INVESTIGATION OF BORON DERIVATIVES' EFFECTS ON BREAST CANCER CELLS PROLIFERATION AND TUMOR-SPECIFIC T CELLS *IN VITRO* BY DISTINCT MECHANISMS

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ABSTRACT

INVESTIGATION OF BORON DERIVATIVES' EFFECTS ON BREAST CANCER CELLS PROLIFERATION AND TUMOR-SPECIFIC T CELLS *IN VITRO* BY DISTINCT MECHANISMS

Breast cancer is the most commonly identified cancer in women throughout the world. Despite the initial clinical response obtained with the widely used conventional chemotherapy, an improved prognosis for breast cancer individuals has been missing in the clinic because of the high toxicity to normal cells, induction of drug resistance, and the potential immunosuppressive effects of these agents. Therefore, our objective was to examine the potential anti-proliferative ability of some boron derivatives (SPP) and (SPT), which showed a promising effect on some types of cancers in the literature, on breast cancer cell lines as well as immuno-oncological side effects on tumor-specific T cell activity. These findings suggest that both SPP and SPT suppressed proliferation and induced apoptosis in MCF-7 and MDA-MB231 cancer cell lines through downregulation of the monopolar spindle-one-binder (MOB1) protein. Contrarily, these molecules increased the expression of CD274 protein through their effect on the phosphorylation level of Yes-associated protein. In addition, they reduced the concentrations of pro-inflammatory cytokines such as IFN-γ and cytolytic effector cytokines such as sFasL, perforin, granzyme A, Granzyme B, and granulysin and raised the level of CD279 surface protein in activated T cells. In conclusion, SPP, SPT, and their combination could have growth inhibitory (antiproliferative) effects and could be a therapeutic option for breast cancer. However, their stimulatory effects on the PD-1/PD-L1 signaling pathway and their effects on cytokines could ultimately account for the observed repression of the charging of effector T cells that have been particularly activated against breast cancer cells. This study paved the way for a future line of research to investigate the anti-carcinogenic effect of the boron derivatives in vivo and the synergistic effect of these boron derivatives with an optimal dose and modified T cells activated against breast cancer cells.

ÖZET

BOR TÜREVLERİNİN MEME KANSERİ HÜCRELERİNİN ÇOĞALMASI VE TÜMÖRE ÖZGÜ T HÜCRELERİ ÜZERİNDEKİ ETKİLERİNİN *İN VİTRO* OLARAK FARKLI MEKANİZMALARLA İNCELENMESİ

Meme kanseri, dünya çapında kadınlar arasında en sık teşhis edilen kanserdir. Yaygın olarak kullanılan konvansiyonel kemoterapi ile elde edilen ilk klinik cevaba rağmen, normal hücrelere yüksek toksisite, ilaç direncinin indüklenmesi ve bu ajanların potansiyel immünosüpresif etkileri nedeniyle klinikte meme kanseri hastalarında iyileştirilmiş bir prognoz eksiktir. Bu nedenle literatürde bazı kanser türleri üzerinde ümit verici etki gösteren bazı bor türevlerinin (sodyum pentaborat pentahidrat (SPP) ve sodyum perborat tetrahidrat (SPT)) meme kanseri hücresi üzerindeki potansiyel anti-kanserojen etkisini araştırmayı amaçladık. hatların yanı sıra tümöre özgü T hücresi aktivitesi üzerindeki immüno-onkolojik yan etkiler. Bu bulgular, hem SPP hem de SPT'nin, monopolar mil-bir-bağlayıcı (MOB1) proteininin aşağı regülasyonu yoluyla MCF-7 ve MDA-MB231 kanser hücre hatlarında proliferasyonu baskıladığını ve apoptozu indüklediğini göstermektedir. Öte yandan, bu moleküller Yes ile ilişkili proteinin (Phospho-YAP (Ser127) fosforilasyon düzeyi üzerindeki etkileriyle CD274 proteininin ekspresyonunu arttırırken, IFN gibi proinflamatuar sitokinlerin konsantrasyonlarını azaltmışlardır. -γ ve sFasL, perforin, granzyme A, Granzyme B ve granülysin gibi sitolitik efektör sitokinler ve aktifleştirilmiş T hücrelerinde CD279 yüzey proteininin ekspresyonunu arttırdı.Sonuç olarak, SPP, SPT ve bunların kombinasyonları büyümeyi inhibe edebilir (antiproliferatif) etkiler ve meme kanseri için potansiyel bir tedavi olabilir. Bununla birlikte, PD-1/PD-L sinyal yolu üzerindeki uyarıcı etkileri ve sitokinler üzerindeki etkileri, nihai olarak, spesifik olarak aktive edilmiş efektör T hücrelerinin yüklenmesinin gözlenen baskılanmasını açıklayabilir. meme kanseri hücrelerine karşı. Bu çalışma, bor türevlerinin in vivo anti-kanserojen etkisini ve bu bor türevlerinin optimal doz ve meme kanseri hücrelerine karşı aktive edilmiş modifiye T hücreleri ile sinerjistik etkisini araştırmak için gelecekteki bir araştırma hattının yolunu açtı.

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APCs	Antigen Presenting Cells
BM	Bone marrow
CAFs	Cancer-associated fibroblasts
CTLA4	cytotoxic T-lymphocyte-associated protein 4
DC	Dendritic Cells
DPBS	Dulbecco's Phosphate Buffered Saline
ER	Estrogen receptors
FBS	Fetal Bovine Serum
Gal-9	Galectin-9
HER2	human epidermal growth factor receptor 2
HHLA2	human endogenous retrovirus-H long terminal repeat-associating protein 2
IC	inhibitory concentration
IFN-γ	Interferon gama
IL	Interleukin
LATS1	large tumor suppressor kinase 1
LDH	Lactate Dehydrogenase
MDSCs	Myeloid-derived suppressor cells
MHC	Major histocompatibility complex
MST1	macrophage stimulating 1
NK	Natural killer cells
PBMCs	Peripheral Blood Mononuclear Cells

PD-1	Programmed cell death protein 1
PD-L1	Programmed Cell Death Ligand-1
PI3K	Phosphoinositide 3-kinase
PR	Progesterone receptor
PSA	Penicillin, Streptomycin, Amphotericin
PTEN	Phosphatase and tensin
RT qPCR	Reverse transcription-quantitative polymerase chain reaction
SAV-1	Salvador protein 1
SPP	Sodium Pentaborate Pentahydrate
SPT	Sodium Perborate Tetrahydrate
T reg	Regulatory T cell
TAZ	Tafazzin Protein
TCR	T-cell receptor
Th1	T helper type 1 cells
Th2	T helper cells 2
TIL	Tumor-infiltrating lymphocytes
TIM-3	T cell immunoglobulin and mucin domain 3
TME	Tumor microenvironment
TNBC	Triple Negative Breast Cancer
TNF-α	Tumor Necrosing Factor α
VISTA	V-domain immunoglobulin containing suppressor of T-cell activation
YAP	yes-associated protein

β TrCP	Beta Transducin repeats Containing Proteins

1. INTRODUCTION

1.1. CANCER

1.1.1. Epidemiology of cancer

Cancer is the abnormally proliferation of the body cells. Cancer can be the primary cause of death by leading to metastases in the body. In the world, a considerable amount of death is occurring because of the cancer. Especially, in the United States, cancer as a public health issue causes 1 of every 4 death (1). Although cancer is one of the primary causes of death, there are many factors that affect the way of cancer. In most cases, cancer survival is based on social and economic factors, healthy behaviours, family medical histories, and the Healthcare provisions level to support early detection, Intervention, and Post-treatment monitoring (2). There are a lot of cancer types according to their originated tissue or organs. According to made population based on cancer survival research in Central America, Africa and Asia (also, including Turkey), 91172 cases are seen as lung cancer and the dead number is 75461, 65722 cases are seen as stomach and the dead number is 43724 and 55520 cases are seen as large bowel cancer and the dead number is 29160 (2). This data shows people who have cancer as lung, stomach and bowel cancer type, were not diagnosed and these cancer types mostly cause the death in people. The highest survival of cancer is seen in Turkey, South Korea, China, and Singapore and the lowest survival of cancer is seen in Uganda and The Gambia. The findings are showed for many cancer types in these countries. The survival rates of various types of cancer were studied in several countries including China, Singapore, South Korea, Turkey, The Gambia, and Uganda. Breast cancer had the highest survival rates of 76-82%, while large-bowel cancer showed the lowest survival rates of 44-60% (2). Gambia and Uganda had the lowest survival rates for all types of cancer, with breast cancer being the only exception at 46% (2).

In 2018, the World Health Organization reported that 18.1 million people worldwide were detected as cancerous patients, and 9.6 million people passed away from cancer (3). The common types of cancer which spread worldwide include lung cancer, female breast cancer and colorectal cancer. These three types of cancer are top three cancer types in all around

the world (3). The following cancer types which lead to cancer types are prostate cancer and stomach cancer. The lung cancer and colorectal cancer are leading cancer types which cause the death (18.4% and 9.2% of total, respectively) because these cancer types cannot be detected early and they have poor prognosis (3).

The data of WHO established the cancer mortality profile for the prostate cancer in Turkey includes 7.0% in males and 9.1% in females (4). In Turkey, scanning and Prompt diagnosis methods for colorectal cancer include the use of faecal immunological test, as well as Bowel cancer screening through colonoscopy or physical examination. (4).

