T.C.
BIRUNI UNIVERSITY

INSTITUTE OF GRADUATE EDUCATION

MOLECULAR BIOLOGY AND GENETICS DEPARTMENT

MOLECULAR AND MEDICAL GENETICS MASTERS PROGRAM

ANTIPROLIFERATIVE AND APOPTOTIC EFFECTS OF PROTON PUMP INHIBITOR LANSOPRAZOLE ON DU-145 PROSTATE CANCER CELLS

Buminhan Özgültekin

SUPERVISOR

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BİRUNİ ÜNİVERSİTESİ

LİSANSÜSTÜ EĞİTİM ENSTİTÜSÜ

YÜKSEKLİSANS TEZ ONAY SAYFASI

Anabilim Dalı : Moleküler Biyoloji ve Genetik

Program : Moleküler ve Tıbbi Genetik (İngilizce) Tezli Yüksek Lisans

Öğrencinin;

Adı ve Soyadı : Buminhan ÖZGÜLTEKİN

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Biruni Üniversitesi Lisansüstü Eğitim Enstitüsü Moleküler ve Tıbbi Genetik (İngilizce) Anabilim Dalında **Buminhan ÖZGÜLTEKİN** tarafından hazırlanan "Antiproliferative and Apoptotic Effects of Proton Pump Inhibitor Lansoprazole on Du-145 Prostate Cancer Cells" adlı tez çalışması jüri tarafından YÜKSEK LİSANS tezi olarak kabul edilmiştir.

Tez Savunma Tarihi: 16 /07 /2021

Jüri Üyesinin Unvanı, Adı, Soyadı	Çalıştığı Kurum	İmza
Prof. Dr. S. Nezih HEKİM	Biruni Üniversitesi	
Prof. Dr. Prof. Dr. Nedret HIZEL	Üsküdar Üniversitesi	
Dr. Öğr. Üyesi Elif Sibel ASLAN	Biruni Üniversitesi	

Biruni Üniversitesi Lisansüstü Eğitim-Öğretim ve Sınav Yönetmeliği'nin ilgili maddeleri uyarınca bu tez jüri tarafından onaylanmış ve Lisansüstü Eğitim Enstitüsü Yönetim Kurulu kararıyla kabul edilmiştir.

Prof. Dr. Leman ŞENTURAN
Lisansüstü Eğitim Enstitüsü Müdürü

DECLARATION

I declare that I have designed and performed all experiments in this thesis study according to good scientific practices. I obtained all the information contained in this thesis under academic and ethical rules. All the information I have used from the literature has been respectively referenced. I also declare that I have not violated any patents and copyrights during the preparation and writing of this thesis.

Buminhan Özgültekin

DEDICATION

Dear my mother and father,
All the people who encourages and supports me...

ACKNOWLEDGEMENT

I would like to thank my thesis advisor Prof. Nezih Hekim for his supports and supervision during my Masters studies.

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I would like to express my gratitude to my friend, Burak Pak, who supported me during my graduate education.

My precious friends who have always been with me,

And to my family, who have always supported me.

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LIST OF SYMBOLS AND ABBREVIATIONS

dH_2O : Distilled water

CO_2: Carbondioxide

µl: Microliter

FBS : Fetal Bovine Serum

PSA : Prostate Specific Antigen

V-ATPase: Vacuolar ATPase

MMP: Matrix metalloproteinase

mTORC: Mammalian target of Rapamycin complex

DMSO: Dimethyl Sulfoxide

PBS: Phosphate Buffer Saline

BCA: Bicinchoninic acid

PI: Propidium Iodide

FITC: Fluorescein isothiocyanate

ROS: Reactive oxygen species

FACS: Fluorescence-activated cell sorting

FDA: Food and Drug Administration

ETC: Electron Transport Chain

ER: Endoplasmic Reticulum

PD-1: Programmed Cell Death Protein 1

UCLA: University of California, Los Angeles

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ABSTRACT

Ozgultekin B. Antiproliferative and apoptotic effects of proton pump inhibitor Lansoprazole on Du-145 prostate cancer cells. Biruni University, Institute of Graduate Education, Department of Molecular Biology and Genetics. Istanbul, 2021.

Cancer has been a major threat on human health since 20th century and is one of the most significant causes of death among people. Prostate cancer is the second most cause of death among elder people. In spite of early detection and new treatments, there is a need for improvements for detection, diagnosis and therapy of prostate cancer. Ion channel blockers are promising cancer drugs not only modulate tumor microenvironment and pH but also overcome chemotherapy resistance and slow or even prevent invasion or metastasis by different mechanisms. Lansoprazole is a proton pump inhibitor that blocks V-ATPase and H+/K+ ATPase channels on cells. In this study, we aim to discover antiproliferative and apoptotic effects of lansoprazole on Du-145 prostate cancer cell lines. To explore these features, MTT assay and trypan blue counting for cytotoxicity were performed. To evaluate Caspase-9 and PARP expression, Western Blotting was done. Apoptosis was analyzed by Annexin/PI flow cytometry. In conclusion, it could be stated that lansoprazole promotes a nonapoptotic caspase-independent cell death on Du-145 castration-resistant prostate cancer cells and suppresses cell proliferation and growth dynamics at 6 hours, at 10 and 100 micromolar doses.

Keywords: Prostate Cancer, Ion Channels, Lansoprazole, Du-145
Supervisor: Prof. Dr. Nezih HEKİM

ÖZET

Özgültekin B. Bir proton pompası inhibitörü olan Lansoprazolün prostat kanseri hücreleri (Du-145) üzerindeki antiproliferatif ve apoptotik etkileri, Biruni Üniversitesi, Lisansüstü Eğitim Enstitüsü, Moleküler Biyoloji ve Genetik ABD. Yüksek Lisans Tezi. İstanbul, 2021.

Kanser, insan sağlığı açısından 20.yüzyıl boyunca önemli bir tehdit olmasının yanı sıra ölümlerin en sık nedenlerinden biridir. Prostat kanseri ise ileri yaş erkeklerdeki ölümün 2. en sık sebebidir. Yeni tedavilere ve erken tanı imkanlarına rağmen tanı ve tarama testlerinde ve tedaviler noktasında iyileştirmelere ihtiyaç vardır. İyon kanalı blokerleri, hem tümör mikroçevresi ve pH ını düzenleyerek hem de kemoterapi direncininin yok edilmesi ve farklı mekanizmalarla invazyon ve metastazı yavaşlatıp hatta durdurabilen ümit verici ilaçlardır. Lansoprazol ise V-ATPaz ve H+/K+ ATPaz ı bloke eden bir proton pompası inhibitörüdür. Bu çalışmada, lansoprazolün Du-145 prostat kanseri hücre hattındaki antiproliferatif ve apoptotik etkilerini görmek istedik. Bu amaçla sitotoksisite analizi için MTT assay ve Trypan mavisi ile hücre sayımı gerçekleştirildi. PARP ve Kaspaz-9 ekspresyonunu tespit edebilmek amacıyla Western Blot yapıldı. Apoptozu değerlendirmek için Annexin/PI flow sitometre analizi yapıldı. Sonuç olarak, lansoprazolün 6.saatte, 10 ve 100 mikromolar dozlarda Du-145 kastrasyona dirençli prostat kanseri hücrelerinde apoptotik olmayan kaspaz bağımsız hücre ölümünü teşvik ettiği ve hücre proliferasyonu ve büyüme dinamiklerini baskıladığı söylenebilir.

Anahtar Kelimeler: Prostat Kanseri, İyon Kanalları, Lansoprazol, Du-145

Tez Danışmanı: Prof. Dr. Nezih Hekim

1. INTRODUCTION AND PURPOSE

Cancer is a complex disease which is characterized by proliferation of aberrant cells in normal tissues and invasion of other tissues and organs by these abnormal cells. Other terms of cancer that are used in literature are malign tumours or neoplasms. Cancer may perturb any part of the human body and it comprises plenty of histological and molecular subtypes which are managed distinctly.

Cancer has been a major threat on human health since 20th century and is one of the most significant causes of death among people. It may be considered as the plague of the 20th century in terms of its catastrophic effects (Siegel et al, 2017). Cancer is the second leading cause of death globally and is estimated to account for 9,6 million death in 2018. Lung, prostate, colorectal, stomach and liver cancer are the most common types of cancer in men, while breast, colorectal, lung, cervix and thyroid cancer are the most common among women(https://www.who.int/cancer/en/).

Prostate cancer is a type of cancer which mainly affects middle age and elder people. According to US statistics, it is the second most cause of death in males(Humprey, 2014). There are some therapies of prostate cancer which could control the disease partially including radiotherapy, chemotherapy and surgery. In spite of this therapeutic modalities, existence of invasion, metastasis and drug resistance phenomena restrain and worsen the therapies.

One of the most important and robust reason of decreased therapeutic efficacy is the tumor microenvironment(Correiaa and Bissell, 2012; Oudin and Weaver, 2016). Ion channels in this environment is responsible for regulation of the metabolism and physiology of not only normal cells but also tumor cells. It has been proven that ion channels like Na+ and K+ channels organize tumor microenvironment(Andersen et al., 2014). In addition, it has been pointed out that Na channels are related with metastasis(Yildirim et al., 2012). Likewise, potassium channels such as Kv11.1 is overexpressed which trigger tumor invasion and angiogenesis(Pardo

and Stühmer, 2014). Another type of ion channel which is prominent in cancer is the V-ATPase channels that are mainly responsible for the acidity of tumor microenvironment. These channels are overexpressed in tumor cells and pump H+ ions outside of them and consequently the extracellular pH may decreases under 7.0. This low pH activates matrix metalloproteinases to degrade and demolish the extracellular matrix. This degradation facilitates the invasion process(Huang et al., 2016; Mc Guire et al., 2016). Additionally, low pH limits a chemotherapy drug to transport inside a tumor cell enough. This is frequently seen in drugs which are weak bases(Wojtkowiak et al., 2011).

In order to control tumor metabolism, there are some new ion channel-specific drugs has been invented in recent years. These drugs frequently antagonize the ion channels or pumps and lead to antiproliferative and apoptotic effects in cancer cells(Arcangeli and Becchetti, 2015). In this research, we are going to conduct a research on Du-145 prostate cancer cells with lansoprazole which is a proton pump (H+/K+ ATPase) and V-ATPase inhibitor. First, Du-145 cells are cultured in 2-D well-plates. After culture, 5 different doses of lansoprazole will be given to Du-145 cells. It is expected that blockage of these channels cause a decrease of proton transport to extracellular matrix that promotes a decrease in pH inside the tumor cells. The low pH inside the cells is expected to provoke apoptosis. After the drug administration, cell viability will be evaluated at 6, 24, 48 and 72 hours by MTT assay and Trypan blue. Furthermore, PARP and Caspase-9 will be analyzed by Western Blot technique. Annexin/PI Flow cytometry analysis will be applied to depict apoptosis or necrosis. Results will be analyzed statistically.

2. GENERAL INFORMATION

2.1. Prostate Anatomy

The prostate gland is a chest-nut formed organ which is about 4 centimeters in length and 3 centimeters in width. It encapsulates the proximal part of urethra, below the urinary bladder and contains plenty of branched tubular glands inside of it. Septal part and smooth muscle spread into the capsul and seperates the tubules. Ducts of these glands open into the urethra. The prostate gland secrete an alkaline, milky liquid. This fluid neutralizes the acidic fluid containing sperm cells, which is composed of metabolic wastes from the sperms. In addition, prostatic fluid facilitates the motility of these cells. Moreover, the prostatic fluid promotes the buffering of the acidic media of the vagina to ease to support sperm cells. This event simplifies sperms to go into the female reproductive tract. The prostate gland release its excretions into the urethra when contraction of the smooth muscles occur (Hole's Human Anatomy and physiology, 17 th edition, Mc Graw-Hill, 2007).

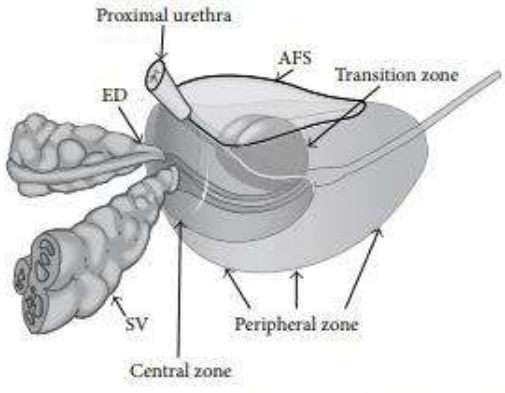


FIGURE 1: Zonal anatomy of the prostate gland. ED: ejaculatory ducts; SV: seminal vesicles; AFS: anterior fibromuscular stroma.

Figure 2.1: An illustration showing prostate anatomy. It is adapted from (Bhavsar *et al*. 2014)

2.2. Embryology of Prostate

A large number of endodermal protrusions of the prostatic part of the urethra expand into the surrounding mesenchyme. The glandular epithelium of the prostate evolves from these endodermal cells, while the mesenchyme associated with the epithelial cells creates stroma and smooth muscles of the organ(Moore, İnsan Embriyolojisi, 1.Baskı, Nobel Tıp Kitabevleri, 2002).

2.3. Histology of Prostate

The prostate gland is a composition of 30-50 branched tubuloalveolar glands. Their ducts drain into the prostatic urethra, which crosses the prostate. The prostate gland contains 3 different zones: The central zone of the gland occupies 25% of its volume. 70% of the gland is shaped by the peripheral zone, which is the primary location of prostatic adenocarcinoma. The transition zone has a significance because it is the part where most of the benign prostate hyperplasia cases embark on.

The tubuloalveolar glands of the prostate gland are shaped by a cuboidal or a columnar pseudostratified epithelium. An intensive fibromuscular stroma encircles the glands. The prostate is surrounded by a fibroelastic capsule abundant in smooth muscle. Septa from this capsule diffuse the gland and seperate it into lobes that are indefinite in males.

The glands also produce prostatic fluid and stock it for ejaculation. Together with the seminal vesicle, the structure and function of the prostate gland is subject to the level of testosterone hormone.

Small globular bodies of glycoproteins, 0.2-2 mm in diameter, are often observed in the lümen of prostatic glands. They are referred as prostatic concretions or corpora amylacea. (Junqueira, Carneiro, Basic Histology Text&Atlas 10th edition, Lange Medical Books Mc Graw-Hill, 2003)

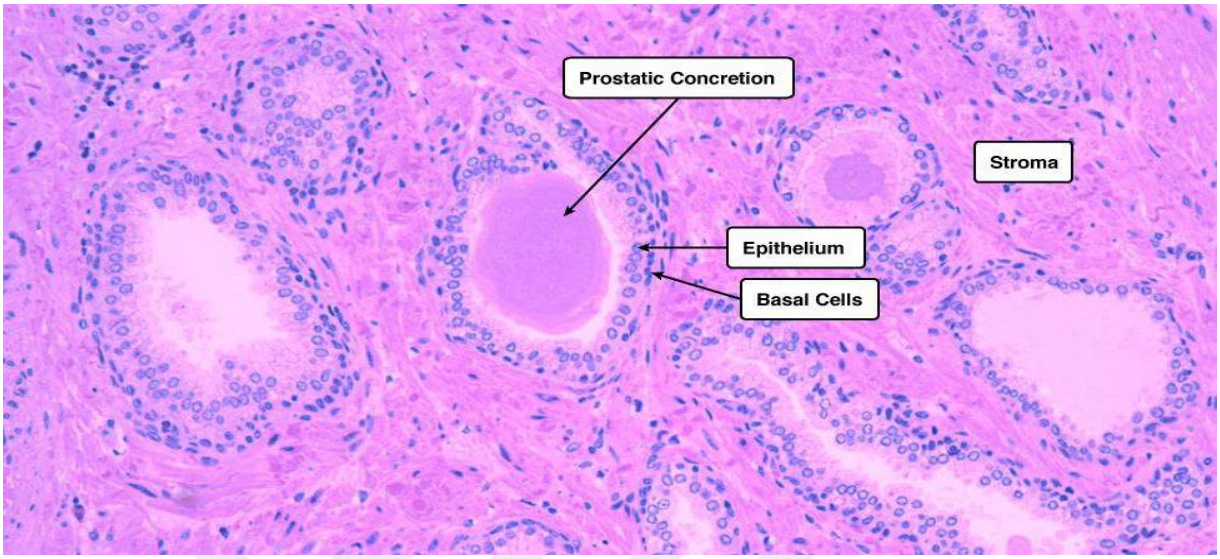


Figure 2.2: The histology of normal prostate is shown in the figure. Picture is adapted from (http://medcell.med.yale.edu/histology/male_reproductive_system_lab/prostate_gland.php,18. 06.18).

2.4. Physiology of Prostate

The prostate gland and seminal vesicles mainly generates ejaculate. Seminal fluid includes a variety of excreted proteins. Generally, the principal functions of the prostate secretion interconnect to gelation, coagulation, and liquefaction of semen. In addition, secretory proteins of prostate are related to the covering and uncovering of spermatozoa and interacts with mucus of the cervix. The particular components of seminal fluid and their specific biological tasks differ between species. For instance, in rodents proteins in the seminal fluid creates the copulatory plug, which may act as a prerservation against superfecundation. The molecular analysis of prostatic and seminal vesicle secretions in humans and other species have been investigated. The dominant secretory products of the human seminal vesicle are seminogelin, fibronectin, and lactoferrin which are the first two appear to be participated in the postejaculatory coagulation of the following ejaculation. Human semen rapidly tranforms a soft coagulum of proteins. This is happened by a gel polymer formed from seminogelin and fibronectin. The presence of transglutaminase in the prostate secretion proposes a task for this enzyme in the formation of the gel. Within a period of 5 to 20 minutes the coagulum dissolves

as a result of the action of prostate-specific antigen (PSA), which is a kallikrein-type serine protease and one of the main secretory products of the prostate gland. PSA expression is normally subjected to the action of androgens on well-differentiated tall columnar prostatic epithelial cells(Hayward and Cunha, 2000).

2.5. Prostate Cancer

Cancer is a disease that happens with distinct genetic and epigenetic alterations. The prevalence of cancer in developing countries is higher than in developed countries, according to the cancer assessment in the world (Figure 2.3). It is important to comprehend the complexity and the molecular mechanisms of cancer. The onset and growth of cancer in humans is characterized by diverse mutations, chromosomal abnormalities, and levels of gene expressions. Increased transcript levels in genomes of a cancer tissue is related with increased expression level of oncogenes and decreased expression level of tumor suppressor genes (Laconi E. *et al*., 2020).

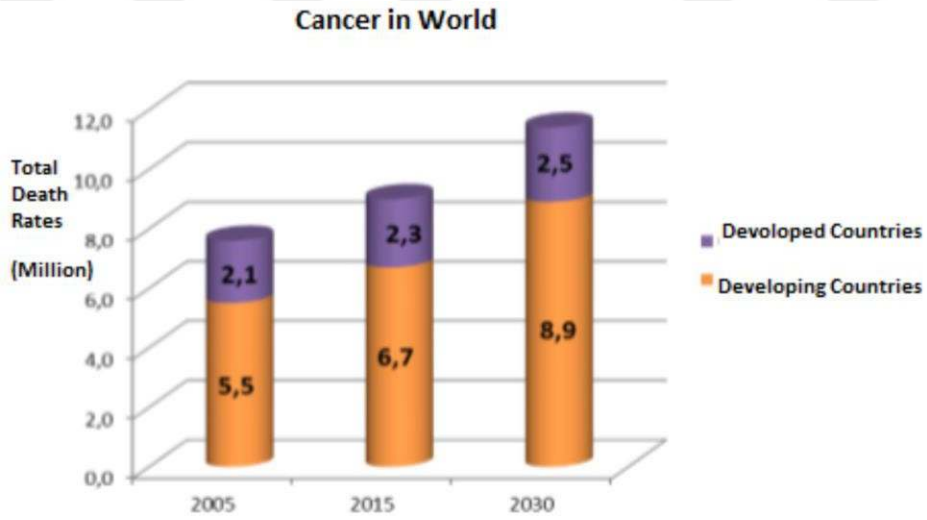
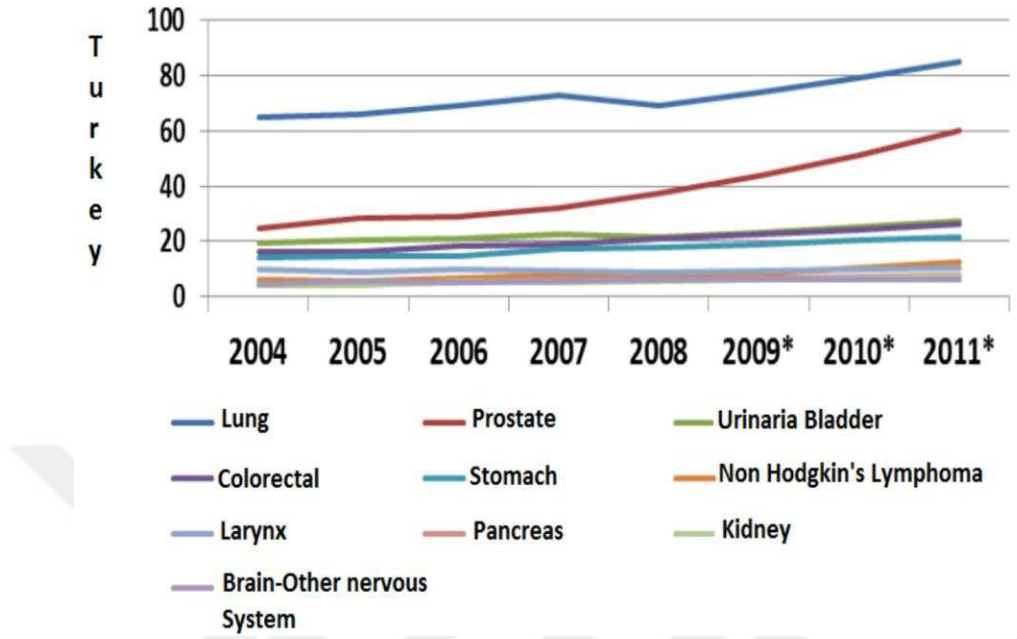


Figure 2.3: Death rates in Developed and Developing Countries. It is adapted from (T.C. Sağlık Bakanlığı Türkiye Halk Sağlığı Kurumu, 2014).



