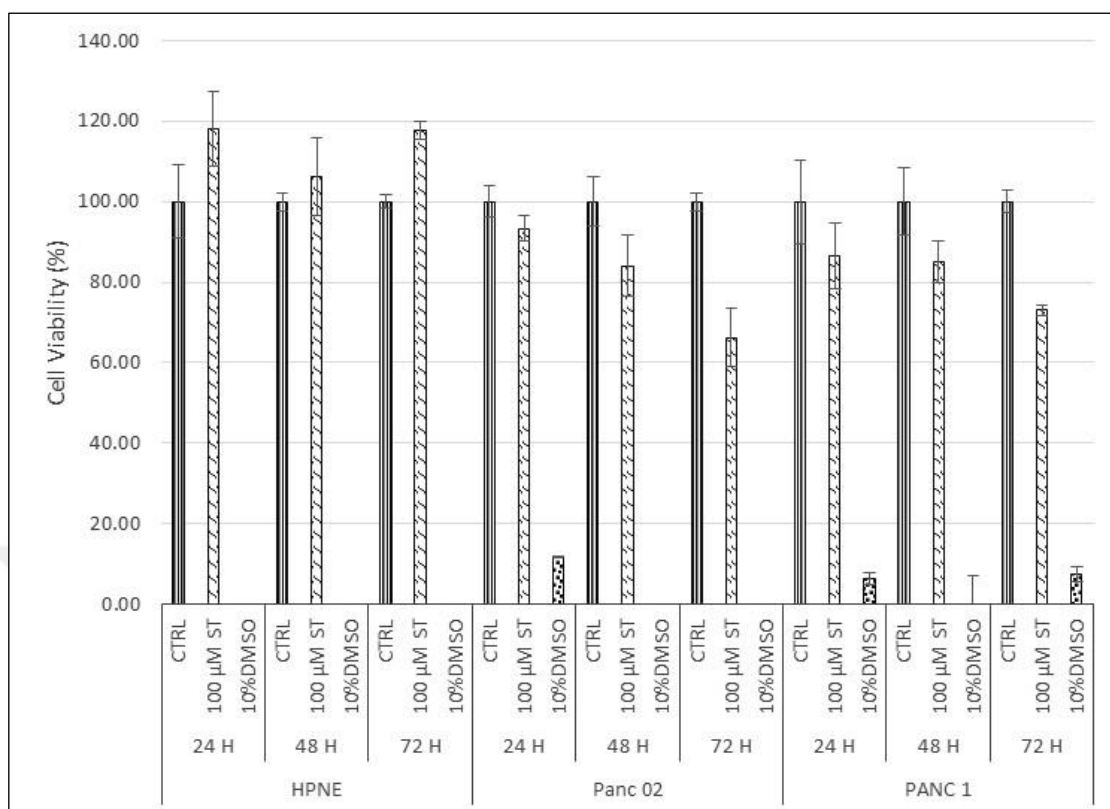


Figure 4.6 Cell growth inhibition determination of ST treatment for 24, 48, and 72 H on HPNE, Panc 02, and PANC-1 cell lines by MTS assay. The data were shown as mean $\pm$ standard deviation. CTRL: Control, ST: Stevioside (100 μ M).

4.8 DETERMINATION OF CELL DEATH MECHANISM BY ANNEXIN V-FITC + PI STAINING

According to time-dependent cell viability results and circulation time of the stevioside in humans [54], cell death mechanisms related to ST treatment were examined in 24 and 48 H by Annexin V-FITC +PI staining by flow cytometry. ST treatment for 24 H increased the early apoptosis almost 2 times, and necrosis 2.5 times in Panc02. In PANC-1, ST treatment for 24 H increases the late apoptosis and necrosis rates up to 1.5 times. In 48 H, treatments, stevioside increased the early apoptosis four times with a 3-fold decrease in Panc 02, and in PANC-1 ST decreased the necrosis rates by twenty percent with an increase in early apoptosis (Fig 4.7).