1.1.2. Cancer Biology & Cell Death Pathways

According to the cancer researches, it was revealed that the cancer disease involves dynamic changes in the genome. The hallmarks of the cancer include six biological hallmarks that produce human tumors. There are several hallmarks of cancer cells that contribute to their growth and spread, including the ability to Continue growth, counteract Growth inhibitors, resist cell death, replicate infinitely, induce angiogenesis, and initiate spreading. These hallmarks work together to support the chronic proliferation of cancer cells. Unlike normal cells, which regulate the production of growth-promoting signals and initiate cell growth and division, cancer cells have lost this control and continue to divide and grow uncontrollably. That control mechanism maintains the function and morphology of the normal tissues. Cancer cells regulate these signals by themselves. Therefore, the proliferation of the cancer cell occurs without any control mechanism (5). Tyrosine kinase domain helps the transmission of the growth-promoting signals to a cell surface receptor. The transmission of the signals stimulates the intracellular signaling pathways. The intracellular pathways regulate and control the cell growth, cell death and cellular energy metabolism (6).

Genetic mutations can cause cancer by affecting various kinds of genes, such as proto-oncogenes, tumor suppressor genes, and DNA repair genes. Such mutations can modify the way these genes work and lead to the progression of cancer (7). Proto-oncogenes take place in the cellular growth. However, when they mutate or become more active, they turn into the oncogenes which are cancer causing genes that triggers cell growth and proliferation and cell survival when they should not (8). Tumor suppressor genes, also called as anti-oncogene, regulate the cell division by slowing it down; also control the mistake of the DNA

repair and apoptosis. When these genes mutate, they do not work properly and cells grow and proliferate uncontrollably (9). DNA repair genes are coded for the proteins that control the error occur in the DNA before cell division. When these genes mutate, additional mutations occur in the other genes. This causes deletions or duplications in some parts of the chromosome and leads cancerous cell (10).

PI3K, mTOR, and AKT are vital regulators of the cell cycle. Together, they form a signaling pathway referred to as the AKT/PI3K/mTOR pathway, which controls the cell division process. When PI3K is activated, it phosphorylates a lipid called phosphatidylinositol 4, 5-bisphosphate (PIP2) to produce PIP3. PIP3 then functions as a crucial secondary messenger that helps transmit signals, causing the activation of AKT. Upon activation, AKT becomes retained to the plasma membrane and stimulates the initiation of mammalian target of mTORC1, which regulates cellular growth by controlling mRNA translation. Additionally, activated AKT helps prevent apoptosis by targeting BCL2 associated agonist of cell death (BAD) (11). Additionally, AKT inhibits GSK3, leading to increased cell proliferation. In summary, this pathway helps to regulate and control cell growth, and proliferation. In the case of over-activation of this pathway leads to reducing in the apoptosis and causes over proliferation and consequently cancer development. Mutation in PI3K and deletion of PTEN are commonly seen in breast cancers (12).

The pathway of Raf/Ras/MEK/ERK is a significant pathway that facilitates communication from the cell surface to DNA. This pathway is consisted of a chain of proteins that are triggered by RTKs, which then activate the SHC. The interaction between GRB2 and SOS triggers Ras activation, which occurs in its GTP-bound state. Activated Raf subsequently phosphorylates and activates MEK (or MAPKK), which, in turn, triggers the activation of ERK. ERK then activates transcription factors that facilitate cell division, suppress apoptosis by phosphorylating BAD, and control cell growth, differentiation, and death. Overall, this path is vital in regulating several cellular processes (13).

BRAF proteins are serine-threonine kinases and are important for regulating cell growth and death. They play a vital part in transmitting signals as a signal transduction molecule from the RAS gene family to the MEK pathway (14). This path is activated by the oncogenic proteins including RAS and RAF, as well as growth factors (15).

The feedback mechanism controls the proliferative signaling pathways. Therefore, if this mechanism is disrupted, it can cause cancer cells to increase their proliferation signal (16). Ras proteins are involved in GTPases and GTP binding proteins. Ras gene becomes activated

when the oncogenes are mutated. The triggering of the Ras genes leads to the activation of the Ras-GTPase activity. The Ras gene when activated, plays a role in promoting cellular growth, Sustaining life and Rapid multiplication (17). PTEN is a phosphatase generated by the PTEN gene that serves as a negative feedback mechanism by blocking the signal cascade of PI3K. It does this by dephosphorylating PIP3 to PIP2. However, when PTEN is mutated, PI3K signal cascade is not blocked and this leads to the formation of various cancer types such as glioblastoma, breast cancer, lung cancer and prostate cancer (18).

Beside tumor formation, the increase in the proliferative signaling can also cause the cell senescence and apoptosis. Generation of oncoprotein in excessive amount can leads to apoptosis or cell senescence (19).

There are several tumor suppressive genes exist that manage the inhibition of the cellular growth and cell proliferation such as; PTEN, TP53 and RB. These genes can be activated with internal or external stimuli. Active tumor suppressor genes can cause cell cycle arrest, induction of apoptosis or senescence. Therefore, these genes found in inactive form in the cancer cells. This can be achieved by the tumor suppressor gene deletion or inactivation of these genes by mutations (20). To control the growth of cells and their division, Retinoblastoma (RB) proteins regulate the intracellular and extracellular signals. Mainly, they receive extracellular signals for the inhibition of the growth (21). TP53 proteins take intracellular signals for the inhibition of the cell cycle process as a result of the damage in the intracellular space. If a cell is unable to be repaired, it goes through a process called apoptosis, which is controlled by proteins known as TP53 (22).

In healthy cell population, the confluency of the population has its limits. The cell can be divided until the population reaches the confluency. This is achieved by the contact inhibition. This inhibition occurs as a result of the cell to cell contact. Cancer cells do not have the contact inhibition (23). The neurofibromatosis-2 (NF2) gene coded for the Merlin protein which is also a tumor suppressor. It mediates the cell proliferation through the contact inhibition. The inhibition occurs by the association of cell surface adhesion molecules with transmembrane receptor tyrosine kinases with the help of the Merlin. It also prevents the reception of the mitogenic signal by the growth factor (24).

1.1.3. Cancer Hallmarks

Cancer cells display a variety of abnormalities in controlling cell division. They can produce their own growth signals, ignore anti-growth signals, evade programmed cell death, replicate indefinitely, stimulate the creation of new blood vessels, metastasize, and resist the immune system. They also follow a different metabolic pathway, have unstable genomes, and can be associated with chronic inflammation. These irregularities help in the growth and advancement of cancer.

- Self-sufficiency in Growth Signals

Healthy cells rely on external signals to initiate their growth and division. However, cancer cells can produce their own signals, which is known as autocrine signaling, and therefore do not require external signals to proliferate (5, 25).

- Anti-growth Signals Insensitivity

Cancer cells do not react to signals that would normally inhibit their growth. This is because the tumor suppressor proteins in cancer cells are mutated, which leads to the loss of their ability to restrict cell division (5, 26).

- Programmed Cell Death Evasion

Cancer cells have the ability to avoid programmed cell death, which is also known as apoptosis (5, 27).

- Limitless Replicative Potential

Normally, healthy cells have restricted number of times they can splite, known as senescence. Contrarily, cancer cells can continue to divide indefinitely because of mutations in their tumor suppressor proteins. This is achieved in part by manipulating telomeres, which control the number of times a cell can divide. Most cancers (about 85%) overexpress telomerase to prevent telomeres from shortening, while 15% use a different mechanism called the Alternative Lengthening of Telomeres (28).

- Sustained Angiogenesis

As a tumor grows and spreads, it demands a fresh inventory of oxygen to support the new cancer cells. To achieve this, new blood vessels is stimulated by the tumor, called angiogenesis (5, 29).

- Metastasis

Cancer cells have the ability to multiply beyond their boundaries and travel from their original location to invade other tissues and spread throughout the body (a process known as metastasis), where they can form new tumors and continue to multiply (5, 30).

- Deregulated Metabolism

Cancer cells use a metabolic pathway called the Warburg effect, where they increase their rate of glucose breakdown and lactic acid fermentation in the cytosol, without going through the normal process of aerobic respiration in the mitochondria. Rather than producing high amounts of ATP, cancer cells prefer to convert pyruvate into the raw materials needed for cell growth and division (5).

- The Immune System Evasion

Cancer cells have created mechanisms to evade or resist the body defense system (5).

- Genome Instability

Cancer cells are typically marked by mutations in various genes and significant chromosomal abnormalities that contribute to the progression of the disease (5).

- Inflammation

Persistent inflammation is frequently linked with different types of cancer. Inflammation can prompt the creation of new blood vessels and activate the immune system. However, this mechanism can also aid the travelling of cancer cells to distent areas of the body by promoting the development of new blood vessels (5).

