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INVESTIGATION THE EFFECT OF PROTEASOME INHIBITION ON DEATH RECEPTORS IN BREAST CANCER CELL LINES

MASTER THESIS

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Tokat Gaziosmanpaşa Üniversitesi Lisansüstü Eğitim Enstitüsü tez yazım kılavuzuna göre, Doç. Dr. Ercan ÇAÇAN danışmanlığında hazırlamış olduğum "Investigation The Effect of Proteasome Inhibition on Death Receptors in Breast Cancer Cell Lines" adlı Yüksek Lisans tezinin bilimsel etik değerlere ve kurallara uygun, özgün bir çalışma olduğunu, aksinin tespit edilmesi halinde her türlü yasal yaptırımı kabul edeceğimi beyan ederim.

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ÖZET

MEME KANSERİNDE PROTEAZOM İNHİBİSYONUNUN ÖLÜM RESEPTÖRLERİ ÜZERİNDEKİ ETKİSİNİN ARAŞTIRILMASI

Khorsheed, Rabar Abdulelah

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Meme kanseri dünyada en sık görülen kanserlerden biridir. Meme kanseri tedavisi için çeşitli modern genetik modülasyon ve modifikasyon yöntemleri geliştirilmiştir. Malignite tedavisi için yeni stratejiler geliştirilse de araştırılması gereken birçok yeni alan bulunmaktadır. Bu tez çalışmasında, iki meme kanseri hücre hattında çeşitli ölüm reseptörlerinin ekspresyon profillerindeki değişiklikleri belirlemek için 26S proteazom inhibitörü bortezomib'i kullandık. Başlangıçta hücreler, kullanılan iki meme kanseri hücre hattının hücre canlılığı üzerinde etkili olan belirli dozları belirlemek için farklı konsantrasyonlarda bortezomib ile tedavi edildi. MTT sonuçları, artan bortezomib konsantrasyonlarının, doza bağlı bir şekilde hücre canlılığını azalttığını ortaya koydu. Ayrıca, apoptoz indüksiyonunda önemli roller oynayan birkaç ölüm reseptörünün ekspresyonundaki bortezomib aracılı potansiyel değişiklikleri de test ettik. Toksik olmayan düşük dozdaki bortezomib uyglamasınınmeme kanseri hücre hatlarında FAS (CD95), DR4 (TRAIL-R1) ve DR5 (TRAIL-R2) ekspresyonunu modüle ettiğini bulduk. Bu veriler, düşük bortezomib konsantrasyonunun, immünojenik hücre ölümüne katkıda bulunabilecek ölüm reseptörlerinin ekspresyonunu modüle ettiğini göstermektedir.

ANAHTAR KELİMELER: Meme kanseri, Proteazom İnhibisyonu, Ölüm reseptörleri, Gen ifadesi

ABSTRACT

INVESTIGATION THE EFFECT OF PROTEASOME INHIBITION ON DEATH RECEPTORS IN BREAST CANCER CELL LINES

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Master's Thesis, Department of Biology

Advisor: Associate Prof. Dr. Ercan ÇAÇAN

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Breast cancer is one of the most common cancers in the world. Various modern methods of genetic modulation and modification have been developed for the treatment of breast cancer. Despite developing new strategies for the treatment of malignancy, many new areas need to be investigated. In this thesis study, we used the 26S proteasome inhibitor, bortezomib, to determine changes in the expression profiles of several death receptors in two breast cancer cell lines. Initially, the cells were treated with different concentrations of bortezomib to determine particular doses that are effective on the cell viability of two used breast cancer cell lines. MTT results revealed that increased bortezomib concentrations decrease cell viability in a dose-dependent manner. In addition, we also tested bortezomib-mediated potential changes in the expression of several death receptors that play important roles in the induction of apoptosis. We found that a low concentration of bortezomib which is not toxic to breast cancer cells treatment modulates expression of FAS (CD95), DR4 (TRAIL-R1), and DR5(TRAIL-R2). These data suggest that a low concentration of bortezomib modulates the expression of death receptors which may contribute to immunogenic cell death.

KEYWORDS: Breast cancer, Proteasome Inhibition, Death receptors, Gene expression

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RABAR A. KHORSHEED

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ABBREVIATIONS

Abbreviation	Explanation
BRCAN	Breast Cancer
CACE	Cancer Cell
BORT	Bortezomib
DeRe4	Death Receptor 4
DeRe5	Death Receptor 5
FasR	FAS receptor
FasL	FAS Ligand
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
APOP	Apoptosis
PRIN	Proteasome Inhibitor
DEDO	Death Effecter Domain
FADEDO	Fas AssociatedDeath Domain
TRADEDO	TNF receptor-associated death domain
DISC	Death Inducing Signaling Complex
PRT	Proteasome
PRTIN	Proteasome Inhibitor
UACE	Ubiquitin ctivating Enzyme
UCOE	Ubiquitin conjugating Enzyme
UPRL	Ubiquitin ligase Enzyme
FBS	Fetal Bovine Serum
PBS	Phosphate-buffered saline
DMSO	Dimethyl sulfoxide
FDA	U.S. Food and Drug Administration
EMEA	European Medicines Agency

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1. INTRODUCTION

Breast cancer commonly develops in the epithelial cells of the breasts. Over the time, these diffuse cancer cells in the other parts and organs of the body come by the lymphatic spreader system. This can lead to distant metastases and can be fatal for the patient. Although skin cancer ranks first in the number of its incidence, breast cancer competes with it in terms of its rank in the world. Depending on the report from WHO approximately around 2.3 million women were effected with breast cancer. In the same year about 650.000 death were recorded from the breast cancer. These data indicate that about 30% of breast cancer patients will loose their life at the some point (WHO, 2021). The incidence of breast cancer in women positively occurs after puberty at any age at an increasing rate later in life which makes a clear change in the difference in the rate of cure for breast cancer around the world, where the estimated 5-year rate of cure is 80% in developed countries, but around 40% in other countries (Coleman *et al*., 2008). These rates have greatly contributed to understanding and awareness of breast cancer, which has led to research funding to advance laboratory discovery in breast cancer and its treatment. It is difficult to determine why a woman develops breast cancer and not others. These causes of breast cancer are almost poorly understood. However, there are scientific factors and exact stats for an approximate reversal to reduce the likelihood of developing breast cancer.

Molecular biology techniques have seen a tremendous upsurge in the treatment of breast cancer in recent decades, particularly in the area of gene expression, modification, and modulation of specific genes and now play an important role in clinical oncology (Lockshin and Zakeri, 2007). Cell death is initiated by two common pathways, which are the exogenous death receptor and intrinsic mitochondrial pathways. In the extrinsic death receptor pathway, the death receptor leads to provide a rapid answer to programmed cell death, and this includes the extrinsic pathway of apoptosis by attaching cell ligands to the receptor, which causes activation of killing selected cells (Fulda and Debatin, 2006).

The death receptor-mediated apoptotic pathway is usually stimulated by binding to the death receptors to their cognative ligands and leads to activation of caspase cascade in the cell (Cacan, 2015). It has been reported that the proteasome inhibitor, bortezomib,

induces apoptosis synergistically together with TRAIL (TNF-related apoptosis-inducing ligand) (Naumann *et al*., 2011). These ligands complete their action through the activity of death receptor surface genes such as FAS (CD95), DR4 (TRAIL -R1), and DR5 (TRAIL -R2).

In this thesis study, we used the FDA-approved 26S proteasome inhibitor, bortezomib, to determine changes in the transcript expression of DR4 (TRAIL-R1), DR5 (TRAIL -R2) and FAS (CD95) in two breast cancer cell lines, MCF-7 and MDA231. Initially, cells were treated with different concentrations of bortezomib, and the cell viability of two breast cancer cell lines was determined by MTT. Here, we aimed to investigate the potential use of bortezomib in the modulation of death receptors and their potential contribution to immunogenic cell death.

2. GENERAL INFORMATION

2.1. History and Epidemiology

Ancient Egyptians were aware of breast cancer as early as 1600 B.C., according to the Edwin Smith Papyrus, a scroll 4.68 meters long that described the first case of the disease (Reuters Staff, 2015). Breast cancer was attributed to an "excess of black bile" by both Hippocrates and Galen, though their theories differed from era to era. Hippocrates, in 460 B.C., was the first to describe breast cancer as a humoral disease. He believes that an increase in black bile is the cause of cancer. If left untreated, solid tumors in ultra-black breast cancer have been observed to burst and release a black fluid. The term "karkinos" was first used to describe the disease. François de la Beau Silvius, a French physician, began questioning old theories at the end of the sixteenth century. Cancer was thought to be caused by a chemical shift in lymph from acidic to pungent. Science gave a huge boost to the development of scientific information and facts in the last century, particularly when breast cancer grading systems were developed in the twentieth and twenty-first centuries (Olson, 2002). It has evolved into a broad entry point for researchers looking for new methods, pathways, and processes to determine the progression of cancer through the growth and spread of breast cancers. One of the most common known cancer in women is breast cancer, accounting for approximately 15% of all cancer deaths and a quarter percentage of all cancer cases (Narayan et al., 2020). Over the past decade, researchers have shown that breast cancer is not just a simple disease, but was shown of a group of changes in molecular genes that arise from breast epithelial cells (Topham and Hewitt, 2009). Aging is the way in which the incidence of breast cancer has increased, clearly, 95% of new cases occur in women over 40 years. Some types of breast cancer occupy more compare than others. Invasive ductal carcinoma is occupying the first rank in breast cancer about more than 50%. (Alkabban and Ferguson, 2021). Invasion of breast tissue with cancer occupies both men and women but in women more compared to men. Breast cancer is counted as the second most common cancer in women (Momenimovahed and Salehiniya, 2009).

In the course of pathogenicity, cells sometimes develop into cancer. The healthy breast has endothelial cells and blood and lymphatic vessels. They develop into cancer cells due to environmental factors or genetic mutations that affect the expression of genes

and disrupt the cell mechanism. During a healthy epithelial cell becomes a cancerous cell, it results in a degeneration of the tissue called dysplasia. Breast cancer begins in different parts of the breast (Henry *et al*., 2020). During a breast cancer cell enters an abnormal division, it goes through three different stages. These stages are classified according to the intensity of the infection. Stage-1 appears normal breast cells and usually grows slowly, while Stage-2 appears less than normal cells and grows faster than Stage-1. Stage3 is exactly different from normal breast cells and usually grows faster than the other stages. The area that carries milk to the nipple is the duct most breast cancers arise in this area, for this reason, they are called ductal carcinoma (Loring and Dershaw, 1997). Ductal carcinoma is the oldest form of breast cancer and is difficult to detect at this stage because there are no symptoms that show up until a routine breast exam or mammogram (X-ray of the breast). Sometimes breast cancer arises in the area that produces milk, this type is called lobular carcinomas. A rare cancer type arises in other parts or other tissues of the breast. Although a lump is an area of arising of many types of breast cancer, 80% of breast cancer cases were first diagnosed with breast masses and more than 80% of breast cancer cases are discovered when people feel the lump with their fingertips (Zhang *et al*., 2012). Many detections of breast cancers perform in different ways including screening mammograms, which detect cancer at an earlier stage, often before it can be palpated and before symptoms appear (Zakelj, 1999).

One responsibility for more cancer deaths than other causes is the spread of breast cancer cells throughout the body. This infection of cancer cells performs through the blood and lymph when breast cancer spreads to other organs of the body (Leung *et al*., 2021). The system of lymph is a complex system that appears as a network of lymphatic vessels scattered throughout the body and connecting the lymph nodes. Lymph is the fluid in the system of lymph inside lymphatic vessels, which carries a variety of tissue, waste material, and cells of the immune system. In the case of breast cancer, cancer cells can invade these lymph vessels and begin to grow in the lymph nodes (Scanlon, 1985). A lot of the lymph vessels in breast tissue drain through their way into the nodes of lymph under the arm, the nodes of lymph around the collarbone and under the collarbone, and the intrathoracic lymph nodes near the breast bone. During the spreading of cancer cells to the lymph nodes, these cells take a huge chance that the

cells will transmit through the lymphatic system and spread to other organs of the body. In some cases, women with cancer cells in their lymph system turn to spread, and some women that have no cancer cells in their lymph system in the future will spread and develop in other organs. Sometimes affected patients with cancer initially affected countless metastases, which is almost always incurable and not treated because of the breast that entering the last stage of the disease, Nevertheless, not all women with cancer cells in their nodes of lymph cause metastases, and some women who do not have cancer cells in their nodes of lymph in the future cause metastases. Metastases vary from patient to patient and some cancer patients initially cause innumerable metastases; these patients are diagnosed with the final stage of the disease, which is almost always incurable (You *et al*., 2019).