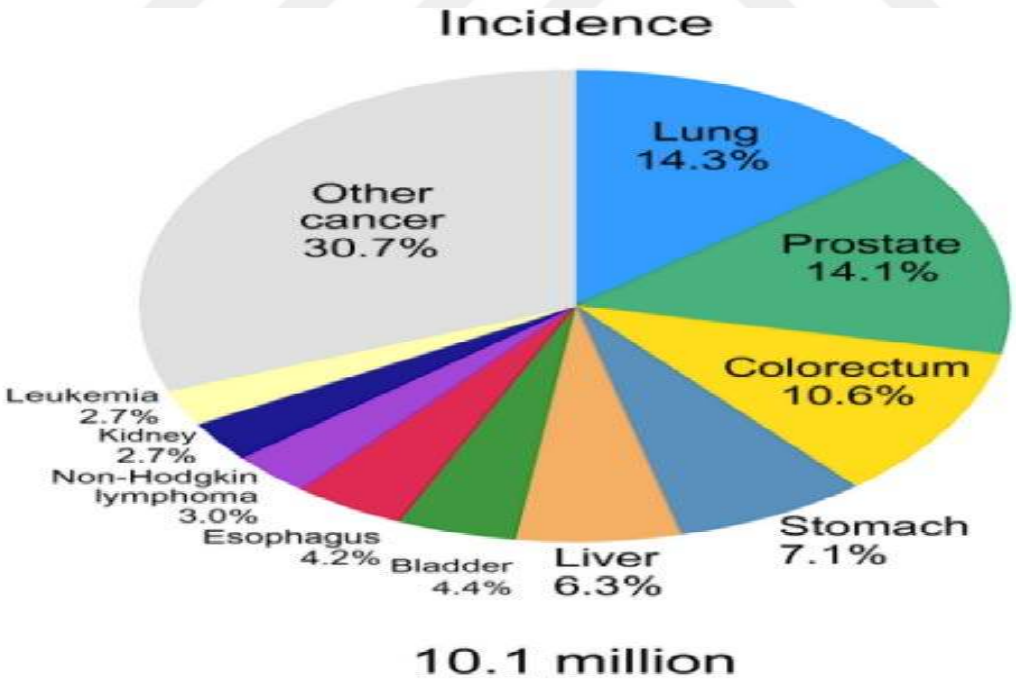


Figure 2.4: The incidence of the 10 most common cancer types among men in Turkey(the incidence at 100,000) and in world(https://www.saglik.gov.tr/TR,11655/saglik-istatistikleri-yilligi-2014.html; Sung H et. al., 2020).

2.5.1 Prostate Cancer Pathology

Pathological diagnosis of prostate cancer is performed by light microscopic examination of hematoxylin and eosin (H&E)-stained tissue sections. The most common prostate tissue samples examined in pathology laboratories are, 18-gauge needle cores, transurethral resections, radical prostatectomy specimens, open prostatectomy specimens and fine-needle aspirates. Needle core biopsies and fine needle aspirates could be utilized to diagnose metastatic prostate cancer. The most preferred type of prostate biopsy is the core needle biopsy.

Prostate cancers are graded between 1 to 5 based on how much the cells are differentiated from normal prostate tissue called the Gleason system.
(https://www.cancer.org/treatment/understanding-your-diagnosis/tests/understanding-your-pathology-report/prostate-pathology/prostate-cancer-pathology.html)

The Gleason grading method is based on architectural arrangements and differences of prostatic carcinoma. The grading diagram depicting Gleason patterns has undergone modifications fromthe original diagram(Humphrey PA., 2017).

Table 2.1: Grading by corresponding Gleason score(https://www.cdc.gov/cancer/prostate/index.htm).

Risk Group*	Grade Group	Gleason Score
Low/Very Low	Grade Group 1	Gleason Score ≤ 6
Intermediate (Favorable/Unfavorable)	Grade Group 2	Gleason Score 7 (3 + 4)
	Grade Group 3	Gleason Score 7 (4 + 3)
High/Very High	Grade Group 4	Gleason Score 8
	Grade Group 5	Gleason Score 9-10

**Risk Groups are defined by the Grade Group of the cancer and other measures, including PSA, clinical tumor stage (T stage), PSA density, and number of positive biopsy cores.*

Grade group 1: Gleason score ≤6
Only individual discrete well-formed glands

Grade group 2: Gleason score 3 + 4 = 7
Predominantly well-formed glands with lesser component of poorly formed/fused/cribriform glands

Grade group 3: Gleason score 4 + 3 = 7
Predominantly poorly formed/fused/cribriform glands with lesser (>5%) component of well-formed glands

Grade group 4: Gleason score 4 + 4 = 8; 3 + 5 = 8; or 5 + 3 = 8
Only poorly formed/fused/cribriform glands, or
Predominantly well-formed glands and lesser component lacking glands, or
Predominantly lacking glands and lesser component of well-formed glands

Grade group 5: Gleason scores 9–10
Lack gland formation (or with necrosis) with or without poorly formed/fused/cribriform glands

Table 2.2: Gleason grades of prostate cancer(Epstein JI et. al., 2016).

The primary pattern of Gleason Score(X+Y) is the most predominant area that is seen on visual inspection. The secondary pattern of Gleason score is the second most common pattern. If only one grade is visualized in the tissue sample, the Gleason grade is multiplied by two to calculate the score. If, a high-grade (pattern 4 or 5) prostate cancer is detected, and a low-grade pattern occupies less than 5% of the tumor, the low-grade component of the tumor should not be included in the score. A Gleason Grade 7 (3+4) has a better prognosis than a Grade 7(4+3).

Percentage of prostate cancer that is high grade Gleason pattern 4 or 5 is an important prognostic indicator(Humphrey PA, 2017).

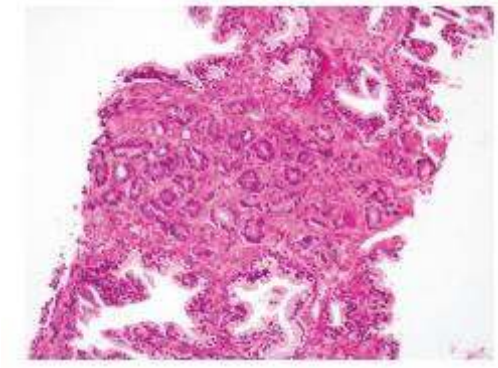


Figure 2.5: Adenocarcinoma of the prostate, Gleason grade 3 + 3 score of 6 (grade group 1)

(Humphrey PA, 2017)

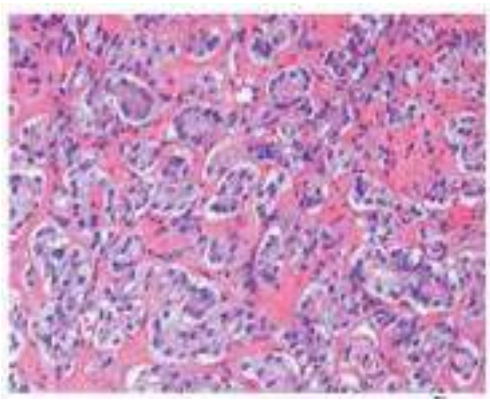


Figure 2.6: Adenocarcinoma of the prostate, Gleason grade 8 (grade group 4)
(http://pathology.jhu.edu/prostatecancer/newgradingsystem.cfm)

Although the adenocarcinoma of the prostate gland is the most common, there are various histopathological types of prostate cancer:

Glandular neoplasms
Acinar adenocarcinoma
- Atrophic
- Pseudohyperplastic
- Microcystic
- Foamy gland
- Mucinous (colloid)
- Signet ring-like cell
- Pleomorphic giant cell
- Sarcomatoid

Intraductal carcinoma
Ductal adenocarcinoma
Urothelial carcinoma
Squamous neoplasms
Adenosquamous carcinoma
Squamous cell carcinoma
Basal cell carcinoma

Neuroendocrine tumors
Adenocarcinoma with neuroendocrine differentiation
Well-differentiated neuroendocrine tumor
Small-cell neuroendocrine carcinoma
Large-cell neuroendocrine carcinoma

Table 2.3: 2016 WHO classification of carcinomas and neuroendocrine tumors of the prostate(https://radiopaedia.org/articles/who-classification-of-prostate-tumours)

In some cases of prostate cancer, there are additional molecular immunohistochemistry staining methods to identify tumor molecular characteristics like PIN cocktail(triple antibody cocktail), ERG, AMACR / p504s, p63, 34 beta E12, NKX3.1, AR, CK7 and CK20(Cox RM. et al., 2018).

2.5.2 TNM Staging System

TNM staging is beneficial for patients with prostate cancer and this staging system is preferred all over the World. It was created by Denoixx in 1940. The staging system is currently organized by 2 organizations with continous revisions (AJCC Cancer Staging Manual, 1997). American Joint Committee on Cancer (AJCC) and the International Union for Cancer Control (UICC) is used to categorize cancers. The T classification points out the size of the primary tumor and are site specific; there is considerable overlap in the cervical node (N) classifications.

The TNM system does not comprise the clinical and biological features of cancer. The locus of the tumor gives the T phase. Lymph node metastasis to the local lymph nodes are utilized for N staging. M explains the presence of a distant metastasis (https://cancerstaging.org/referencestools/deskreferences/Documents/AJCC5thEdCancerStagingManual.pdf).

CATEGORY	CRITERIA
Clinical (cT)	
T category	
TX	Primary tumor cannot be assessed
T0	No evidence of primary tumor
T1	Clinically inapparent tumor that is not palpable
T1a	Tumor incidental histologic finding in 5% or less of tissue resected
T1b	Tumor incidental histologic finding in more than 5% of tissue resected
T1c	Tumor identified by needle biopsy found in one or both sides, but not palpable
T2	Tumor is palpable and confined within prostate
T2a	Tumor involves one-half of one side or less
T2b	Tumor involves more than one-half of one side but not both sides
T2c	Tumor involves both sides
T3	Extraprostatic tumor that is not fixed or does not invade adjacent structures
T3a	Extraprostatic extension (unilateral or bilateral)
T3b	Tumor invades seminal vesicle(s)
T4	Tumor is fixed or invades adjacent structures other than seminal vesicles, such as external sphincter, rectum, bladder, levator muscles, and/or pelvic wall
Pathologic (pT)	
T category	
T2	Organ confined
T3	Extraprostatic extension
T3a	Extraprostatic extension (unilateral or bilateral) or microscopic invasion of bladder neck
T3b	Tumor invades seminal vesicle(s)
T4	Tumor is fixed or invades adjacent structures other than seminal vesicles, such as external sphincter, rectum, bladder, levator muscles, and/or pelvic wall
N category	
NX	Regional lymph nodes were not assessed
N0	No positive regional lymph nodes
N1	Metastases in regional lymph node(s)
M category	M criteria
M0	No distant metastasis
M1	Distant metastasis
M1a	Nonregional lymph node(s)
M1b	Bone(s)
M1c	Other site(s) with or without bone disease

WHEN T IS...	AND N IS...	AND M IS...	AND PSA IS...	AND GRADE GROUP IS...	THEN THE STAGE GROUP IS...
cT1a-c, cT2a	N0	M0	<10 ng/mL	1	I
pT2	N0	M0	<10 ng/mL	1	I
cT1a-c, cT2a	N0	M0	≥10, <20 ng/mL	1	IIA
pT2	N0	M0	≥10, <20 ng/mL	1	IIA
cT2b-c	N0	M0	<20 ng/mL	1	IIA
T1-2	N0	M0	<20 ng/mL	2	IIB
T1-2	N0	M0	<20 ng/mL	3	IIC
T1-2	N0	M0	<20 ng/mL	4	IIC
T1-2	N0	M0	≥20 ng/mL	1-4	IIIA
T3-4	N0	M0	Any	1-4	IIIB
Any T	N0	M0	Any	5	IIIC
Any T	N1	M0	Any	Any	IVA
Any T	Any	M1	Any	Any	IVB

Abbreviation: PSA indicates prostate-specific antigen. [a]Note that, when either PSA or grade group is not available, grouping should be determined by T category and/or either PSA or grade group, as available.

Figure 2.7: TNM Staging of prostate cancer(Buyyounouski et al., 2017).

2.5.3 Prostate Cancer Etiology and Risk Factors

Early prostate cancer is frequently asymptomatic(https://www.cancer.columbia.edu/cancer-types-care/types/prostate-cancer/about-prostate-cancer). Advanced prostate cancer may cause signs and symptoms:

Trouble when urinate like dysuria or urgency

Decreased force in the stream of urine

Blood in the urine, semen

Back and bone pain

Effortless weight loss

Erectile dysfunction

(https://www.mayoclinic.org/diseases-conditions/prostate-cancer/symptoms-causes/syc-20353087)

Known risk factors of prostate cancer are older age(>65), ethnicity(African American), family history of prostate cancer, genetic changes(BRCA1+ and BRCA2+, Men with Lynch Syndrome) and obesity. Less clear risk factors include high fat diet, smoking, chemical exposures, prostatitis and sexually transmitted infections like gonorrhea or chlamydia (https://www.cancer.org/cancer/prostate-cancer.html).

2.5.4 Prostate Cancer Screening

Early detection and improvements and advances in cancer therapy have resulted in a dramatic decline in prostate cancer deaths(approximately 50 percent) since 1990s. Best technique to detect the prostate cancer is screening of the disease.

Screening likely increases the detection and eventually diagnosis of prostate cancer of any stage. The most common and accepted screening method is detecting and screening for blood PSA levels(every 1-2 years). However, clinicians have to decide precisely if a man is really need to be detected for prostate cancer because PSA screening probably has a minimum effect on mortality of prostate cancer and there is no effect on all-cause mortality. In addition, overdiagnosis and overtreatment are critical problems for patients especially who are >70 years and a life expectancy is less than 10 years. It is known that prostate cancer is a very slowly progressed disease in most patients which may not affect their life time. Most of the patients who are not screened with a PSA may die from other causes or diseases. Moreover, therapies for prostate cancer may cause complications including urinary incontinence or erectile dysfunction(Ilic et al., 2018).

In some cases, screening may reduce the risk for distant-stage prostate cancer which is beneficial for them. In general, patients are encouraged to decide for themselves whether the benefits of screening is beneficial for them or not. Patients and clinicians should collaborate about shared decision-making and when initially discuss prostate cancer screening as well as during subsequent screening discussions. For men who choose a prostate cancer screening, a PSA blood test is simply recommended. Digital rectal examination (DRE) is generally not suggested as a screening procedure for prostate cancer (https://www.seattlecca.org/prevention/prostate-cancer-early-detection).

For men with average risk, discussion of screening for prostate cancer should initate at age 50-55. For high risk men(BRCA carriers, African American men, family history of prostate cancer under 65 years) should be screened at age 40-45 years. Men at higher risk may be more likely to benefit from screening tests. However, there is relatively little evidence explaining this situation and these men should be informed that the potential benefits and risks of early

screening are vague. The most advantageous age group for prostate cancer screening is between 55 to 70 years because prostate cancer is mostly diagnosed at mid 60s. For men with a life expectancy of at least 10 years, most clinicians offer screening up to age 70 years. Some clinicians propose to continue screening until age 75 years if the patient request it. There is a general agreement about not screening men who suffer from comorbidities that restrain life expectancy to less than 10 years(https://www.uptodate.com/contents/table-of-contents/oncology/prostate-cancer).

2.5.5 Prostate Cancer Treatment

There are several treatment modalities for prostate cancer patients depending on their age and life time expectancy, disease stage(level of PSA, Gleason score, Grade Group) and recurrence of cancer(Grozescu T and Popa F, 2016; Komura K. et al., 2018).

Watchful waiting: This approach is suggested for elder men who do not have any signs or symptoms of prostate cancer or have other health problems or diseases and for men whose prostate cancer is detected during screening. Watchful waiting is closely following up the patient without giving any treatment until signs or symptoms appear or change. Treatment is initiated to alleviate symptoms and improve quality of life(https://www.ncbi.nlm.nih.gov/books/NBK65915/).

Active surveillance is a closely follow-up condition of patients without giving any treatment if there are no alterations in their test results. It is utilized to discover early signs that the condition is getting worse. In active surveillance, patients are suggested various examinations and tests, including digital rectal exam, PSA test, transrectal ultrasound, and transrectal needle biopsy, to control if the cancer is growing. When the cancer is detected to proliferate then appropriate treatment is initiated(https://www.mskcc.org/cancer-care/types/prostate/treatment/active-surveillance).

Surgery: Radical prostatectomy, Open radical prostatectomy, Laparoscopic radical prostatectomy, Robot assisted laparoscopic radical prostatectomy(Da Vinci), Transurethral resection of the prostate(TURP) are the most common surgical procedures for prostate cancer.

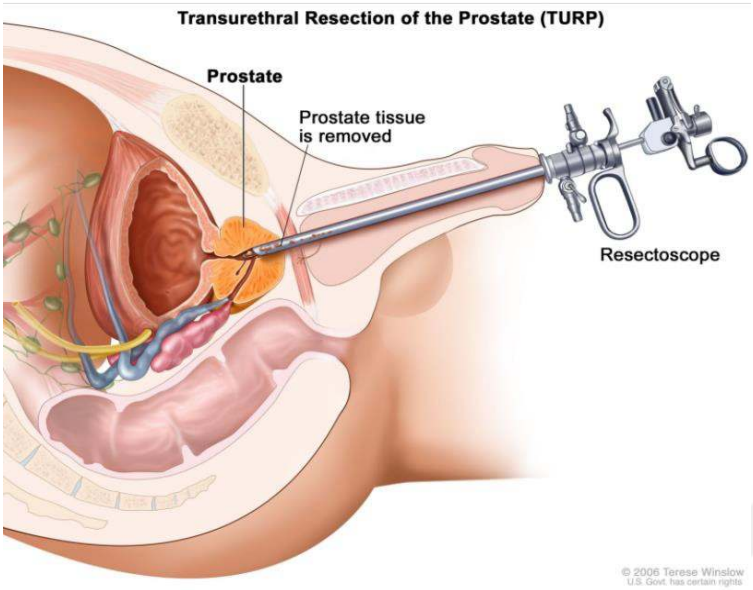


Figure 2.8: TURP surgery(https://www.cancer.gov/types/prostate/patient/prostate-treatment-pdq).

Complications related with surgery including impotence, bladder rectum fistule and inguinal hernia may be seen(https://www.cancerresearchuk.org/about-cancer/prostate-cancer/treatment/surgery/problems).

Radiation Therapy that are mostly preferred are external radiation therapy, hypofractioned Radiation Therapy, internal Radiation Therapy(Brachytherapy) and Radiopharmaceutical Therapy. External radiation therapy is assisted with a computer to centre tumor by building a 3D picture of the tumor. This supports high dose of radiation hit to tumor and hinders healthy tissue around tumor. Internal radiation therapy(Brachytherapy) utilize radioactive seeds or other instruments that are placed tumor closely. This is helpful for low risk, early stage localized prostate cancer. Radioactive seed release most of their radiation in the first 3 months placed after into the prostate and radioactivity effect prolongs up to 8-10 months. After this period, the amount of radioactivity is minimal or negligible(https://www.mayoclinic.org/diseases-conditions/prostate-cancer/diagnosis-treatment/drc-20353093).

Alpha emitter radiation therapy is a radiopharmaceutical therapy to treat bone metastases. Radium-223 used for this therapy is administred into blood and gathered in bone to kill cancer cells(Komura K. et al., 2018).

Hormone therapy is preferred to interfere and block the androgen action, receptor binding or hormone production. Abiraterone acetate can prevent prostate cancer cells from producing androgen hormones. It is utilized and preferred for men with advanced prostate cancer that has not responded well with other hormone therapy. Orchiectomy is a surgical procedure to remove one or both testicles. The principal origin of male hormone testosterone isblocked with this procedure. Estrogens also have the effect of blocking the testicles from producing testosterone. However, estrogens are rarely suggested recently in the treatment of prostate cancer because estrogen therapy could increase the risk of serious side effects. Luteinizing hormone-releasing hormone agonists can block the testicles to produce testosterone(Leuprolide, goserelin, and buserelin). Antiandrogens can interfere and block the activity of androgen hormones. Flutamide, bicalutamide, enzalutamide, apalutamide, and nilutamide are the antiandrogens that are used. Ketoconazole, aminoglutethimide, hydrocortisone, and progesterone are drugs that can intercept with the activity of adrenal glands to produce androgens. There are many unwanted adverse effects of hormone therapy including impaired sexual function and libido, increased bone fragility(Gamat M and Mc Neel G., 2017; Teo MY. Et al., 2019; Komura K. et al., 2017).