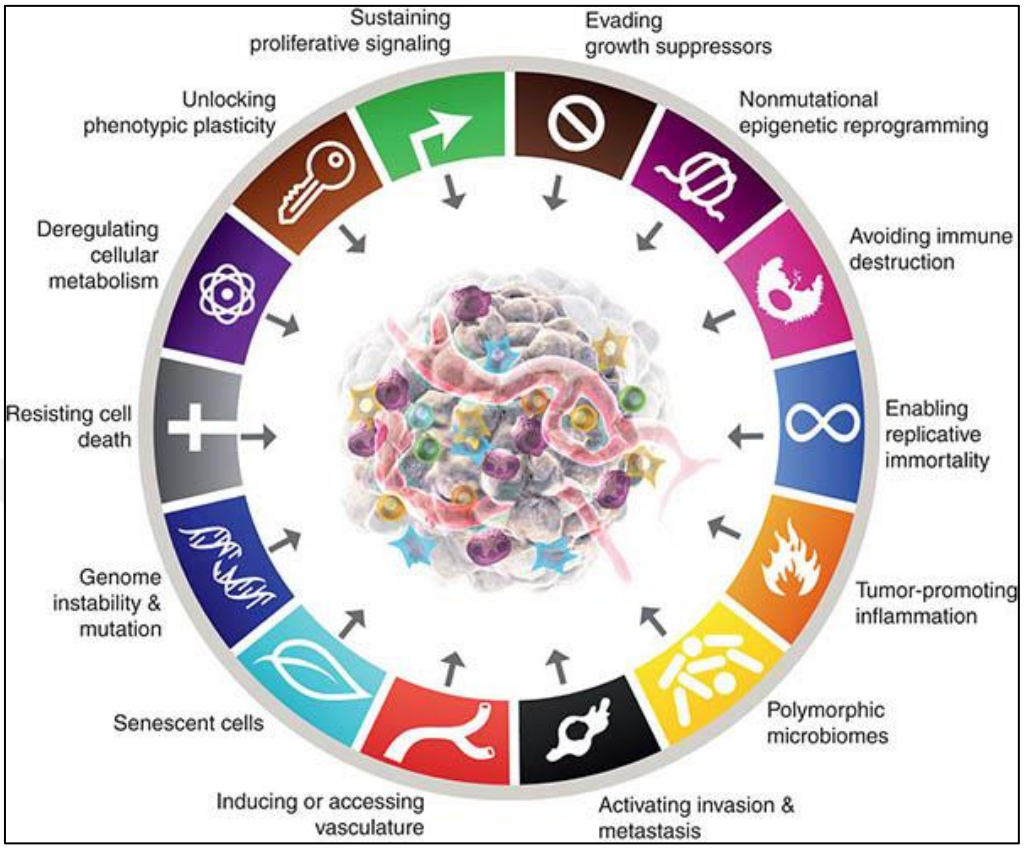

Figure 1.1. Hall marks of cancer (31)

1.1.4. Apoptosis

Apoptosis is an essential mechanism that occurs during both fetal progression and in adult tissues. Commonly physiological cell death occurs with the apoptosis instead of necrosis (32). Necrosis can be defined as accidental cell death caused by external factors (33). Distinct characteristics are exhibited by cells that undergo apoptosis, including the compaction of chromatin, division of the nucleus, reduction in cell size, and the creation of bulges on the cell's outer membrane. When there is an issue with the control of apoptotic cell death, diseases or disorder can arise such as cancer. The molecular mechanism that is responsible for the apoptosis is the intracellular proteases family, caspases. Besides these cell death proteases, there are inhibitors and activators of these molecules. Activation of the cysteine protases that cleaves their substrate from the aspartic acid residue causes the morphologic changes and apoptosis. Under normal situations they are in the form of zymogens (34).

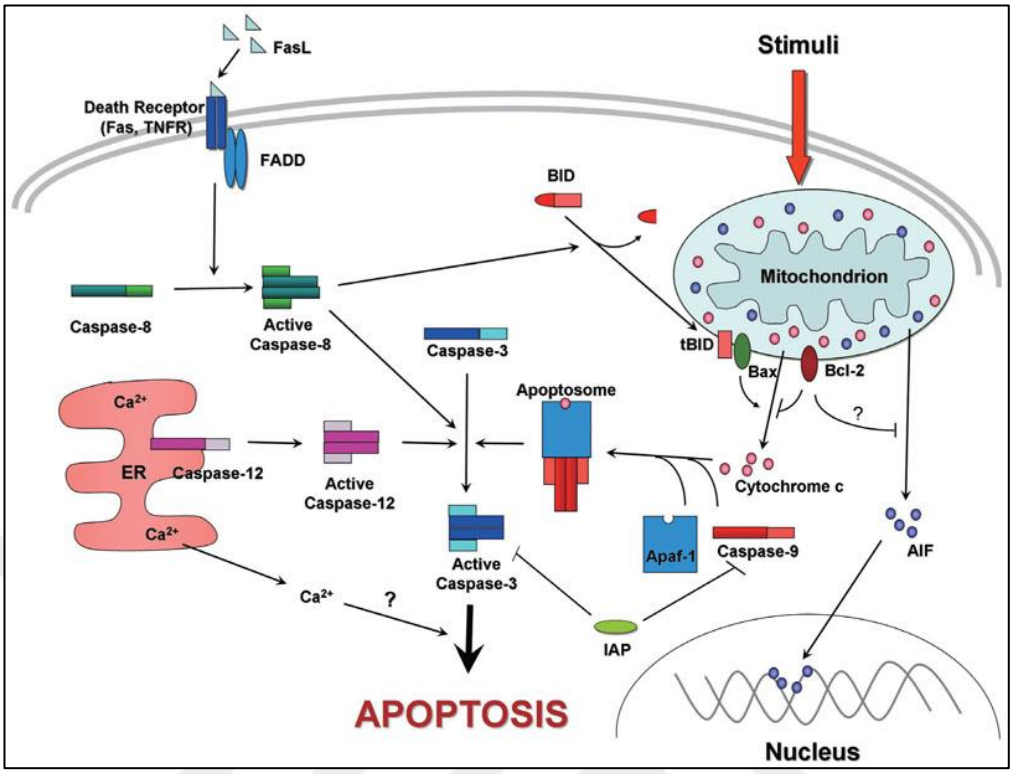

Figure 1.2. The figure of apoptosis pathways (35)

In the apoptotic process, cells stimulated with two different ways; intrinsic and extrinsic. In the intrinsic apoptotic program, the apoptotic signals that induce cell death takes place inside the cell (35). In the extrinsic apoptotic program, these signals stimulate the cells from the outside of the cell. In the intrinsic program, the cell death inducing signals interact with the mitochondria (35). The Bcl-2 protein family is responsible for determining whether a cell undergoes apoptosis or not. It is located on the mitochondrial membrane and regulates the permeability of the outer membrane of mitochondria, resulting in the discharge of intermembrane space proteins in an irreversible manner from the mitochondria. This release activates caspases and triggers apoptosis. Thus, Bcl-2 plays an Vital role in the intrinsic pathway of apoptosis (36). Cells can undergo apoptosis, which is either prevented or induced by the Bcl-2 protein family. The downstream caspases are classified into 3 groups, consisting of pro-apoptotic pore-formers (including BAX, BAK, and BOK), anti-apoptotic proteins (including BCL-2, BCL-XL, BCL-W, MCL-1, and BFL-1/A1), and pro-apoptotic BH-3-only proteins (including BAD, BID, BIM, and others) (37) (38). During apoptosis, pores form in the mitochondrial membrane, allow cytochrome c to be released into the cytosol. This leads to the release of an apoptosome that activates casp-9, which subsequently triggers casp-3, casp-6, and casp-7, ultimately causing apoptosis. However, anti-apoptotic proteins

prevent the discharge of cytochrome c into the cytoplasm. The intrinsic pathway is a crucial focus for cancer therapies as it aims to induce the death of cancer cells. In these treatments, the objective is to target the overexpression of Bcl-2 proteins and non-functional pro-apoptotic molecules (39).

The extrinsic pathway of apoptosis involves receptors with death domains such as Fas receptors and TNF receptors that bind to Fas ligand, a signaling molecule (40). The extrinsic pathway of apoptosis is initiated by external signals such as FasL, TRAIL/Apo2L, and TNFα, which bind to TNF-related apoptosis inducing ligand receptors (TRAIL) on the cell surface. When enough receptors accumulate, the death domain is activated, causing the formation of the death induced signaling cascade (DISC). Casp-8 is then activated by the DISC, and it plays a significant role in the extrinsic pathway and its interaction with the intrinsic pathway (41). In the extrinsic pathway, caspase 3 is triggered by caspase 8. Caspase 3, once activated, degrades the inhibitor of the nuclease enzyme, releasing the nuclease to cleave nucleic acid and induce cell death. Additionally, caspase 8 can activate Bid protein, leading to the production of tBid. tBid then activates BAX and BAK resulting in the discharge of cytochrome c and subsequent induction of the intrinsic pathway (42).

1.2. BREAST CANCER

The breast cancer is crucial cancer type that majorly seen in women but rarely can be seen on men and early diagnosis may save life. In 2020, 2.3 million women diagnosed with breast cancer and 685 thousand deaths were reported (43). Breast cancer incidence rates vary across regions, with Africa and Asia having lower rates compared to Australia and Southern Europe which have higher rates. Mortality to incidence rate ratios also vary across regions, with more developed countries having lower ratios (the lowest recorded ratio being 0.16 in North America) and less developed countries having higher ratios. From 1975 to 2010, breast cancer incidence rates increased in developed countries due to factors such as reproductive patterns, exogenous hormones, and increased breast cancer screening (44).

Breast cancer incidence is strongly correlated with age, with over 60% of cases individuals above 60 and less than 5% diagnosed in those under 40. Additionally, breast cancer

incidence varies among different ethnic and racial groups. In the US, for example, white women have a higher incidence rate than black women (45).

Breast cancer has different stages, with the earliest stage being in situ which has the highest chance of being cured. Metastasis can occur at any stage of breast cancer. Studies indicate that using hormones produced by the ovaries can significantly increase the breast cancer risk (44). The breast cancer risk is connected to endocrine activity, especially early onset of menstruation and late onset of menopause. Research based on 117 epidemiological studies has found that a one-year delay in menarche lowers the breast cancer risk by 5%, while a yearly increase in age at menopause raises the risk by 2.9%. Early pregnancy lowers the breast cancer risk, and women of young age who have been pregnant are at a 50% lower risk compared to older women. Breastfeeding also lowers the breast cancer risk, and females who breastfeed for longer durations have a lower risk (46).