2.2. Diagnosis and Treatment Breast Cancer

The first way to recognize breast cancer is through a physical exam, followed by several techniques including mammogram, ultrasound, and tissue biopsy. One way to find out if a lump in the breast or abnormal tissue is cancer is to have a biopsy. A breast biopsy is a test of tissue, or sometimes fluid, is taken from the suspicious area, it can be definitively determined if the suspicious area is cancer (Bick, 2020). The cells taken are investigated under a microscope and future tested to determine if breast cancer is present.

Options for breast cancer treatment are developed by recognizing a variety of scientific paths (Figure 2.1). Surgical treatment is one option; depending on the severity of the cancer tissue, there are several sub-surgical treatments available, including lumpectomy, which removes cancer and a clear margin around the tissue, and mastectomy, which removes the entire breast (Nava *et al*., 2015). Chemotherapy is another option. This treatment uses chemicals as a drug to kill cancer cells or inhibit their division, causing cancer cells to stop growing (Furue, 2003). When the chemical drugs enter the blood, they can arrive at cancer cells throughout the body. After surgical treatment in the remaining some of the cancer cells, Radiation therapy is given and sometimes in combination with chemotherapy. High radiation beams may be used to mortality of cancer cells and stop them from growing (Abshire and Lang, 2018). Hormone therapy used after all cancer treatments reduces the risk of developing breast cancer in patients

with early hormone-sensitive breast cancer and may also effectively reduce the risk of growth and progression of metastatic breast cancer in patients with hormone-sensitive tumors (Conner, 2007). Hormone receptor-positive occupy Most breast cancer types, which means these types seek hormones for growing and spreading. Hormone therapy acts as a preventer of cancer cells from absorbing normal hormones. Targeted drug therapy that causes blockage of the growth of breast cancer cells in specific therapy, for example, blocking the action of an unwanted and distorted protein in certain genes of breast cancer cells (Masoud and Pagès, 2017).

In recent decades, survival rates for breast cancer have increased. This is largely due to factors such as a better understanding of the disease, earlier detection and new approaches to treatment. Extensive promotion of breast cancer awareness and funding for research has also led to advances in the diagnosis and treatment of breast cancer.

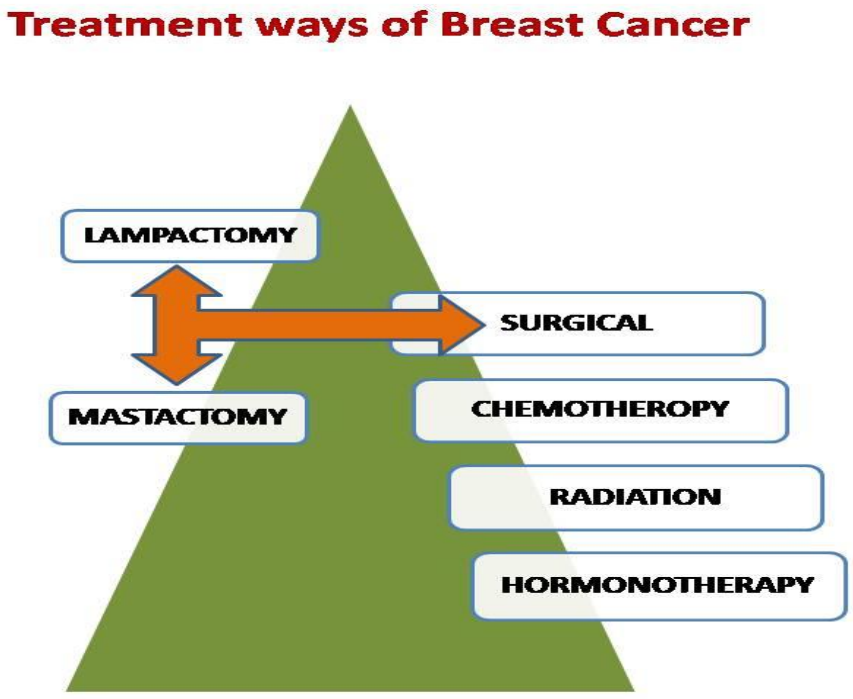


Figure 2.1. Diagram showing the different methods used to treat breast cancer.

2.3. Molecular types of Breast Cancer

Breast cancer is classified into five major subtypes based on molecular gene expression profiles: HER2-positive, luminal A, luminal B, triple-negative/basal-like2 and normal-like breast cancer, which is very similar to luminal A (Eliyatkn *et al*., 2015).

The Luminal A is defined by the presence of both estrogen and progesterone receptors, as well as the absence of EGFR-2 (Human epidermal growth factor receptor 2). It also has low levels of the Ki-67 protein, which plays a role in cancer cell growth control. The MCF-7 cell line, for example, is progesterone receptor-positive and thus assigned to the luminal A molecular subtype (Comşa *et al*., 2015). Luminal A is low-grade, tends to slow growth, and has the best prognosis.

Luminal B type is characterized as estrogen receptor and progesterone receptor-positive, human EGFR-2 positive with high levels of Ki-67. Luminal B types cancers generally grow slightly faster than A one and have a slightly worse prognosis.

Triple-negative breast cancer is both estrogen receptor and progesterone receptor-negative, and also human EGFR-2 negatives. This sort of cancer is more common in women with a BRCA1 type gene mutation, especially younger and black women. For example, the MDA-MB-231 breast cancer cell line is both estrogen receptor and progesterone receptor-negative and is therefore assigned to the triple-negative molecular subtype of breast cancer (Theodossiou *et al*., 2019). Human EGFR-2-enriched breast cancer is both estrogen receptor and progesterone receptor-negative, and human EGFR-2 positives. Human EGFR-2 -enriched breast cancer can grow faster than luminal this tends to have a slow prognosis, but has most successfully been treated with targeted therapies that target the human EGFR-2 protein.

Breast cancer that looks like normal breast cancer is similar to luminal A cancer. It has hormone receptors (estrogen and progesterone receptors), is HER2-negative, and has low levels of the protein Ki-67, which helps control cancer cell growth rate. Although normal-like breast cancer has a favorable prognosis, it has a slightly worse prognosis than luminal A breast cancer. Researchers and scientists have developed different types of breast cancer cell lines based on these molecular types of breast cancer to aid scientists in the treatment of breast cancer. These cell lines have been used to open

closed gates in several experiments that could lead to a new treatment for certain types of breast cancer.

2.3.1. MCF-7 cell line

MCF-7 is a famous used breast cancer cell line founded in 1973 by Dr. Soule and colleagues at the Michigan Cancer Foundation. The cell lines were isolated from the pleural effusion of a 69-year-old woman with metastatic disease were MCF-7 cells (Soule *et al*., 1973). She underwent a surgical mastectomy of her right breast tumor 7 years before the start of primary cell culture and underwent a successive radical left mastectomy for her left malignant adenocarcinoma. Four years later, after treatment with radiation and hormone therapy, the patient was treated with high doses of the synthetic agent estrogen diethylstilbestrol and the disease was monitored three times to obtain a tumor response hormone. Two months later, samples were brought from the pleural effusion for laboratory tests. Over time, MCF-7 cells provided unique data on the practical knowledge of patient care more than any other breast cancer cell line. MCF-7 cells are invasive ductal breast cancer cells with many genetic phenotypic differences, MCF-7 cell line is hormone-dependent, both estrogen and progesterone positive (Gest *et al*., 2013). MCF-7 cells exhibit many features of normal breast epithelial cells, including the ability to form multicellular three-dimensional aggregates that can mature into mass cells with irregular nuclei, strong cell-cell junctions.

2.3.2. MDA-MB-231 cell line

MDA-MB -231 is a breast cancer cell line that was derived from the pleural effusion of a 51-year-old Caucasian woman with metastatic adenocarcinoma of the breast and is one of the most commonly used cell lines in medical research laboratories. The MDA-MB-231 cell line, which expresses high levels of epidermal growth factor receptors and is hormone-independent, is one of the prototypes for the study of hormone-independent breast cancer (Godden *et al*., 1992). MDA- MB -231 is a highly aggressive, invasive, poorly differentiated triple-negative breast cancer cell line because it lacks estrogen receptor and progesterone receptor expression and human epidermal growth factor

receptor 2 amplification (Liu *et al*., 2003). The cell line MDA-MD-231 has been well established for bone metastasis research. Subclones of MDA-MB-231 cells have also been isolated that preferentially transduce into the bone, brain, and lungs of mice then intraventricular injection has also been isolated.

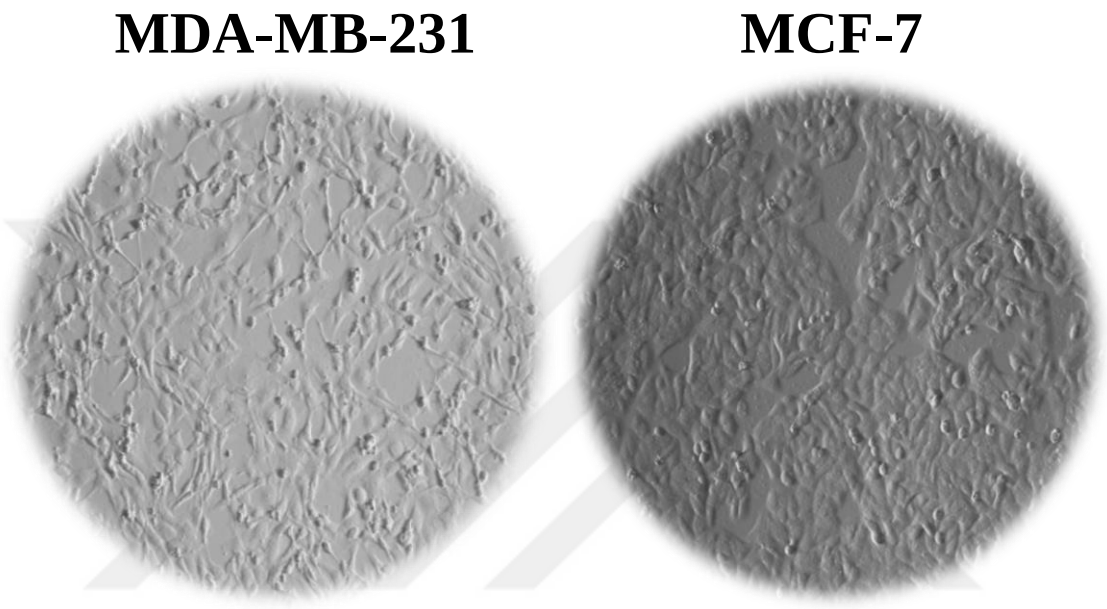


Figure 2.2. Illustration morphology of the two breast cancer cell lines MDA-MB-231 and MCF-7 cell lines under a fluorescence microscope using 10X.

2.4. Apoptosis Approach and Its Abnormality

The process of programmed cell death occurs in multicellular organisms known as Apoptosis (Ameisen, 2002). This process just does not occur in abnormal cells but occurs also in normal cells development (Schultand and Harrington, 2003). Scientists are convinced apoptosis is an active physiological process of cell death that kills cells and regulates the elimination of individual cells from living tissue. Apoptosis is an important cellular process that prevents abnormal cells from living causes cells to die (Engelberg-Kulka *et al*., 2006). Also, the elimination of affected cells is regulated by apoptosis in the human body. To maintain cell populations in tissues during

development and aging some molecular reactions take place these reactions organize in systematic mechanisms by apoptosis (Elmore, 2007). During apoptosis, there is a written plan for the pathways and proteins in the cell that are activated to kill the cell without producing a lot of havoc. During the development of organs, apoptosis gives a role in differentiation for instance in the development of the hand, without participating in apoptosis the hand appears like a paddle and webs between the fingers such as bat hand or duck foot, apoptosis makes these cells oscillate and give off differentiated fingers.

Some defense mechanisms that occur in the body return to apoptosis responsibility, such as in immune responses (D'Arcy, 2019). There are different types of stimuli in different conditions, this means that not all cells necessarily die in response to the same stimulus in terms of apoptosis stimuli regulation. The processes for maintaining the body, such as getting rid of cells that are not needed and are potentially dangerous to the rest of the organism, Cells that are not needed may never have had a function, or they may have lost their function, or they may have competed with other cells and lost makes as to think that apoptosis is one of the most important regulatory processes. Those cells have been in the body so long that they have worn out and need to produce way for new, young cells these aging cells renewing by the activity of apoptosis. Thus apoptosis can be normal and the lack of apoptosis can lead to cancer (Pfeffer and Singh, 2018). In some cases of nervous system disease, apoptosis leads to several series processes as result in many apoptoses causing neurodegenerative diseases (Radi, 2014), or where in other cases cells die when they shouldn't die, cells receive messages from someplace, which do not understand receive messages, for the most part, telling them to die, this happens in a certain part base of the brain, will resulting in Parkinson's disease (Miller and O'Callaghan, 2015). This is also characteristic of Alzheimer's disease and several other neurodegenerative diseases.