Chemotherapy: Main chemotherapeutic agents that are used in prostate cancer is taxanes and platins. Docetaxel is a microtubule stabilizing semisynthetic alkaloid which promotes cell cycle arrrest and apoptosis. It is the standard chemotherapy agent for castration resistant prostate cancer. Carboplatin is a platinum based chemotherapeutic that acts as intercalation. Most frequent side effects of carboplatin are myelosuppression(Dasari S. et al., 2014).

Immunotherapy: Sipuleucel-T is a FDA-approved dendritic cell immunotherapy which activates antigen presenting cells to activate T cells to attack tumor. Sipuleucel-T is preferred to treat metastatic castration-resistant prostate cancer(Kantoff PW. et al., 2010; Janiczek M. et al., 2017).

Clodronate and zoledronate are bisphosphonates that limit bone disease when cancer has metastatized to bone. Men who are treated with antiandrogen therapy or orchiectomy are at an increased risk of bone loss. Among these men, bisphosphonate drugs decrease the risk of bone

fractures. Bisphosphonate drugs are studied in the prevention or slowing the growth of bone metastases in clinical trials. Treatments for bone pain or metastases are managed by algology, external radiotherapy, Strontium-89, Denosumab(Monoclonal Antibody), Biphosphonates and Steroids(Teo MY. et al., 2019).

Novel treatments in clinical trials: Cryosurgery, Hyperthermia therapy, HIF ultrasound therapy, Proton therapy, photodynamic therapy and other therapies that focus on ion channels and tumor metabolism and microenvironment. Cryosurgery is a treatment that utilizes a device to freeze and kill prostate cancer cells. Ultrasound assists to find the area that will be treated. Cryosurgery may promote impotence and bladder-rectum fistule.

High-intensity–focused ultrasound therapy is a treatment that uses ultrasound to kill cancer cells. Proton beam radiation therapy is a type of high-energy, external radiotherapy that aims to destroy tumors with proton energy.

Photodynamic therapy uses a photoactive drug and a specific wavelength laser to destroy cancer cells. The drug that is given is activated with laser and it gathers more in cancer cells than in normal cells. Photodynamic therapy causes minimum damage to healthy tissue. It is preferred primarily to treat tumors on or just under the skin or in the lining of internal organs(Ahdoot M. et al., 2019 ; Hurwitz M. et al., 2011).

2.5.6 Diagnosis of Prostate Cancer

PSA blood test is the primary screening technique for prostate cancer. An elevated PSA test has to be evaluated according to age group thresholds at first:

Age	PSA Reference Range
40-49	up to 2,5 ng/ml
50-59	up to 3,5 ng/ml
60-69	up to 4,5 ng/ml
70-	up to 6,5 ng/ml

Table 2.4: Reference Range of PSA for Age Groups

If the PSA level is higher than the reference range then patient should be investigated by an urologist to perform a digital rectal examination(DRE). DRE gives an opinion about prostate abnormalities such as a mass in the prostate gland. This examination also helps clinicians to identify the shape and size of the prostate gland(https://www.mayoclinic.org/diseases-conditions/prostate-cancer/diagnosis-treatment/drc-20353093).

There are another screening tests for prostate cancer diagnosis for example urinary PC3, free/total PSA ratio(< %15), PSA density, PSA velocity, PHI index and 4K Score.

An increased PSA level, Abnormal DRE and clinical suspicion of prostate cancer especially merits a prostate biopsy either a transrectal(TRUS) or transperineal(TPUS). In recent years, multiparametric MRI is also preferred before and during biopsies(mpMRI-guided biopsy). However, the gold standart diagnostic test is TRUS biopsy(Descotes JL., 2019).

2.6 Molecular Phenomenas of Cancer

2.6.1 Cell Death

Cell death is an intricate process and it is delicately controlled by organisms. There are different types of cell death mechanisms like apoptosis, necrosis, autophagic cell death, necroptosis, ferroptosis, pyroptosis, anoikis, parthanatos, netotic cell death, lysosomal dependent cell death, alkaliptosis or oxeiptosis. Morphologically, cell death can be mainly classified into four distinct types: apoptosis(type 1), autophagic cell death(type 2), necrosis(type 3), and entosis(Green DR. and Llambi F., 2015; Pentimalli F. et al., 2019; Ke B. et al., 2016)

2.6.1.1 Apoptosis(Type 1 Cell Death)

The apoptosis was initially identified by Kerr, Wyllie, and Currie in 1972. They revealed and explained a distinct type of cell death mechanism morphologically(Kerr JFR et al., 1972). The process of apoptosis is highly conserved among multicellular organisms and is genetically strictly organized and controlled (Lockshin R. and Zakeri Z., 2004). Apoptosis is the process which a cell stops to proliferate and divide. The cell enters a novel process which ultimately results in the controlled death of the cell without scattering of its internal contents into the extracellular environment. Apoptosis is also referred as programmed cell death or cellular suicide. The beginning and triggering of apoptosis is dependent on the activation of a type of cysteine-aspartic proteases known as caspases. There are two types of caspases which are the initiator caspases and the executioner caspases (Elmore S., 2007). Once cell damage is determined,the initiator caspases (caspases 8 and 9) are activated from inactive procaspases and continue to activate the executioner caspases (caspases 3, 6 and 7). The activation of the executioner caspases starts a series of events that results in DNA fragmentation from activation of endonucleases, destruction of the nuclear proteins and cytoskeleton, crosslinking of proteins, the expression of ligands for phagocytic cells and the formation of apoptotic bodies. Apoptosis is also characterized by blebbing of cell membrane, shrinkage of cell, fragmentation of nucleus, condensation of chromosomes (Martinvalet et al., 2005; Poon et al., 2014; D'Arcy, 2019)

There are two main apoptotic pathways which are the intrinsic and the extrinsic pathways. The intrinsic apoptotic pathway is dependent on factors released from the mitochondria and is started either from a positive or negative pathway. The pathway is activated by different intracellular

stimulus like oxidative stress, DNA damage, cytotoxic stimuli, radiation, hypoxia, growth factor or hormone deficiency. Negative signals arise from the absence of cytokines, hormones and growth factors in the immediate environment of the cell. Without these prosurvival signals, pro -apoptotic molecules within the cell, such as puma, noxa and bax that are normally inhibited become active and initiate apoptosis. Intrinsic pathway is based on the formation of apoptosome which is composed of procaspase-9, Apaf-1, and cytochrome c. Bcl-2 family members, such as Bax, Bak, Bcl-2, and Bcl-xL, also control the release of cytochrome c by regulating membrane permeabilization of mitochondria(Green DR and Llambi F., 2015; D'Arcy, 2019).

When apoptosis is induced either by positive or negative stimuli, changes are triggered in the mitochondrial membrane. The result of this process is the opening of the mitochondrial permeability transition (MPT) pore. Once the MPT pore is open, proapoptotic proteins (including cytochrome c, Smac/Diablo and HtrA2/ Omi) are able to leak from the mitochondria into the cytoplasm and activate apoptosis(Cain et al., 2002; D'Arcy, 2019). Cytochrome c promotes apoptosis by binding to the WD domain of APAF1 monomers that results in a conformational change in APAF1 exposing a nucleotide binding and oligomerization domain which is able to bind deoxy ATP (dATP). This binding induces an additional conformational change in APAF1, exposing both its caspase recruitment domain(CARD) and oligomerization domains, thus allowing several APAF1s to assemble into a complex known as an apoptosome. The apoptosome contains in its open centre several exposed CARD domains, which then recruit and activate several procaspase 9 proteins. The activated caspase 9 enzymes are able to promote and activate the executioner procaspase 3, which in the form of active caspase 3 can fully induce and trigger apoptosis(Acehan et al., 2002; D'Arcy, 2019).

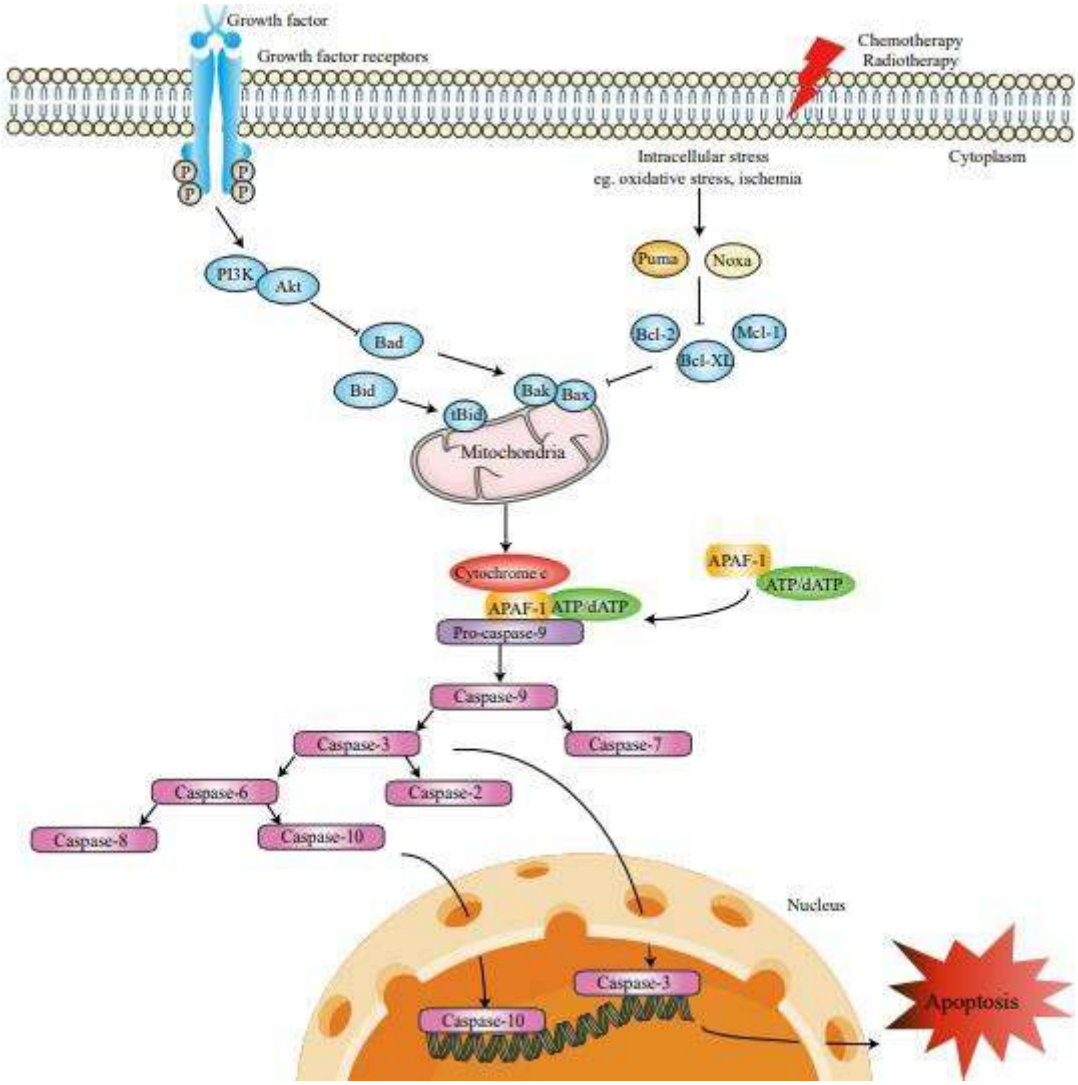


Figure 2.9: Intrinsic pathway of apoptosis(https://www.creative-diagnostics.com/intrinsic-apoptosis-pathway.htm)

The extrinsic pathway of apoptosis is started with the binding of death ligands (Fas ligand (FasL), TNF-related apoptosis inducing ligand (TRAIL), and TNF-α) to death receptors of the TNF receptor superfamily.

Upon binding of ligand to receptor, the death receptors bind via their intracellular death domain with a corresponding protein motif in adapter proteins such as Fas-associated death domain (FADD) and TNF receptor-associated death domain (TRADD). These adaptor proteins also

have another protein interaction domain, called the Death Effector Domain (DED). Pro-caspase-8 also contains DED, which interacts with the DED of FADD (Khosravi-Far and Esposti, 2004). At this time, a death inducing signaling complex (DISC) is developed and DISC then either activates downstream effector caspases (caspase-3, 6 and 7) to directly stimulate and promote cell death or cleaves the Bcl-2 family member Bid into tBid to activate the mitochondria-mediated intrinsic apoptotic pathway. This activation leads to an autocatalytic activation of procaspase-8(Boatright et al., 2003; Su et al, 2015; Goldar S. et al., 2015).

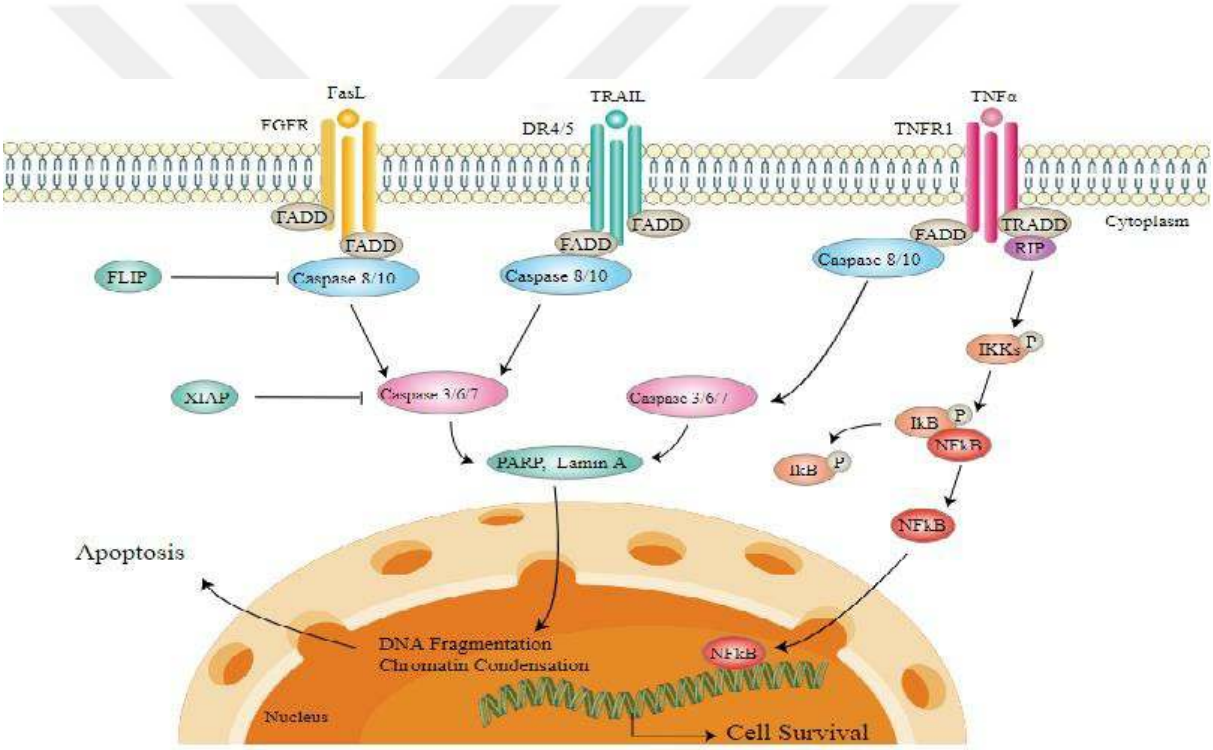


Figure 2.10: Extrinsic pathway of apoptosis(https://www.creative-diagnostics.com/extrinsic-apoptosis-pathway.htm).

Different factors like p53, cellular inhibitor of apoptosis proteins (cIAPs), and NF-κB, have been stated to be involved in the regulation of apoptotic pathways.

p53 is a transcription factor that stops the cell cycle in the G1 phase when DNA damage occurs in the cell and this gives an extra time for cell to repair its DNA. If the damage is beyond the

repair, it increase the production of Bax, Apaf-1 and Fas and suppresses Bcl-xL and Bcl-2 to induce apoptosis.

Proapoptotic members of Bcl-2 family including Bad, Bax, Bid, BclXs, Bak, Bim, Puma, and Noxa are proteins that are located in the cytosol and they also induce apoptosis by increasing the secretion of Cytochrome-c and AIF(Apoptosis inducing factor). Antiapoptotic members of Bcl-2 are Bcl-2, Bcl-xL and Mcl-1. These proteins are located in the outer membrane of mitochondrium, endoplasmic reticulum and nuclear membrane(Vousden KH and Lu X, 2002).

2.6.1.2 Autophagy and Autophagic Cell Death(Type 2 Cell Death)

Autophagy is a mechanism that causes intracellular macromolecules and organelles to be taken into a vesicle and directed to lysosomes where they combine with the lysosome and cause their degradation. While short-lived proteins are degraded in the ubiquitin-proteosome system, long-lived proteins and intracellular organelles are degraded by the autophagy system, and the end products such as amino acids are regained for reuse by the cell. The term autophagy means self eating of cell. In recent studies, autophagy plays an effective role in regulation of metabolism, cell differentiation, aging, cell death and destruction of intracellular pathogens. Autophagy is also related with cancer, infectious and neurodegenerative diseases(Gozuacik D. and Kimchi A., 2004).

Autophagy is mainly divided into 3 groups: Macroautophagy, microautophagy and chaperone-mediated autophagy. Macroautophagy is generally known as autophagy. Autophagy occurs at a certain level in many cells and plays an important role in the breakdown of proteins and damaged organelles. Microautophagy is the collapse of the lysosome membrane and the direct ingestion of the cytoplasm by the lysosome and the digestion of its contents within the lysosome. Chaperone-mediated autophagy provides selective transport of KFERQ motif proteins to the lysosome membrane(Ozarslan et al., 2011).

There are four main steps of autophagy: activation and elongation(phagophore), maturation(autophagosome), lysosome fusion and degradation(Li F. et al., 2019).

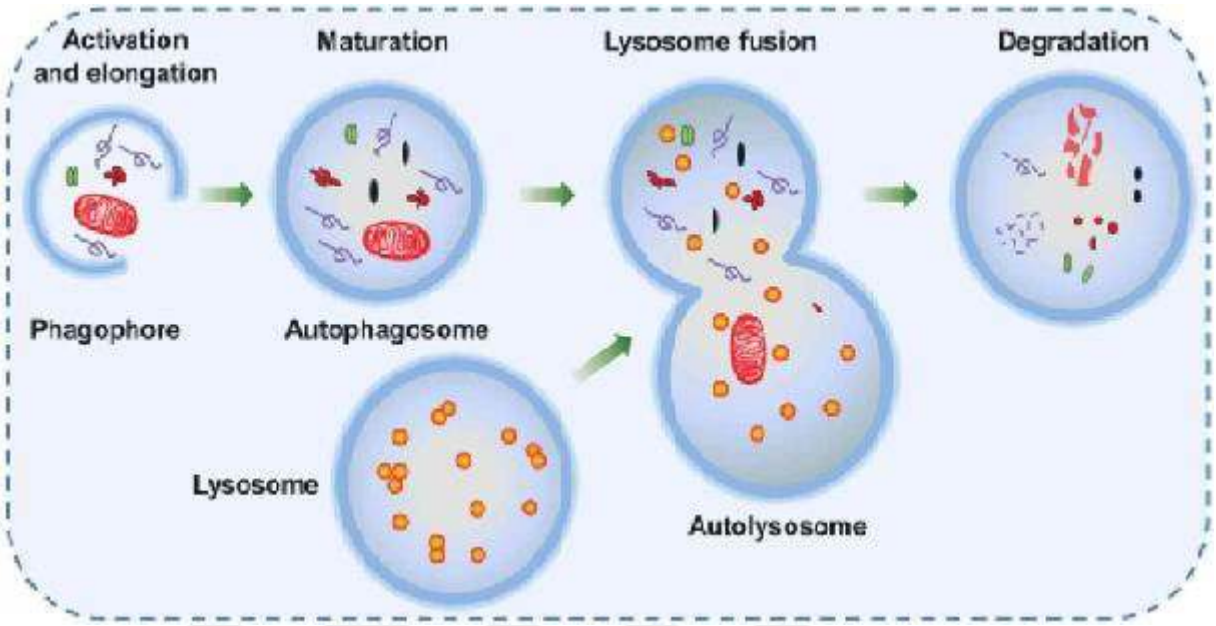


Figure 2.11: Steps of autophagy(Li F. et al., 2019).

Proteins that regulates and control autophagy are known as autophagy related proteins(Atg) which are first identified during researches about yeasts.

Basic molecular mechanism of autophagy can be summarized in four steps as Atg1-Atg13-Atg17 kinase complex; class III phosphoinositol 3 phosphate(PI3F) kinase, Atg6 protein (Beclin-1 in mammals) complex, which regulates the activity of Vps34, two ubiquitin-like systems and Atg9 and the loop system. Starvation, hypoxia, decreased growth factor signals and various stress conditions stimulates autophagic activity. In the control of autophagic activity, mTOR protein complex which is a kinase plays an essential role. mTORc, controls cellular growth and protein synthesis in the cell. Suppression of mTORc activates autophagy in both yeasts and mammals. Under stress conditions like hunger, mTOR activity decreases and Atg13 becomes dephosphorylated which activates autophagy. On the other hand, in the condition where the food is abundant, the yeast mTOR phosphorylates Atg13 and suppresses autophagy.