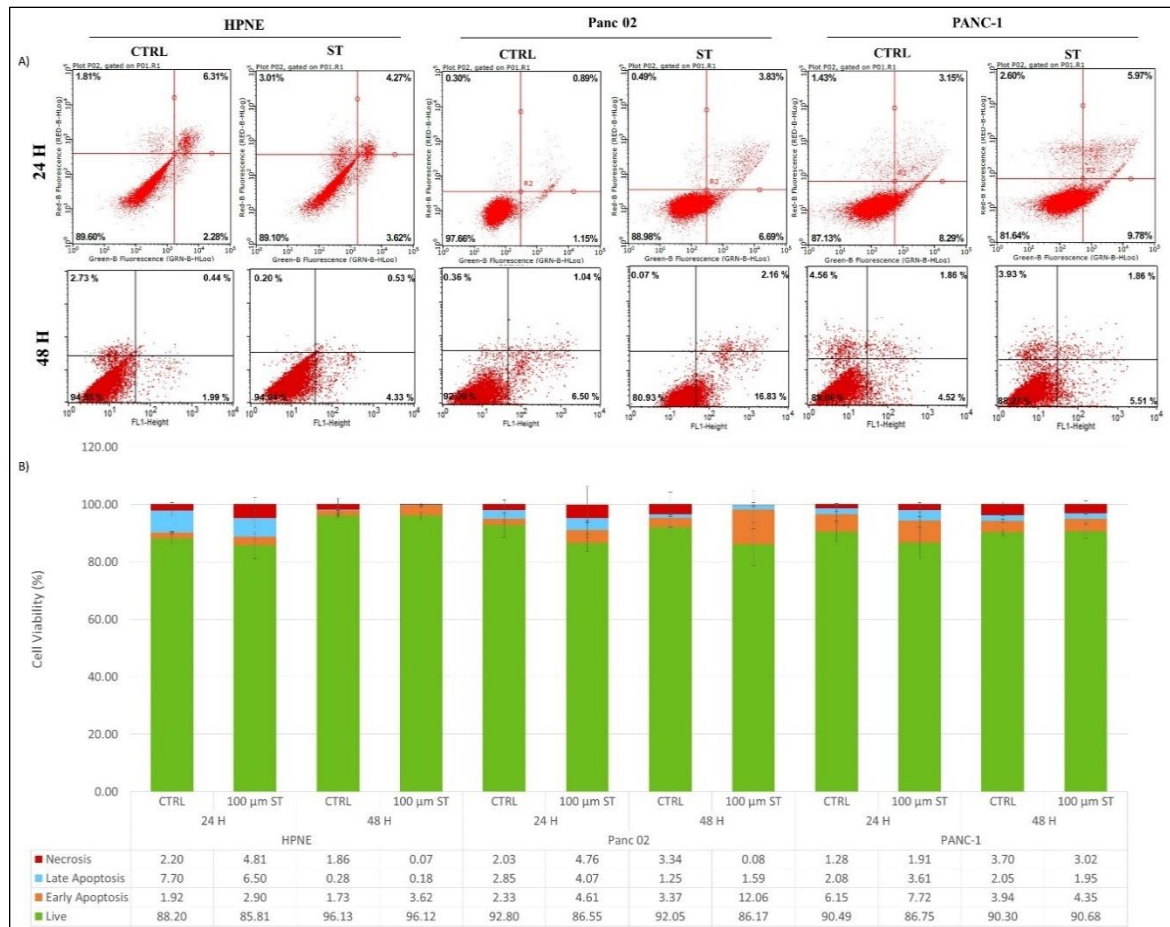


Figure 4.7 Cell death mechanisms of HPNE, Panc 02, and PANC-1 after ST treatment for 24 and 48 H were determined by FACSCalibur™ with Annexin V-FITC + PI staining. A) Cell death mechanism analysis by flow cytometry, lower left: living cells, lower right: early apoptosis, upper right: late apoptosis, upper left: necrosis B) Percentage analysis of living, apoptotic, and necrotic cells after ST treatment.

4.9 DETERMINATION OF INTRACELLULAR ROS LEVELS OF PDAC CELLS TREATED WITH ST

ROS are important molecules for maintaining cellular homeostasis and signaling. Under normal physiology, high ROS levels or oxidative stress cause DNA damage and abnormalities in signaling mechanisms which triggers cell death mechanisms. Intracellular ROS levels were determined by DCFDA/H2DCFDA staining by flow cytometry after 24 H ST treatment. There was no significant ROS level change observed in cancer cells (Panc 02,

PANC-1) compared to normal cells (HPNE) with ST treatment. However, ST treatment caused ROS levels to almost double in all cell lines (Fig 4.8).

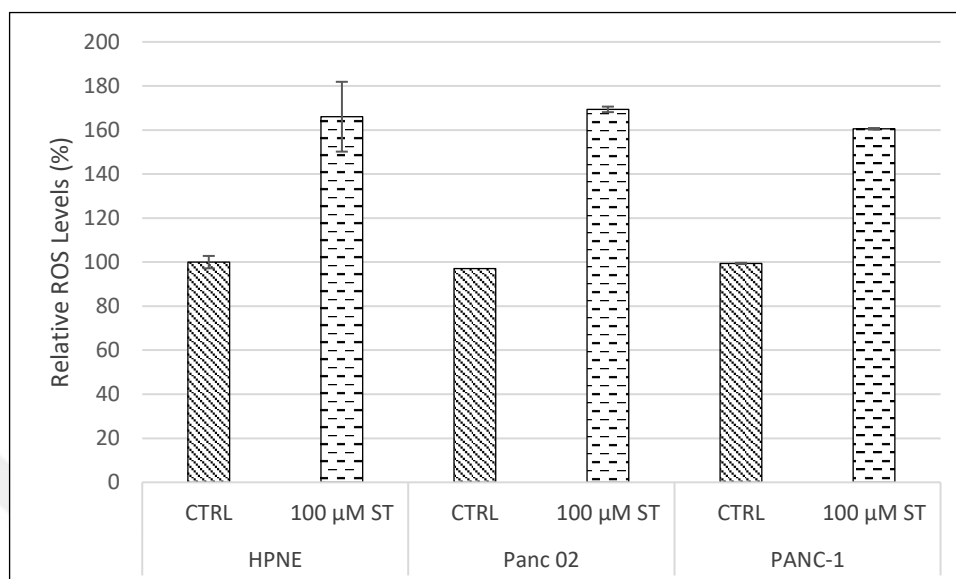


Figure 4.8 Relative changes in intracellular ROS levels after 24 H ST treatment of HPNE, Panc 02, and PANC-1 determined with DCFDA/H2DCFDA staining by flow cytometry. The data is shown as mean $\pm$ standard deviation.

4.10 LIVE CELL IMAGING OF MITOCHONDRIAL MEMBRANE POTENTIAL AND MITOCHONDRIAL MASS OF PDAC CELLS BY CONFOCAL MICROSCOPY

ST treatment-related mitochondrial changes were visualized by Rhodamine 123 for $\Delta\Psi_m$ and MitoTracker Green FM for mitochondrial mass by confocal microscopy in HPNE, Panc 02, PANC-1. MitoTracker showed that mitochondrial mass was decreased, and the tubular structure of mitochondria changed to a spherical in all cell lines. Rhodamine 123 stain showed that $\Delta\Psi_m$ was not affected by ST treatment in HPNE and decreased in Panc 02 cells. In contrast, $\Delta\Psi_m$ was increased in PANC-1 cells (Fig 4.9 A, B, and C). FCCP is a synthetic mitochondrial uncoupler used as a Rhodamin 123 negative control [71]. The ratio of $\Delta\Psi_m$ to mitochondrial mass was analyzed by using flow cytometry results (Fig 4.10). $\Delta\Psi_m$ to mass ratio showed that ST treatment increased the $\Delta\Psi_m$ per mitochondrion in HPNE and PANC-1 two times, however, in Panc 02 potential per mitochondrion decreased to almost 3-fold (Fig 4.9 E).