The cell is broken down by several proteins called caspases during programmed cell death. Caspases are important enzymes that carry out apoptosis and activate it (Creagh *et al*., 2003). Caspase enzymes are cysteine-dependent, aspartate-directed proteases that come in twelve different types. The active site of the caspase enzyme contains a cysteine residue, which is required for the enzyme's protease activity. These caspases must be activated to cause apoptosis. Caspases are activated to ensure that cellular

components are degraded in a controlled manner and that cell death occurs with minimal impact on surrounding tissue (Rathore *et al*., 2015).

The inactive form of caspase is pro-caspases which synthesize caspase after appropriate stimulation (Chehade et al., 2021). Dimerization and often oligomerization of the pro-caspases, followed by cleavage into a small and a large subunit causes in involves of activation of caspases. A combination of the large subunits and small subunits will form an active heterodimer caspase. The active enzyme is often present in the biological environment as a heterotetramer, whereby a pro-caspase dimer is cleaved together to form a heterotetramer (Shi, 2004).

Signals used by the cells, and ligands will express this duty, to perform their duty in growth and stopping their growth. Receptors by proteins on the cell surface transmit these signals (Heldin *et al*., 2016). Receptors and ligands search each other to communicate themselves. Receptors are composed of three domains which are the domain of extracellular binding ligand, a domain of transmembrane, and a domain of intracellular. Communication of a ligand to the extracellular domain activates the receptor tyrosine kinase, following activation of other proteins by phosphorylating the amino acid tyrosine. Communication of the ligand and the receptor cause for sending a signal to the domain of intracellular, and causes activating the relating enzyme and triggering a cascade of signals to the nucleus that promotes cell growth and division or stops the growth (Trenker and Jura, 2020).

Caspase activation is initiated by the cleavage of caspases. The cleavage of initiator and executor caspases are different from each other. Initiator caspases cleave autoproteolytically, while executor caspases are cleaved by initiator caspases. Caspase 2, Caspase 8, Caspase 9, Caspase 10 are starter caspases, while killer caspases are Caspase 3, Caspase 6, and Caspase 7. When the initiator caspase will activate, they further cause a chain of reactions that activates other killer caspases. The killer caspases degrade more than 600 cellular components to trigger the morphological changes for apoptosis (Sollberger *et al*., 2014).

The caspase activation process is regulated by two pathways: the extrinsic pathway and the intrinsic pathway (Parrish *et al*., 2013). The first will occur via the extrinsic pathway, as the initiating signal originates from outside the cell, beginning in T

lymphocytes. Fas ligand is a surface molecule found on these lymphocytes. When the Fas ligand comes into contact with Fas receptors on the target cell's surface, the extrinsic pathway is activated. The combination of T cell death receptor ligands and target cell Fas receptors causes apoptotic stimuli to be signaled, which activates the death triggering stimuli complex (DISC) (Figure 3) and caspases, which kill the target cells (Cacan and Ozmen, 2020). These signaling pathways are the starting point for some biochemical changes such as activation of caspases, degradation of proteins, changes in membrane permeability and sensitivity, and recognition by phagocytic cells.

The extrinsic pathway is called the death receptor pathway because the starter signal comes from outside the cell starting in the T lymphocytes immune cells and natural killer cells that activated to kill damaged cells by being responsible for various death receptors such as (death receptor 5, death receptor 4 and Fas receptor) (Kumari *et al*., 2013). Death receptors are ligand-activated cell surface receptors that transmit apoptosis signals. Tumor necrosis factor-related programmed cell death-inducing factor ligand and its death receptors 4 and death receptors 5 are responsible for inducing cells. They do so by stimulating a group of related receptors called death receptors. Death receptors are tumor necrosis factor receptor superfamily cell surface receptors that bind to tumor necrosis factor-related programmed cell death-inducing factor ligand and mediate apoptosis (Walczak *et al*., 1997; Wiley et al., 1995). This gene encoded the protein which is a member of the TNF receptor superfamily and contains the domain of death of an intracellular domain. This receptor can be activated by tumor necrosis factor-related programmed cell death-inducing factor ligand and transmits the apoptosis signal (Shetty *et al*., 2002) and (Mendoza *et al*., 2008). The receptor of Fas is a protein encoded by the gene of FAS (Nagata, 2004) and (Lichter *et al*.,1992). Death receptors contain an intracellular death domain that installs adapter proteins such as the tumor necrosis factor receptor-related death domain and Fas-associated death domain, as well as cysteine proteases such as caspase 8 (Schneider and Tschopp, 2000). They support T cells in killing damaged target cells.

One way that cancer cells can be viable longer and have more time to accumulate mutations is through loss control of apoptosis. Cancer cells cause apoptosis dysfunction by increasing or decreasing the expression of genes of stopping apoptosis or genes of starting apoptosis. Cancer cells can inhibit apoptosis by destroying the activity of genes

of stopping apoptosis or genes of starting apoptosis (Shahar and Larisch, 2020). Thus, cancer cells become resistant to the apoptosis process by upregulating signals of stopping apoptosis (e.g., Bcl-2, Akt, Mcl-1, etc) and downregulating signals of starting apoptosis (e.g., Bax, Bak, Bad, etc.), followed by implication of apoptosis. One of the new ways of the prestige of carcinogenesis is that showing roles in the inhibition of development and progress of cancers is suppression of apoptosis (Kerr and Searle, 1972). A great chance to prevent from spreading of cancer cells is confirmed by the whole Introduction to cancer. Cancer makes unbalance between the division of cells and the death of cells in the body. Disorder in apoptosis shows a great role in cancer and allow cancer cells to remain and elongate their life. Cancer cells have a fantastic capacity to produce, transport, and modify proteins. The transmission of unwanted signals by cancer cells leads to the loss of dysregulation of cell proliferation from apoptosis or invade of programmed cell spreading that allow cancer to spread and generate angiogenesis (Berkel and Cacan, 2020; Kucuk *et al*., 2021; Berkel and Cacan, 2021). Proteasome activity in cancer cells is generally higher than normal cells and is more sensitive to the pro-apoptotic effects of proteasome inhibition. Therefore, The proteasome is a strong candidate for cancer treatment (Almond and Cohen, 2002).

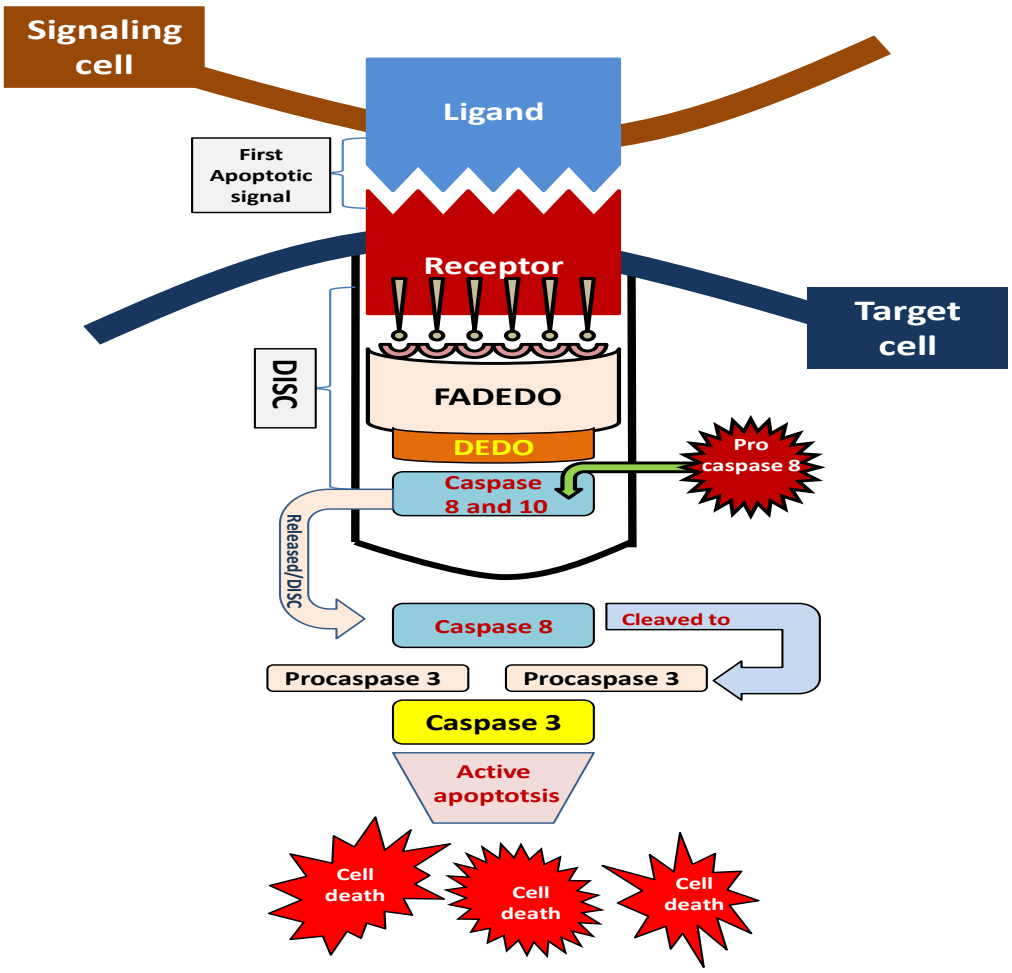


Figure 2.3. Initial apoptotic signal through ligation between the death receptor ligands of T cells and Fas receptors of target cells, which activate a series of caspases to induce the death triggering stimuli complex (DISC).

2.5. Proteasome and Proteasome Inhibitor

Proteins are extremely important molecules that are essential for cell structure and function. By maintaining an active, healthy balance between the breakdown of unwanted proteins and the production of new proteins, this renewing process is necessary to prevent damaged proteins from aggregating and becoming toxic to cells. This recycling process relies mainly on structures known as the proteasome (Tanaka,

2009). The proteasome is a complex of protein that cleaves spread proteins by proteolysis. The proteasome is catalytic of a multi-enzymatic complex present in every periodic cell. Proteasomes are located in the cytoplasm and nucleus. Proteasomes are like cylinders or barrels in shape that open at one end to let tagged proteins in and at the other end to let degraded protein pieces out. Damaged proteins are found by a long polypeptide o 76 amino acids called ubiquitin, which marks the proteins that need to be degraded (Cooper, 2000). They bind many ubiquitin molecules to the target protein that needs to be degraded and go through a process known as ubiquitination (Guo and Tadi, 2021). At first, proteins must be degraded by the proteasome, They must be labeled to prevent the destruction of normal proteins. Therefore, ubiquitination must occur for proteins that are targets of the proteasome and must be destroyed. Some ubiquitin residues are attached to the protein. As a result, the tagged protein is recognized by these regulatory caps of the proteasome, and the protein is found and passes through the large cylinder of the proteasome. Only tagged proteins are engulfed by proteasomes. Once the protein is engulfed, the protein unties and enters the interior of the proteasome where it is degraded.

Sometimes proteasomes can become overwhelmed when cells produce large amounts of unwanted proteins. In this case, a different resolution system is used in which the tagged proteins aggregate into structures called aggresomes. The formation of aggresomes is a highly regulated process in which misfolded proteins arrange themselves at a single aggregated site (Corboy *et al.*,2005). Involved in this are other structures called lysosomes, which get rid of the proteins instead of recycling them.

In general, the ubiquitin-proteasome process is ATP-dependent and degrades misfolded proteins and functional proteins through a variety of enzymatic activities (Peth *et al*., 2013). It consists of a multistep enzymatic cascade that includes the 20S proteasome, ubiquitination activating enzyme (UACE), ubiquitination conjugating enzymes (UCOE), ubiquitination protein ligases (UPRL), 20S proteasome, and deubiquitinating enzymes (Neutzner, 2012). The formation of peptide bonds, occurs by the hydrolysis of an ATP molecule. The hydrolysis of an ATP molecule serves to generate sufficient energy to carry out the process of attaching ubiquitin to the target protein. Any unwanted protein degradation must first be initiated by a UACE ubiquitin-activating enzyme. ATP is needed for performing the process of activating protein ubiquitin then

send to the UCOE enzyme. This enzyme performs its duty as a convoy for the ubiquitin to the UPRL enzyme. The UPRL enzyme performs its duty as a regulator to the substrate of the target protein and the active UCOE form that contact with the UPRL enzyme. Both the targeted protein and ubiquitin are loaded on the enzyme UPRL, They are positioned close enough to each other for ubiquitin to translocate to the target substrate of the protein. Repeated several times of this process resulted in the formation of a series of polyubiquitin that breakdown the protein (Li and Ye, 2008). These can potentially be targeted to inhibit cancer and other diseases. Dangerous proteins and normal proteins must be breakdown by the proteasome when accumulate in the endoplasmic reticulum and cytosol (Rao and Bredesen, 2004). This accumulation eventually leads to Endoplasmic reticulum stress stimulating apoptosis.