Equivalent to this protein complex in mammals is ULK:Atg13: FIP200(200 kDa focal adhesion kinase family interacting protein). It has been shown that ULK1 and ULK2 proteins accompany the initiation of autophagy by interacting directly with mTOR and AMPK (Adenosine monophosphate-activated protein kinase) in mammals.

Class I phosphatidylinositol 3-kinase (PI3K)/ protein kinase B (Akt/PKB), is one of the most effective signaling pathways in the control cell growth in response to mitogenic stimuli. Class I PI3K, PI(3,4)P2 and PI (3,4,5)P3 contribute to the activation of the Akt/PKB pathway.Active Akt activates mTOR that blocks autophagy. PTEN, which is a a tumor suppressor protein, works in the opposite direction with the PI3K/Akt pathway to increase autophagy. Mutations in the PTEN molecule have been shown to suppress autophagy. Class III PI3K Vps34, a kinase distinct from PI3Ks, and its product, PI3-phosphate, play an important role in the regulation of autophagy. PI3K complex consists of Atg6 (Beclin-1 in mammals), Atg14, Vps15 and PI3K Vps34 proteins and it initiates autophagic vesicle formation(Gozuacik D. and Kimchi A., 2004; Levy JM. et al., 2017; Onorati A. et al., 2018; Guo JY. and White E., 2016).

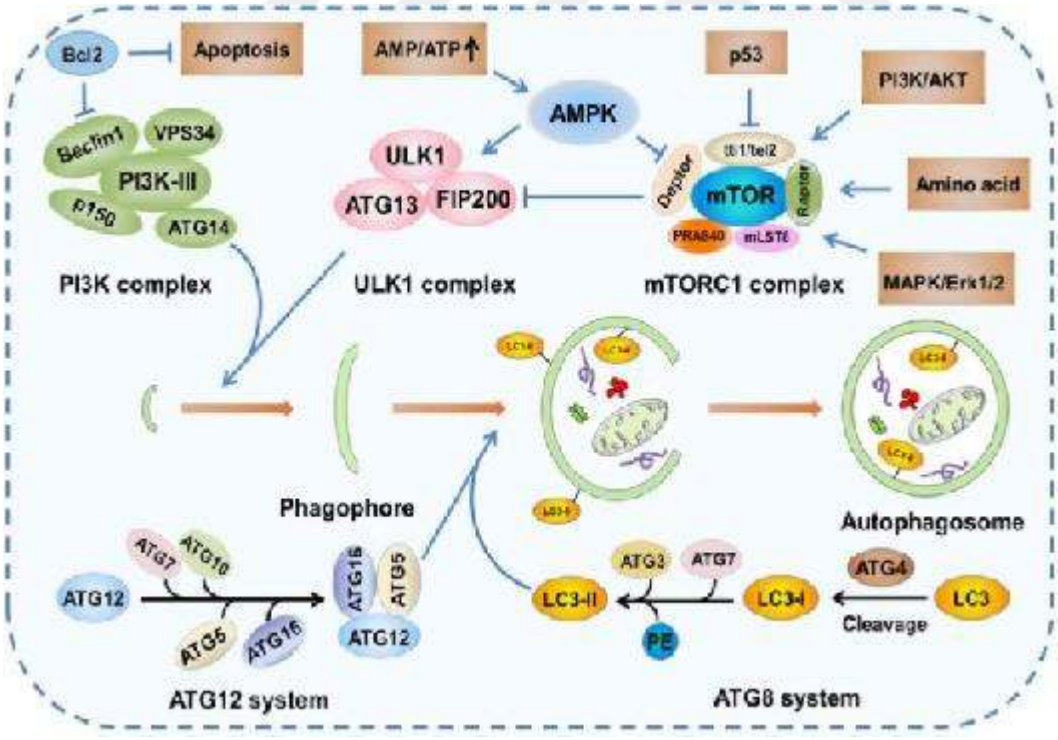


Figure 2.12: Mechanism of autophagy(Li F. et al., 2019).

The most prominent morphological change in Autophagic or type 2 cell death is the presence of vesicles formed in the cytoplasm, surrounded by two or more layers of membranes, containing fragments of cytoplasm and/or organelles such as mitochondria, endoplasmic reticulum (ER). These vesicles fuse to the lysosome and cause their cargo to be broken down by lysosomal enzymes. Nucleus specific changes like chromatin condensation can be seen at later phases instead of apoptotic cell death. Death is caspase-independent and apoptotic bodies do not form. Also, phagocytosis of dead cells occurs later and irregularly.(Lockshin R. and Zakeri Z., 2004; Green DR. and Llambi, 2015; Orrenius S. et al., 2015).

2.6.2 Cell Cycle/Cell Cycle Arrest

The cell cycle consists of Interphase (G1, S, G2 phase) and Mitosis (M phase). The G1 phase, in which the RNA and proteins necessary for the cell are synthesized, is followed by the S phase, in which the chromosomal DNA of the cell is copied. During G2, the cell continues to grow and prepare for division. During the M phase, the duplicated chromosomes are condensed, sister chromosomes separate to opposite poles, and the cell divides in two(Schafer K, 1998).

Cells that divide continuously do not exit the cell cycle, but progress through the G1, S, G2, and M stages until they receive the necessary signals to stop their growth. If the cell stops proliferating, it enters the G0 phase of the cell cycle. During G0, the cell is metabolically active but cannot grow or divide. Cells in the G0 are often stimulated to re-enter the cell cycle by signals such as growth factors and hormones.

In some cases, abnormal cell surface receptors or cytoplasmic signal transduction molecules send continuous growth signals to the nucleus even in the absence of external stimuli. These signals are those that normally inhibit cell proliferation in tissues.

The main checkpoints of the cell cycle are the G1/S, G2/M and M checkpoints(Gao SW. and Liu F., 2019).

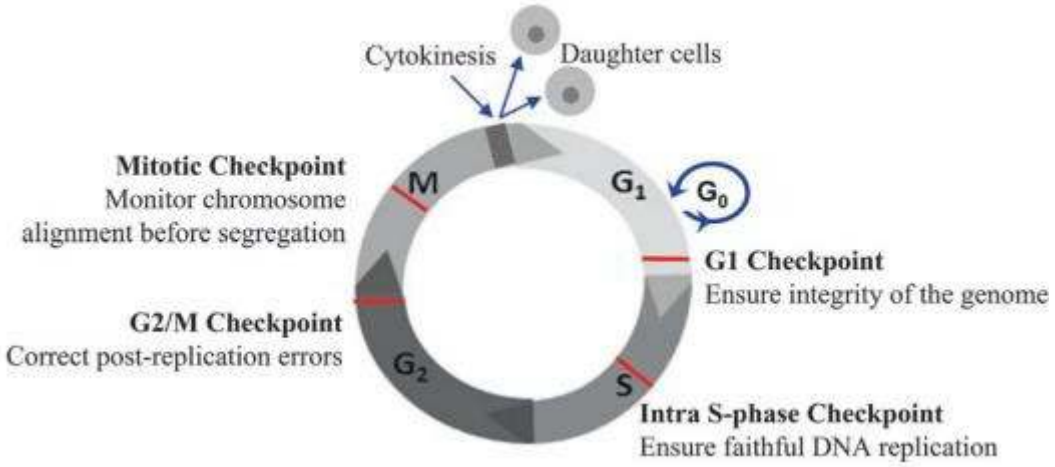


Figure 2.13: Cell Cycle Checkpoint(Encyclopedia of Cancer, Springer-Verlag Berlin Heidelberg 2017)

At the G1/S checkpoint, the cell controls its size and decides whether its DNA is damaged. If the cell has failed to reach the appropriate size or its DNA is damaged, the cell cycle is stopped until these conditions are corrected. If the cell size and DNA integrity are normal, the G1/S checkpoint is passed and the cell progresses to the S stage.

The second important checkpoint is the G2/M checkpoint, where physiological conditions in the cell are monitored for entry into mitosis. If DNA replication or repair of any DNA defect is incomplete, the cell cycle is stopped until these processes are complete.

The third checkpoint occurs during mitosis is the M checkpoint. At this control point, both the formation of the spindle fibers and the connection of the spindle fibers with the kinetechores are controlled. If the spindle fibers are not formed properly or their attachment is not appropriate, mitosis is stopped(Barnum KJ. and O'Connell M., 2014).

Two classes of proteins, specifically cyclins and cyclin-dependent kinases (CDKs), play a role in the regulation of the cell cycle. Cells synthesize and break down cyclin proteins in certain situations throughout the cell cycle.

CDKs phosphorylate critical target proteins that are necessary for the cell cycle to pass to the next stage. They are expressed continuously during the cell cycle. In their inactive forms, they become phosphorylated and activated by binding to the protein family called 'cyclins'.

Cyclins are synthesized at certain stages of the cell cycle. Their function is to activate CDKs. More than fifteen cyclins have been identified; Cyclins D, E, A, and B appear in the cell cycle, respectively, and bind to one or more CDKs(Lim S. and Kaldis P., 2013).

Another element that has a critical role in the cell cycle; Phosphorylates Retinoblastoma Protein (RB). Phosphorylation of RB functions as a molecular switch for the cell cycle. In its hypophosphorylated form, RB forms a tight complex with the transcription factor E2F, preventing cell replication. Phosphorylation of RB causes dissociation of the complex and removal of the transcriptional activity barrier on E2F(Sherr CJ and Mc Cormick F., 2002).

The next decision point in the G2/M phase of the cell cycle is the G2/M transition. This complex regulates events in mitotic prophase.

Cell cycle inhibitors control the cell cycle by tightly controlling the activities of cyclin-CDK complexes. There are two main classes of CDK inhibitors; Cip/ Kip Family, INK4/ ARF Family. These inhibitors function as tumor suppressors and are often found in a modified state in tumors. There are three main elements of the Cip/Kip Family; p21, p27 and p57. They bind to complexes formed between cyclin and CDK and inactivate them.(Fahmi M. and Ito M., 2019)Transcriptional activation of p21 is under the control of p53. p53 is a tumor suppressor gene and is mutated in the majority of human cancers. p53 triggers checkpoint controls that slow or stop cell cycle progression of damaged cells or induce apoptosis(Chen J., 2016).

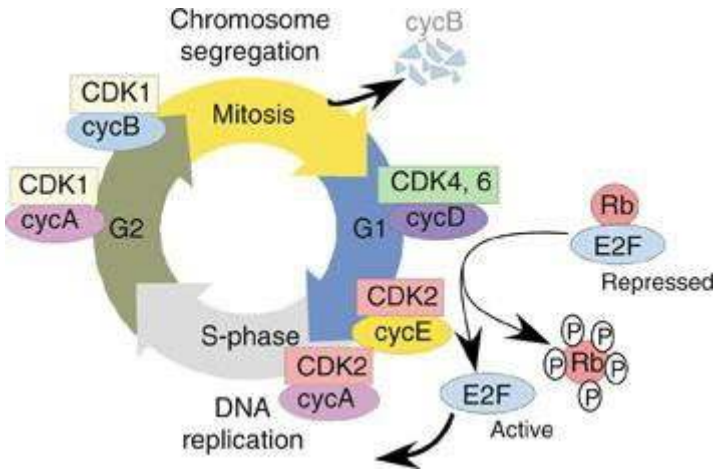


Figure 2.14: Cell cycle and its control by cyclins and CDKs. (https://link.springer.com/referenceworkentry/10.1007%2F978-3-540-38918-7_38)

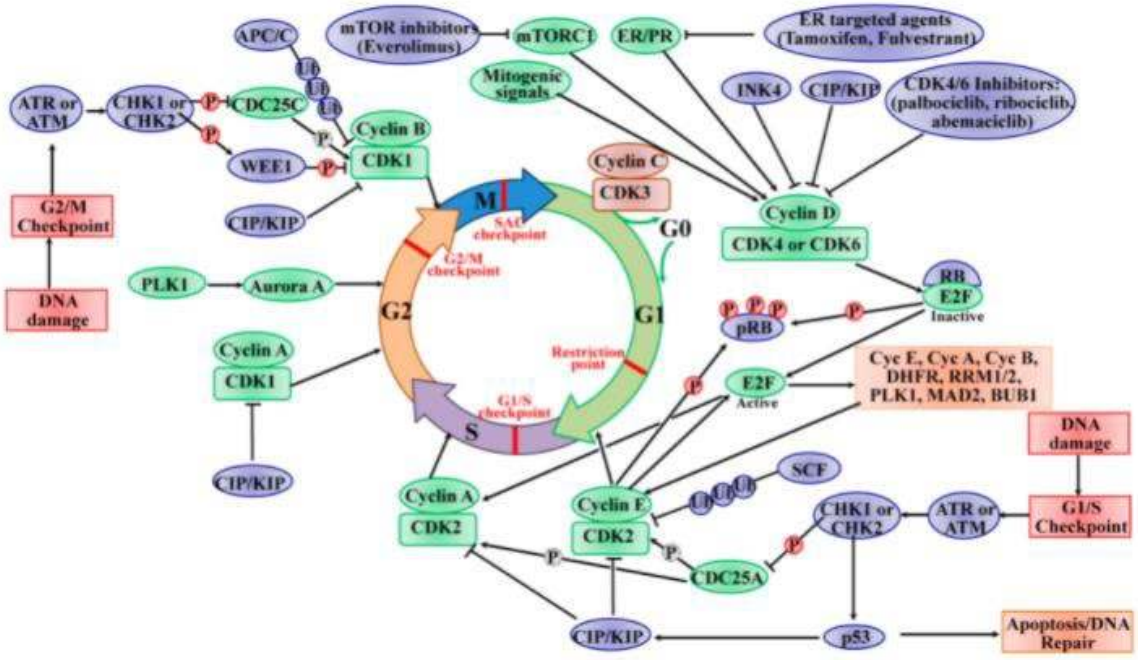


Figure 2.15: Cell Fates after DNA Damage: Signaling cascades activated in response to DNA damage also activate DNA repair pathways. If the DNA damage is severe, apoptosis or cell death mechanisms will be activated (https://wou.edu/chemistry/courses/online-chemistry-textbooks/ch450-and-ch451-biochemistry-defining-life-at-the-molecular-level/chapter-12-dna-damage-repair-and-mutations/).

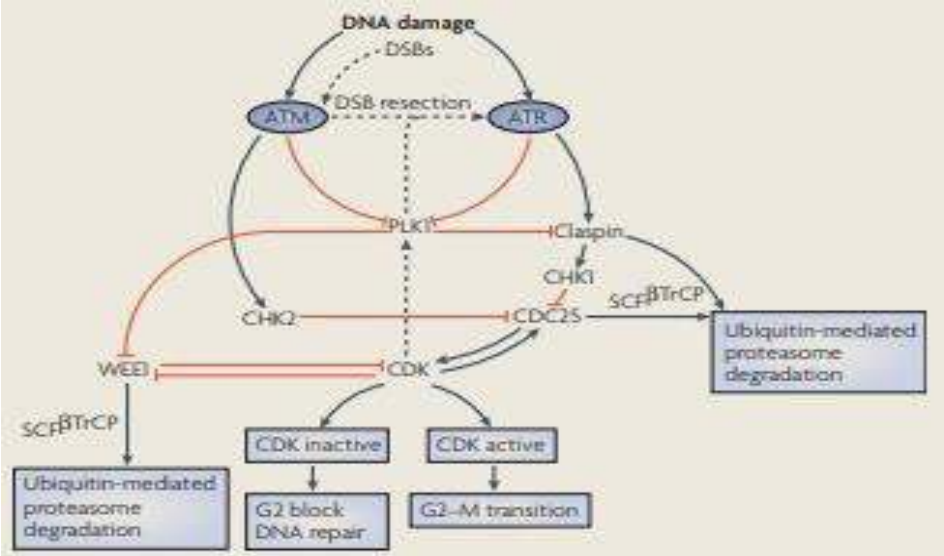


Figure 2.16: Checkpoint-mediated cell cycle arrest and recovery(Branzei et. al., 2008).

2.6.3 Tumor Microenvironment:

Tumor microenvironment is one of the most important factors regulating tumor angiogenesis, tumor invasion and metastasis. The microenvironment is a structure known to play a crucial role in tumor progression and is composed of many important components, including tumor parenchyma cells, fibroblasts, mesenchymal cells, blood and lymph vessels, tumor infiltrating immune cells, chemokines, and cytokines. Tumor microenvironment includes immune system cells, tumor vasculature and lymphatic vessels, apart from malignant cells(Hui L. and Chen Y., 2015).

In the tumor microenvironment, especially the proliferation of vascular endothelial cells and the formation of new blood vessels contribute to the nutrition of the tumor, triggering tumor development and metastasis formation(Wang M. et al., 2017) .

Fibroblasts in the tumor microenvironment are activated by the paracrine effect of the tumor, and these cells, characterized by the production of α-smooth muscle actin, are termed cancer-associated fibroblasts (CAFs). CAFs arise from local fibroblasts but can also arise from pericytes and vascular smooth muscle cells, bone marrow-derived mesenchymal cells. Cancer-associated fibroblasts (CAF) stimulate angiogenesis, triggering tumor growth and metastasis(Sounni N. and Noel A., 2013).

Tumor associated macrophages (TAM) are recruited into tumors via chemoattractants and produce various chemoattractants such as colony stimulating factor-1, SDF-1, monocyte chemotactic protein-1 and VEGF to fight cancer cells. TAMs also suppress T-cell-mediated anti-tumor immune response by expressing IL-10 and TGF-Beta.

Proliferation of lymph endothelial cells and increased lymphatic vessel density also help tumor metastasis(Spano D. and Zollo M., 2012).

Tumor-infiltrating lymphocytes (TILs) are a relatively common finding in solid tumors. TILs are a heterogeneous cell population composed of T and B lymphocytes. Therefore, depending on the effects of these cells, they can show immunostimulating or suppressive effects. Cells such as myeloid suppressor cells, T regulator cells and dendritic cells can weaken the anti-tumor immunity by suppressing cells such as cytotoxic T lymphocytes in the tumor microenvironment(Quail DF. and Joyce JA, 2013).

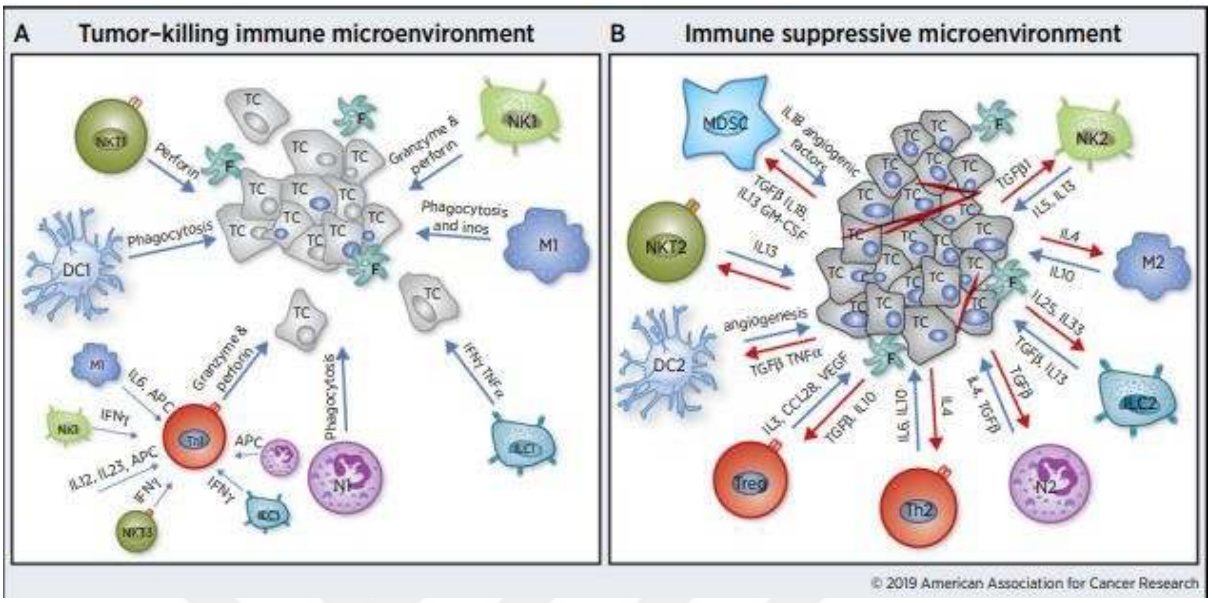


Figure 2.17: Crosstalk in the tumor microenvironment(Am Assoc for Cancer Research, 2019).

The extracellular matrix (ECM) in the tumor microenvironment consists of many proteins such as laminin, fibronectin (FN), type IV collagen, Entactin / nidogen and proteoglycans. It not only provides a physical skeleton for cells in the tumor microenvironment, but also plays a dynamic role in the spread and development of cancer cells. In particular, the adhesion of cells to the ECM plays a key role in the migration of cells into or out of the tumor microenvironment. The ECM also contains growth factors such as angiogenic factors and chemokines that interact with cell surface receptors and enable the cell to acquire resistance-elasticity. The degradation, density, stiffness, and topographic structure of the ECM also affect the behavior of tumor cells. For example, increased matrix deposition (desmoplasia) caused by CAFs can increase the invasive ability of tumor cells by affecting the plasticity and structure of the stroma of ECM (Sounni and Noel, 2013).