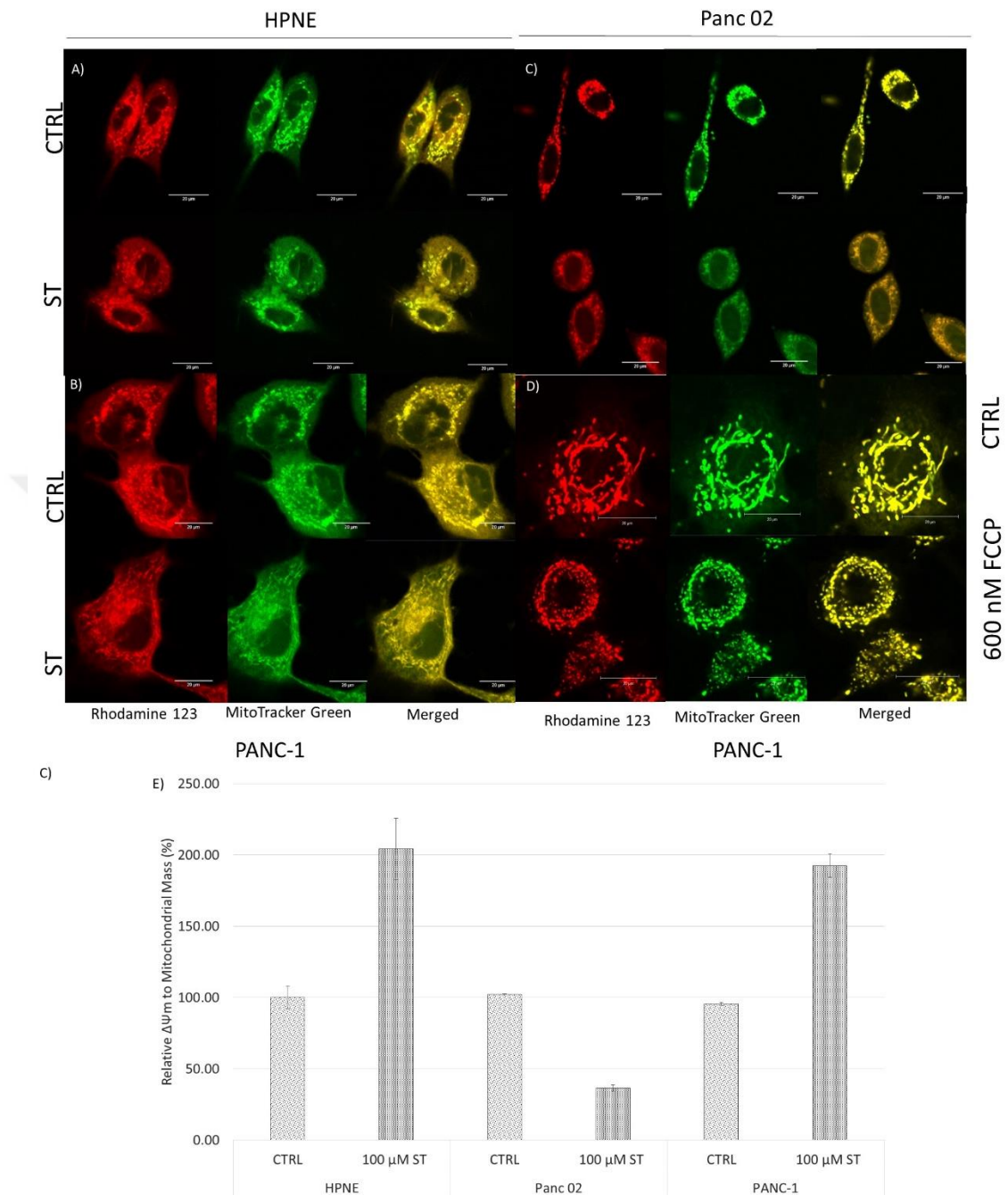


Figure 4.9 Mitochondrial membrane potential ($\Delta\Psi_m$) and mass were detected by Rhodamine 123 and MitoTracker Green FM staining, respectively by Zeiss Confocal microscope, and the ratio of mitochondrial membrane potential to mass was quantified by flow cytometry Rhodamine 123 and MitoTracker Green FM staining. 24 H stevioside treatment of HPNE (A), Panc 02 (B), PANC-1 (C), 2 H 600 nM FCCP treatment (D), relative changes in mitochondrial potential to mitochondrial mass of 24 h ST treatment of HPNE, Panc 02, and PANC-1.

4.11 QUANTITATIVE ANALYSIS OF MITOCHONDRIAL MEMBRANE POTENTIAL AND MASS BY FLOW CYTOMETRY

ST treatment-related quantitative mitochondrial changes were analyzed by Rhodamine 123 for $\Delta\Psi_m$ and MitoTracker Green FM for mitochondrial mass by flow cytometry in HPNE, Panc 02, PANC-1. Rhodamine 123 stain showed that $\Delta\Psi_m$ was not affected by ST treatment in HPNE and decreased almost 4-fold in Panc 02 cells. In contrast, $\Delta\Psi_m$ was increased 1.5 times in PANC-1 cells (Fig 4.10 A). MitoTracker showed that mitochondrial mass was decreased in all cell lines. Especially in normal cell line HPNE, mitochondrial mass decreased to 50% (Fig 4.10 B).