Breast cancer individuals commonly die due to the spread of cancer to other organs or lymph nodes. Factors such as aging, genetic mutations, and family history influence the occurence of breast cancer. Breast cancer risk is elevated by BRCA1 and BRCA2 genes mutations. Early detection through screening methods can result in a high cure rate of 90%. Breast cancer can be treated through various methods such as radiation therapy, chemotherapy, and surgery to remove lymph nodes or metastatic tissue. However, the treatment of breast cancer can be expensive (47).

The categorization of breast cancer is based on the transmembrane receptors, such as estrogen and progesterone receptors, and the amplification of the oncogene/protein HER2 (48). The most common type of breast cancer is Luminal A, which is featured by the presence of estrogen and/or progesterone surface receptors but the absence of HER2 receptors, and has a better prognosis. Luminal B breast cancers have a higher proliferation rate and are more aggressive, as they may have estrogen and/or progesterone receptors as well as HER2 receptors. HER2+ but ER and/or PR- tumors have a worse tumor grade and often involve lymph nodes, but treatment with the anti-HER2 antibody Herceptin has improved prognosis. Triple-negative breast cancer (TNBC) lacks all three receptors and is often more aggressive with a poorer prognosis, and currently has no targeted therapies available (49).

1.2.1. Chemotherapy in breast cancer

Breast cancer is a prevalent cancer type globally. Research indicates that chemotherapy enhances the overall survival rate in women with early-stage breast cancer, although the extent of improvement decreases with age. Systemic therapy is used to detect this disease in its early stages (50). The systematic adjuvant provides decline in the rate of recurrence and also improvement for the long term survival. It also controls the micro metastatic disease. Although the effect of the adjuvant radiotherapy, adjuvant chemotherapy and hormonal therapy occur after 5 year, these therapies decrease the mortality rates. However, metastatic disease treatments remain a major challenge but it generally chemo sensitive which provide symptom control and long survival (51).

It is recognized that about 20% of breast cancer cases involve the overexpression of the HER-2/neu protein and its associated gene. Trastuzumab, a humanized mAb that is generated to antagonize the extracellular area of the HER-2 protein, significantly prolong survival time of the breast cancer patient that has the over expression of HER-2 (52). HER-2 stimulation increases the cellular growth, angiogenesis and the apoptotic signal inhibition (53).The use of trastuzumab is worldwide and therefore is accepted as standard therapy. Patient specific therapies depend on the tumor's molecular characteristics (54). Studies show that the use of trastuzumab with the HER-2+ breast cancer individuals decreases the risk of recurrence (55).

1.2.2. Adjuvant therapy

The adjuvant therapy involves a systematic treatment that includes surgery, medical treatments and radiotherapy. The main purpose of adjuvant therapy is to decrease the recurrence risk as much as possible and to extend survival. Various types of adjuvant therapeutic choices exist for primary breast cancer, including endocrine therapy, chemotherapy, and biological treatment. Additionally, these all therapies can be combined. The therapy should be decided according to the cancer behavior and tumor burden (56).

1.2.3. Chemotherapy for metastatic breast cancer (MBC)

The widely used chemotherapeutic agents for the early breast cancer therapies as adjuvant and neo-adjuvant treatments are anthracyclines and taxanes. These agents reduce the recurrence rate in the breast cancer. Although these adjuvant therapies in the early stage breast cancer provide improvements, it creates complexities for the women that have metastatic disease since they have already exposed to the anthracyclines and taxanes (57). Anthracyclines are the antibiotics that used for the cancer treatment as a chemotherapy drug. They kill the cancer cells by damaging the DNA. Daunorubicin, doxorubicin and epirubicin are the examples of the anthracyclines (58). Especially epirubicin and doxorubicin are frequently used for the breast cancer treatments despite of their toxicities. The pegylated liposomal doxorubicin is packed inside of a liposome and it provide preferential accumulation of the doxorubicin in the tumors (59).

Taxanes are chemotherapeutic agents against the metastatic breast cancer treatment. Paclitaxel and docetaxel are the widely used examples. Taxanes disrupt the microtubule function of the cancer cells and inhibit the cell division by preventing depolymerization. Therefore, they are called as mitotic inhibitors (60). According to the literature, the combination of taxanes with standard chemotherapy has been found to increase survival rates and decrease the cancer recurrence. However, side effects may arise with the use of this drug such as neutropenia and neuropathy (61).

1.2.4. The Breast Cancer Immune System

Recently, numerous researches have been done to investigate the influence of the immune system on the onset and advancement of cancer (62). The immune status is utilized as an indicator to assess the possibility of developing primary, recurrent or secondary breast cancer, and relapsed breast cancer (63). The immune system is composed of the part of innate immunity, which comprises NK, and the adaptive immuninty, consisting of T cells, T reg cells, B cells, beside their cytokine secretion, and both are crucial in the 1^{ry} and 2^{ry} prevention of breast cancer (63).

Firstly, the innate immune system in the earlier stages can recognize cancerous cells and eliminate them by mechanism known as immune-surveillance mechanism (62). The

neighbouring cells to the cancer cells are induced to call the NK cells (innate lymphoid cells), the primary drivers of immune-surveillance, through tissue damage signals, such as IFN-γ (64).

T lymphocytes are responsible for producing cytokines crucial to the immune system response. Individuals with breast cancer exhibit reduced numbers of CD4+ and CD8+ T lymphocytes producing type I cytokines (e.g. interleukin-2, IFN-γ, or TNF-α) and type II cytokines (e.g. interleukin-4) in comparison to healthy individuals. This suggests that breast cancer patients, even those in the early stages, experience immune system dysfunction (63).

T reg cells are a distinct type of T-cells that can suppress both humoral and cell-mediated immune responses. In cancer, T reg cells can become active and prevent the immune system from effectively responding to tumor antigens (63).

The relationship between the cancer cells and defence system goes through 3 stages: Eradication, balance, and evasion. In the 1st stage, the defense system has the ability to eradicate all cancer cells. In the 2nd stage, the defense system can only impede the multiplication of cancer cells. In the final stage, cancer cells can resist the immune system's control and evade immune-mediated elimination (65), This phase is named the ‘escape’ phase. Cancer cells can achieve this by reducing surface MHC 1 and costimulatory molecules, as well as expressing immunosuppressive molecules (66). Several cell types are involved in eliminating tumors, including mature DCs, activated macrophages (M1), NK cells, CD8+ T cells, CD4 Th1 cells, and B cells. Conversely, there are also cells that have a pro-tumor effect, such as MDSCs, CD4+ Tregs cells, regulatory B cells, CD4+ Th2 cells, and activated macrophages (M2) (67, 68).

1.3. T CELLS

T cells are a crucial part of the defense system that plays an important part in immune function maintenance, responding to pathogens, and forming memory. These cells can recognize and respond to various pathogens through their T cell receptors located on their surface. T cells develop from bone marrow precursors and mature in the thymus before relocating to different parts of the body. There are 3 primary subsets of peripheral T cells: naive, memory, and regulatory T cells, each with distinct functions. Naive T cells react to

new antigens, memory T cells provide sustained immunity, and regulatory T cells prevent excessive immune reactions. When encountering antigens presented by DCs, naive T cells produce interleukin 2, which triggers cell proliferation, differentiation, and the release of cytokines and cytotoxic mediators to clear the pathogen (69).

Immune cells, including (NK) cells, macrophages, B cells, and T cells, encircle cancer cells. These immune cells that infiltrate the tumor are known as tumor-infiltrating lymphocytes (TIL) and are divided into helper (CD4+) and cytotoxic (CD8+) types, expressing surface activation markers such as CD25 and the transferrin receptor (70).

CD8 T cells can fight against tumors by targeting specific pathogenic antigens. The defense system's anti-carcinogenic effects are attributed to this response. TILs are capable of directly destroying cancer cells or indirectly through the discharge of cytokines such as INF-g, GM-CSF, and TNF-α (71).

The function of TIL can be inhibited by various factors, including an increase in regulatory T lymphocyte activity, the presence of inhibitory cytokines, and alterations to the MHC molecules on tumor cells (72).

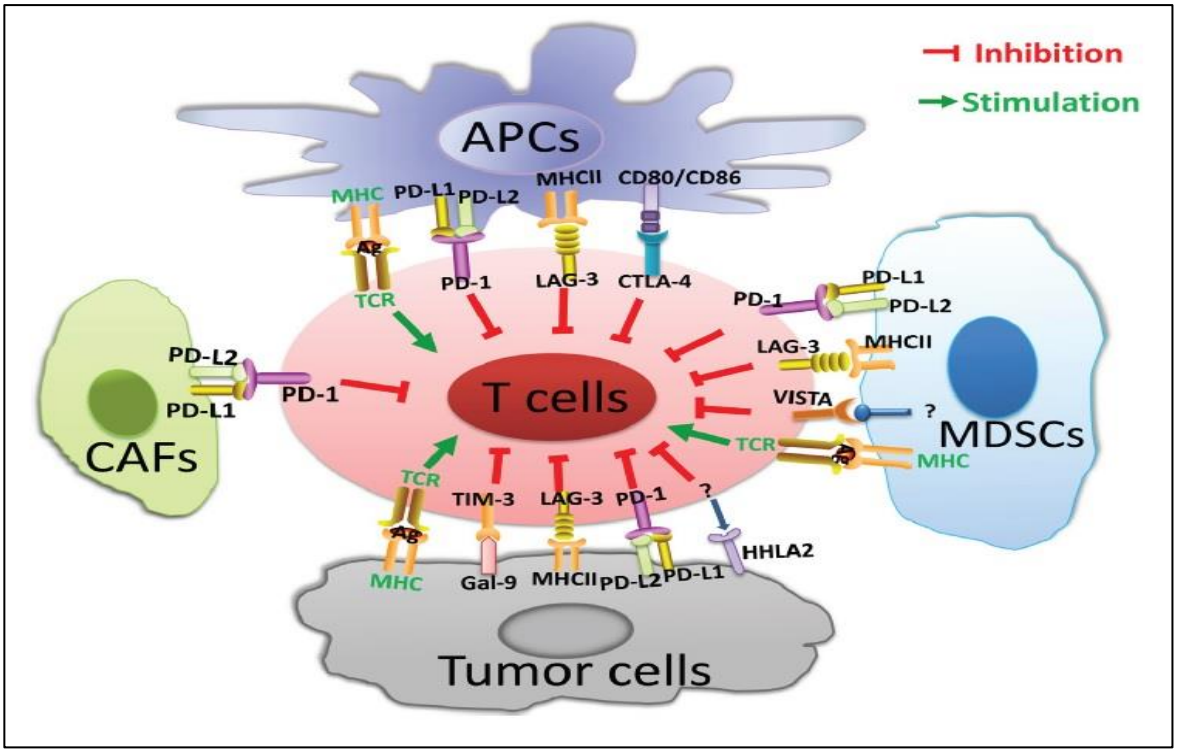

Figure 1.3. Microenvironment of Tumor and immune checkpoints pathways (73).