Acidic extracellular pH is another important feature of the tumor microenvironment. Acidic extracellular pH is thought to occur as a result of the production of lactate, which is seen as a result of anaerobic glycolysis of cancer cells. Transporters such as Na+ /H+ exchanger, H+ -lactate co-transporter carrying H+ -lactate, monocarboxylate carriers, proton pump (H+ -ATPase), V-ATPase contribute to acidic extracellular pH by providing extracellular hydrogen release(Arcangeli A., 2011).

Carbonic anhydrase enzyme also contributes to the acidic extracellular pH, as excessive carbon dioxide (CO2) production is observed in tumor cells via the pentose phosphate pathway. Acidic extracellular pH activates various lysosomal enzymes. It also induces expression of various pro-metastatic factor genes(Andersen A., 2014).

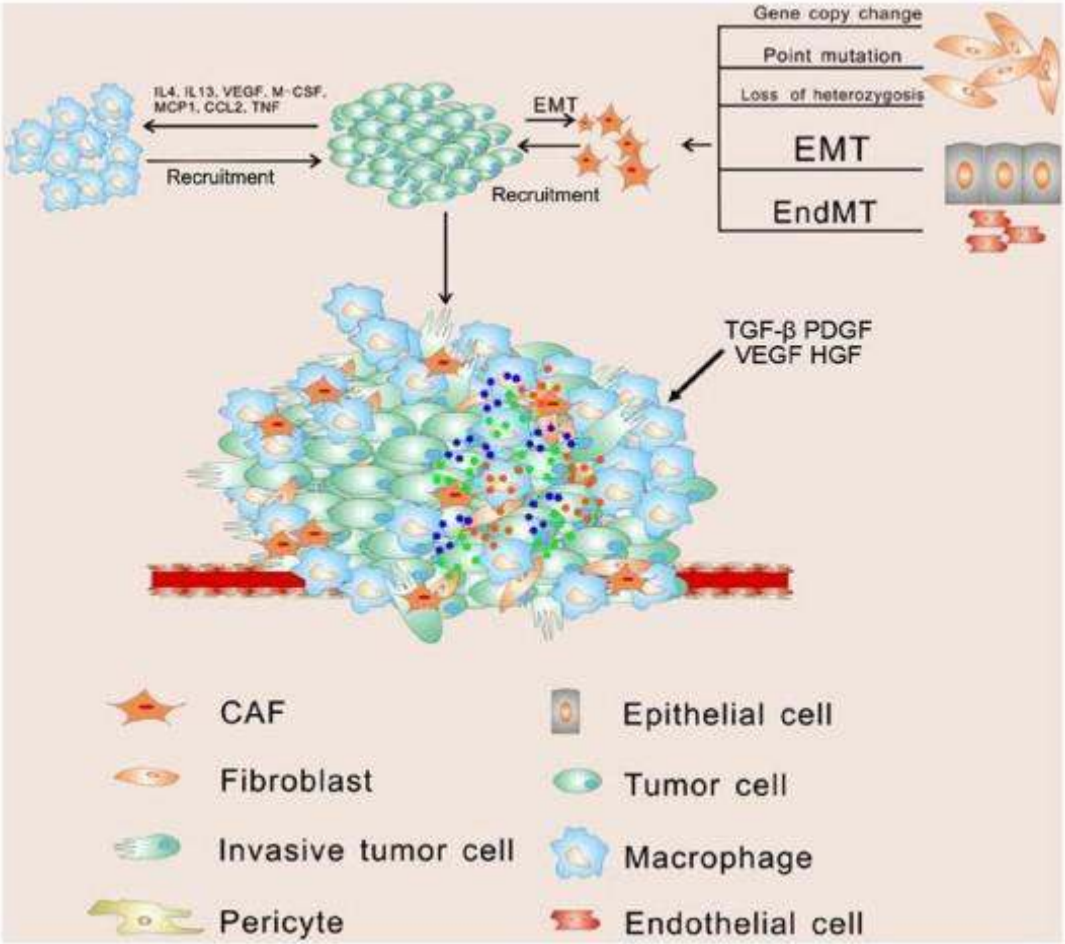


Figure 2.18: A general view of tumor microenvironment(Yuan et al, 2016).

Especially in recent studies, it has been revealed that the expression and activity level of ion channels such as Na+, K+, H+/K+ ATPase and V-ATPase increase considerably in different tumor tissues and tumor microenvironments. It has been determined that these channels can both trigger tumor development and increase the possibility of tumor invasion or metastasis (Onkal

R. and Djamgoz MBA., 2009, Whitton et al, 2018). Na+/H+ Exchanger (NHE), monocarboxylate transporters (MCT), carbonic anhydrase and V-ATPase channels, which decrease the tumor microenvironment pH by causing proton release out of the cell are of particular importance. Among these channels, V-ATPase proton channels, which are overexpressed both on the cell surface and on the lysosomal membrane, have a critical importance in maintaining the pH and acidity of the tumor microenvironment(Michel V. Et al., 2013). It is known that low microenvironment pH (<7.0) increases the activity of proteases such as MMPs in the tissue, thus contributing to tissue destruction and invasion and metastasis of the tumor(Mc Guire et al., 2016). In addition, low pH can cause chemotherapy resistance (Wojtkowiak JW. Et al., 2011).

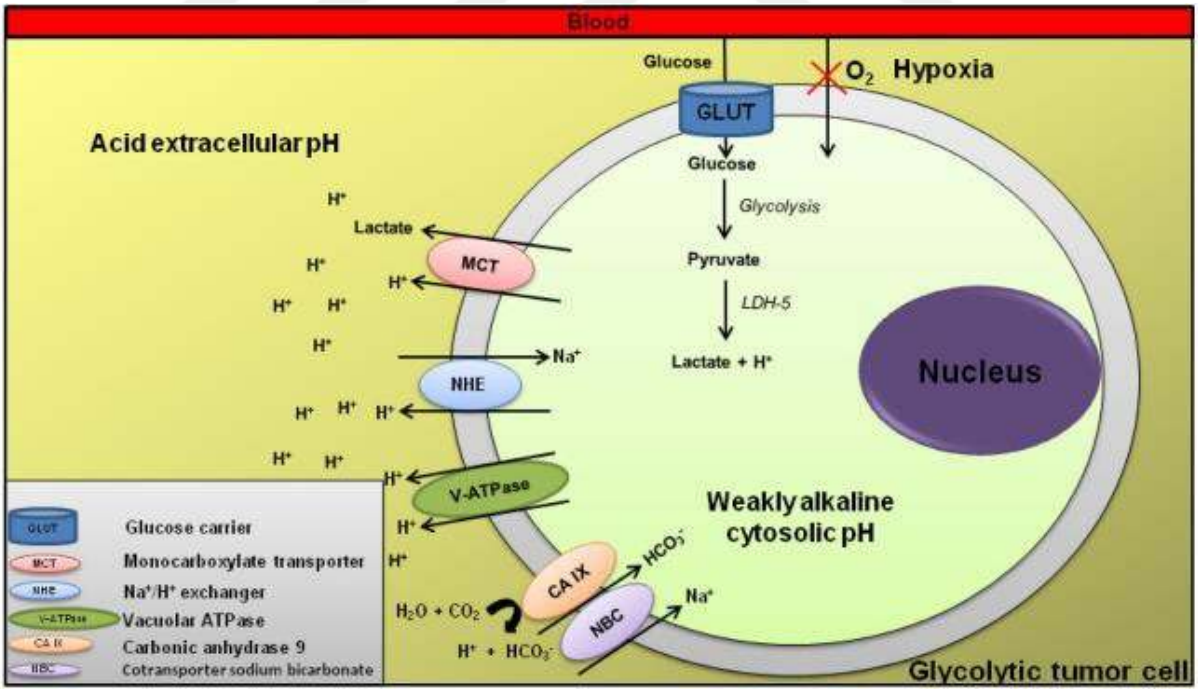


Figure 2.19: Hypoxia-induced extracellular tumor microenvironment acidosis model. (Taylor, 2015)

2.7 Cytotoxic Effects of Ion Channel Blockers and Proton Pump Inhibitors on Cancer

In recent years, various anticancer drugs for ion channels have been developed, and both in vitro and clinical studies have been conducted for single or combined therapy.

Studies on various cancers such as melanoma, breast cancer and pancreatic cancer with V-ATPase inhibitors such as bafilomycin and V-ATPase and proton pump inhibitors such as lansoprazole, pantoprazole, omeprazole, the antiproliferative and cytotoxic effects of these drugs have been determined (De Milito A. et al., 2009; Ihraiz WG. et al., 2020; Udelnow A. et al., 2011; Michel V. et al., 2013). In addition to these features, it has been observed that chemotherapy resistance is eliminated by distinct mechanisms like increased drug uptake and distribution, especially when combined with a chemotherapy drug. For example, in a breast cancer study in which lansoprazole and doxorubicin were given together, it was found that doxorubicin resistance disappeared and drug efficacy increased (Yu M. et al., 2015; Ferrari S. et al., 2013; Spugnini EP. et al., 2011; Patel K et al., 2013). In a similar study with cisplatin and vinblastine, similar results were obtained with proton pump inhibitors (Luciani F. et al., 2004). In conclusion, proton pump inhibitors seem to be quite effective in both a cytotoxic effect against tumors and in eliminating the resistance to existing chemotherapeutics. The resulting cytotoxic effect occurs by affecting apoptotic or autophagy-related death pathways depending on the cancer type (Tan Q. et al., 2015; Canitano A. et al., 2016; Graham RM et al., 2013). Apart from these effects, it is a group of drugs with a well-known side-effect profile since it has been used clinically for many years for digestive system disorders such as gastritis. Proton pump inhibitors, which convert to their active form in an acidic environment after being taken into the body as prodrug, are sulfonamide-derived drugs containing benzimidazole ring and are generally well tolerated. Following activation, they function by covalently binding to the H+/K+ ATPase and V-ATPase channels (Strand DS. et al., 2017).

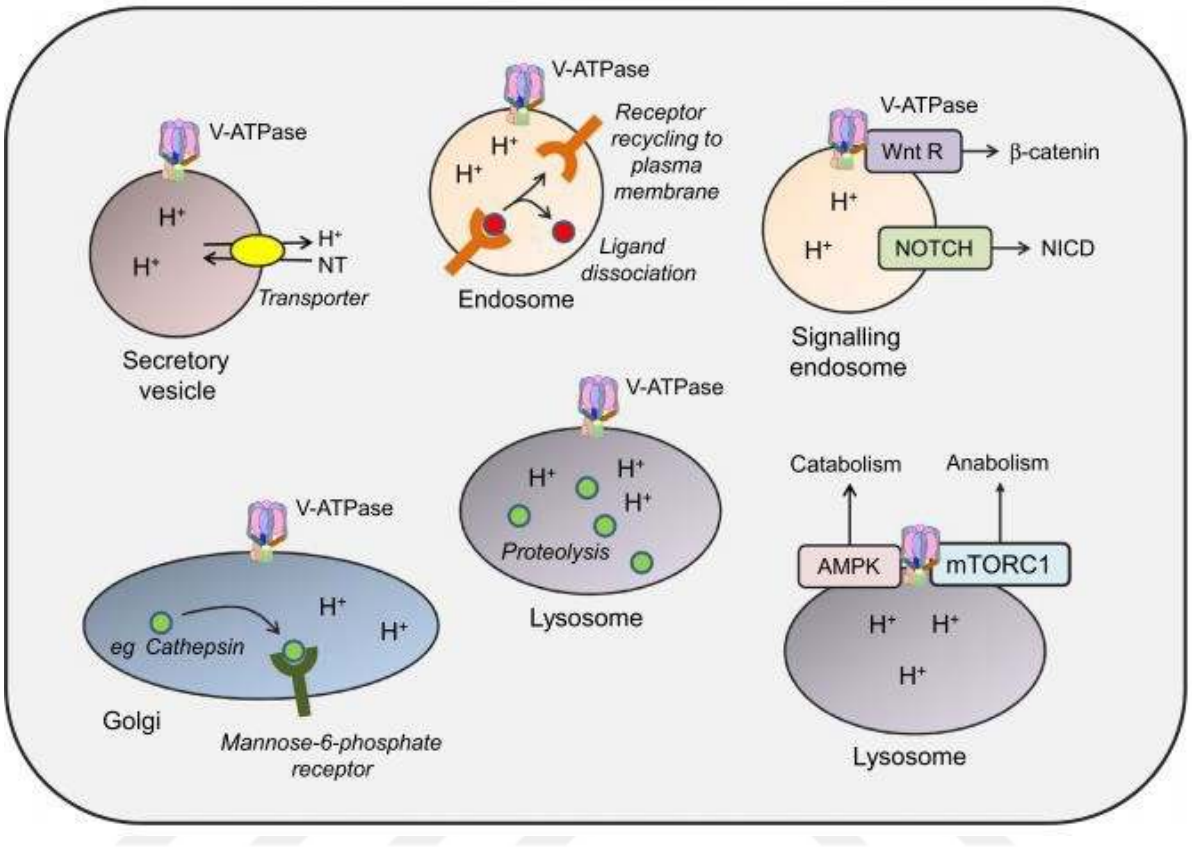


Figure 2.20: V-ATP ase channels on different organelles of cells(Whitton et. Al, 2018).

3. MATERIALS AND METHODS

3.1 Materials and Equipments

- Nanodrop spectrophotometer (Thermo Scientific-ND 2000c, Germany)
- Micropipette (Thermo Scientific, Germany)
- BioSafe Avanti J-30I Centrifuge
- Mini Centrifuge (Thermo Scientific, Germany)
- Vortex(Wise Mix-VM10, Korean)
- Spin Down
- 0.2-1.5 ml Eppendorf (Nest Biotechnology, China)
- FACS Calibur 4CS Flow Cytometer
- Vertical Laminar Flow Cabinet
- CO2 Incubator
- Inverted Fluorescent Microscope
- Mini-PROTEAN Tetra Cell ,10-well,1.0mm
- PowerPac Power Supply
- Trans-Blot Cell With Plate Electrodes and Super Cooling coil
- Mini Trans- Blot electrophoretic Transfer Cell
- Human Du-145 prostate cancer cell line(ATCC)
- Lansoprazole %98 TLC Powder (Sigma Aldrich)
- Other consumables needed for molecular applications

3.2 Methods

3.2.1 Preparation of Lansoprazole

Lansoprazole with a molecular weight of 369.36 g/L was supplied in the form of 98% TLC powder and stored under appropriate conditions. As a result of the literature review, it was determined that it was best dissolved in DMSO (30 mg/ml). The 1st intermediate stock solution was prepared by dissolving lansoprazole at 30mg/ml (0.8 M) with DMSO. Afterwards, the 2nd intermediate stock solution was prepared by diluting it with RPMI medium at a ratio of 1/80. The necessary calculations were made for Blank, Control (RPMI + 0.1% DMSO), Positive Control (RPMI + 1% TX-100) groups and 10, 25, 50, 100 and 200 micromolar lansoprazole concentrations to be used in the experiments, and control and experimental groups were formed.

3.2.2 Cell Culture

Human Du-145 prostate cancer cell line(ATCC) was used in this study. RPMI-1640 cell medium(2mM L-glutamine, 4.5g/L glucose, 1mM Sodium pyruvate, 10 mM Hepes and 1.5g/L NaHCO3) was taken as ready and 10% FBS and 1% penicillin/streptomycin was added into the culture medium.

Thawing and proliferation of cells: The cryo tube containing Du-145 cells, stored at -80C, was heated in a 37ºC water bath. The heating process was carried out quickly, in about 2 minutes. In the laminar flow; Cells were taken from cryo tubes sterilized with 70% ethanol and transferred to a 15 ml Falcon tube containing fresh culture medium. The falcon tube was centrifuged at 1500 rpm for 5 minutes and the supernatant was removed. Bottom cells were resuspended with fresh culture medium. The cells taken into the cell culture dish were grown in an oven at 37ºC and 5% CO2.

Counting and Passaging of Cells: When the cells reached 80% confluency, hemocytometric counts and passages were performed. In the first step of the passaging process, the medium in the cell culture dish was removed and washed twice with PBS so that the serum residues in the dish did not inhibit the trypsin enzyme to be used in the next step. After washing, PBS was removed, trypsin/EDTA enzyme solution was added to the culture dish and the cells were

expected to be completely separated from the surface. Enzyme activity of trypsin was stopped by adding 3 ml of fresh culture medium to the culture dish. The cell suspension was collected in 15 ml falcon tubes and centrifuged at 1500 rpm for 5 minutes. The cell pellet in the tube was counted after being resuspended with fresh culture medium and the desired number of cells was seeded into new cell culture dishes. Cells were proliferated by incubating at 37°C and 5% CO2 in an incubator.

Trypan blue dye was used for counting and detection of viable cells. While this negatively charged dye cannot enter living cells with intact cell membranes, it can enter non-living cells with impaired membrane structure. For the dyeing process; In Eppendorf, 50µl of cell suspension and 0.4% trypan blue dissolved in 50µl of PBS were added and mixed. After waiting for 5 minutes, 10 microliters of this mixture were placed in each counting camera on the Neubauer counting slide and unstained live cells were counted under the microscope.

After counting under the microscope, the number of viable cells in 1 milliliter was calculated using the formula below.

(Total of cells counted/ Squares counted) x Dilution Factor x 10^4/ml

3.2.3 MTT Assay:

MTT Solution: 50 mg of MTT was dissolved in 10 mL of Dulbecco's Phosphate Buffered Serum Physiological. The prepared solution was filtered through a membrane filter into a sterile, opaque tube. The prepared MTT solution was stored at +4°C, protected from light.

Cells were incubated at 37°C for 24 hours in an incubator with 5% CO2 and 95% humidity. At the end of the incubation, the substance solutions were discarded. 90 µL of the medium and 10 µL of the prepared 5 mg/ml MTT solution were added to each well. Cells were incubated for another 3 hours in an oven containing 37°C, 5% CO2 and 95% humidity.

At the end of the 3-hour incubation period, the MTT solution was discarded. To dissolve the formazan crystals formed in the wells, 100 µL of dissolving solution was added to each well. The plate was shaken on a horizontal shaker for 15 minutes to terminate dissolution. The color

intensity formed in the wells after shaking was measured at 570 nm in a spectrofluorometer device. Because the dye degrades in light, each step of the experiment was performed in as dark as possible. Cell viability was calculated by multiplying the ratio of the absorbance value obtained for the each lansoprazole dose to the control absorbance value by 100. Experiments were repeated three times.

3.2.4 Immunoblotting and Measurement of Protein Expressions by Western Blotting

3.2.4.1 Protein isolation from cells

Control and Lansoprazole-treated cells in cell culture dishes were removed with trypsin, transferred to separate falcon tubes and centrifuged at 1500 rpm for 5 minutes at 4°C. Cells washed twice with PBS were transferred to clean eppendorf tubes and suspended with lysis buffer. Eppendorfs were kept in ice for 30 minutes to allow the cells to lysis. Meanwhile, mechanical fragmentation with an injector was also applied. Then it was centrifuged for 25 minutes at 14 000 rpm at +4°C. After centrifugation; The supernatant portion containing the proteins in the ependorfs was carefully separated and transferred to new ependorfs and stored at -20°C.

3.2.4.2 Protein quantification

The amount of obtained proteins was measured by the Bicinchoninic Acid (BCA) protein determination method. This method is based on the reduction of Cu+2 to Cu+1 (Biuret reaction) of proteins in an alkaline medium and then the spectrophotometric measurement of the colored complex given by Cu+1 with BCA at 562 nm.

First of all, albumin standards were prepared with distilled water at concentrations of 0, 25, 125, 250, 500, 750, 1000, 1500, 2000 µg/ml. After 10 µl of samples and standards were placed in duplicate in the wells of the 96-well plate, 200 µl of BCA reagent was added to them. It was kept in an incubator at 37 °C for 30 minutes. At the end of the incubation, absorbance values of the wells were measured in a microplate reader at 562 nm. Standard curve was created with the obtained values and protein concentrations of the samples were calculated using this curve:

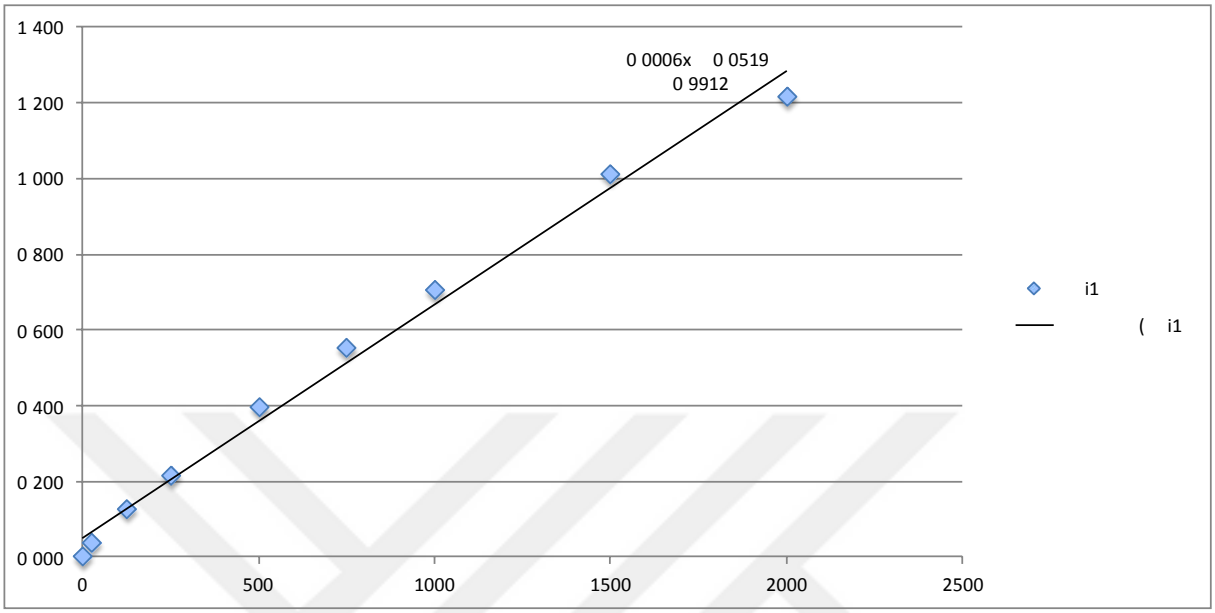


Figure 3.1: Graph of absorbance versus standard protein concentration used in the BCA protein determination method.