Cancer cells naturally produce more proteins than normal cells because they grow rapidly (Cooper, 2000). During cancer the ubiquitin protease system may become overzealous, causing beneficial proteins to be inappropriately destroyed or retained in some way and potentially harmful proteins to accumulate in toxic amounts. This leads to disruption or malfunction of the ubiquitination system. To prevent proteins from accumulating, the body must rely on proteomes to function properly. So drugs that stop proteomes from working are a very useful way to kill cancer cells by preventing the proteins to build up and disaggregate, which eventually becomes toxic and causes the cancer cells to die. Proteasome inhibitor drugs that work in this way have been developed and used to treat cancer cells such as bortezomib (Crawford *et al*., 2011; Dou and Zonder, 2014).

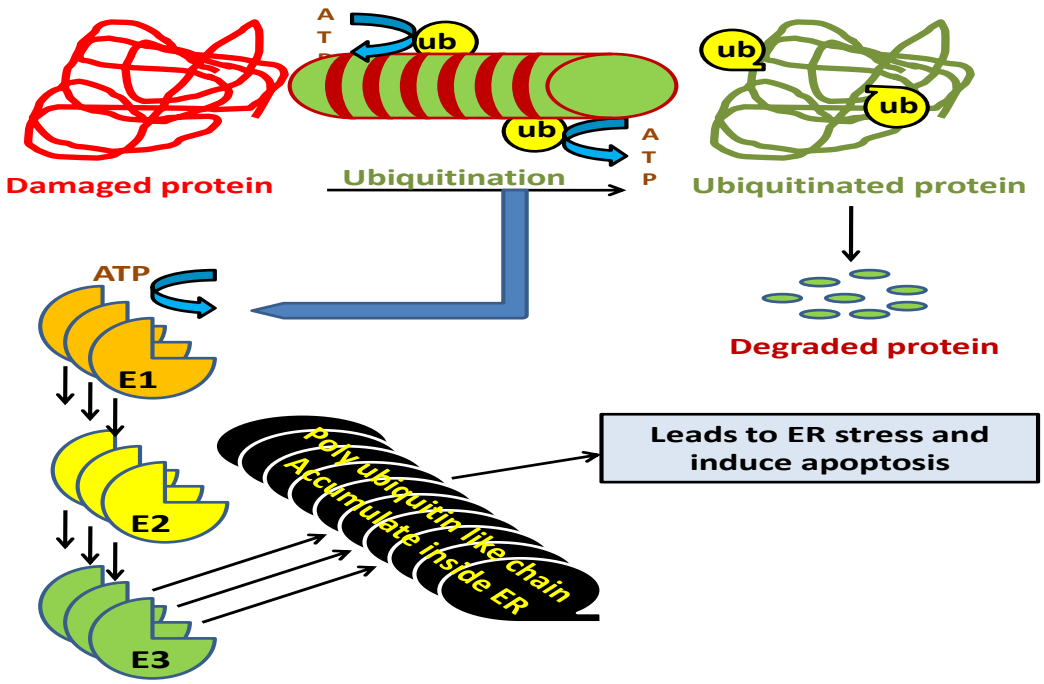


Figure 2.4. Mechanism of proteasome activity with enzyme derivatives during degradation of damaged proteins. Ubiquitination is the most important process contributing to protein degradation.

2.6. Role of Bortezomib

Bortezomib is a derivative of a dipeptide boronic acid that acts on inhibition of 26S proteasome. The group boronic acid in bortezomib binds with the active site of the threonine hydroxyl group in the β5-subunit that blocks chymotrypsin. Bortezomib is used to treat different types of cancers (Hambley *et al.*,2016). The active function of bortezomib is a proteasome inhibitor. It blocks the proteasome system then causes the cell to die (Chen *et al.*, 2011). Proteasome inhibitors bortezomib disrupt cancer cells more than normal cells by its more sensitivity to cancer cells. Bortezomib may provide better treatment of cancer cells when used with ionizing radiation in sub lithal dose, this becomes a barrier to enhanced death receptor-mediated apoptosis and anti-tumor

immune attack, leading to modulation of gene expression (Cacan *et al*., 2015). Bortezomib enhances cytotoxicity when combined with other drugs. This suggests that combination treatments with bortezomib have potential benefits in reducing cancer cells (Berkel *et al*., 2020). The success of therapeutics depends mainly on the ability. Targeting overexpressed anti-apoptotic proteins or stimulating the expression of proapoptotic molecules may induce apoptosis. Therefore, bortezomib is particularly effective in destroying cancer cells.

Bortezomib was developed in 1995 and belongs to the antineoplastic agent class of drugs (Lu *et al*., 2006). Bortezomib is a cancer drug that is selective and cytotoxic against human cancers, with only minor side effects on normal cells. It also induces apoptosis in cancer cells that are resistant to chemotherapy. Bortezomib was the first proteasome inhibitor against cancer, approved by the FDA and the European Medicines Agency in 2003 under the trade name VELCADE for the treatment of multiple cancer types (Kane *et al*., 2003). Bortezomib was approved for the first-line treatment of patients with multiple cancers in the United States in 2008. This approval was based on a multicenter, international, open-label trial in previously untreated patients with symptomatic multiple cancer types (US Food and Drug Administration).

2.7. Mechanism of Breast Cancer tumor suppression gene

The resting phase of the cell cycle is occupied by a normal breast cell. Through the growth phase, the cell's organelles and mitochondria are duplicated, and the cell enters the cell cycle. The replication of DNA is carried out during the synthesis phase (Baserga, 1968). After the synthesis phase, the cell enters the growth 2 phase and prepares for division in the mitosis phase, dividing into two identical cells. In mitosis, cells produce two genetically identical nuclei and the rest of the cell can divide by cytokinesis to produce two identical cells (Maton *et al*., 1997).

These new cells re-enter the cell cycle or return to the resting phase. The cell cycle has checkpoints to prove that there are no mistakes with division. The growth phase checkpoint is the first checkpoint at the end of the growth phase to make sure that the cell has no problems in the DNA and in the cell itself. The second checkpoint is the

growth 2 phase checkpoint, which is to prove that the cell has no mistakes before it enters mitosis. The third checkpoint in the mitosis phase is to prove that the cell cycle has a continuous progression from the growth phase, growth 2 phase, and mitosis phase. During entering the cell cycle, the cell starts for production of proteins that are responsible for the progression of the cell cycle. These proteins are cyclins and Cyclin-dependence kinases, they perform their duty by controlling the drivers of the cell cycle (Lim and Kaldis, 2013). The cell starts producing Cyclin-dependence kinases and cyclin. In the early growth phase, Cyclin-dependence kinase 4 and Cyclin-dependence kinase 6 are produced, They bind to Cyclin D. This causes a reaction in the cell that causes E2F to bind to the retinoblastoma protein (Yang and Sladek, 1997). While E2F is released, it acts as a transcription factor that allows the cells to enter the synthesis phase. During finishing the growth phase, there are Cyclin-dependence kinase 2 and cyclins. At the synthesis phase, the cell produces Cyclin-dependence kinase 1, Cyclin-dependence kinase 2, and cyclin A. In the growth 2 phase, Cyclin-dependence kinase 1 and cyclin B are produced by the cell. These cyclins and Cyclin-dependence kinases enhance the cell to the resting phase through the cell cycle. Low amounts of cyclins and Cyclin-dependence kinases allow the cells to not rest phase through the cell cycle. However, high amounts of cyclins and Cyclin-dependence kinases cause cells to continuously enter the cell cycle make uncontrol the growth of cells, Cells enter to uncontrol division follows cancer. When genetic mutations occur, cancer may occur. Mutations can occur in DNA (Brown, 2002). There is a variety of mutations that occurs in the cell including mark point mutations, which is one change in a nucleotide, or DNA amplification, furthermore, in which a single gene is amplified, can lead to chromosomal rearrangement, in which one chromosome is attached to other. Cancer is also caused by epigenetic modifications such as methylation and acetylation of genes, which means silence of certain genes, genes become more active and cause a mutation that turns a normal cell into a cancer cell this is the cell enters the cell cycle and gets uncontrolled cell growth through the checkpoints of the cell cycle (Cacan *et al*., 2015). This uncontrolled cell growth is mainly caused by the activation of oncogenes and the inactivation of tumor suppressor genes (Pierotti *et al*., 2003; Dopeso *et al*.,2018; Berkel and Cacan 2021a-b).

During entering the cell the cell cycle in the growth phase usually contains DNA, the RAC gene that produces the RAC protein (Didsbury *et al*., 1989). It is an intracellular protein located below the plasma membrane and is a natural growth factor receptor. When the cell enters the cell cycle, it requires growth factor stimulation, growth factor stimulation stimulates growth factor receptor. When RAC is activated, the RAC protein activates a chain of intracellular phosphorylation of other proteins, eventually leading to transcription factor activation. Once that transcription factor is activated, it essentially goes to the DNA and reads the genes to create proteins for cell growth, specifically to make proteins that allow the cell to transition from growth phase to synthesis phase and those proteins are the cyclins and Cyclin-dependence kinases. Thus a mutation in the RAC gene leads to a mutation in the other genes and the cells overproduce proteins, leading to uncontrolled growth and cancer (De *et al*., 2019). Behind the cyclins and Cyclin-dependence kinases MYC gene, proteins are normally produced in the body that is important for cell growth, cell survival and cell activity. Mutation of the MYC gene leads to more cell growth, more cell activity, and more cell survival. Hyperactivation of these oncogenes allows a cell to bypass cell cycle checkpoints and allows the cell to grow in an uncontrolled manner.

Normally, the cell has a mechanism to prevent abnormal cells from continuing the cell cycle - this is where tumor suppressor genes play a role. The tumor suppressor gene is a gene that regulates a cell during cell division and replication. Tumor-suppressor genes or antioncogenes are genes that protect the cell from a single event or multiple events leading to cancer (Velez and Howard, 2015). Nuclear phosphoprotein gene p53 is one of the tumor suppressor genes while the cell contains damaged DNA, it produces p53 proteins that act as transcription factors, reading the damaged DNA and creating proteins for cell, such as the nuclear phosphoprotein p21 protein, which inhibits cyclins and Cyclin-dependence kinases, inhibiting the drivers of the cell cycle. The nuclear phosphoproteinp53 also produces proteins that are important for cell repair and during the cell is arrested and can repair itself, repair of the damaged DNA follows. The nuclear phosphoprotein p53 protein also produces proteins that are important for apoptosis (Aubrey *et al*., 2018). When tumor suppressor genes such as nuclear phosphoprotein p53 are inactivated, the cell does not produce proteins for cell arrest, the cell does not produce proteins for cell repair and the cell does not produce proteins for

apoptosis, become abnormal cell that enters the cell cycle and pass the checkpoint, going to continuously grow and proliferate.