The defence system can identify the antigens demonstrated by breast cancer cells, but they are not very effective in triggering an immune reaction. Tumors can avoid immune detection through different mechanisms inducing immune suppression. Breast cancer cells can suppress the immune response by using T cell co-inhibition, where CTLA-4/CD152 plays a key role. CTLA-4/CD152 is overexpressed in activated T cells, and it works to block T cell activation by sending direct inhibitory signals and by blocking CD28 binding (74). In addition, CD274 and PD-L2 (CD273) which bind CD279 (75), which play as both co-stimulatory and co-inhibitory (76).

1.4. PD-1/PD-L1

Inhibitory receptors of the defence system, such as CTLA4 and CD279, initiate immunosuppressive signaling pathways when present on immune cells. CD279, a part of the CD28/CTLA4 family, operates as a receptor for CD274 and CD273, which are members of the B7 family (77). The major impact of the CD279/CD274 signaling path is to prevent TCR and co-stimulatory signals. CD274 can be found in several hematopoietic cells, such as DCs, T cells, macrophages, B cells, and neutrophils, as well as some non-hematopoietic tissues, like the cardiac tissues, pancreas, vascular endothelial cells, muscle, liver, lung, eye, and epithelial cells (78). In cancer, the expression of CD274 has significant role in evading the defence system.

The identification of CD274 and/or CD273 expression in cancer cells may have significant implications for cancer treatment and prognosis. Researches have revealed a link between the severity of cancer and the presence of CD279 on TILs (79) and the poor patient outcome. When CD274, present on cancer cells, interacts with the CD279 receptors on cytotoxic CD8+ T cells, it triggers apoptosis in these cells (80).

While normal breast tissue has low levels of CD274 expression, breast cancer tissue samples and cell lines have shown abundant expression of this protein. A study was conducted on 44 breast cancer individuals to evaluate the levels of CD274 expression and compare them with clinic-pathological parameters (79). In breast cancer, the high level of CD274 expression on cancer cells is related Grade III tumor of high size and tumors that do not express estrogen receptors (ER) or progesterone receptors (PR).

The discovery of the CD279 and CD274 axis has been a meaningful innovation in cancer treatment (81).

Studies on the impact of chemotherapeutic agents on the expression of CD274 have revealed that doxorubicin decreases CD274 expression on the cell surface but increases it in the nucleus. However, inhibiting the PI3/AKT pathway can prevent this up-regulation (82). In contrast, etoposide, fluorouracil, and paclitaxel have been found to up-regulate CD274 expression (83). These results recommend that further investigation is needed to comprehend the effects of chemotherapy on these immune checkpoints before and after therapeutics (83).

MDA-MB231 cells reduce the leveln of CD279 in CD4+ CD25+ T cells (84), whereas ER+ breast cancer cells such as the MCF-7 cell line increase the expression of CD279 in CD4+/CD25- T cells but have no effect on CD4+/CD25+ lymphocytes (84, 85). Yang Zheng et al. also found that Co-culturing MCF-7 and MDA-MB231 cells led to high CD274 expression and significant inhibition of T cell activation and cytokine release (86). Chemotherapeutic agents such as doxorubicin, etoposide, fluorouracil, and paclitaxel can affect CD274 expression in breast cancer cells. (82). Similarly, etoposide, fluorouracil, and paclitaxel can induce CD274 surface expression in human breast cancer cells (87). Current researches have emphasized the use of anti-CD279/CD274 drugs in breast cancer treatment, especially for the triple-negative subtype. The outcomes have shown encouraging results when used independently or alongside conventional therapies (88). Pembrolizumab, a CD279 inhibitor, has shown sustained anti-cancer effects and a manageable toxicity profile in a group of individuals with metastatic triple-negative breast cancer who had previously undergone treatment, indicating its potential as a monotherapy (89).

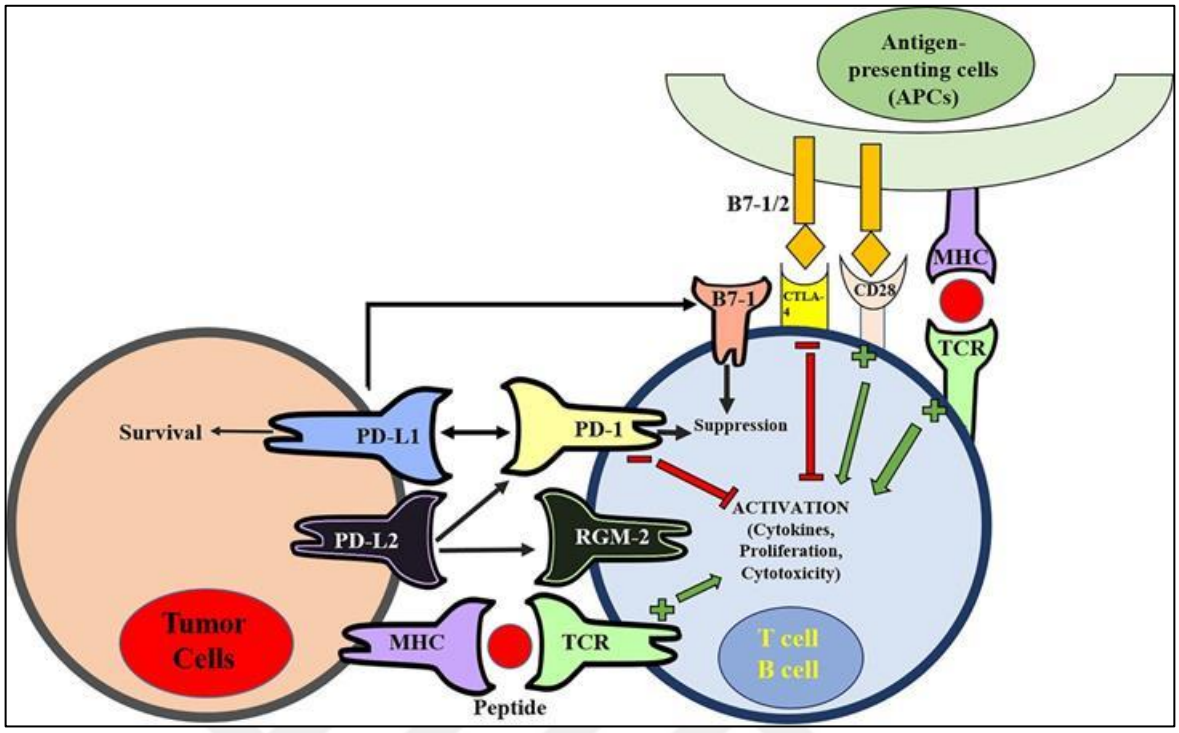

Figure 1.4. A snapshot of the PD-1/PD-L1 pathway PD-1/PD-L1 Pathway (90).

1.5. HIPPO PATHWAY

Healthy cells undergo contact inhibition, a process in which cell proliferation stops upon contact with adjacent cells. On the other hand, cancer cells do not exhibit contact inhibition and continue to proliferate even when in contact with neighboring cells, resulting in the formation of multi-layered foci (91). One of the distinguishing characteristics of cancer is the lack of contact inhibition (5).

The Hippo pathway is an important mechanism in controlling contact inhibition, and it is conserved across a variety of species from insects like Drosophila to mammals (92).

YAP, a family of transcriptional co-activators, is a powerful oncoprotein that plays a significant role in controlling cell duplication in different types of stem/progenitor cells and cancers (93). YAP family co-activators have been identified as significant factors in promoting cell proliferation and tissue overgrowth (94). YAP protein functions by interacting with to the DNA-binding transcription factor Scalloped (Sd), which is also known as TEAD in humans. This interaction leads to the regulation of transcription for downstream genes that initiate cell amplification and prevent death (95). The Hippo signaling pathway

in human cells involves LATS1/2 kinase phosphorylating the YAP protein, which prevents its movement into the nucleus. Phosph-YAP is then bound to 14-3-3 family proteins and remains in the cytoplasm (96).

The Hippo pathway in mammals involves the activation of Mst1/2, which activates Sav1 and Mob1. This then leads to the stimulation of Lats1/2 kinases through phosphorylation. Once activated, Lats1/2 kinases can phosphorylate YAP/TAZ, inhibiting their translocation to the nucleus and retaining them in the cytoplasm. β-TrCP then degrades the phosphorylated YAP/TAZ, leading to apoptosis (97). Contrarily, if the Hippo pathway is not functioning, YAP/TAZ molecules lose their phosphate groups and move into the nucleus, where they interact with transcription factors like TEAD and SMAD (95). Other upstream regulators of the Hippo pathway include WWC1 (similar to mammalian Kibra), Merlin (similar to mammalian NF2), and mammalian FRMD6 (92).