3.2.4.3 SDS-PAGE (Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis)

A 10% polyacrylamide electrophoresis gel was prepared using the materials and ratios shown in the table.

	% 5 Stacking Gel	% 10 Seperating Gel
Distilled Water	**2,7 ml**	**3,96 ml**
1,5 M Tris(pH 8,8)	**-**	**2,5 ml**
1 M Tris(pH 6,8)	**500 ul**	**-**
30% Acrilamide/Bisacrilamide	**670 ul**	**3,34 ml**
10% SDS	**40 ul**	**100 ul**
10% APS	**40 ul**	**100 ul**
TEMED	**4 ul**	**4 ul**

Table 3.1: Required chemicals for the preparation of SDS-PAGE gel.

After the gels were prepared, they were placed in the electrophoresis tank. ¼ of sample loading buffer was added to 25 µg protein taken from each sample and denatured by heating at 95°C for 5 minutes. Denatured samples and protein marker (ladder) were loaded onto the gels placed in the tank. By adding running buffer to the tank, the proteins were separated at 110 volts constant current for 10 minutes and then at 100 volts constant current.

3.2.4.4 Measurement of Protein Expression Levels by Western Blot Method

Proteins separated according to their molecular weights by SDS-PAGE were transferred to nitrocellulose membrane by wet transfer system. After the transfer process, the proteins were stained with Ponceau S stain. Then, the membrane was blocked with TBST containing 5% milk powder for 1 hour at room temperature in a shaker. Afterwards, the membrane was incubated in a shaker at +4°C overnight with the specific primary SOD-2 antibody prepared with a 5% milk powder-TBST mixture at a ratio of 1:500. The next day, the membrane was washed 3 times with 1X TBST. After removing unbound antibodies, the membrane was incubated with HRP-conjugated secondary antibody prepared at a ratio of 1:5000 using a 1% milk powder-TBST mixture for 1 hour at room temperature in a shaker. After the membrane was washed 3 times with 1X TBST and treated with ECL solution at room temperature for 5 minutes, the bands were visualized with the ChemiDoc XRS system device. Band intensities were analyzed with the Image Lab program and all proteins were normalized to their respective GAPDH protein bands as a loading control.

3.2.5 Annexin/PI Flow Cytometry Analysis

Cells or particles in suspension are passed through a chamber illuminated by laser light by flow cytometry. The signals given by the cells as they pass in front of the light are collected and analyzed. The source of the signals can be physical characteristics of the cell such as size and granularity; There may also be various fluorochromes attached to the cell. Thus, information about various properties of the cell or particle such as immunophenotype, DNA content, enzyme activities, cell membrane potential and viability can be collected. As each cell or particle passes through the laser beam, the deflected laser light and the fluorescent light emitted by the cells are

brought together and converted into analog signals, separated by optical filters and mirrors according to different wavelengths. These signals are digitized and transferred to the screen as histograms. The histogram is a visual representation of the frequency distributions of the measured parameters.

Cells must be live or stationary at the time of measurement and individual cells must be suspended in the liquid. The cells in suspension must pass through the laser beam in a continuous flow. Each cell deflects some of the laser light and at the same time emits fluorescent light because they are excited by the laser, that is, they are loaded with extra energy. Apoptosis, known as programmed cell death, can be studied at various stages according to its developmental stages. It has been determined that in the early stages of apoptosis, cells extrude their phosphatidylserine structures from the cell membrane. In the later stages, pores are formed in the membrane with the deterioration of the cell membrane structure. The apoptotic process is one of the cell death pathways, which is characterized by breaks in the cell DNA, bleb formations containing some of the membranes and organelles. Annexin V, which provides staining of phosphatidylserines released in the cell membrane during the activation of the apoptosis pathway, is displayed in the FL1 panel since it is FITC conjugated, while Propidium Iodide (PI), which stains the cell nucleus, is displayed in the FL2 panel by staining the late stage of apoptosis and necrotic cells. As a result, unstained cells are considered viable, cells stained only with Annexin V are considered early apoptotic, cells stained with both dyes are considered late apoptotic, and cells stained with PI alone are considered necrotic.

Staining of the cells with Annexin V/PI was determined. Cells were seeded at 320 000 cells/well in 6-well plates containing 2 ml of medium and incubated for 24 hours at 37°C for adhesion. After treatment with determined doses of lansoprazole, apoptosis and necrosis rates at 6 hours were analyzed by flow cytometry device. After cells were harvested into eppendorfs, they were washed twice with cold PBS. Then, it was centrifuged at 1500 rpm (2 819 x g) for 5 minutes and the supernatant was removed. AnnexinV binding buffer was added so that the concentration of the cell pellet was 106 cells/mL, 100 µL (105 cells) was taken in it, 5µl Annexin V, 5µl PI were added. The tubes were incubated for 15 minutes at room temperature in the dark. Afterwards, the tubes, in which 400 µl of binding buffer was added, were placed on ice and analysis was performed by flow cytometry.

3.2.6 Statistical Analysis

Data of this study was analyzed using the GraphPad Prism 7 program. The results of the control and treatment groups were compared with the ANOVA test. Values with $p < 0.05$ were considered statistically significant.

4. RESULTS

4.1 MTT Assay after Lansoprazole Administration

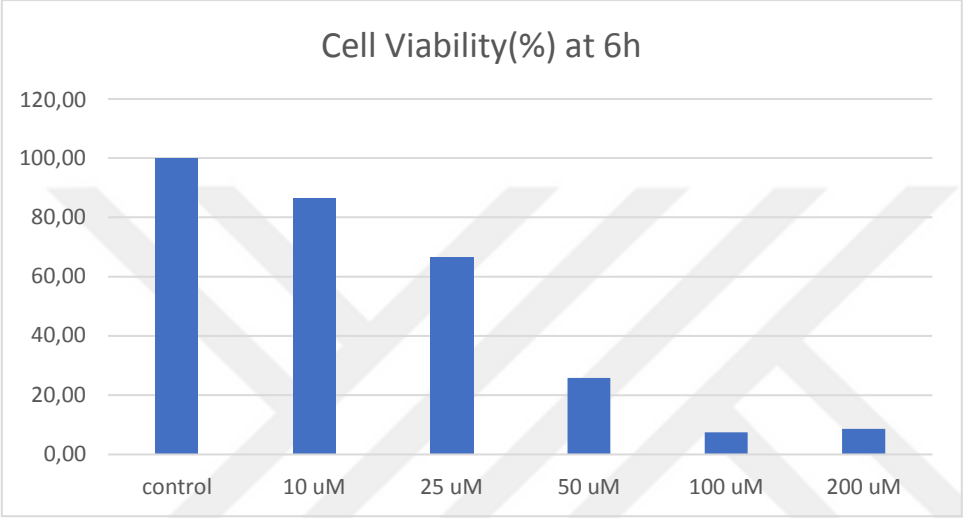


Figure 4.1: MTT Assay Results of Lansoprazole(10, 25, 50, 100, 200 uM and Control) at 6h. Viability percent of DU-145 cells by doses of 10, 25, 50, 100, 200 uM Lansoprazole are % 86, % 66, % 25, % 7 and % 8 respectively.

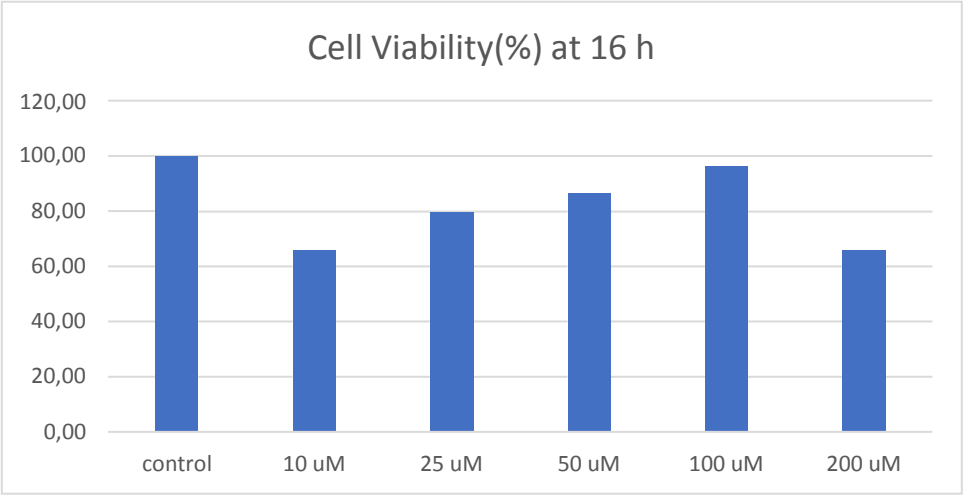


Figure 4.2: MTT Assay Results of Lansoprazole(10, 25, 50, 100, 200 uM and Control) at 16h. Viability percent of DU-145 cells by doses of 10, 25, 50, 100, 200 uM Lansoprazole are % 65, % 79, % 86, % 95 and % 65 respectively.

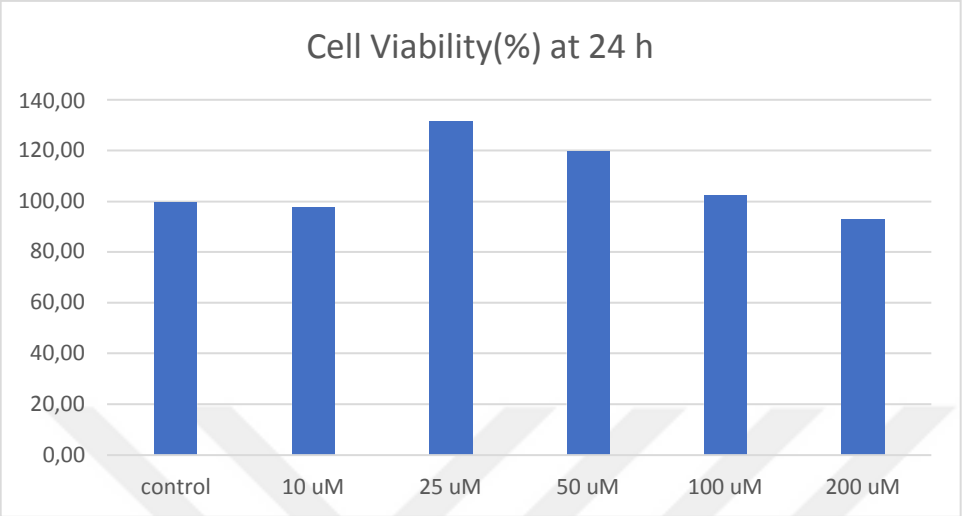


Figure 4.3: MTT Assay Results of Lansoprazole(10, 25, 50, 100, 200 uM and Control) at 24h. Viability percent of DU-145 cells by doses of 10, 25, 50, 100, 200 uM Lansoprazole are % 97, % 131, % 119, % 102 and % 93 respectively.

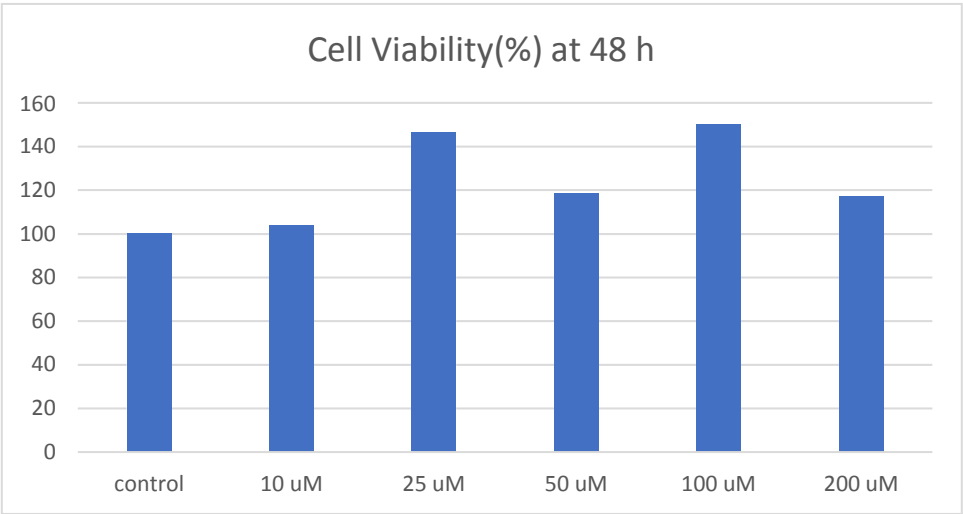


Figure 4.4: MTT Assay Results of Lansoprazole(10, 25, 50, 100, 200 uM and Control) at 48h. Viability percent of DU-145 cells by doses of 10, 25, 50, 100, 200 uM Lansoprazole are % 103, % 146, % 118, % 150 and % 117 respectively.

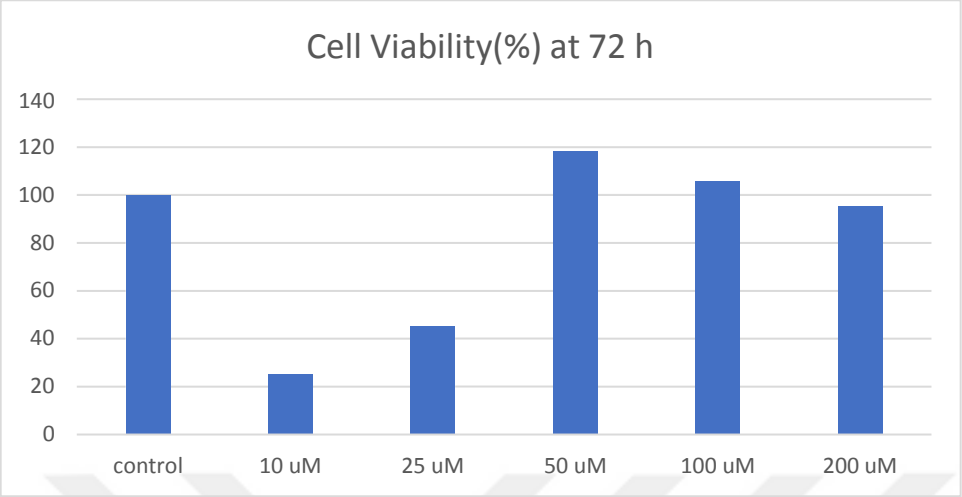


Figure 4.5: MTT Assay Results of Lansoprazole(10, 25, 50, 100, 200 uM and Control) at 72h. Viability percent of DU-145 cells by doses of 10, 25, 50, 100, 200 uM Lansoprazole are % 25, % 45, % 118, % 105 and % 95 respectively.

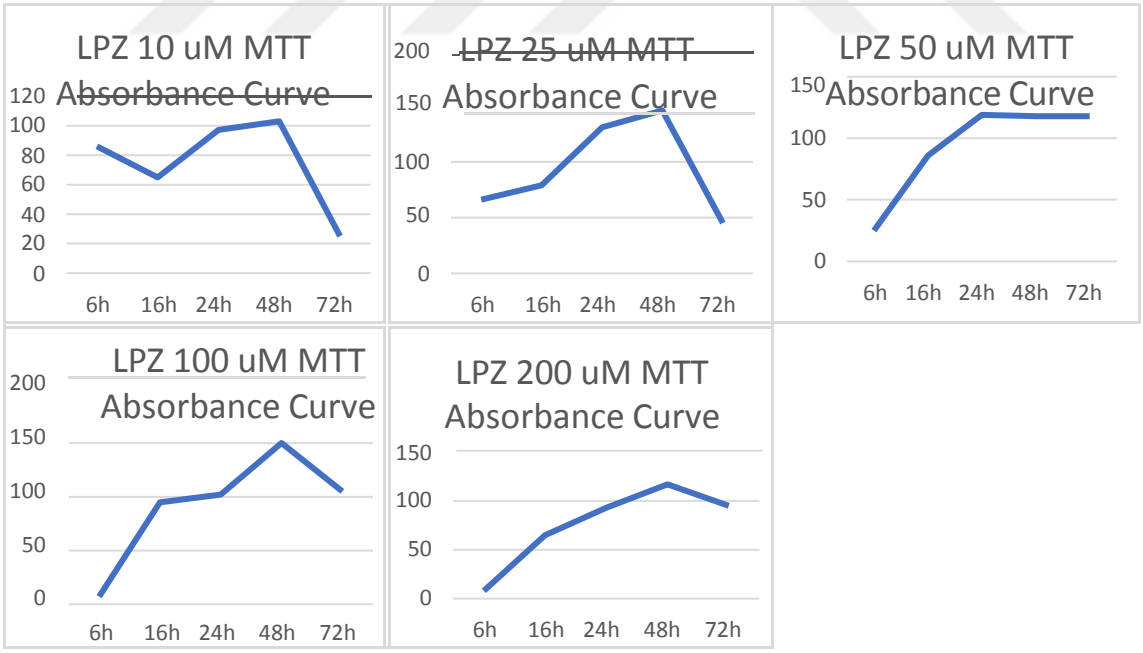


Figure 4.6: Lansoprazole 10, 25, 50, 100 and 200 uM MTT Assay Absorbance Curves at 6, 16, 24, 48 and 72 hours. Generally, there is a similar curve pattern among different doses. At early hours(6 and 16 h) curve has an ascending pattern. During 24 and 48 hours, rise of the curve decreases and the curve draws a plateau. After 48 hours, curve has a descending pattern.

4.2 Cell Viability Analysis with Trypan Blue

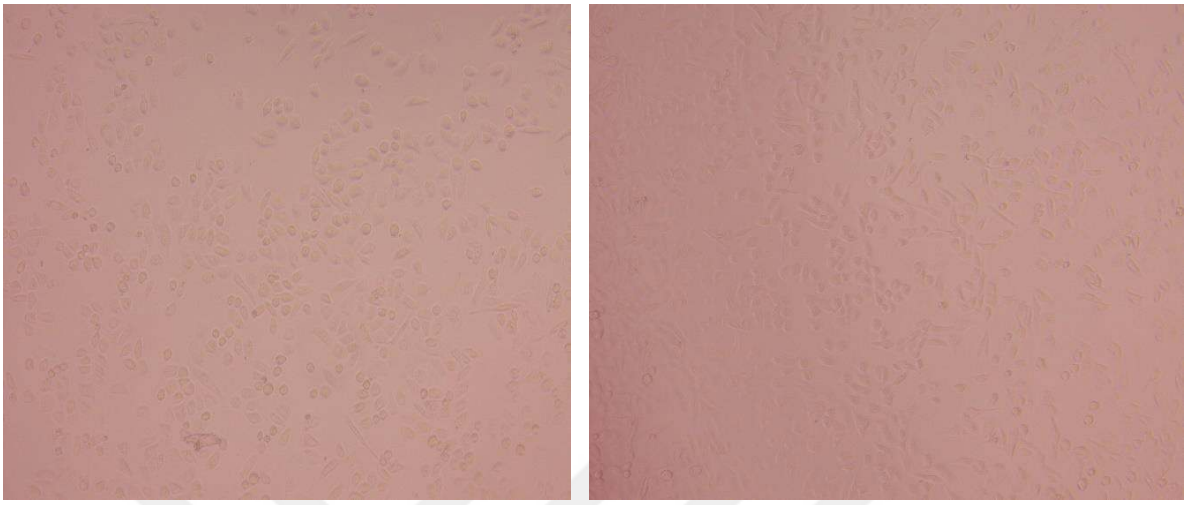


Figure 4.7: Inverted fluorescent Microscopic view of Du-145 Cells Before Lansoprazole Administration. Cells were adhered to the flask and elongated.