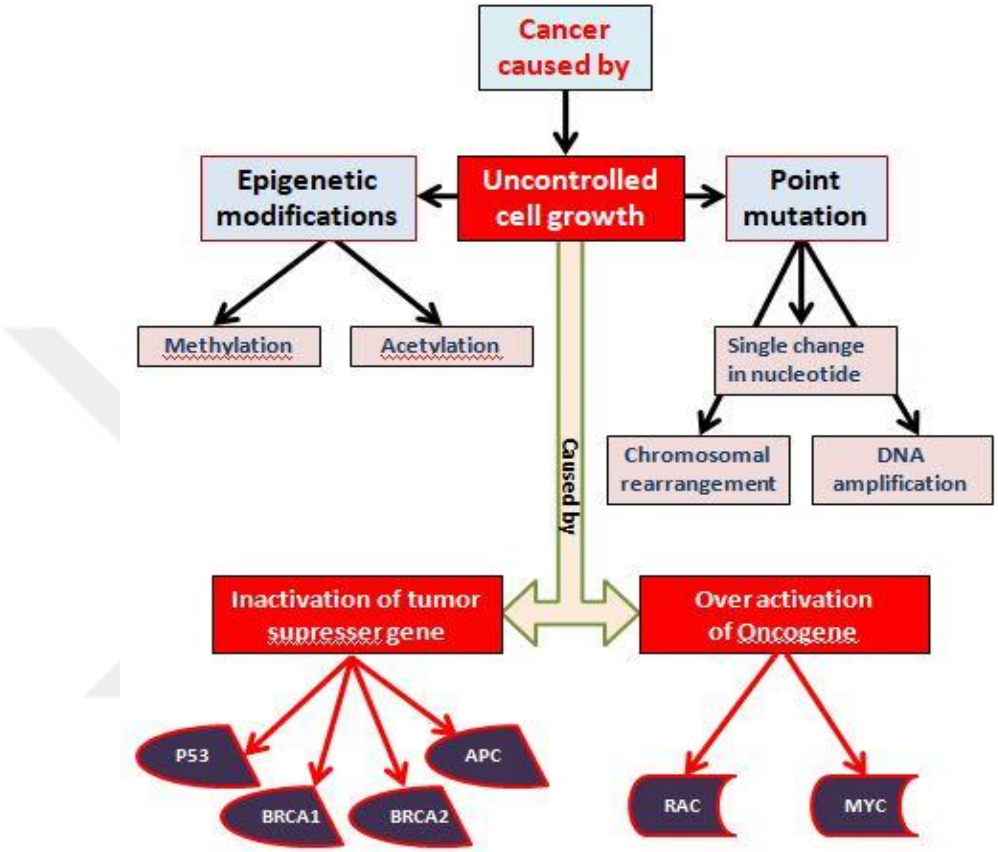


Figure 2.5. Cancer results from uncontrolled cell growth of a series of alterations that include point mutations, epigenetic modifications, and abnormalities of breast cancer genes.

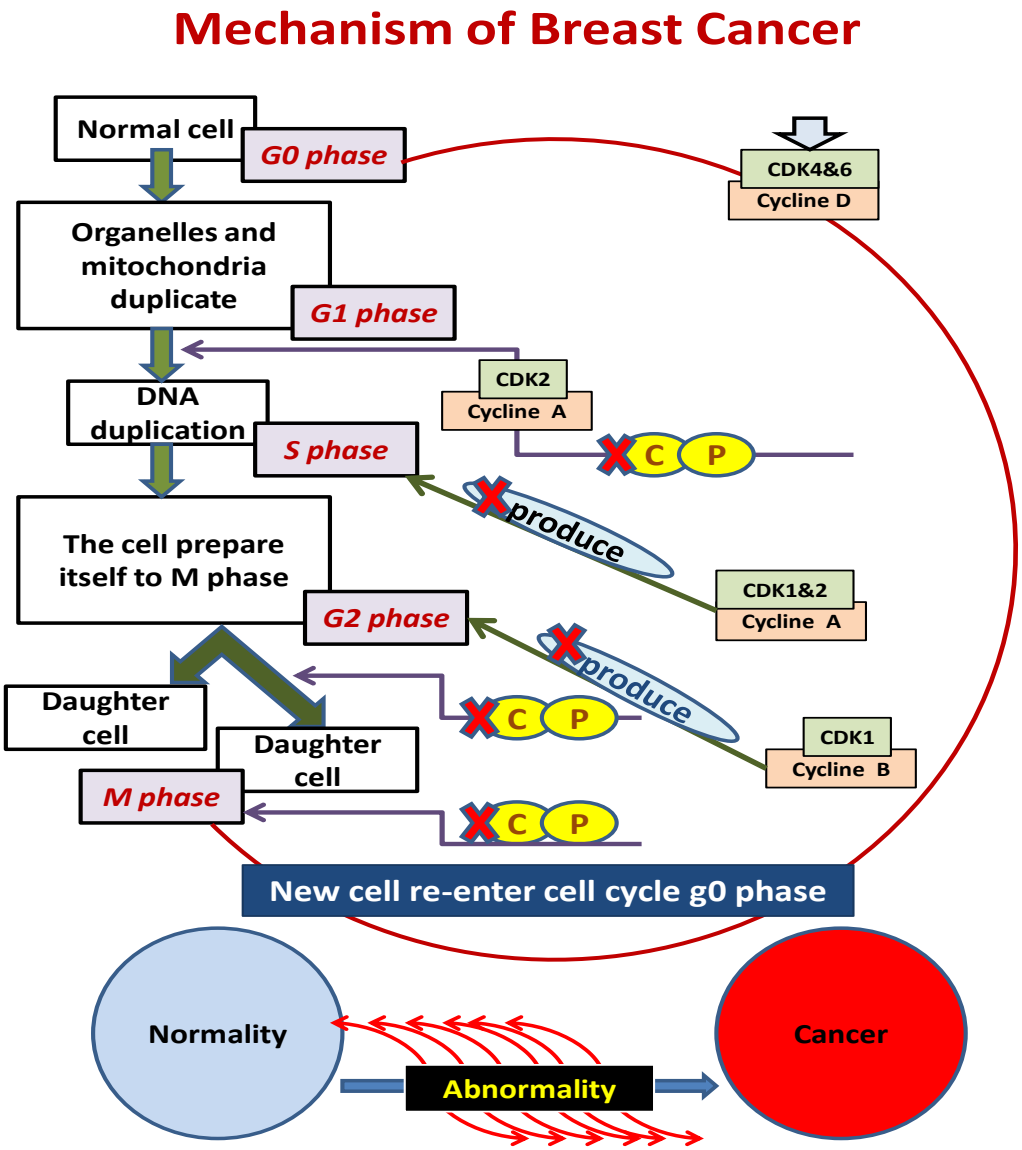


Figure 2.6. Mechanism of carcinogenesis by abnormalities and interruptions of cell cycle checkpoints. Disruption of each of the three different checkpoints and their derivatives results in abnormalities and causes cancer.

3. MATERIAL AND METHODS

3.1. Cell Culture

The cell lines MCF-7 and MDA-MB-231 were used. For 500ml of media, MDA-231 cells were cultured in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum (FBS), 5 mM L-glutamine, and 5 mM penicillin/streptomycin, while MCF-7 cells were cultured in colored RPMI medium supplemented with 10% FBS, 5 mM Sodium bicarbonate, and 5 mM penicillin/streptomycin. Both cell lines were placed in a humidified incubator with 5% CO2 at 37°C and a pH level adjusted between 7.2 and 7.5. Vacuum filters were used to filter the media, which were then stored in the refrigerator for later use.

The UV light was turned on for about 30 to 60 minutes before and after each cell culture session in the cell culture room and hood. The level of contamination was monitored regularly. All cell culture experiments were conducted in an ESCO class II type A2 cell culture hood. The surfaces in the cell culture hood were cleaned with 70% ethanol and 10% bleach.

MCF-7 frozen cells were thawed in RPMI medium and MDA-MB-231 frozen cells were thawed in 10 mL DMEM medium and transferred into 50mL canonical tubes using Pasteur pipettes, then centrifuged at 3000 rpm for 3 min to remove the DMSO present in the freezing media and its effect.

After discarding the supernatant, the cell pellets were added to 10 mL media, and the cells were distributed into the T25 cell culture flask. For further passages, after washing the flask with 10 mL of PBS, confluent cells were detached by adding 5 mL of pre-warmed trypsin for cell culture flasks. The flasks were incubated at 37 ºC for approximately 3-5 minutes until the cells were completely detached from the surface of the flasks and then 10 ml of pre-warmed media were added to the flask. After pipetting with serological pipettes, the media were transferred to 50-mL canonical tubes and centrifuged at 3000 rpm for 3 minutes. After discarding the supernatant, the cell pellets were dissolved in 10 mL of media, and the cells were seeded onto the plates at the desired dilution, taking cell density into account. The total volume of media in a T75

flask was made up to 20 mL. The confluence of the cells was continuously checked to avoid over confluence.

3.2. Chemotherapeutic Agents

Bortezomib was purchased from LC Laboratories (Woburn, MA 01801, USA). Bortezomib was dissolved in DMSO then further diluted to different concentrations (5nM, 10nM, 50nM, 100nM, and 250nM) in PBS solution, PBS, and DMSO+PBS controls were included in the assays. Bortezomib stock solutions were kept at – 20 ºC and were avoided in the storage of drugs.

3.3. Cell viability Cytotoxicity Screening (MTT assay)

The viability of cancer cells under the influence of drugs was measured using the MTT ((3-(4, 5-dimethyl thiazolyl-2)-2, 5-diphenyltetrazolium bromide) assay. Normally active cells metabolize MTT yellow tetrazolium and the color changes to purple formazan due to the action of dehydrogenase NADPH enzymes.

For the MTT assay, 96-well plates were used in which 7500 cells per well were seeded in a 200µl medium. 24 hours after seeding the cells, treatment with the drug (bortezomib) was performed in 6 replicates for each concentration and the cells were incubated for 48 hours. MTT powder was dissolved in phosphate buffered saline (PBS) and filtered using filters with a pore size of 0.2 microns in a cell culture hood to make the MTT solution (5 mg/ mL). The MTT solution was kept frozen at -20 oC in the dark. The MTT solvent was made by diluting MTT in 90% serum-free RPMI. In the MTT assay, the medium was carefully aspirated from the wells and each well was washed with 100µl PBS. Then, 10% diluted MTT in 90% serum-free RPMI was added to each well. (The final MTT concentration in the wells was 0.5 mg/mL) and the plates were incubated in a humidified 5% CO2 incubator at 37 ºC for 4 hours. After incubation, 75 µL was removed from each well. Then, 75µL of DMSO was added to each well and the plates were incubated in the dark on an orbital shaker for 1 hour. Spectrophotometric measurement was performed using Multiskan Go microplate reader (ThermoScientific) at two different wavelengths of 540 and 570 nm and absorbance values were used for

data analysis. The spectrophotometric signal of formazan correlates with the number of cells.

Table 3.1. Table shows steps to perform MTT viability assay.

Material	**Volume**	**Incubation period**	**Purpose**
7500 cells	200µl	24 hours	Viability
Bortezomib	5, 10, 50, 100 and 250nM	48 hours	Treat cells
PBS	100µl	non	Washing cells and remove dead cells
MTT dye	100µl	4 hours	Change color notification, and dehydrogenase NADPH enzymes by mitochondria
DMSO	75µl	1.5 hour	Remove 75µl MTT, replace by 75µl DMSO

3.4. Cell Migration and Wound Healing Assay (Scratch Assay)

The migration of cells and the healing of wounds are both convincing indicators of cell vitality. Many pathological processes, such as inflammation and cancer, can be triggered and involve cell migration. We seeded 800000 cells per well in 6-well plates (Costar, Corning, flat bottom), scratched each well with a 200µl-pipet tube tip size to create a scratch line, divide the circle of each well into two equal radii, and then incubated the cells for 24 hours. The cells in each well were treated with different concentrations of bortezomib the next day, and the cells were then incubated for another three days to observe cell migration towards the scratch line.