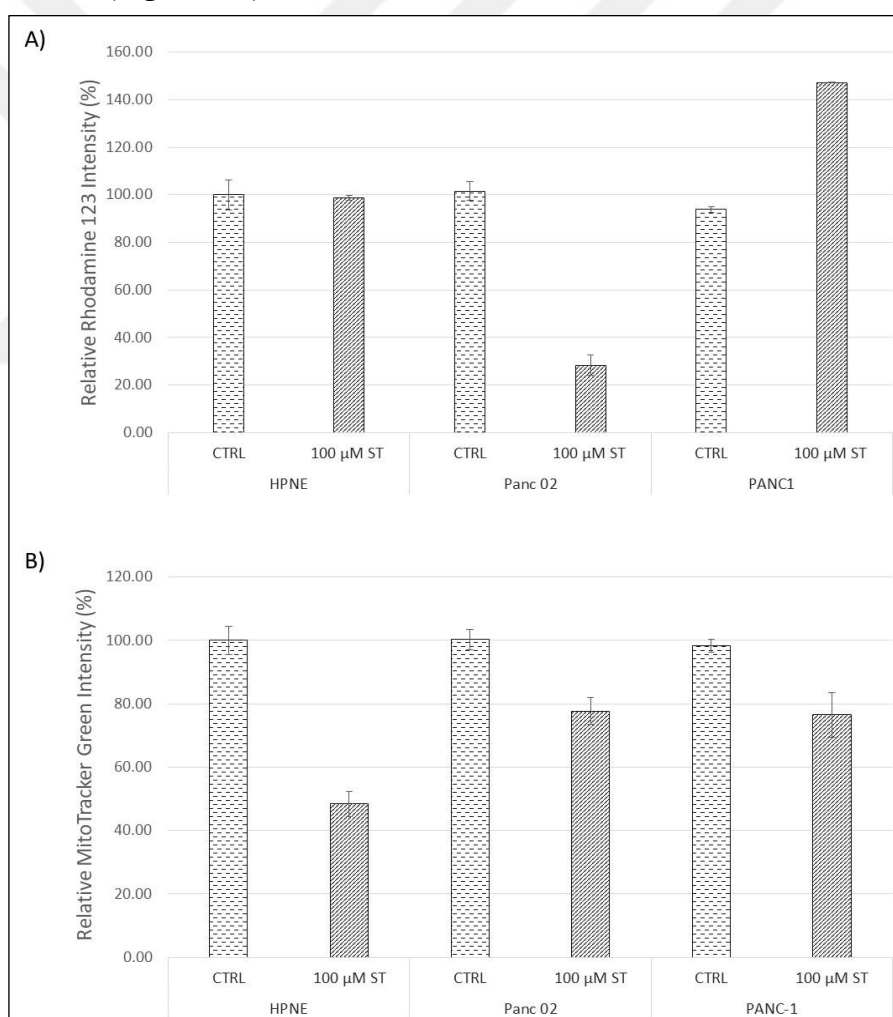


Figure 4.10 Quantitative mitochondrial membrane potential and mass were analyzed by Rhodamine 123 and MitoTracker Green FM staining, respectively by flow cytometry. Relative changes after 24 h ST treatment in $\Delta\Psi_m$ (A), mitochondrial mass (B). All data is displayed as mean $\pm$ standard deviation.

4.12 MEASUREMENT OF TOTAL ATP LEVELS

According to mitochondrial changes, ST treatment reduces mitochondrial mass in all cell lines and $\Delta\Psi_m$ in Panc 02 with an increase in PANC-1. Intracellular ATP levels were calculated by ATP chemiluminescence assay and the signal obtained from ATP standards. The catalysis of ATP oxidation was performed by luciferase to calculate the concentration of the ATP. ATP levels were found PANC-1, Panc 02, and HPNE from highest to lowest, respectively. In both HPNE and Panc 02 ATP levels increased 1.2- fold, in contrast, ATP levels decreased 33 % in PANC-1 cells (Fig 4.11).