New research indicates that TAZ and YAP have a role in controlling the level of CD274 in cancer cells. TAZ can attach itself to the CD274 promoter through the use of the TEAD family of transcription factors, leading to a boost in the production of CD274. These results emphasize the significance of the Hippo pathway in the immune evasion strategies employed by cancer cells (98).

Recent studies have found a robustly +ve association YAP and CD274 level in human cancer cells, suggesting that as YAP levels increase, CD274 levels also increase. Examples include BRAFi-resistant melanoma cells and NSCLC, where YAP is shown to up-regulate CD274 and facilitate immune system evasion (99, 100)). Additionally, TAZ-S89A and YAP-S127A have been found to induce CD274 protein expression in MCF10A cells. These outcomes indicate that this route plays a significant function in directing cancer immune evasion (98).

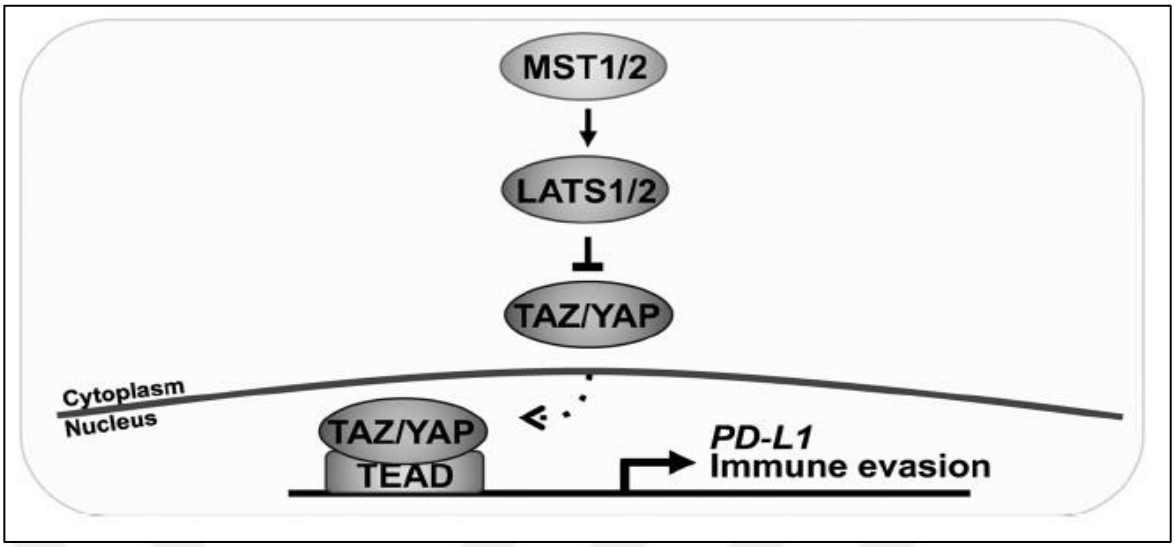

Figure 1.5. Scheme shows the relation between the Hippo pathway and CD274 expression (98).

1.6. BORON AND BORON DERIVATIVES

Boron (B) is a chemical element called the metalloids found in different compounds with diverse biological functions such as some antibiotics, including tartrolon, borophycin, boromycin, and aplasmomycin. Boron-containing molecules have been explored as potential therapeutic agents, such as the antimalarial and antibacterial agent diazaborine, antibacterial oxazaborolidines, and the antifungal agent benzoxaborole AN2690 (101). Additionally, the boronic acid-containing anti-carcinogenic drug Bortezomib (sold under the brand name Velcade®-PS-34) is also in use (102). Bortezomib is an anti-carcinogenic drug that contains boronic acid. It induces apoptosis and disrupts cell cycle regulation by blocking nuclear factor-kB (NF-kB), deregulating intracellular calcium metabolism, and activating caspase and apoptosis pathways (103). Studies show that Bortezomib is cytotoxic to different cancer cell lines, including MCF-7 and EMT-6 (104), myeloma (105), lymphoma (106), ovarian (107), pancreatic (108)and head and neck cancers (109).

Most Boron compounds used as cancer therapy are under the category of Neutron Capture Therapy (BNCT). BNCT involves the use of boron derivatives to kill cancer cells. In this approach, boron derivatives are taken up by cancer cells and then irradiated with a thermal neutron beam. The heated boron carriers then destroy the cancer cells. BNCT has been used to cure high-grade gliomas, melanomas, hepatic metastasis, and head and neck tumors (103).

Numerous boron-containing molecules have derivatives, including SPP and SPT. A study demonstrated that SPP can enhance the movement ability and SOD (superoxide dismutase) activity of fibroblasts derived from humans. These findings indicate that SPP could be beneficial for the healing of wounds (110).

The impact of boron on the immune response has been investigated, and it was found that a dose of 2 mg/kg/day decreased inflammation in rats with arthritis, indicating a regulatory role for boron in treating inflammation. Recent research has also suggested that boric acid can enhance the secretion of chemical mediators required for host defense against infection. In In LPS-stimulated mice, boric acid promoted the growth of CD4+ T cells and CD19+ B cells (111).

Immunoliposomes have been suggested as potential boron carriers for BNCT in treating locoregional recurrences of HER2+ breast cancer subtypes (112). New research suggests that boric acid can enhance the release of chemical mediators that have a significant function in the body's resistance against infections. In mice treated with LPS, boric acid has been shown to increase the growth of CD4+ T cells and CD19+ B cells (111). There are multiple types of boron-containing molecules, including SPP and SPT. Our previous research found that both SPP and SPT exhibit antiproliferative effects against small lung cancer cells and hepatocellular carcinoma (113, 114).

The target of this project was to examine the potential anti-cancer properties of two boron derivatives, SPP and SPT, on breast cancer cells. The objective of the study is to assess the impact of these boron derivatives on CD279/CD274 expression and investigate their effects on activated T cells. This in vitro study may provide a basis for future in vivo studies to further examine the anti-cancer impacts of these boron products on breast cancer.

2. MATERIALS AND METHODS

2.1. CELL CULTURE

The MCF-7 (ER-positive) and MDA-MB 231 (triple-negative) human breast cancer cell lines were obtained from ATCC, USA, and cultured in DMEM medium (Invitrogen®) with 10% FBS and 1% PSA (Life Technologies™, USA). The healthy MCF 10A epithelial cell line was grown in MEBM™ (LONZA) with the MEGM™ SingleQuots™ Kit (Cat No: CC-4136). The cells were incubated at 37°C with 5% CO2 and 80% relative humidity.

2.2. CELL VIABILITY ASSAY (MTS ASSAY)

The study measured the viability of three types of cells (MCF-7, MDA-MB-231, and MCF 10A) by adding various doses of two chemicals, SPP (from Ulkar Chemistry) (500, 1000, 2000, 3000, 4000, and 5000 µg/ml) and SPT (from Merk®) (500, 400 µg/ml, 300, 200, 100, 50), and a combination of the two (500/50 µg/ml SPP/SPT) for 72 h to the cells. The cells were incubated for 24, 48, and 72 hours, and then the cells' viability percentage was measured using a colorimetric assay called the MTS reduction assay. This assay uses a Cell titer 96® Aqueous MTS reagent (Promega®) to measure the viability by detecting the amount of formazan product that is produced when MTS is bioreduced by the cells. The target of the study was to get the optimal dose for each chemical that maximizes the killing of cancer cells while minimizing damage to healthy cells. (115). The viability percentage MCF-7, MDA-MB-231, and MCF 10A cells was determined by seeding approximately 5 x 10^3 cells in a ninety six-well plate after incubation with different doses. The formazan absorbance was detected at 490 nm (116) via a microplate reader (Biotek Instruments ELx800 Absorbance Micro-plate Reader). GraphPad software was used to determine the IC50 of SPP and SPT molecules and the dose that caused fifty percent cell death.

2.3. ANNEXIN V AND CELL DEATH ASSAY

The MCF-7 and MDA-MB231 cells were incubated with the IC50 (the dose that prevents 50% of cell development) of SPP, SPT, and a combination of the two for 24, 48, and 72

hours. Then the apoptotic cells measured using the Muse® benchtop flow cytometer and the Annexin V and Dead Cell Assay Kit (Cat no; MCH100105, Merck). This kit allows for the measurement of apoptotic cells by labeling them with Annexin V, a protein that binds to PS on the surface of apoptotic cells. The kit also includes a dye that stains dead cells.

Apoptosis is a crucial process that regulates cell growth and division. It is triggered by specific signals that cause various physiological effects, such as moving a membrane component called phosphatidylserine from inside the cell to the surface, breaking down certain proteins, and compromising the integrity of the membrane in later stages (117, 118) (119, 120) (121).

Annexin V is a protein that can attach to a molecule called phosphatidylserine (PS) in a way that depends on the presence of calcium (122, 123). PS is typically found on the inside of the membrane of the cell, but throughout early stages of apoptosis, it is relocated to the cell membrane outer surface. Annexin V can interact with the PS molecules on the outer surface of the membrane and detect cells passing through apoptosis (124, 125) (126, 127) (128, 129).