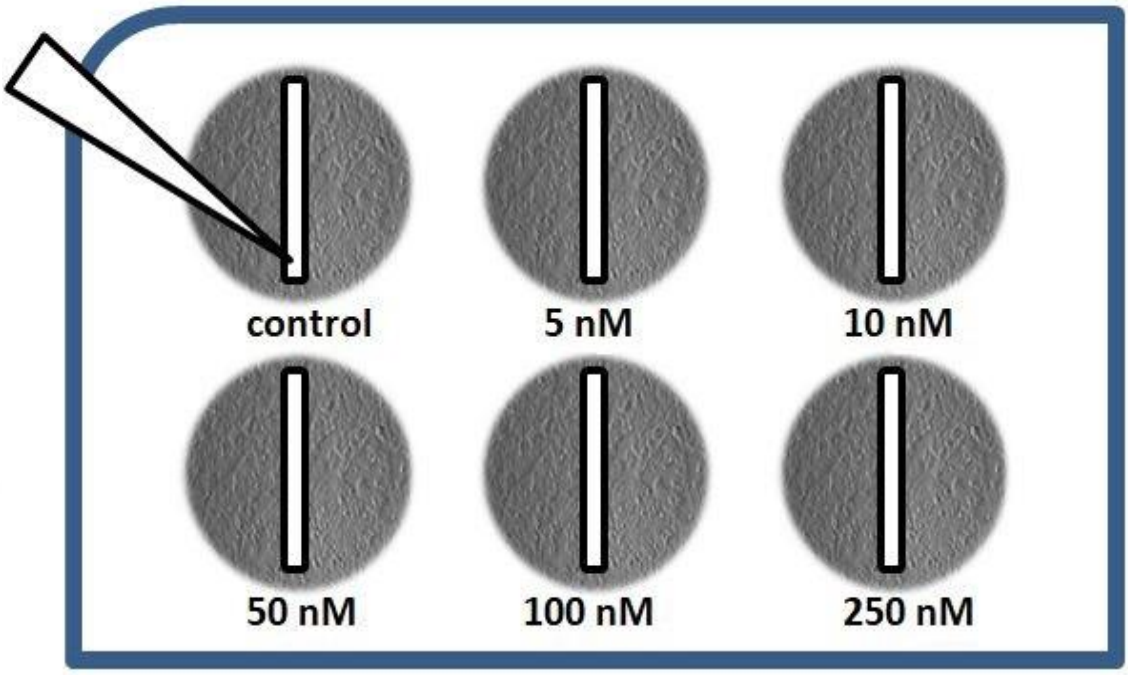


Figure 3.1. Method for drawing the scratch line to endorsement cell migration assay. Here we observed the cells for 3 days to determine the migration of the cells toward scratch line

3.5. RNA Extraction

RNA extraction was performed as described in Ali *et al*, 2013. Briefly, 600.000 cells were seeded per well in 6-well plates (Costar, Corning, flat bottom) and the cells were incubated for 24 hours. The two lowest concentrations (10nM and 5nM) were administered and the cells were further incubated for 24 hours. Then media were removed from each well and the wells were washed with cold PBS. After washing the cells, cell harvesting was started by adding 1 ml (1000µl) of QIAzol lysis reagent (Qiagen, Germany) to maintain the integrity of RNA due to the highly effective inhibition of RNase activity during cell breakage and cracking and dissolution of cell components in the process of sample homogenization. Into each well and the plates were stored at room temperature for 5 min (with pipetting and shaking during the incubation period to detach all cells from the surface of the plate). The cells were then transferred to a 1.5 ml Eppendorf tube and 200µL chloroform (Amresco, USA) was

added to each tube to separate the aqueous phase containing RNA from the organic phase containing other organic particles of the cells and the tubes were shaken for one minute. After the tubes were placed in the tube rack for 3 minutes, centrifugation was performed at 12,000 rpm for 15 minutes at 4°C. Then, the top layer containing RNA molecules was carefully transferred to a new 1.5-ml tube, avoiding other layers containing other cell particles and 500 µl isopropyl alcohol (Amresco) was added for each tube, briefly shaking the tubes up and down by hand and then incubated for 10 minutes at room temperature. Due to the presence of NH 4+, the addition of isopropyl alcohol keeps the free nucleotides in solution, separating them from the precipitated RNA, After the incubation period, the tubes were centrifuged at 12,000 rpm for 10 minutes at 4°C.

The supernatant was then aspirated and discarded with care. Each tube received 1 ml of 75 percent absolute ethanol (Isolab, Germany) dissolved in 25 percent RNA-free water, and the tubes were centrifuged at 7500 rpm for 5 minutes at 4 C. The RNA pellet in the tubes was air dried to remove all ethanol residues after the supernatant was completely removed (the tubes were placed on a tube rack in a sterile hood environment). Finally, the RNA pellets were redissolved in 40 l RNase-free water, and 1 µl of their concentrations were measured using a spectrophotometer at two different wavelengths in two different replicates (260 nm and 280 nm). Concentrations were calculated by taking the average of each replicate in specific absorbance minus the average of RNAase replication at 280nm. The average of the 260nm replicates was divided by the average of the 280nm replicates to determine the RNA purity ratio.

Isolated RNA samples were stored at -82 ºC in the freezer before being used in qRT-PCR experiments. Extensive freeze-thaw cycles were avoided so as not to compromise RNA quality. RNA isolation experiments were performed in a fume hood to avoid inhalation of evaporating chemicals.

3.6. Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

LightCycler capillaries (20 µL, Roche) were used with the Roche LightCycler 1.5 instrument (Roche Diagnostics GmbH, Roche Applied Science, Mannheim, Germany). Samples were prepared for RT-PCR reverse transcription reaction by adding qRT-PCR Master Mix (10 µL, 1x), forward primer (1 µL), reverse primer (1 µL), template RNA

(50 ng/reaction), RNase-free water (to the full standard volume 20 µL). Samples in two replicates were read with one-step real-time RT-PCR by amplifying the replicates using the thermal cycle protocol analysis mode (denaturation, amplification and cooling). One cycle reverse transcription (50 ºC, 25 min), one cycle denaturation (95 ºC/15 min), three-step amplification (95 ºC/15 sec/40 cycle), annealing (detection, 60 ºC, 30 sec, 40 cycle), amplification (72 ºC, 35 sec, 40 cycle) and cooling (37 ºC, 30 sec, 1 cycle).

Table 3.2. Volumes of each reaction used in qRT-PCR experiments.

Material	**Volume for one reaction**	**Concentration**
Extracted RNA	4µl	20ng/µl
qRT-PCR Master Mix	10 µl	1X
Reverse primer	1µl	µM
Forward primer	1µl	µM
RNase water	4µl	100% free

Table 3.3. Program style using in lightcycler qRT-PCR database

Analysis mode	**Number of cycles**	**Analysis mode**	**Temprature (ºC)**	**Duration (minute)**	**Acquisition mode**
Rev. Transcription	1	None	50	25	None
Denaturation	1	None	95	15	None
Amplification	40	Quantification	95	15	None
			60	30	None
			72	35	Single
Cooling	1	None	37	30	None

The instrument software was used to acquire the data.

Relative RNA quantification was performed using the following formula:

R = 2-{Cp(sample) - Cp(control)}.

For normalization in this study GAPDH primers were used in all RT-PCR experiments. In each RTPCR run sample, duplicates were used for each condition in addition to duplicates in which GAPDH primers were used for the same conditions. Relative RNA quantification analysis based on Cp values was performed in R. Primers were purchased from MacrogenPrimers, were dissolved in nuclease-free water by pipetting without vortexing and stored at -20 C. Primers were used in RT-PCR experiments as follows:

Table 3.4. Primers were used in the quantitative real-time PCR experiment.

Receptor	Forward and Reverse primers
FAS	Forward: 5′-GCGATGAAGAGCATGGTTTAG-3′ Reverse: 5′-GGCTCAAGGGTTCCATGTT-3′
DR4	Forward: 5’- CTGAGCAACGCAGACTCGCTGTC -3’ Reverse: 5’- TCAAAGGACACGGCAGAGCCTGTG -3’
DR5	Forward: 5’- GGGAGCCGCTCATGAGGAAGTT -3’ Reverse: 5’- GGCAAGTCTCTCTCCCAGCGTCTC -3’
GAPDH	Forward: 5′-AAGGTGAAGGTCGGAGTCAA-3′ Reverse: 5′-AATGAAGGGGTCATTGATGG-3′

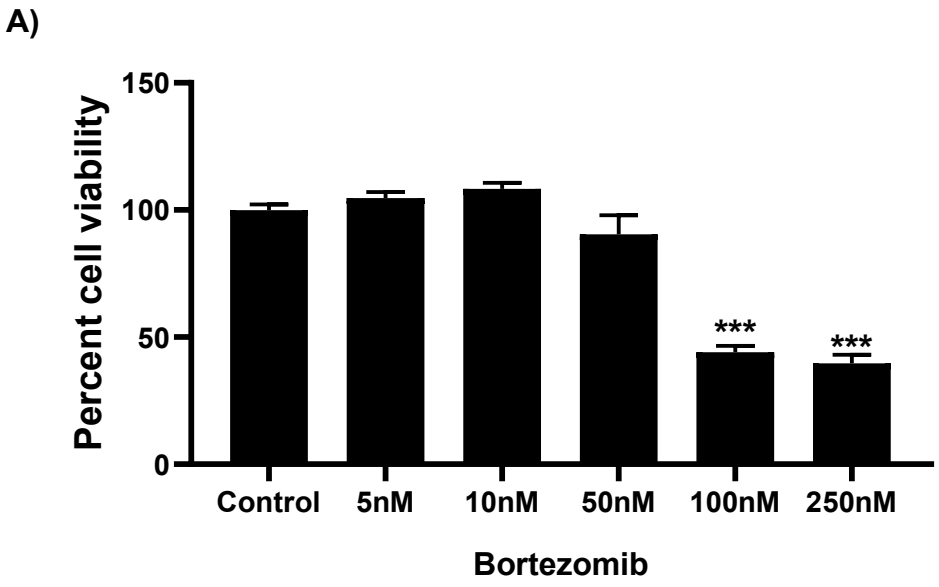
3.7. Analysis and Visualization

The data in the charts determining the results came from a variety of molecular biology tests. Microsoft Excel was used to make measurable comparisons. To determine the p-value, realistic merit was determined using Student's t-test and standard deviation comparison test consistent with ANOVA (analysis of variance). The data in these practical parts of the thesis were obtained from three biological replicates for getting more details on the statistical analysis.

4.RESULTS

4.1. Bortezomib Decreased Viability of Breast Cancer Cell Lines in a Dose Dependent Manner

In this study, we measured different concentrations of bortezomib on the two breast cancer cell lines MDA-231 and MCF-7 to determine sensitivity and viability (Figure 4). The cells were treated with 5, 10, 50, 100, and 250nM concentrations of bortezomib and incubated for 48 hours. Following the incubation period, MTT was performed. The results showed that both MCF-7 and MDA-231 cells remained viable at 5nM and 10nM, while viability decreased with increasing concentrations of bortezomib, which was particularly evident at 100nM and 250nM. While the majority of tumor cells remained viable after treatment with bortezomib at 5nM and 10nM, 50nM of bortezomib treatment killed about 20%. The cell viability decreased by 30-40% at 100nM and 250nM. During the 50nM concentration of bortezomib, there was a small difference in treatment sensitivity, with the resistance of the MCF -7 cell line being slightly stronger than that of the MDA-231 cell line. Depending on these cell viability experiments, two different concentrations of bortezomib 5nM and 10nM were selected for drug treatment and further experiments because the cell viability of these two concentrations was not significant for both the MCF -7 and MDA-231 breast cancer cell lines.



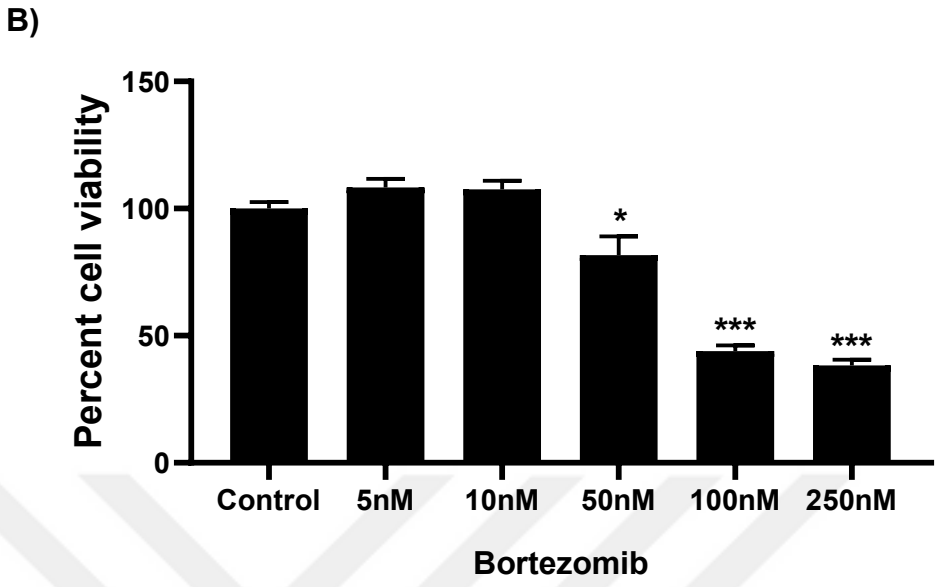


Figure 4.1. Response to bortezomib: sensitivity and viability of breast cancer cell lines, (a) MCF -7 and (b) MDA-MB -231. Cells were treated with different concentrations of bortezomib (5, 10, 50, 100, and 250 nM) for 48 hours compared to the control group (no treatment with PBS) and the percent viability of cells was measured by MTT assay, Experiment was repeated three times with similar results. Statistical analysis was performed by calculating the t-test according to the control groups in the GraphPad program (***: P <0.0005, **: P< 0.005, *: P< 0.05).

4.2. Effect of Bortezomib on Breast Cancer Cell Lines Migration

Bortezomib was used at different concentrations in each well. Concentrations were high (control, 5nM, 10nM, 50nM, 100nM and 250nM). We stated that the viability concentrations were 5nM and 10nM, respectively, depending on the cell migration. Cancer cells migrated in a variety of ways, both individually and collectively. As shown in Figure 4.2, individual and collective cell migration was determined and mediated. The cells migrated towards the scratching line after 24 hours, slightly decreasing the depth of the line, while the depth was significantly less after 48 and 72 hours. Because the dead cells detached and floated completely in the scratch, cell viability in 50nM, 100nM, and 250nM was significantly lower than in 5nM and 10 nM.