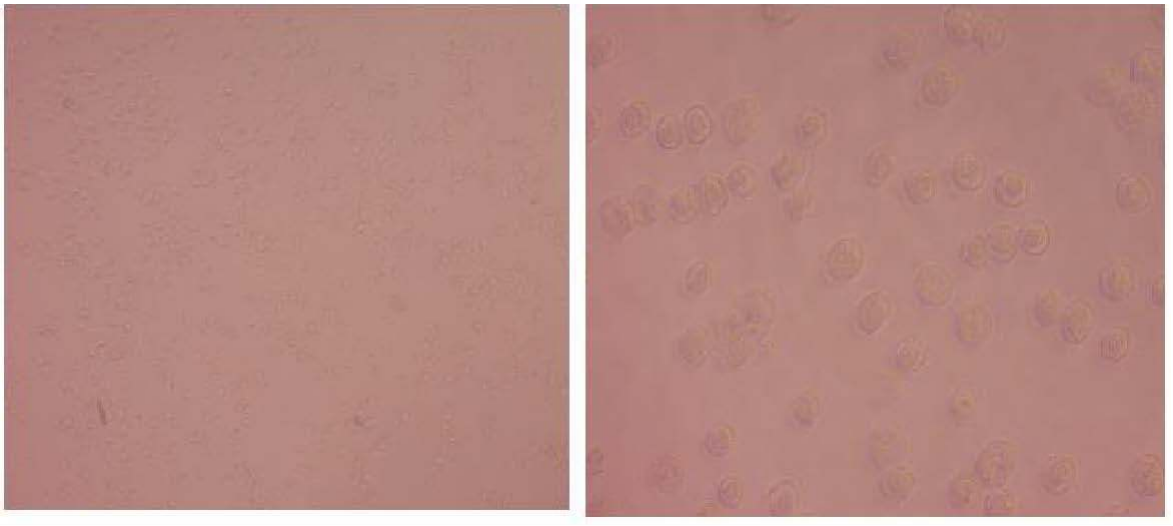
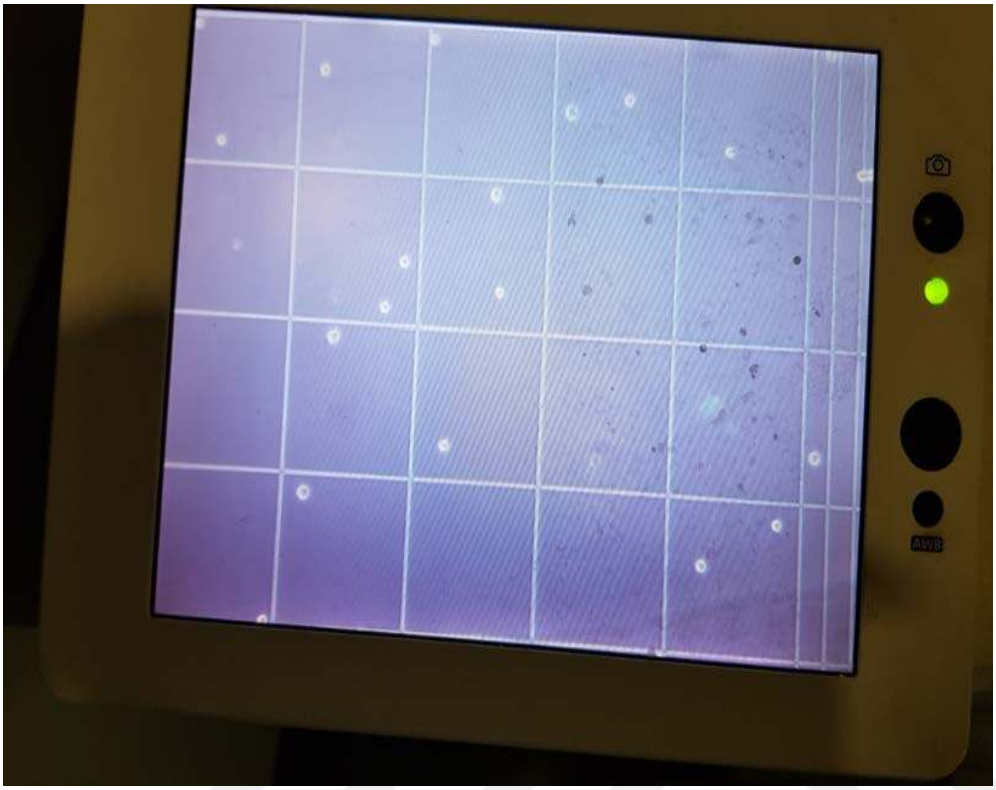


Figure 4.8: Inverted fluorescent Microscopic view of Du-145 Cells After Lansoprazole Administration. Cells were round shaped.



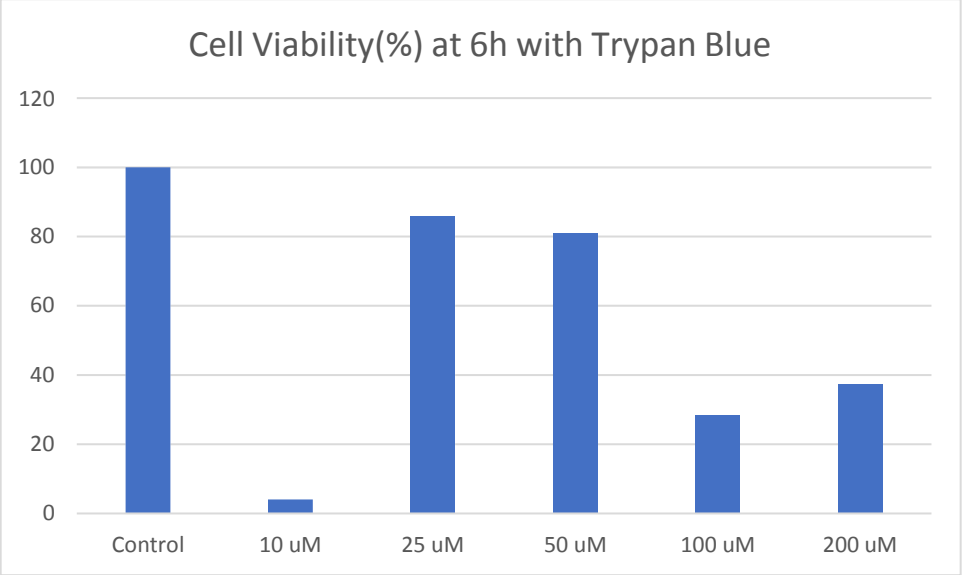


Figure 4.9: Cell Viability with Trpyan Blue Cell Counting after Lansoprazole administration(10, 25, 50, 100, 200 uM and Control) at 6h. The most effective doses of Lansoprazole are 10 and 100 uM, % 4 and % 28,5 respectively.

4.3 Western Blot Analysis of Caspase-9 and PARP

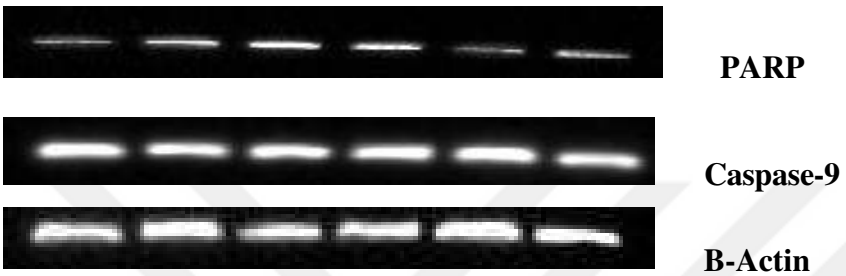


Figure 4.10: PARP and Caspase-9 Western Blotting of Du-145 Cells after Lansoprazole administration(10 and 100 uM) at 6h. PARP and Caspase-9 expressions were increased but not significantly($p > 0,05$).

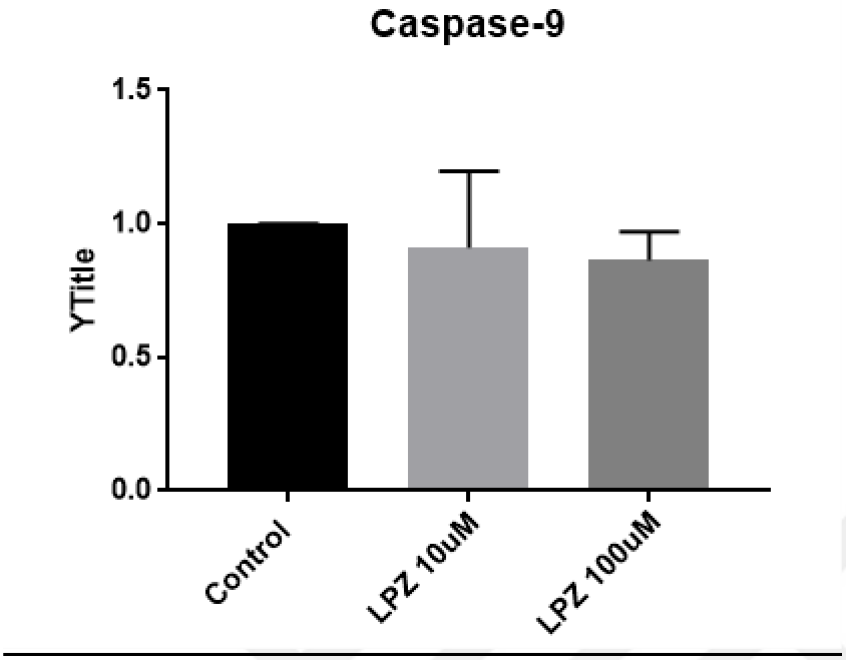


Figure 4.11: Caspase-9 Levels of Du-145 Cells after Lansoprazole administration(10 and 100 uM) at 6h($p > 0,05$)

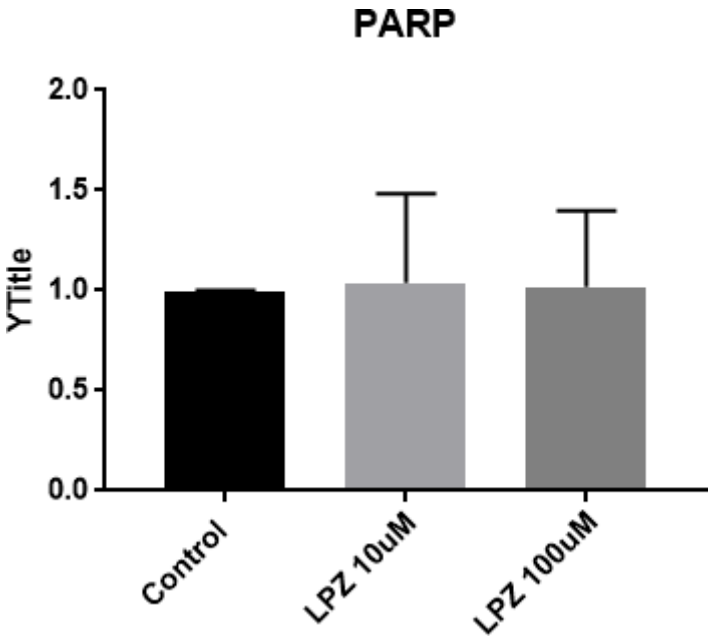


Figure 4.12: PARP Levels of Du-145 Cells after Lansoprazole administration(10 and 100 uM) at 6h($p > 0,05$)

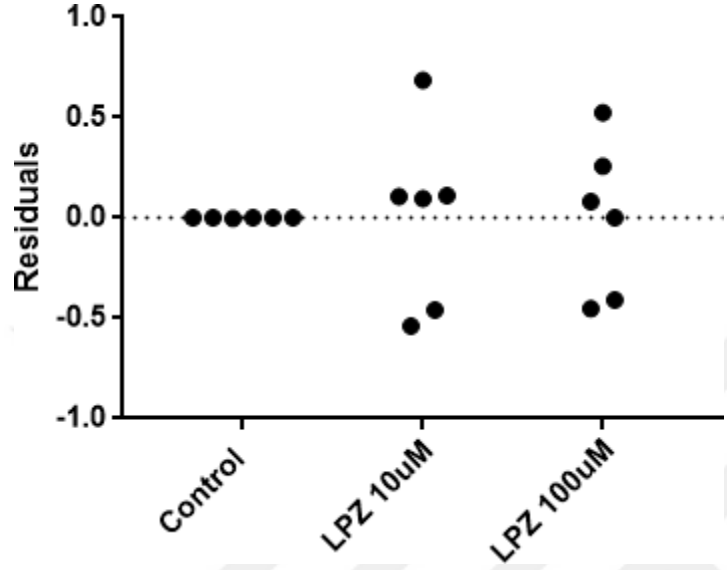


Figure 4.13: Ordinary one-way ANOVA analysis of PARP Levels of Du-145 Cells after Lansoprazole administration(10 and 100 uM) at 6h($p > 0,05$).

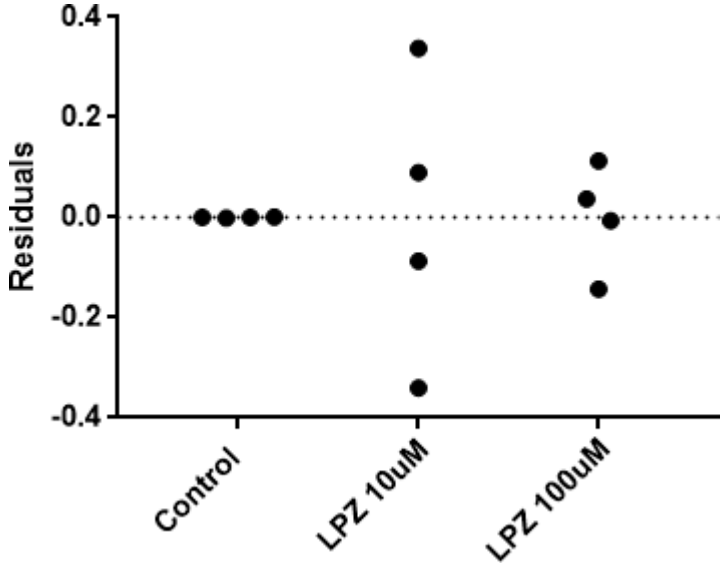


Figure 4.14: Ordinary one-way ANOVA analysis of Caspase-9 Levels of Du-145 Cells after Lansoprazole administration(10 and 100 uM) at 6h($p > 0,05$).

4.4 Annexin-PI Apoptosis Analysis by Flow Cytometry

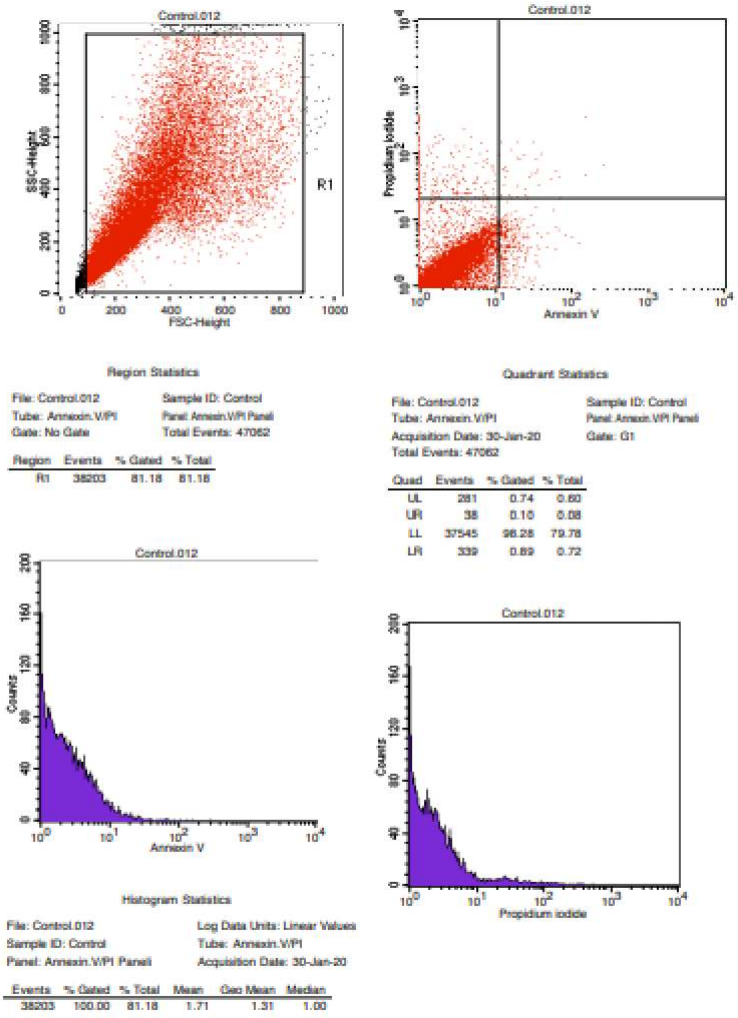
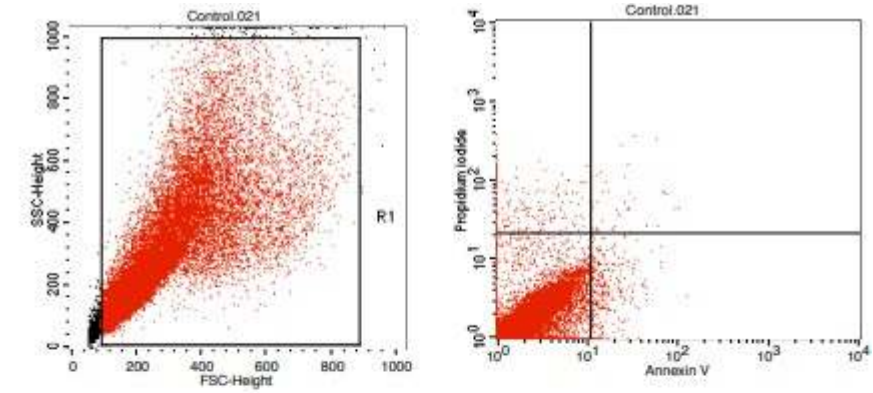


Figure 4.15: Analysis of Apoptosis by FACS with Annexin-PI staining at 6h(Control).



Region Statistics

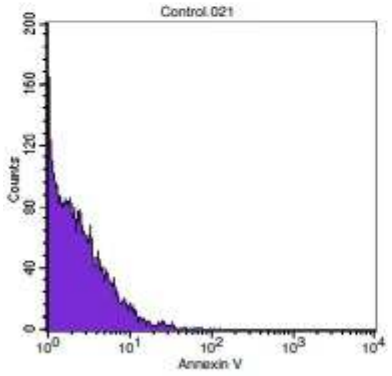
File: Control.021 Sample ID: Control
Tube: Annexin.V/PI Panel: Annexin.V/PI Panel
Gate: No Gate Total Events: 53563

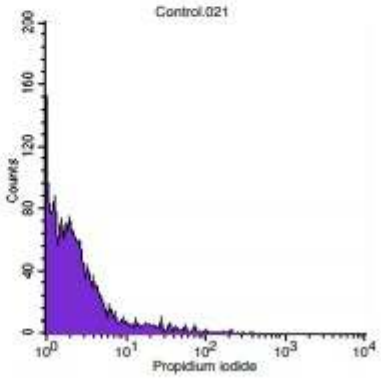
Region	Events	% Gated	% Total
R1	42317	79.00	79.00

Quadrant Statistics

File: Control.021 Sample ID: Control
Tube: Annexin.V/PI Panel: Annexin.V/PI Panel
Acquisition Date: 30-Jan-20 Gate: G1
Total Events: 53563

Quad	Events	% Gated	% Total
UL	281	0.66	0.52
UR	39	0.09	0.07
LL	41645	98.41	77.75
LR	352	0.83	0.66





Histogram Statistics

File: Control.021 Log Data Units: Linear Values
Sample ID: Control Tube: Annexin.V/PI
Panel: Annexin.V/PI Panel Acquisition Date: 30-Jan-20

Events	% Gated	% Total	Mean	Geo Mean	Median
42317	100.00	79.00	1.66	1.30	1.00

Figure 4.16: Analysis of Apoptosis by FACS with Annexin-PI staining at 6h(Control).

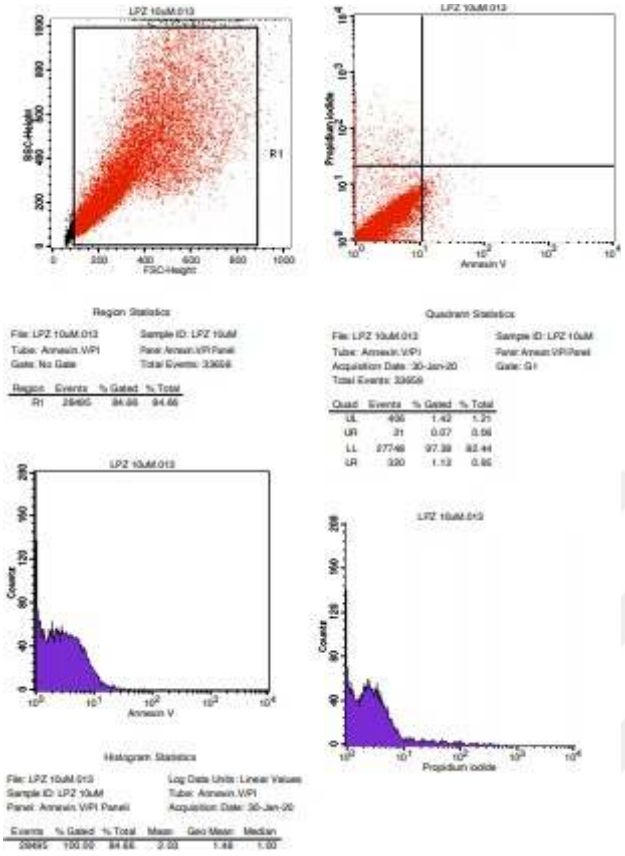


Figure 4.17: Analysis of Apoptosis by FACS with Annexin-PI staining at 6h(Lansoprazole 10 uM).