The Assay uses Annexin to detect PS on the outer membranes of cells undergoing apoptosis. The assay also includes another marker called 7-AAD, which indicates the integrity of the cell membrane. The described assay has the capability of detecting and distinguishing four types of cells based on their properties. These include cells that are not undergoing apoptosis, which can be identified as -ve for both 7-AAD and Annexin V. Cells in early stage of apoptosis are +ve for Annexin but –ve for 7-AAD. Cells in Late stage of apoptosis show +ve signals for both 7-AAD and Annexin, and nuclear debris which is mostly -ve for Annexin but +ve for 7-AAD. This allows for accurate and specific detection of cells undergoing apoptosis and helps to distinguish them from other types of cells in the sample (130). Based on the protocol, the cells were rinsed with PBS and the supernatant was removed by Centrifugal sedimentation to leave 100 µl of PBS. Then, 100 µl Reagent was added to each sample to resuspend the cells. After that, the mix was incubated for twenty minutes in the darkness, and Muse® benchtop flow cytometry was carried out.

2.4. CELL CYCLE ANALYSIS

Cancer cells were incubated with the IC50 of SPP and SPT molecules and their combination for 72 hours. The Muse™ Cell Cycle Kit along with propidium iodide (PI) and RNase A was used in cell cycle analysis using the Muse® benchtop flow cytometer. The kit allows for the identification and quantification of cells in the G0/G1, S, and G2/M phases of the cell cycle. The test distinguishes phases according to the intensity of PI staining of DNA content, which increases as cells begin to cycle and synthesize chromosomal DNA. The G2/M cells fluoresce twice as strongly as the G0/G1 population, and eventually split into two cells. The assay protocol involves washing the cells with PBS and collecting them by discarding the supernatant. The cells are then fixed chilled 70% ethanol, rinsed with PBS, and remıxed with 200 µl Reagent. The mix is incubated at room temperature for thirty minutes, in darkness and analyzed using the Muse™ Cell Analyzer.

2.5. RT-QPCR

In this experiment, cancer cells were incubated with different molecules for 72 h and the RNA was isolated using a NucleoSpin® RNA kit. The RNA samples were then analyzed for purity and quantity using a NanoDrop-ND-100. One microgram of RNA was used to synthesize cDNA with a QuantiTect Reverse Transcription Kit. The cDNA samples were mixed with primers and iTaq™ Universal SYBR® Green Supermix for quantitative assessment. The results were normalized to GAPDH using the $2^{-\Delta\Delta CT}$ method and analyzed using the CFX96® PCR system and CFX Manager Software. The experiments were conducted following the instructions of manufacturer. This assay was carried out to evaluate the level of the downstream oncogenes of the Hippo pathway, which have a significant function in cell growth (CYR61, CTGF, GADD45, TAZ, and BIRC5) and the apoptotic mediators (CASP3, CASP9, BAK1, BAX, BCL2, and TP53) using the primers of the sequences shown in **Table 2.1**.

Table 2.1. The protocol of the qRT-PCR Reaction

Component	**10µl reaction**
SYBR™ Green PCR Master Mix	5µl
Forward primer	1µl
Reverse primer	1µl
Template DNA	2µl
Nuclease-Free Water	1µl

Table 2.2. The recommended thermal cycling conditions

Step	**Temperature °C**	**Time**	**Number of cycles**
Initial denaturation	95°C	2 min	1
Denaturation	95°C	15 sec	40 cycles
Annealing/Extension	60°C	1 min	

Table 2.3. The forward and reverse sequences of the primers

Primer	**Forward sequence**	**Reverse sequence**
Human GAPDH	CAAGAGCACAAGAGGAAGAGAG	CTACATGGCAACTGTGAGGAG
Human Tp53	AGGGATGTTTGGGAGATGTAAG	CCTGGTTAGTACGGTGAAGTG
Human Bax	TTGCTTCAGGGTTTCATCCA	ACACTCGCTCAGCTTCTTG
Human BAK1	GCCCATTCCCACCATTCT	CAGGTCCTAGTCCCATCTCTTA
Human CASP3	GCTGCCTGTAACTTGAGAGTAG	GTATGGAGAAATGGGCTGTAGG
Human CASP9	CACAGGGTCTGCTCTTTCTC	CATTCATCTGTCCCTCTTCCTC
Human Bcl-2	CTGGTGGACAACATCGCCCT	TCTTCAGAGACAGCCAGGAGAAAT
Human TAZ	GGCTGGGAGATGACCTTCAC	CTGAGTGGGGTGGTTCTGCT
Human CTGF	AGGAGTGGGTGTGTGACGA	CCAGGCAGTTGGCTCTAATC
Human CYR61	CCTTGTGGACAGCCAGTGTA	ACTTGGGCCGGTATTTCTTC
Human BIRC5	CCGGTTGCGCTTTCCTTTC	CGCACTTTCTCCGCAGTTTC

Human GADD45	GATGCCCTGGAGGAAGTGCT	GAGCCACATCTCTGTCGTCGT

2.6. PROTEIN ISOLATION AND WESTERN BLOT EXAMINATION

Proteins were isolated from each sample, incubated in a normal complete medium as a negative control, and the other incubated samples with the IC_{50} of SPP and SPT molecules and the combination for 72 h using radioimmunoprecipitation assay (RIPA) Lysis and Extraction Buffer (BioBasic®) /Halt™ PPI Cocktail (100X) (Thermo Fisher Scientific®). The RIPA cocktail was vortexed with the cell pellets and mix incubated on ice frequently for 20 min. The mixture was sedimented for twenty minutes at 14,000 xg at 4°C. Following sedimentation, the upper liquid containing protein was recovered and the pellet was thrown away. Protein samples were preserved at -80°C for later use. The protein was quantified and normalized via the Pierce™ BCA Protein Assay Kit (#23225) before proceeding to western blot analysis. Proteins were heated to 95°C for five minutes with 6x Laemmli SDS sample buffer, (#J61337, Alfa Aesar, Waltham, MA, USA). Equal quantities of protein samples were applied onto NuPAGE™ 4-12% Bis-Tris Protein Gels (Cat No: NP0329BOX) separated by electrophoresis using Mini Gel Tank (Cat No: A25977). The gel was first run at 110 V for about 15 minutes until the proteins migrated through separating gel, and subsequently at approximately 145 V for one hour, and finally transferred to PVDF membranes (#1620177, Bio-Rad, CA) by wet transfer technique, which then were blocked for one hour with 1X Tris-Buffered Saline with 0.01 percent Tween (TBS-T) with 5 percent Blotting-Grade Blocker (#1706404, Bio-Rad, CA). The membranes were exposed to 1^{ry} antibodies at +4 °C overnight before washing with TBST and exposed to the HRP-labeled 2^{ry} antibody at 37 °C for 1 hr. After rinsing with TBST, the bands were illuminated using Pierce™ ECL Western Blotting Substrate (Cat No: 32209) in the ChemiDoc XRS+ System and Image Lab software for analysis (Bio-Rad), and their intensities were calculated using ImageJ software. The primary antibodies and dilutions were used as the following: GAPDH (1:1000; #5174S; CST®,), Caspase-3 (D3R6Y) Rabbit mAb (1:1000; #14220S; CST®), Purified anti-p53 Antibody (1:500; #628202;Biolegend), Bax (E4U1V) Rabbit mAb (1:1000; #41162; CST®), Bak (D4E4) Rabbit mAb (1:1000; #12105; CST®), KIBRA (1:250; #ab107637; Abcam), SAV1 (1:1000; #ab105105; Abcam), MOB1 (1:1000; #13730; CST®), MOB1-p (1:1000; #8699S; CST®), Phospho-YAP (1:1000; #4911S; CST®), Anti-

mouse IgG, HRP-linked Antibody (1:3000; #7076P2; CST®), Anti-rabbit IgG, HRP-linked Antibody (1:3000; #7074P2; CST®) .

2.7. FLOW CYTOMETRY DETECTION OF PD-L1 IN MCF-7, MDA-MB-231

To get a single-cell suspension, cells treated with SPP, SPT, and their combination were rinsed with PBS buffer and sedimented. The pellets were then incubated with 100 µL PD-L1 1^{ry} antibody (1:500, CST®, #13684) in staining buffer (0.5% BSA PBS buffer) and incubated for one hour at room temperature. After washing with PBS, the pellets were mixed with a fluorochrome-conjugated secondary antibody (Alexa Fluor® 647 conjugate #4414) for thirty minutes away from light. The samples were rinsed again with PBS, centrifuged, remixed with 200 µL of PBS, and acquired using a Flow Cytometer (CytoFLEX S Flow Cytometry).

2.8. PREPARATION AND ACTIVATION OF MONOCYTE-DERIVED DENDRITIC CELLS

We used in the study commercially purchased PBMCs from STEMCELL™ TECHNOLOGIES (Cat No: 70025), obtained from healthy donors that were collected from peripheral blood leukapheresis samples and cryopreserved. The PBMCs were incubated in RPMI 1640 complete medium in a six-well tissue culture plate at 1×10^6 cells/mL for four hours at 37 °C in a humidified 5% CO_2 incubator (131). The adherent monocyte fraction was cultured with 50 ng/mL GM-CSF, 100 ng/ml LPS, 20 ng/ml IFN-γ, and 20 ng/ml interleukin-4 for three days to differentiate into immature DCs. These DCs were then cultured for more 48 hours with 20 µg RNA and250 µg total lysate isolated from the targeted cancer cells.