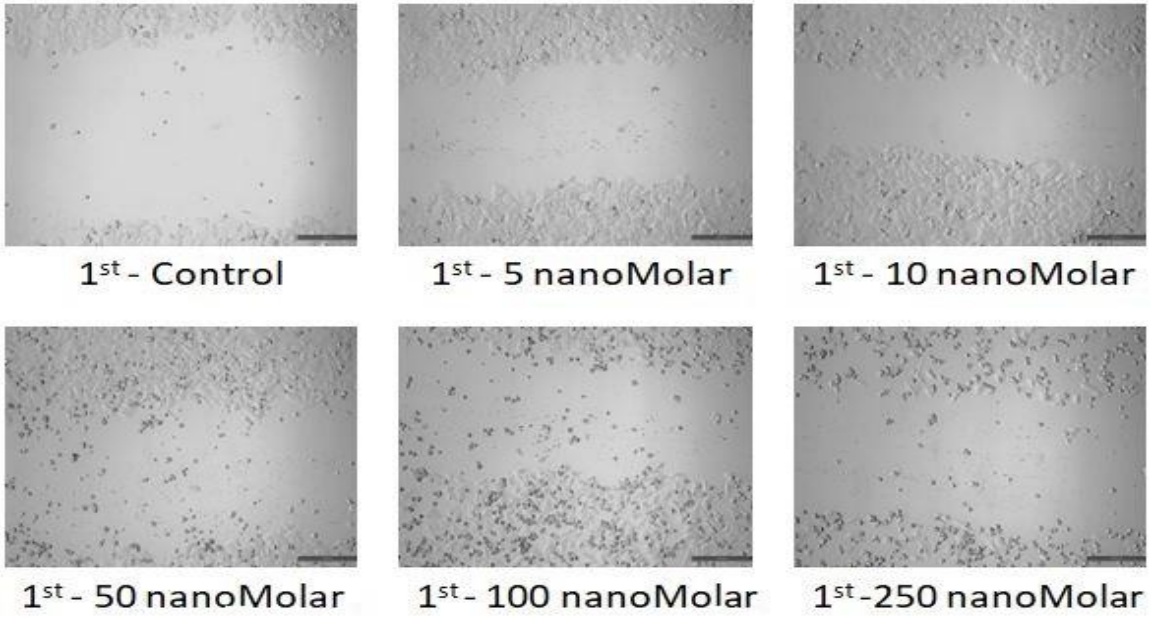


Figure 4.2.1. After 24 hours, cell migration towards the scratching line to show the viability of breast cancer cell lines showed that Control, 5nM, and 10nM migrated due to viability of cells, whereas 50nM, 100nM, and 250nM have died cells.

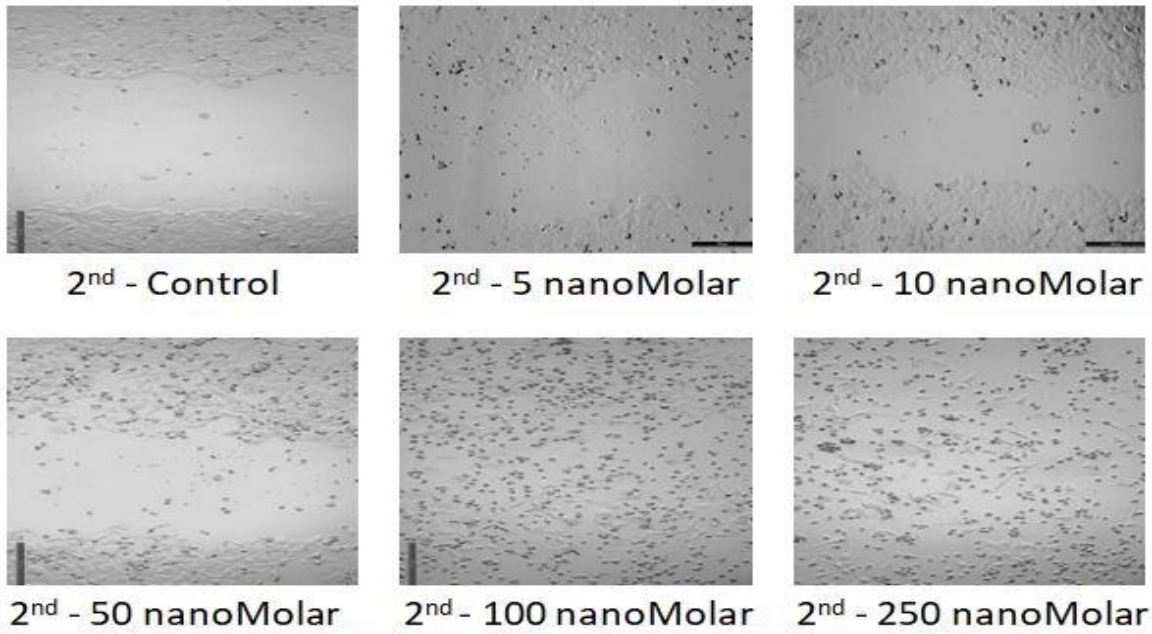


Figure 4.2.2. After 48 hours, cell migration towards the scratching line to show the viability of breast cancer cell lines showed that Control, 5nM, and 10nM migrated due to viability of cells, whereas 50nM, 100nM, and 250nM have died cells.

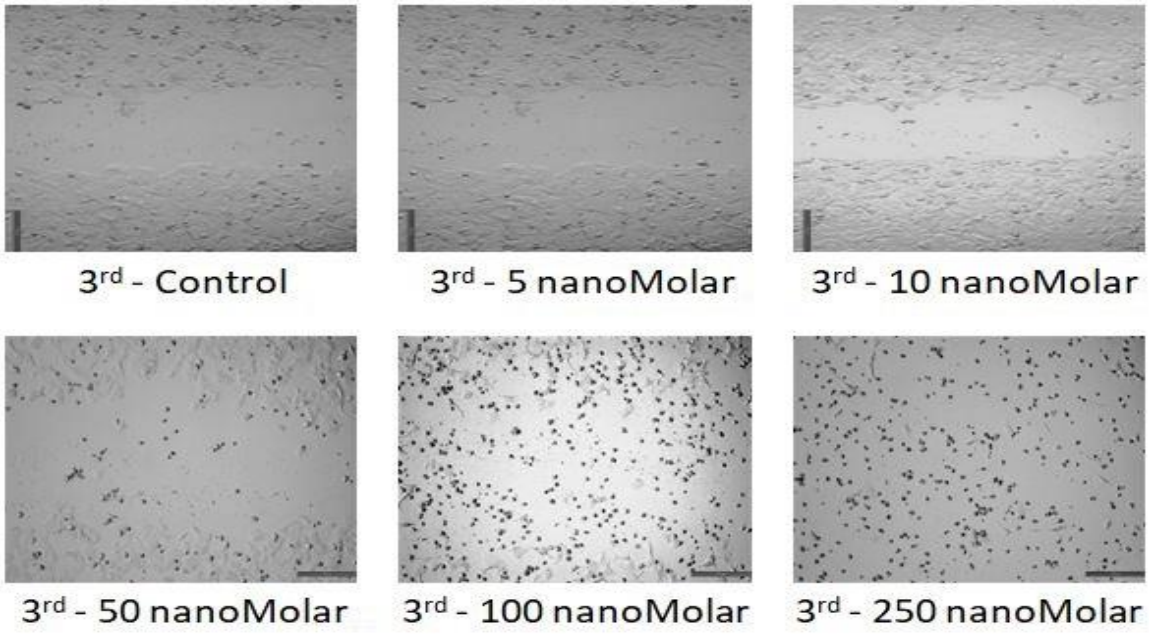


Figure 4.2.3. After 72 hours, cell migration towards the scratching line to show the viability of breast cancer cell lines showed that Control, 5nM, and 10nM migrated due to viability of cells, whereas 50nM, 100nM, and 250nM have died cells.

4.3. Effect of Bortezomib on Death Receptors Expression in MDA-MB-231 and MCF-7 Breast Cancer Cell Lines

After observing the cell viability of both breast cancer cell lines depending on the specific concentration (5nM and 10nM), we investigated the effect of bortezomib on three different receptor genes FAS, DR4, and DR5 to reveal apart of the future role of bortezomib in breast cancer treatment.

The role of bortezomib in death receptor expression has been investigated. We began our study investigation by treating cells with either 5nM or 10nM bortezomib to determine the transcript expression of death receptors in the two breast cancer cell lines, MDA-231 and MCF-7. The mRNA expression of DR4, DR5, and FAS was then quantified by qRT-PCR.

4.3.1. Effect of bortezomib on expression of FAS receptor

Here we examined that the expression of FAS receptor was significantly increased in both MDA-231 and MCF -7 breast cancer cell lines, as compared to the control treatment. When we treated the cells with different concentrations of 5nM and 10nM bortezomib, the expression of the Fas receptor increased, but in different ranges of both concentrations. The expression of the Fas receptor significantly increased and doubled compared to control in the MDA-231 breast cancer cell line. Also in the MCF -7 breast cancer cell line, FAS expression significantly increased and doubled when cells were treated with 5nM bortezomib. While at 10nM bortezomib, FAS receptor expression significantly increased and doubled compared to control in the MDA-231 breast cancer cell line. The same was true for the MCF -7 breast cancer cell line, where FAS expression significantly increased about 75%.

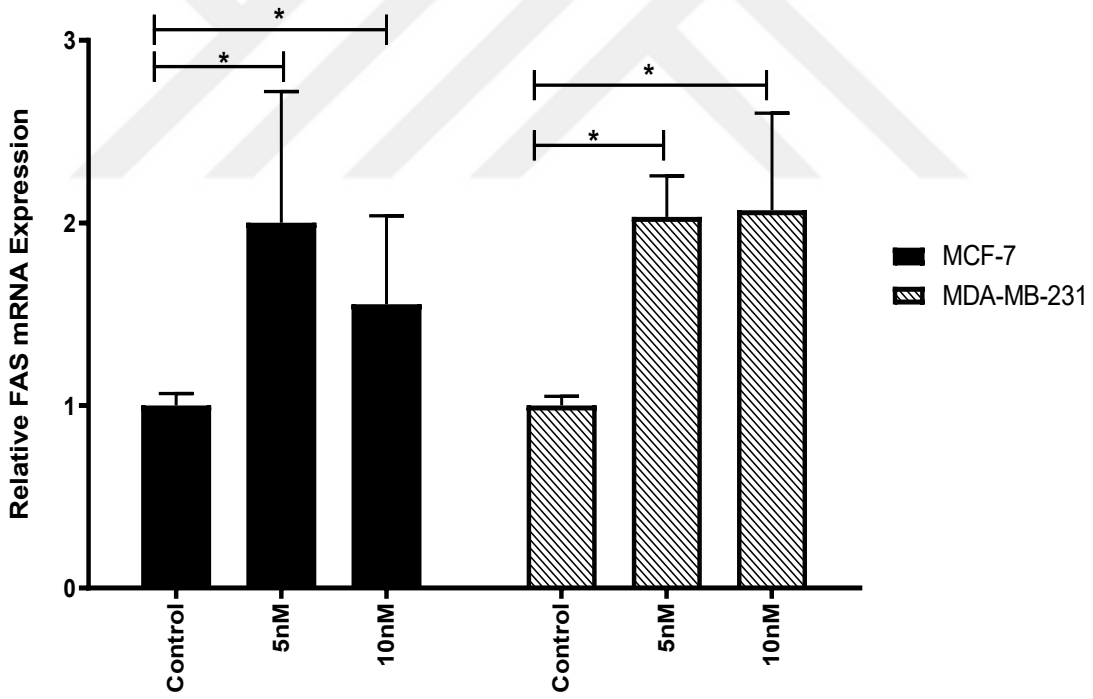


Figure 4.3.1. Bortezomib increases the expression of FAS transcripts in breast cancer cell lines. The expression of FAS was measured after cells were treated with (5nM and 10nM) bortezomib for 24 hours and compared to the control group (no treatment with PBS). qRT-PCR with primers was used to quantify the data. The data on the graph represents the average of three independent experiments.