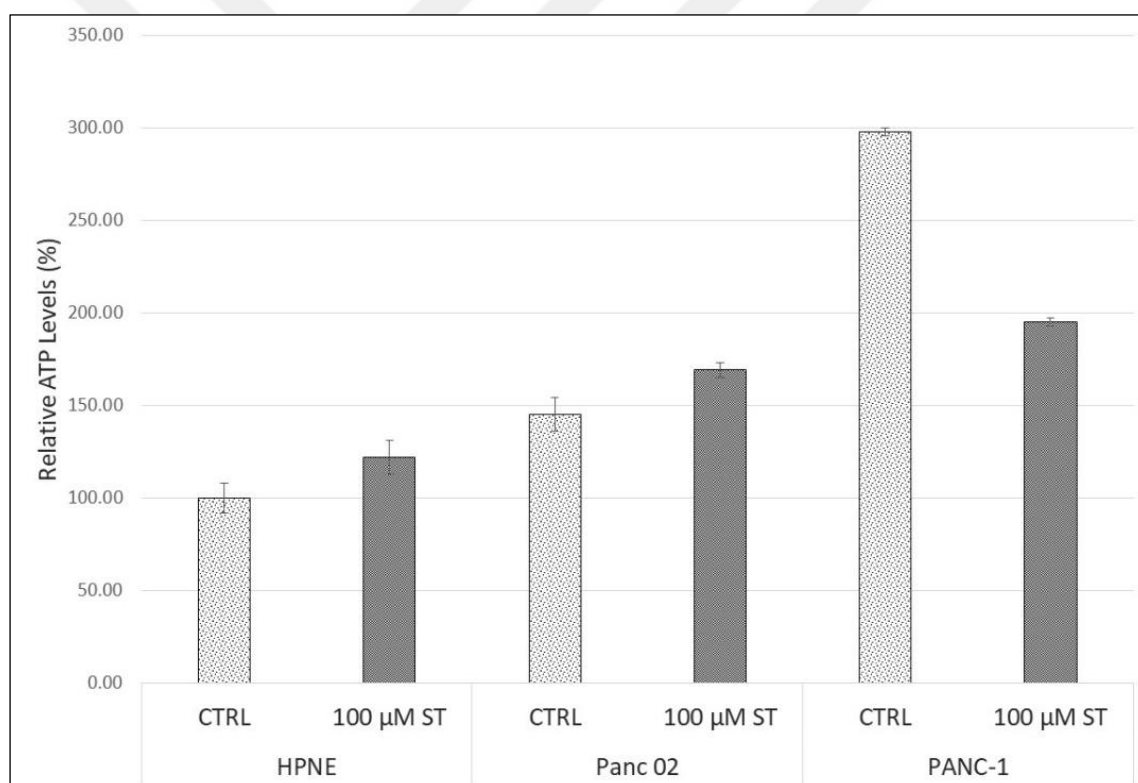


Figure 4.11 Changes in the cellular ATP levels after 24h ST treatment by chemiluminescence measurement. All data is demonstrated as mean $\pm$ standard deviation

4.13 DETERMINATION OF CHANGES IN OXPHOS COMPLEXES EXPRESSION LEVELS IN ST-TREATED PDAC CELLS

Protein complexes of OXPHOS were examined to understand the effect of ST treatment on mitochondrial bioenergetic. OXPHOS complexes expression levels were determined via immunoblotting assay. The results were normalized separately both to HSP60 and GAPDH. On the mitochondrial level, complex III expression level was increased 1.5-fold in HPNE and decreased 5-fold in Panc 02 cells treated with ST, also, complex IV expression was decreased in Panc 02. In the PANC-1 cell line, expression levels of complex I, II, and V were increased 4, 6, and 1.3-fold, respectively related to ST treatment (Fig 4.12). According to cellular level expression levels of GAPDH, ST treatment increased the complex III expression 1.3-fold in HPNE and decreased 0.8, and 0.4-fold in Panc 02 and PANC-1, respectively. In addition, treatment increased the complex V levels in Panc 02 1.4-fold and complex I levels in PANC-1 3-fold.

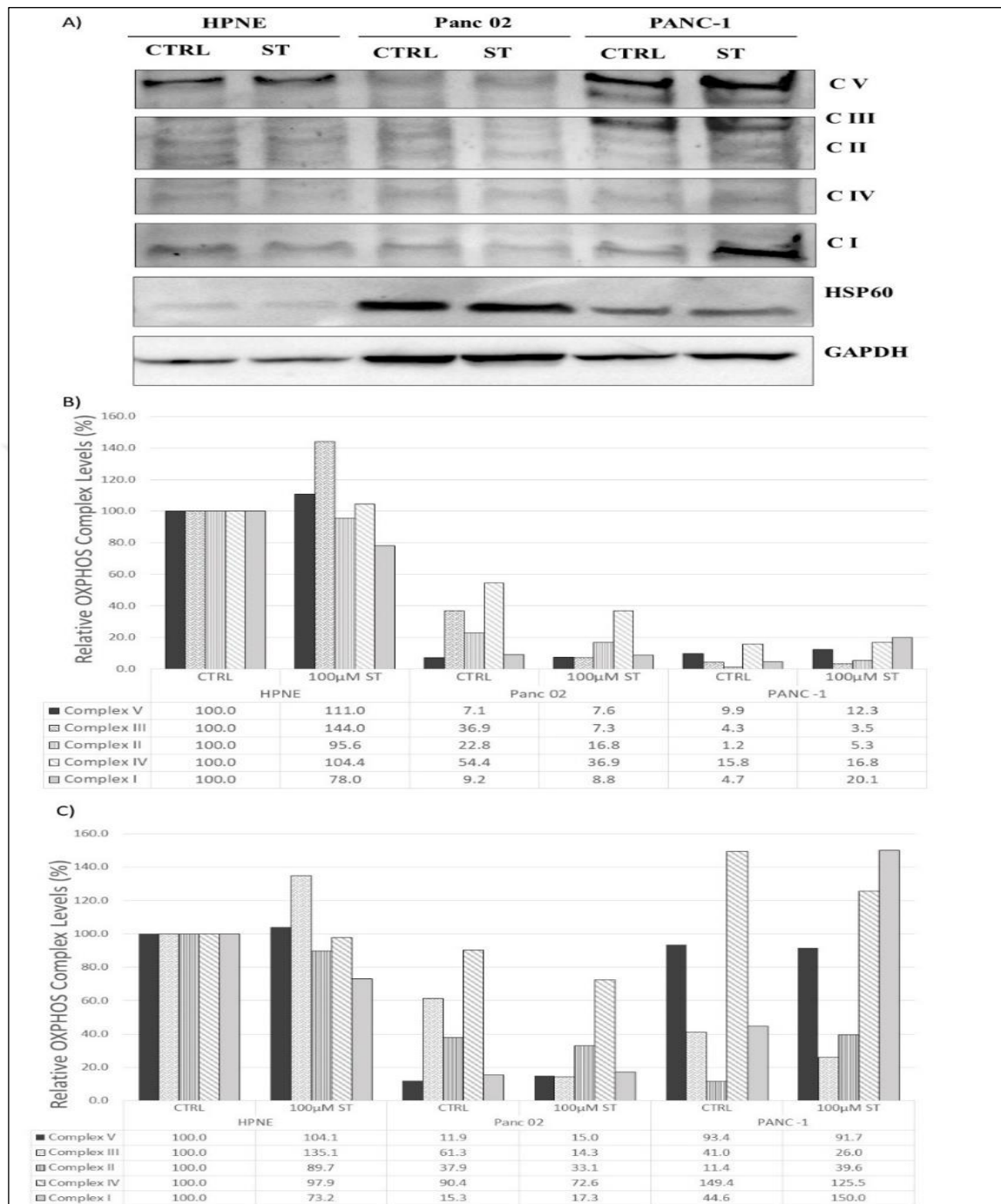


Figure 4.12 The changes in the OXPHOS complexes levels were examined by immunoblotting. Immunoblotting results of OXPHOS complexes complex 1 (CI), complex II (CII), complex III (CIII), complex IV (CIV), and complex V (CV) (A). Relative changes in the OXPHOS complexes compared with HSP60 (B), GAPDH (C).