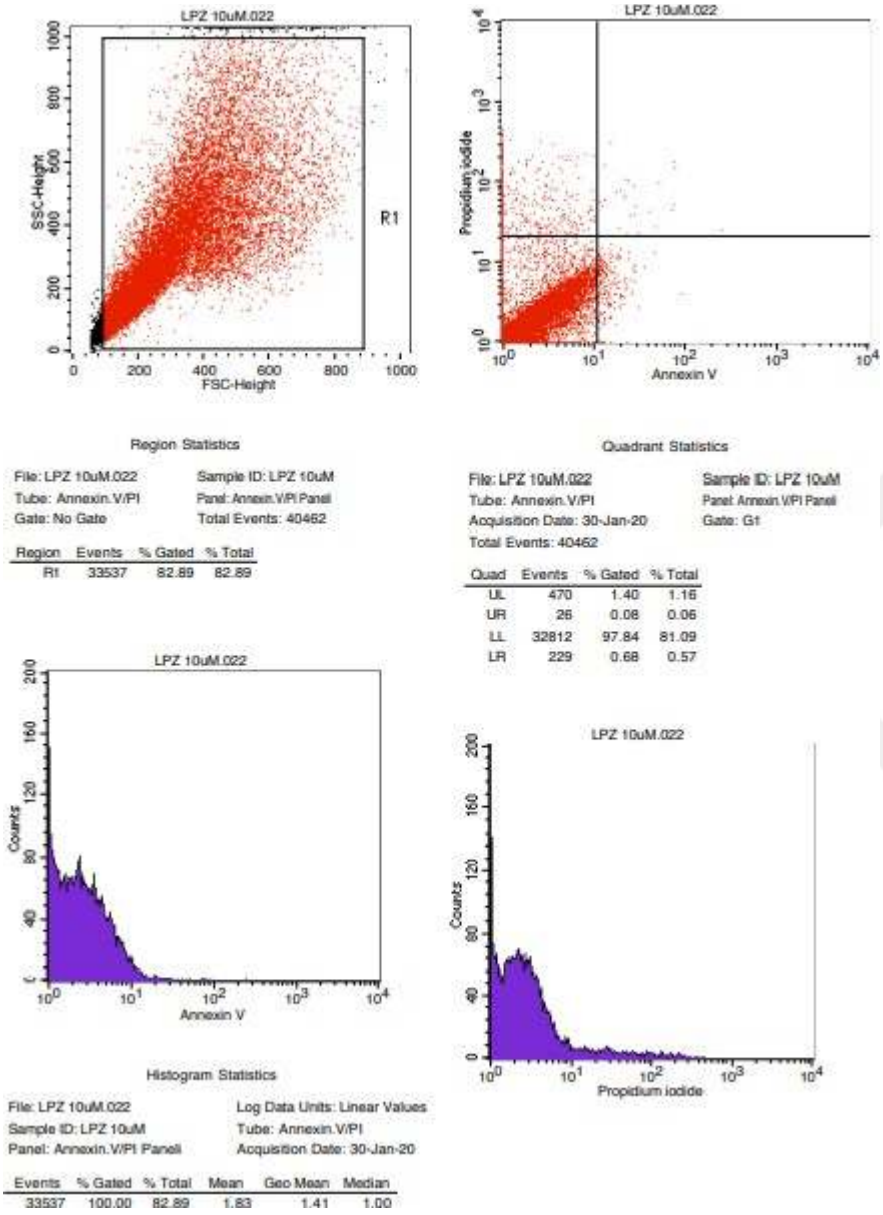


Figure 4.18: Analysis of Apoptosis by FACS with Annexin-PI staining at 6h(Lansoprazole 10 uM).

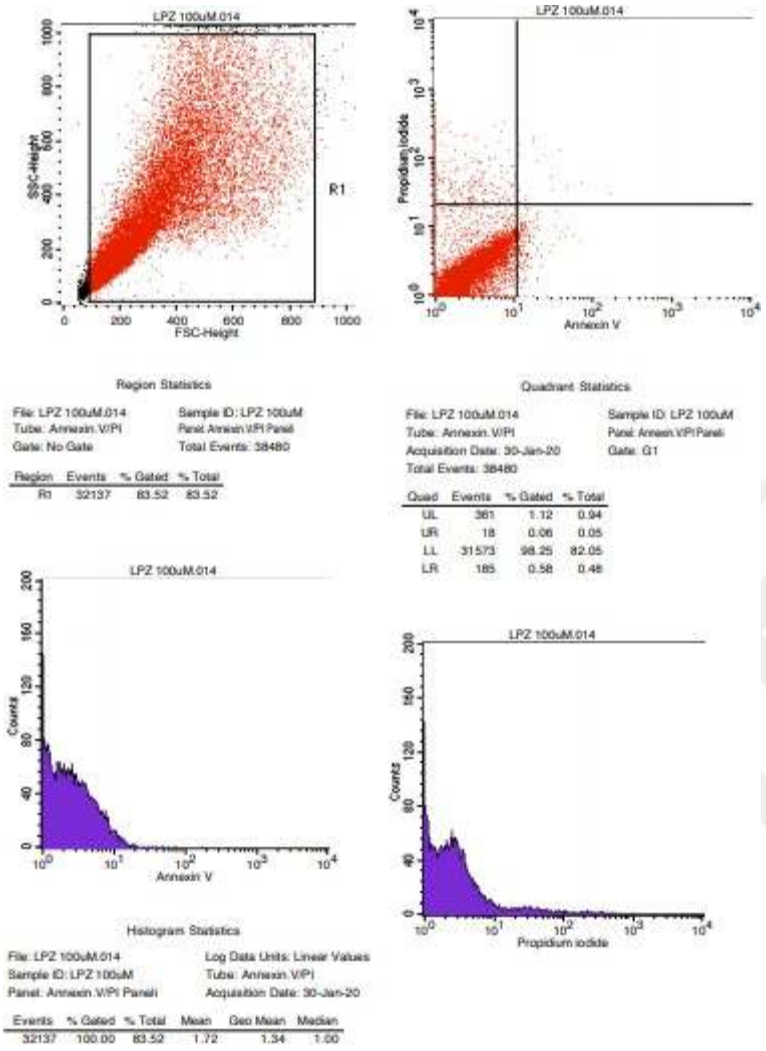


Figure 4.19: Analysis of Apoptosis by FACS with Annexin-PI staining at 6h(Lansoprazole 100 uM).

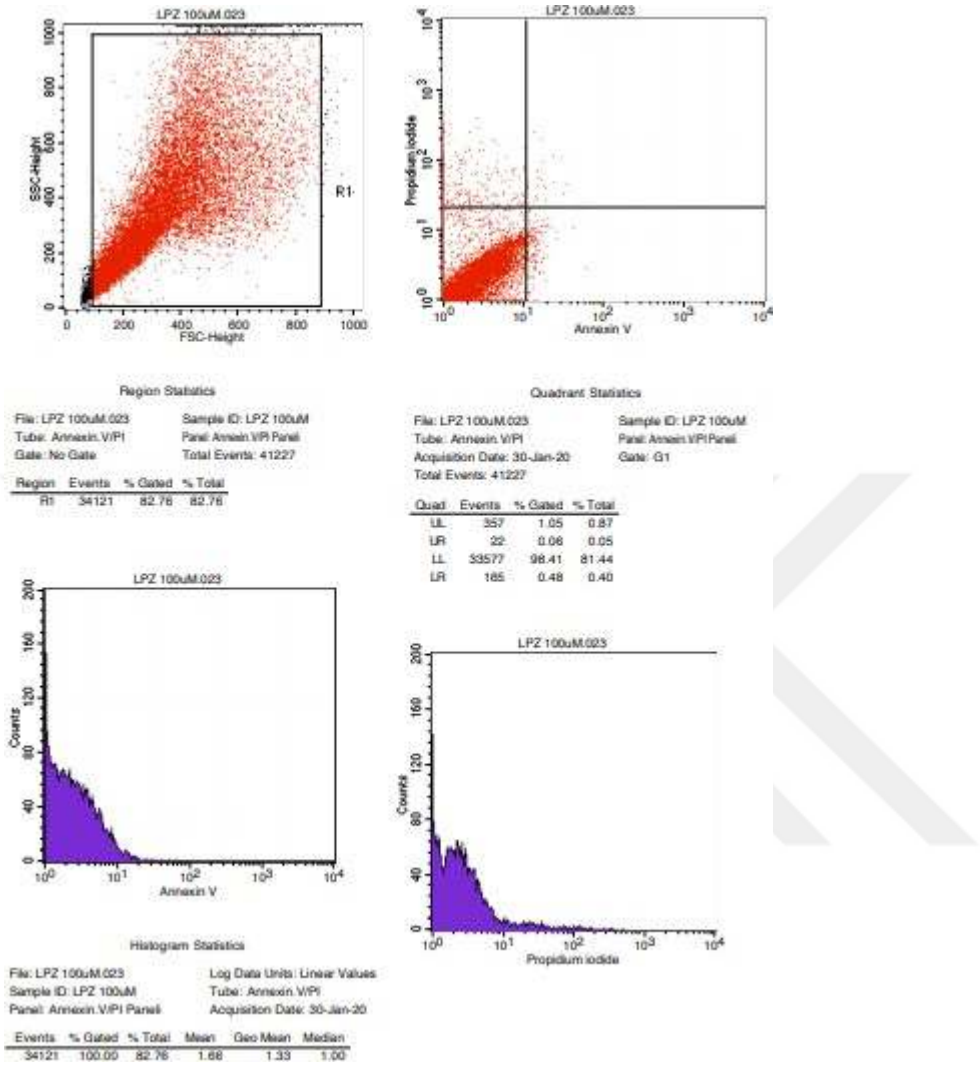


Figure 4.20: Analysis of Apoptosis by FACS with Annexin-PI staining at 6h(Lansoprazole 100 uM).

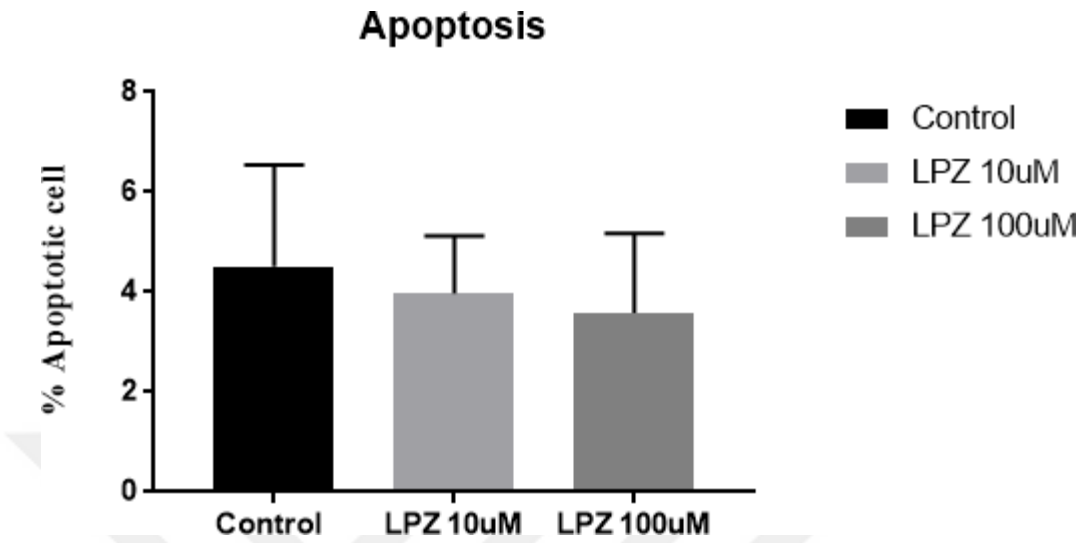


Figure 4.21: Apoptosis Rates of Lansoprazole 10 uM and 100 uM doses analyzed FACS with Annexin-PI staining at 6h(p >0,05).

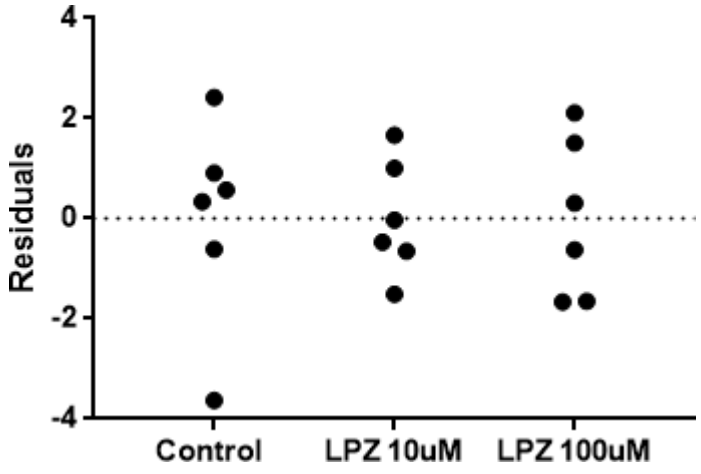


Figure 4.22: Ordinary one-way ANOVA analysis of apoptosis on Du-145 Cells at 6h(p >0,05).

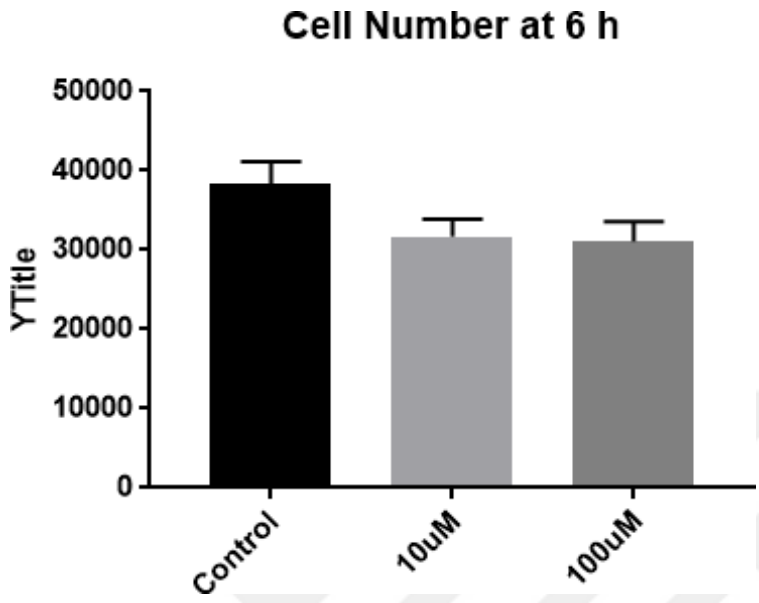


Figure 4.23: Events/Total Du-145 Cell numbers after Lansoprazole 10 uM and 100 uM administration at 6h. Cell growth is significantly inhibited(p <0,05 Ordinary one way ANOVA, GraphPad Prism 7).

5. DISCUSSION, CONCLUSION AND RECOMMENDATIONS

5.1 Discussion

Cancer has been a major threat on human health since 20th century and is one of the most significant causes of death among people. The incidence of new cases of cancer are rising as well. It is the second leading cause of death globally and estimated that almost 10 million people die from cancer in 2020(Sung H. Et al, 2021). The most common cancers that are diagnosed in men are lung, prostate, colorectal, stomach and liver cancers. On the other hand, breast, colorectal, lung, cervix and thyroid cancer are most frequently diagnosed among women. Not only cancer patients suffer from their disease but also health systems, economies of governments are affected. With the help of early detection of cancer and novel treatments, survival rates are increasing but there is a need for improvements related with detection, diagnosis and therapy of cancers(https://www.who.int/health-topics/cancer#tab=tab_1).

Prostate cancer is a type of cancer that primarily hits middle-aged and elder people. According to US statistics, it is the second most cause of death among males(Humprey, 2014). There are both conventional and new therapies for prostate cancer which improves both survival of patients from disease and life quality. For instance, FDA-approved immunotherapeutic agent sipuleucel-T is an autologous dendritic cell vaccine that is preferred for metastatic castration resistant prostate cancer. According to IMPACT research, the median survival time was increased 4.1 months with sipuleucel-T. Drug-given patients survival time was 25.8 months comparing to 21.7 months for placebo group(Kantoff PW. et al, 2010). Another drug, pembrolizumab which is an anti-PD-1 antibody that is efficent for androgen deprivation therapy(ADT) resistant prostate cancer is approved by FDA in 2017. Although these innovations helped both clinicians and patients during disease process, some patients do not respond to these new treatments and disease progression was detected(Graff J et. al., 2016). This is probably because of tumor microenvironment and pH dynamics which affects therapy efficency. In addition, this dynamics contribute to drug resistance which possibly alleviates the respond of patient to therapies.

In this research, we wanted to understand antiproliferative and cytotoxic effects of lansoprazole. To do this, we initiated with MTT assay analysis. Variability of MTT assay results that we found may be related with superoxide and ROS production in Du-145 cells. At early hours like 6 and 16 hours after lansoprazole administration, MTT absorbance curve has an ascending pattern generally. During 24 and 48 hours, the curve draws almost a plateau for each doses of lansoprazole. After 48 hours of lansoprazole, the curve goes to a descending pattern from plateau. In addition, it is possible that mitochondrial electron transport chain(ETC) complex 3 can be inhibited by lansoprazole. It is revealed that the drug has an inhibitory action on the complex 3 on Mycobacterium tuberculosis which facilitates extra ROS production in mitochondria(Rybniker J. Et al., 2015). This is not identified for human mitochondria yet but we know that ETC complex is evolutionary conserved. Thus, lansoprazole could be a similar effect on human mitochondria as well. Moreover, antiproliferative and cytotoxic effects of lansoprazole may be stronger at early hours like 6 and 16 hours. It is known that degradation half life of lansoprazole is about 18 hours at pH 7 in aqueous solutions at 25 degrees(https://pubchem.ncbi.nlm.nih.gov/compound/Lansoprazole). It is also revealed that proton pump inhibitors modulate or inhibit autophagy, promote ER stress and slow cell cycle on cancer cells in different studies(Cao et. Al., 2018; Geeviman K. et al., 2018). This effects can contribute to MTT assay results. A different perspective on MTT assay results of this research is that some chemicals could give false positive or variable MTT assay results(Karakas D. et al., 2017). Another research conducted in UCLA is about variable or false MTT results of green tea polyphenols which includes ring structures in their chemical structures on LnCaP prostate cancer cells and MCF-7 breast cancers cells(Wang P. et al., 2010). Although MTT assay problems has not been defined for lansoprazole yet, it is possible for lansoprazole because it has a ring structure on its chemical structure as well.

After MTT assay, we identified the cytotoxic, apoptotic and antiproliferative effects of lansoprazole specifically at 6 hours. This is because the drug has a half life of degradation of 18 hours at pH 7 at 25 C. We wanted to monitor the early phase(6 hours) effect of lansoprazole on Du-145 prostate cancer cells.

We visualized cells that stained with trypan blue and counted the stained dead cells as previously mentioned before. The most effective doses of Lansoprazole are 10 and 100 uM, cell viability of % 4 and % 28,5 respectively. We chose the most effective doses(10 and 100 uM) to perform PARP and Caspase-9 Western immunoblotting and Annexin/PI Flow cytometry apoptosis analysis.

Western Blotting of PARP and Caspase-9 at 6 hours with lansoprazole 10 and 100 uM are not significantly expressed in our study($p > 0,05$). This is likely that analysis were conducted at 6 hours which maybe early for PARP and Caspase-9 overexpression, technical reasons or caspase-independent cell death mechanisms of proton pump inhibitors in the literature. In addition, changes related with these parameters(PARP and Caspase-9) differs between cell types and subtypes, cytotoxic agents that are used in the experiment and the time of onset of their expressions could be dependent on time. It would be more descriptive if we performed experiments for 24 and 48 hours.

According to the lansoprazole 6h Annexin-PI Flow cytometer analysis results, it is seen that the drug has a suppressive effect (antiproliferative) on the growth dynamics of prostate DU-145 cells. It is known that proton pump inhibitors and lansoprazole suppresses autophagy, lysosomal functionality by V-ATPase inhibition. Moreover, a possible ER and mitochondrial stress, or the direct or indirect effects of the drug on major growth pathways such as mTORC1 (autophagy) may be the cause of this effect. However, we did not detect apoptosis or necrosis by Annexin/PI flow cytometry analysis at 6 hours.

Cytotoxic effect of lansoprazole at 6 hours is most probably related with its nonapoptotic, autophagy related death mechanism which is also identified in other proton pump inhibitors like pantoprazole, omeprazole(Udelnow A. Et al., 2011).

5.2 Conclusions

In conclusion, in line with the 6th hour data of lansoprazole admistration of Du-145 castration-resistant prostate cancer cells, we can state that lansoprazole promotes cell death likely to be caspase-independent and suppresses cell proliferation and growth dynamics at 6 hours, at 10 and 100 micromolar doses.

5.3 Recommendations

This early period data may have the following advantage: lansoprazole creates the metabolic weaknesses mentioned before and also changes the biochemical structure of the extracellular matrix and tumor microenvironment (extracellular pH, lysosomal activity, activity levels of enzymes such as MMP and similar proteases, etc.), breaking chemotherapy resistance, preventing invasion previously defined in literature. Its use in combination therapy to slow down metastasis should also be considered. The effect of lansoprazole on cell growth may produce a strong additive effect in combination chemotherapy. Of course, there is a need for larger studies and possible application areas.

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PLAGIARISM REPORT

ANTIPROLIFERATIVE AND APOPTOTIC EFFECTS OF PROTON PUMP INHIBITOR LANSOPRAZOLE ON DU-145 PROSTATE CANCER CELLS

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