4.3.2. Effect of bortezomib on the expression of DR4

As the expression of FAS receptor, the expression of DR4 in breast cancer cell lines MDA-MB-231 and MCF -7 differed when cells in control were treated with PBS without treatment with bortezomib, while cells treated with different concentrations of 5nM and 10nM bortezomib had a different expression of DR4 receptor at each concentration. The cells treated with 5nM bortezomib confirmed that the expression of DR4 receptor decreased approximately 40% compared to control in both MDA-231 and MCF -7 breast cancer cell lines, while the cells treated with 10nM bortezomib increased significantly of the expression of DR4 receptor by approximately 50% compared to control in MDA-231 breast cancer cell line. The same is true for the MCF -7 breast cancer cell line, where DR4 expression was non significantly increased by approximately 25%.

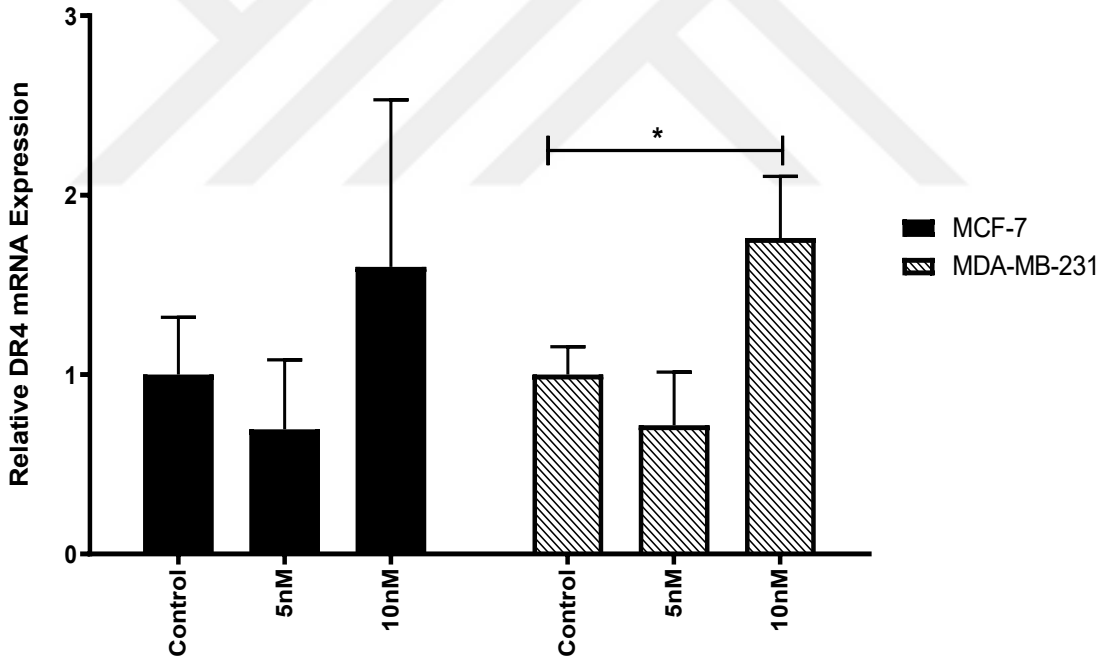


Figure 4.3.2. Response to bortezomib on DR4 expression. Cells were treated with (5 nM and 10nM) bortezomib for 24 hours compared to the control group (no treatment with PBS) and the expression of DR4 was measured by qRT-PCR.

4.3.3. Effect of bortezomib on the expression of DR5

The expression of DR5 was significant in breast cancer cell lines MDA-231 and MCF-7, the cells in control with PBS without treatment with bortezomib. The cells treated with 5nM bortezomib showed different expression of DR5 in MDA-231 breast cancer cell line compared to control with MDA-MB-231 and MCF -7 breast cancer cell line, in which the expression of DR5 in MCF -7 breast cancer cell line significantly increased by about 50% whereas no expression occur in MDA-MB-231 breast cancer cell line. Also, when the cells treated with 10nM bortezomib, DR5 receptor expression increased significantly by approximately 60% in the MDA-231 breast cancer cell line, while DR4 expression increased significantly by approximately 100% in the MCF -7 breast cancer cell line compared to control in both MDA-231 and MCF -7 breast cancer cell lines.

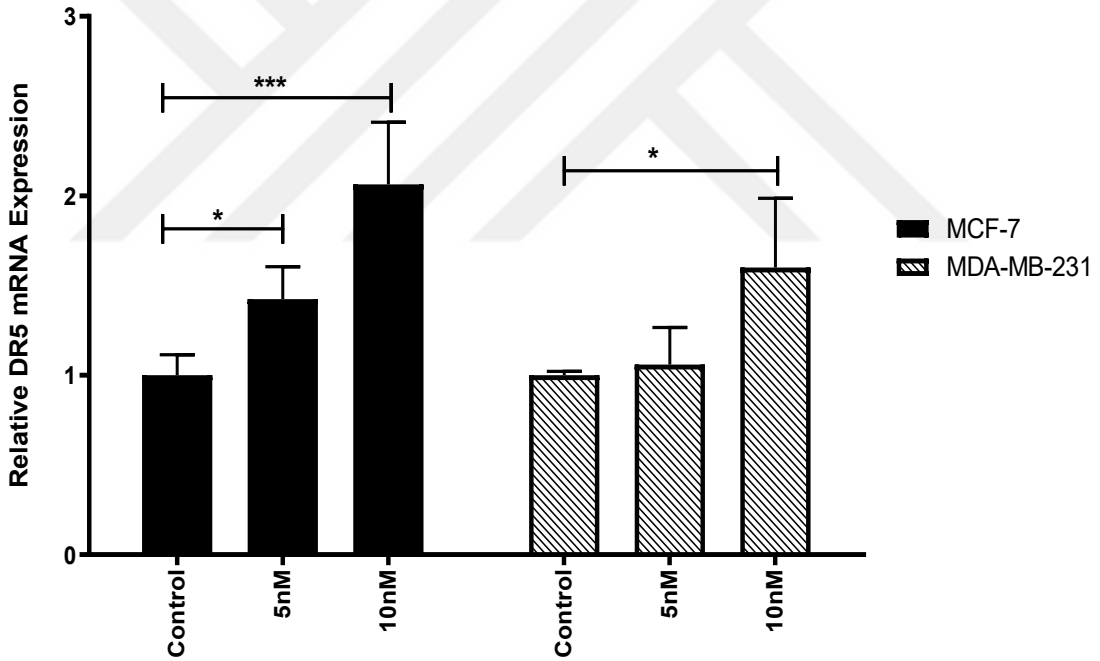


Figure 4.3.3. Response to bortezomib on DR5 expression. Cells were treated with (5 nM and 10nM) bortezomib for 24 hours compared to the control group (no treatment with PBS) and the expression of DR4 was measured by qRT-PCR.

5. DISCUSSION

Many different in vitro approaches to repairing the expressions of cancer cells have been initiated in the current era, leading to the discovery of various types of treatment therapies. The effect of overcoming drug resistance to the treatment itself, which is extremely important for the management of cancer cell development by regulating their resistance and appropriate treatment prognosis in vitro and in vivo, prompted the development of new treatments with a variety of treatments. Bortezomib is a type of anticancer drug defined as a proteasome inhibitor. Proteasomes are present in cells and break down proteins that are not needed or damaged in the cell. Bortezomib partially blocks the 26S proteasomes which lead to the accumulation of some type of proteins. The proteasome inhibitor, bortezomib, induces signaling toward different gene receptors by changing their intracellular peptides, to modulate death receptors that stimulate apoptosis programmed cell death. The proteasome inhibitor, bortezomib, also stimulates multiple signaling cascades (Yang *et al*., 2012).

Proteasome inhibitors have been shown to bind to (DR4) and (DR5) receptors, and these receptors are found on the cell surface. Besides the TNF domain, they possess the proteasome inhibitor-binding site. When a specific TRAIL receptor is cleaved, it can bind via the proteasome inhibitor binding site to the TRAIL ligand (Voortman *et al*., 2013). DR4 and DR5, shown that contains a functional visceral death domain motif and can deliver an apoptotic signal to proteasome inhibitors through death domain binding to the Fas-associated death domain protein, which contains a death effector domain, which is involved in caspase 8 activation (Suliman *et al*., 2013).

Previous studies have been shown to increase sensitivity to TRAIL-induced apoptosis in several human cancers. Another study found that bortezomib plays a direct role in the modulation of Fas ligand, confirming that immunogenic T-cell transfer combined with a tested bortezomib regimen reduced lung tumor nodules and improved survival. They show that when compared to a suppressive regimen close to the maximum tolerated levels of bortezomib, T-cell function persists in vivo after administration of the bortezomib therapeutic regimen. They also show that Bortezomib Improves Adoptive T-cell Therapy by Sensitizing Cancer Cells to FasL Cytotoxicity (Shanker *et al*., 2015).

Depending on stimulation of the death receptors bortezomib stimulates FAS (CD95), DR4 (TRAIL-R1), and DR5 (TRAIL-R2), and activation of caspase-8 at the death signaling complex (DISC) leads to proteolytic processing that performs modulation of death receptor apoptosis pathway.

In this thesis study, we first wanted to determine the specific concentration of bortezomib on breast cancer cell viability (5 nM, 10 nM, 50 nM, 100 nM, and 250 nM). We found that 5 and 10 nM of bortezomib did not cause any toxicity on both breast cancer cell lines. Our results revealed that increased bortezomib concentrations decrease both MCF-7 and MDA-231 cell viability.

Any type of cell death that causes an immune response is called immunogenic cell death. After signaling and target cells have been joined together, apoptosis, or programmed cell death, can occur, triggering an immune response. These occur when some death receptors contribute to this action and duties. Dead receptors are being targeted for treating various types of cancer cells by contacting with the proteasome inhibitor bortezomib, which has been approved in various studies that contribute both extrinsic and intrinsic pathways in apoptosis-induced cell death. Bortezomib modulates TRAIL-induced apoptosis by linking the death receptor to the mitochondrial pathway of neuroblastoma cells (Naumann *et al*., 2011). For instance, FAS (CD95), DR4 (TRAIL-R1), and DR5 (TRAIL-R2) death receptors have a great role to perform the immunogenic activity. It has been reported that Fas, DR4, and DR5 expression is deregulated in ovarian and colorectal cancer cells (Cacan, 2016; Cacan and Ozmen, 2020). It has also been reported that enhancing the expression of FAS and TRAIL receptors decreases ovarian cancer chemoresistance (Cacan, 2017). Therefore, focusing on expression profiles of death receptors, including Fas may contribute to immunogenic tumor cell death.

Bortezomib treatment increased mRNA levels of a number of proapoptotic proteins, including the death receptor protein, which can be occupied by apoptosis-inducing tumor necrosis factor (Johnson *et al*., 2006). Normally bortezomib is a proteasome inhibitor and also has a great role in the modulation of gene expression. Gene expression profile of bortezomib was revealed also in different studies of mRNA expression related to apoptosis, one investigation was revealed by apoptotic

transcription associated with activation of gene transcription Changes in global gene and miRNA expression and analysis of miRNA–mRNA interactions identified after bortezomib exposure in human neuroblastoma cells to determine the pathways affected by this agent in this cell type, this exposure of bortezomib result in molecular mechanisms of bortezomib action contribute to the expression of miRNA and Interaction miRNA–mRNA (Łuczkowska *et al*., 2020).

In our study, we found that a nontoxic concentration of bortezomib may treat and modulate the expression of FAS (CD95), DR4 (TRAIL-R1), and DR5 (TRAIL-R2) in breast cancer cell lines. Our data suggest that a low concentration of bortezomib modulates the activity of death receptors which may contribute to immunogenic cell death. Further, We started by investigating the effects of bortezomib on gene expression profiles. We show that bortezomib treatment significantly increases the expression of FAS (CD95), DR4 (TRAIL-R1), and DR5 (TRAIL-R2) death receptors by increasing their transcription on gene expression. Our data makes an important contribution to breast cancer treatment by using the proteasome inhibitor bortezomib to modulate and control the expression of death receptors Fas (CD95), DR4 (TRAIL-R1), and DR5 (TRAIL-R2) on cancer cells. Our results suggest that bortezomib treatment can be used to treat breast cancer by inducing increased sensitivity to cancer cells. Sufficient expression of death receptors on cancer cells may increase the capacity and sensitivity to cancer cells and modulate death receptors expression.

6. CONCLUSION

This study suggests that the proteasome inhibitor bortezomib may enhance cancer immunogenicity and increase with induction activity of anti-cancer immun cells resulting in increased sensitivity to death receptor-mediated apoptosis. Overall, the results suggest that treatment with the proteasome inhibitor bortezomib can modulate death receptor expression and can be used as a potential treatment regimen for the treatment of breast cancer. However, futher comprehensive *in vitro* and *in vivo* studies need to be performed.

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