4.14 DETERMINATION OF THE CHANGES IN LDHA LEVELS IN PDAC CELLS WITH ST TREATMENT

The last step of glycolysis is to convert pyruvate to lactate by LDHA. According to docking high-affinity results, intracellular ATP levels, and mitochondrial changes, variations in the LDHA expression level were examined by immunoblotting. Normalization of the data was performed according to GAPDH expression levels. The highest glycolysis rate was found in HPNE normal cells and decreased 0.4-fold with ST treatment. In addition to HPNE, LDHA expression levels were increased 1.7-fold in Panc 02 cell line (Fig 4.13)

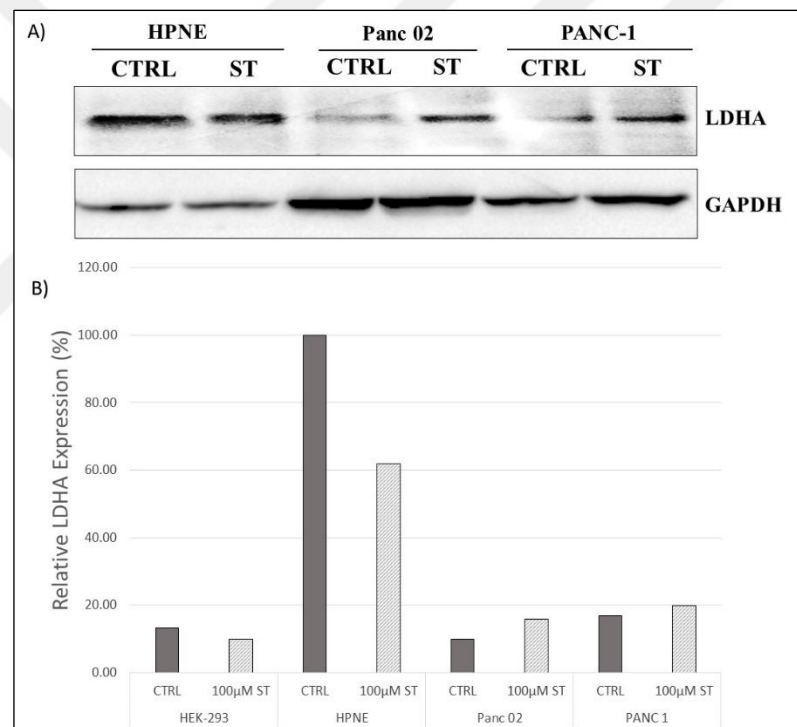


Figure 4.13 The changes in the LDHA levels were examined by immunoblotting. Immunoblotting results of LDHA (A). Relative changes in the LDHA compared with GAPDH (B).

5. DISCUSSION

Pancreatic cancer global burden dramatically increased since the 1990s. It is predicted to be a leading cause of mortality associated with cancer [36]. PDAC is the seventh cause of cancer-related deaths with a poor prognosis and a 5-year survival rate of 5%[2]. Even with the recent developments in molecular oncology and tumor immunology, combinations of chemotherapy remain central therapy as the first line. ST is an extract of stevia and diterpene glycoside containing three molecules of glucose with a steviol backbone [47]. Studies have been shown that ST demonstrates anti-hyperglycemic and anti-diabetic effects and a promising nutrient for cancer therapy. The effect of stevioside on PDAC cells Panc 02 and PANC-1 and normal pancreatic ductal cell HPNE was investigated.

The targets of ST in cancer types are unclear. Therefore, in the first part of the thesis, *in silico* protein-ligand docking study was performed for different cellular pathways important for cancer progression such as for glycolysis GLUT1 and LDHA, apoptosis caspase-3, angiogenesis VEGFA, and autophagy LC-3 was chosen. Next, *in vitro* analysis of PDAC cells was performed. Docking results showed that binding of ST to the active site of proteins is favored according to ΔG , however, the fitness of the ST to the docking site is decreased compared to known substrates and/or inhibitors analyzed (Table 4.1). Stevioside is a compound that contains 3 glucose molecules, as a result, ST can compete with glucose which is used in glycolysis for transport across the membrane by GLUT1 in theory. Results of docking showed ST uses the same amino acids as glucose in the active site (Fig 4.1 and Table 4.2) and almost doubled the binding affinity compared to glucose. Pyruvate is the substrate of LDHA that is converted to the final product of glycolysis lactate and GNE-140 is the inhibitor of LDHA whose active site is adjacent to the NADH [69]. ST showed a high binding affinity with LDHA and more similar chemical interaction with GNE-140 compared to pyruvate which means ST might act as an inhibitor of LDHA according to *in silico* docking results (fig 4.2 and Table 4.3). Docking studies of caspase-3 showed ST has a higher docking affinity compared with its inhibitor however full fitness score decreased significantly. In addition to affinity, there was no common amino acid detected in ST and Ac-DEVD-CHO docking which means the stevioside active site is different from the caspase-3 inhibitor. In addition to docking analysis, the study performed by Chen *et al.* showed that caspase-3 was upregulated by ST and triggered apoptosis in bladder cancer

cells, and the role of ST in caspase-3 upregulation was controlled by caspase-3 inhibitors showing that apoptosis triggered by ST inhibited by caspase-3 inhibitors [60]. As a result, according to this docking analysis, stevioside can directly affect caspase-3 activation and expression levels. Angiogenic biomarker VEGFA-ST docking was also performed and compared with SOR docking. Active site amino acids of both ligands are 60% identical which shows stevioside might have an inhibitory effect on angiogenesis. Diterpene structures can both induce apoptosis or autophagy [72, 73], therefore, LC-3 docking studies were performed for ST, but no inhibitor or substrate is used due to a lack of direct inhibitor or substrate of LC-3. Results showed ST can bind to LC-3 with higher affinity compared to caspase-3 both as full fitness and delta G scores.

In the second part, *in vitro* analysis of ST was performed using normal and cancer pancreatic ductal cells. Time-dependent cell growth inhibition of the ST in HPNE, Panc 02, and PANC-1 was observed by the MTS assay. In HPNE, no growth inhibitory effect was observed related to ST in all 24-, 48- and 72-H treatments. In Panc 02, cell viability decreased to 93% in 24 H, 84 percent in 48, and 66% in 72 H ST treatment. In PANC-1 cells, cell viability decreased to 86 percent in 24 H, 85 percent in 48 H, and 73 percent in 72 H treatment (Fig 4.6). MTS results of the ST showed no significant growth-inhibitory effect in PDAC cells, IC50 was not obtained by changing treatment time and the highest inhibition was observed in 72 H. However, at 72 H, stevioside becomes completely metabolized and removed from the body in human studies [54]. As a result, 72-H results are not applicable for the treatment, and cell mechanism observations continue with 24 and 48-H ST treatment.

To examine and quantify the cell death mechanisms Annexin V+PI staining was performed on all cell lines with 24- and 48-H ST treatment. Annexin V + PI indicates two types of death mechanisms apoptosis and necrosis. Previous studies showed stevioside triggers the intrinsic pathway of apoptosis in bladder [60], breast[58], and colon [61] cancer. Annexin V +PI results showed that in normal cells HPNE percentage of the living cells did not change and stevioside related death mechanism is not triggered. However, in PDAC cell Panc 02, ST triggered apoptosis and in Panc 02 total rate of apoptosis increased 1.6- fold in 24 H and 3-fold in 48 H compared to non-treated control cells. Also, ST treatment increases the early apoptosis rate 2-fold in 24 H and 4-fold in 48H treatment and the necrosis by two-fold in 24 H but this increase is eliminated at 48 H. In PANC-1, no change was observed between control and ST treatment in living cells, and total apoptosis rates remain close in both 24 H

and 48 H treatments, on the other hand, in 24 H treatment late apoptosis increased compared to control 1.5-fold and in 48 H treatment early apoptosis rate increased 1.1- fold with a 0.8-fold decrease in the necrosis (Fig 4.7). Changing necrosis rates in cells might be due to elevated cellular stress and ROS formation caused by treatment. However, Annexin V+PI results showed no significant trigger of cell death mechanisms in PDAC cells in both 24 and 48 H. Since there was no significant change in cell viability between 24 and 48 H results of PANC-1 and Panc 02, 24 H treatment was used in the later analysis except cell death mechanism.

Terpene structures especially diterpenes are related to increased cellular ROS levels [73, 74]. Previous studies showed stevioside increases ROS levels significantly in breast [58], colon [61], and bladder carcinoma [60]. In our study, stevioside caused an elevation in the cellular stress levels and increased the intracellular ROS levels 1.7-fold in all cell lines and compared to the control group however no significant variation between normal and pancreatic cancer cell lines was observed (Fig 4.8).

The mitochondrial function was examined due to changes in the cellular ROS level changes as mitochondrial mass and membrane potential. Mitochondrial mass was analyzed with MitoTracker Green and membrane potential was analyzed with Rhodamine 123 by confocal microscopy for visualization and flow cytometry for quantification. ST treatment decreases the mitochondrial mass significantly and tubular visualization of mitochondria becomes spherical in all cell lines (Fig 4.9). Especially in HPNE cells mitochondria mass was halved (48%) and in PDAC cells decrease was close and around 77% (Fig 4.10). Mitochondrial membrane potential was visualized and quantified with Rhodamine 123. In HPNE cells no effect on mitochondrial potential was observed with ST treatment. However, in Panc02 and PANC-1 cells changes in $\Delta\Psi_m$ were observed. In Panc 02 cells, mitochondrial membrane potential is disrupted by ST treatment and as a result, $\Delta\Psi_m$ was decreased significantly to 28%. In contrast, $\Delta\Psi_m$ was increased to 147% in PANC-1 cells. Previous studies also support the changes in $\Delta\Psi_m$ and showed depolarization of the mitochondria and significantly decrease the potential breast cancer [58], bladder cancer[60], and colon cancer[61] with dose-dependent ST treatment. According to flow cytometry results, mitochondrial membrane potential per mitochondrion was calculated. In HPNE, the ratio of $\Delta\Psi_m$ to mitochondrial mass was increased and taken together with the MTS and ROS level changes, ST treatment possibly provides removal of the defective mitochondria in the cell.

Both decrease in mass and mitochondrial potential cause possible energy crisis in Panc 02 cells when taken together with the MTS results. In PANC-1 however, a decrease of the mass with the increase in $\Delta\Psi_m$ causes the ratio of the $\Delta\Psi_m$ /mitochondrial mass to increase causing a possible overload of energy production per mitochondrion and energy crisis.

Cancer cells require higher ATP levels to continue to fulfill metabolic needs and cancer progression to metastasis compared to normal cells. Due to changes in the mitochondrial function with ST treatment, cellular ATP levels were examined. Panc 02 cell line is a non-metastatic pancreatic cell line that contains KRAS mutation and there is no literature found for energetic pathway [75]. In contrast, the PANC-1 cell line is a metastatic type of PDAC that contains a p53 mutation with high invasion capability and fulfills the cellular energy demand by OXPHOS [76, 77]. Results of the ATP assay showed in both HPNE and Panc 02 ATP levels were increased because of ST treatment and PANC-1 ATP levels were decreased significantly (Fig 4.11). Increased ATP levels of HPNE support the proposition of defect mitochondria removal related to ST treatment along with the $\Delta\Psi_m$ /mitochondrial mass ratio. Also, the significant decrease in the PANC-1 cells showed ST treatment related to an energy crisis due to increased mitochondrial function and overload to produce ATP by OXPHOS. In Panc 02 cells ATP levels were increased contrary to expected because of the significant decrease in $\Delta\Psi_m$ /mitochondrial mass. This unexpected result in Panc02 might have been observed due to the Warburg effect which is changing expression levels of LDHA and OXPHOS by increasing glycolysis rate.

Expression levels of OXPHOS and LDHA after ST treatment were examined by immunoblotting assay due to understand the energy balance between glycolysis and oxidative phosphorylation. Increased accumulation of ROS leads to defective OXPHOS complex functions and antioxidant mechanisms. Immunoblotting results of OXPHOS complexes examined according to cellular protein levels by GAPDH and mitochondrial protein levels by HSP60 normalization. In HPNE cells, CIII levels which are responsible for increased almost 1.5-fold both mitochondrial and cellular levels. In Panc 02 cell line, complex III which is responsible for ROS and gradient formation decreased significantly (5-fold) and CIV decreased 1.5-fold compared to HSP60 expression levels. In PANC-1, CI and CV that are related to ATP production increased significantly both compared to cellular and mitochondrial levels. In addition to CI and V, ST treatment increased complex II, which is responsible for NAD^+ and FAD replenishment for TCA cycle function, and levels

significantly increased because of ST treatment [78]. Changes in the OXPHOS complexes expression levels relatively show that the antioxidant mechanism is disrupted with ST-related increased ROS accumulation in PDAC cells. In addition to defects in antioxidant mechanism, ST-related energy crisis might be led to increased expression of ATP synthase (CV) and CI (Fig 4.12).

LDHA is an essential biomarker to indicate the Warburg effect. Lactate is the final product of glycolysis in which pyruvate is converted to lactate by LDHA. An immunoblotting assay was performed to observe LDHA expression levels in normal and cancer pancreatic ductal cells. LDHA levels were decreased due to ST treatment in HPNE cells supporting increasing ATP levels in Figure 4.11 and increased mitochondrial function in Figure 4.9E. In contrast, in Panc 02 cells LDHA levels increased 1.7-fold which means ST-related mitochondrial loss and energy crisis cause energy balance to shift glycolysis to generate required energy for cancer cell survival which can support the increase in the ATP levels in Figure 4.9E. This metabolic switch might mean a decline in cancer progression and a promising result for PDAC therapy. In PANC-1 cells no effect on LDHA levels was observed due to ST treatment supporting Figure 4.9E.

6. CONCLUSION AND FUTURE ASPECTS

In this study, the molecular mechanism of ST was investigated in normal and cancer pancreatic ductal cells. In conclusion, stevioside showed no anti-proliferative or cytotoxic effect on HPNE cells and leads to the removal of defective mitochondria. In addition, ST disrupts the mitochondrial integrity in the non-metastatic Panc 02 cell line and switches energy metabolism OXPHOS to glycolysis by increasing the LDHA expression levels. Finally, PANC-1 ST increased the mitochondrial inner membrane potential per mitochondrion with a decrease in mitochondrial mass leading energy crisis.

For further studies, metabolic profiles such as extracellular acidification rate and oxygen consumption rate should be examined by Seahorse XF analyzers. Also, pancreatic cancer cell line types should be increased such as SU.86.86 (metastasis in the liver), BxPC-3 (highest invasive ability among pancreatic cells), and MIA PaCa-2 (non-metastatic, similar to PANC-1) [76, 77].

Enhanced glycolysis is considered a dominant alteration in tumorigenesis of pancreatic cancer; however, the promotion of metastasis requires enhanced OXPHOS in PDAC [64, 65]. Therefore, the energy crisis-related metabolic switch to glycolysis from OXPHOS by ST treatment might be a promising result for PDAC therapy to decline metastasis progression without affecting normal cells. Migration tests should be performed to reveal the effect of the metabolic switch led by ST on metastasis.

Also, to understand changes in the OXPHOS complexes related to ROS formation, an antioxidant defense system should be investigated. SIRT3 is considered a tumor suppressor and regulator of mitochondrial biogenesis and bioenergetics by regulation of ROS levels, ETC-dependent ATP production, and glycolysis inhibition [79–81]. For this purpose, expression levels of SIRT 3 can be investigated. In addition to SIRT3, common antioxidant biomarker Nrf2 expression levels and NAD^+/NADH ratio can be examined. In addition, docking studies of GLUT1 showed ST can compete with its substrate glucose. The expression levels of GLUT1 and glucose uptake may be studied to understand the mode of action of ST in glycolysis.

Finally, to increase cytotoxicity and rate of programmed cell death of the ST, combination therapies can be studied such as stevioside can be used with metformin which is a known

complex I inhibitor used commonly on diabetes and has a protective effect on pancreatic cancer to disrupt mitochondrial integrity and inhibit the ATP synthesis [82]. Moreover, co-treatment with first-line chemotherapeutic drugs like gemcitabine may also be studied to increase the efficiency of the drug.

In summary, pancreatic cancer progression depends on the mechanism of bioenergetics. Early-stage PDAC cells demonstrate the Warburg effect and with the increasing demands at the metastatic stage of PDAC ATP production shift to the enhanced OXPHOS to acquire the required ATP for progression. This metabolic change and mitochondria, need a higher attention to increase PDAC treatment efficiency and decline tumor progression.



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