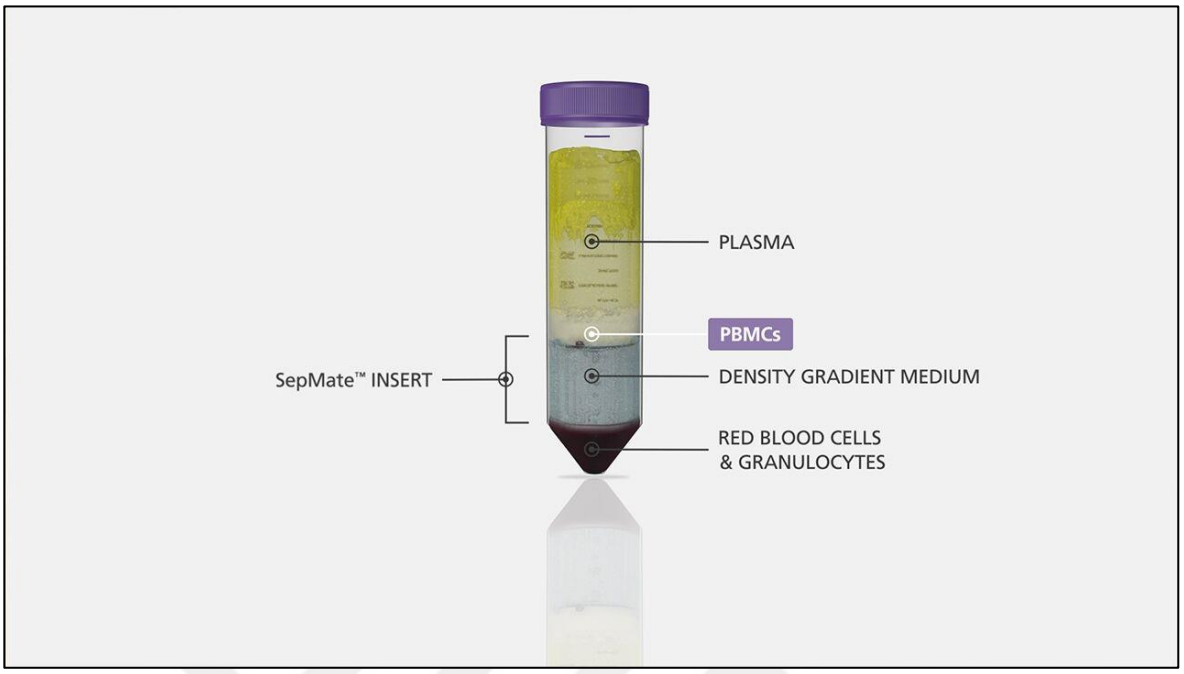

Figure 2.1. SepMate™-50 (IVD), Tube for density gradient centrifugation for in vitro diagnostic (IVD) applications.

2.9. ISOLATION AND ACTIVATION OF PRIMARY T CELLS

To isolate primary human T cells, the EasySep Human T Cell Isolation Kit (STEMCELL™ TECHNOLOGIES) was used for immunomagnetic negative selection of the non-adherent fraction of PBMC. The EasySep Human T Cell Isolation Kit is a technology used to isolate T cells from peripheral blood mononuclear cells (PBMCs) or other cell sources. The kit utilizes immunomagnetic negative selection to isolate untouched T cells without the need for a specialized laboratory or equipment. The principle behind the kit is based on the use of magnetic nanoparticles coated with antibodies specific to non-T cells, such as B cells, NK cells, monocytes, dendritic cells, and erythrocytes. These antibodies bind to non-T cells, and magnetic separation is then applied to remove these cells from the mixture, leaving untouched T cells behind.

The process involves a series of steps that include preparing the PBMCs or other cell source, adding the EasySep Human T Cell Enrichment Cocktail, and then adding the magnetic particles coated with antibodies specific to non-T cells. The mixture is incubated for a specified amount of time to allow the magnetic particles to bind to non-T cells. The sample

is then placed on a magnet, and the non-T cells are pulled towards the magnet, leaving untouched T cells in the supernatant. The isolated T cells can then be further processed or analyzed for downstream applications.

The EasySep Human T Cell Isolation Kit provides a fast, efficient, and reliable method for isolating T cells from a variety of sources. The kit is designed to isolate T cells with high purity, yield, and viability, while minimizing the depletion of T cells.

T cells were then activated and expanded with T Cell TransActTM (αCD3/CD28 beads, Miltenyi) in TexMACS™ medium containing 20 ng/mL human interleukin-2, 20 ng/ml human interleukin -7, and 20 ng/ml human interleukin-15. The T cells were characterized with CD3, CD8, and CD4 surface markers. The activated T cells were then incubated with matured DCs for two days to become active specifically against the target cancer cell line.

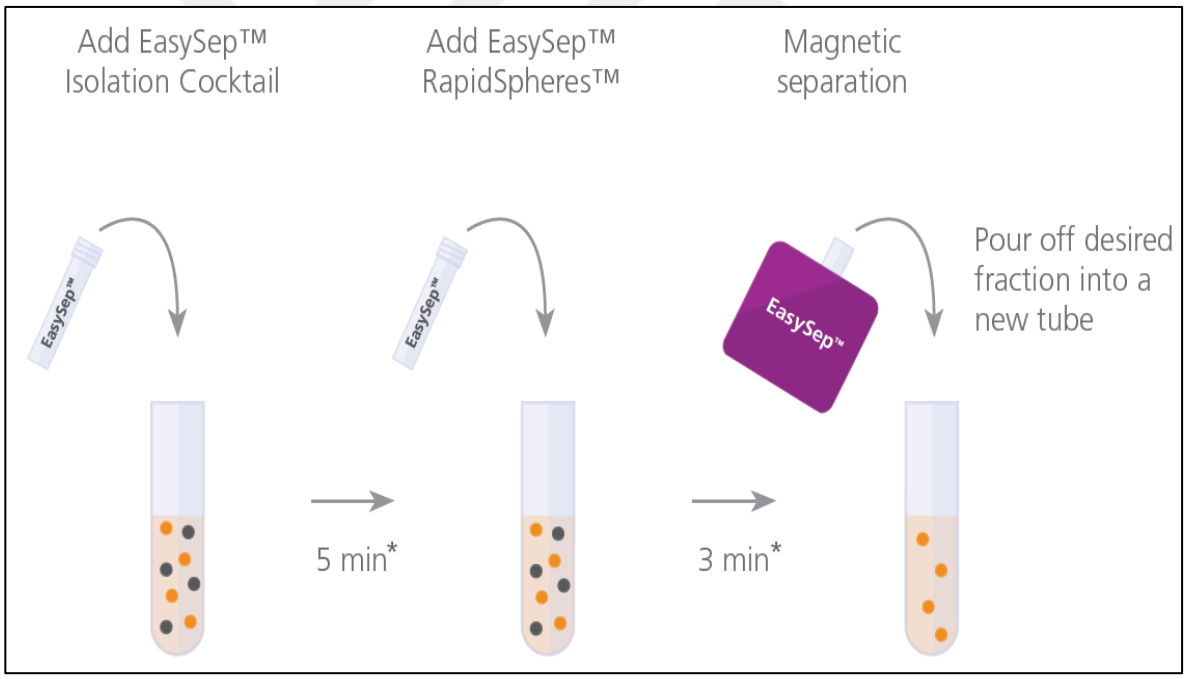

Figure 2.2. EasySep™ 8-Minute Human Cell Isolation Kits.

2.10. Co-Culture Assay

To inspect the impact of breast cancer cells and the combined SPP/SPT molecules on T cell activity, activated T cells were incubated with MCF-7 and MDA-MB231 breast cancer cell lines at a ratio of 30:1 (effector:target) for 72 hours. The experiment was done in the presence

or absence of 500/50 µg/ml SPP/SPT. The culture time and cancer cell to T cell ratio were optimized in preliminary experiments to ensure effective inhibition/killing.

2.11. Analysis of PD-1 Expression on T cells Using fFow cytometry

The study examined the level of CD279 on T cells co-cultured with breast cancer cell lines alone or with 500/50 µg/ml SPP/SPT. The T cells were isolated from the supernatant by centrifugation, washed in PBS, and incubated with PD-1 1^{ry} antibody (1:100, Cell Signaling Technology®, #86163) for 1 h, washed and then with a 2^{ry} antibody (Alexa Fluor® 647 Conjugate #4414) for thirty minutes. Finally, the samples were acquired using a Flow Cytometer (CytoFLEX S, Flow Cytometry).

2.12. LACTATE DEHYDROGENASE COLORIMETRIC ASSESSMENT OF CYTOTOXICITY

The lactate dehydrogenase (LDH) assay is a widely used cytotoxicity test to determine the activity of LDH, an enzyme that is discharged from damaged cells, in cell culture supernatants. The principle is based on the fact that when cells are damaged or undergo cell death, LDH is discharged from the cells into the surrounding culture medium. In the context of T cell cytotoxicity assays, the LDH assay is used to measure the amount of LDH released from target cells that have been lysed by effector T cells. The assay is typically performed by co-culturing the active T cells with the cancer cells, and then measuring the amount of LDH released into the culture supernatant after a certain period of time. The LDH assay relies on the fact that LDH initiates the pyruvate formation from lactate, accompanied by reduction of NAD+ to NADH. The LDH assay uses this enzymatic reaction to measure the amount of LDH in the culture supernatant.

The LDH assay typically involves the following steps:

- Incubation of effector T cells with cancer cells
- Collection of culture supernatant after a certain period of time
- Addition of a reaction mixture containing lactate and NAD+
- Incubation of the reaction mixture at room temperature or 37°C

- Measurement of the absorbance of the product at 490 nm

The amount discharged of LDH from the target cells is proportional to the quntity of cell lysis that has occurred, which in turn reflects the effector T cells cytotoxic activity.

In this study, T cells and cancer cells were co-cultured either alone or with SPP/SPT. After 72 hours of co-culture, the supernatants were collected for LDH activity analysis using a commercially available kit (Cayman Chemical, Cat no: 601170). Cells were seeded based on the E/T ratio, and hundred microliter of cell-free supernatant was mixed with hundred microliter of the reaction mixture and analyzed for Light absorption at 490 nm using a Varioskan LUX multimode microplate reader. The results were reported as LDH activity-associated absorbance (132).

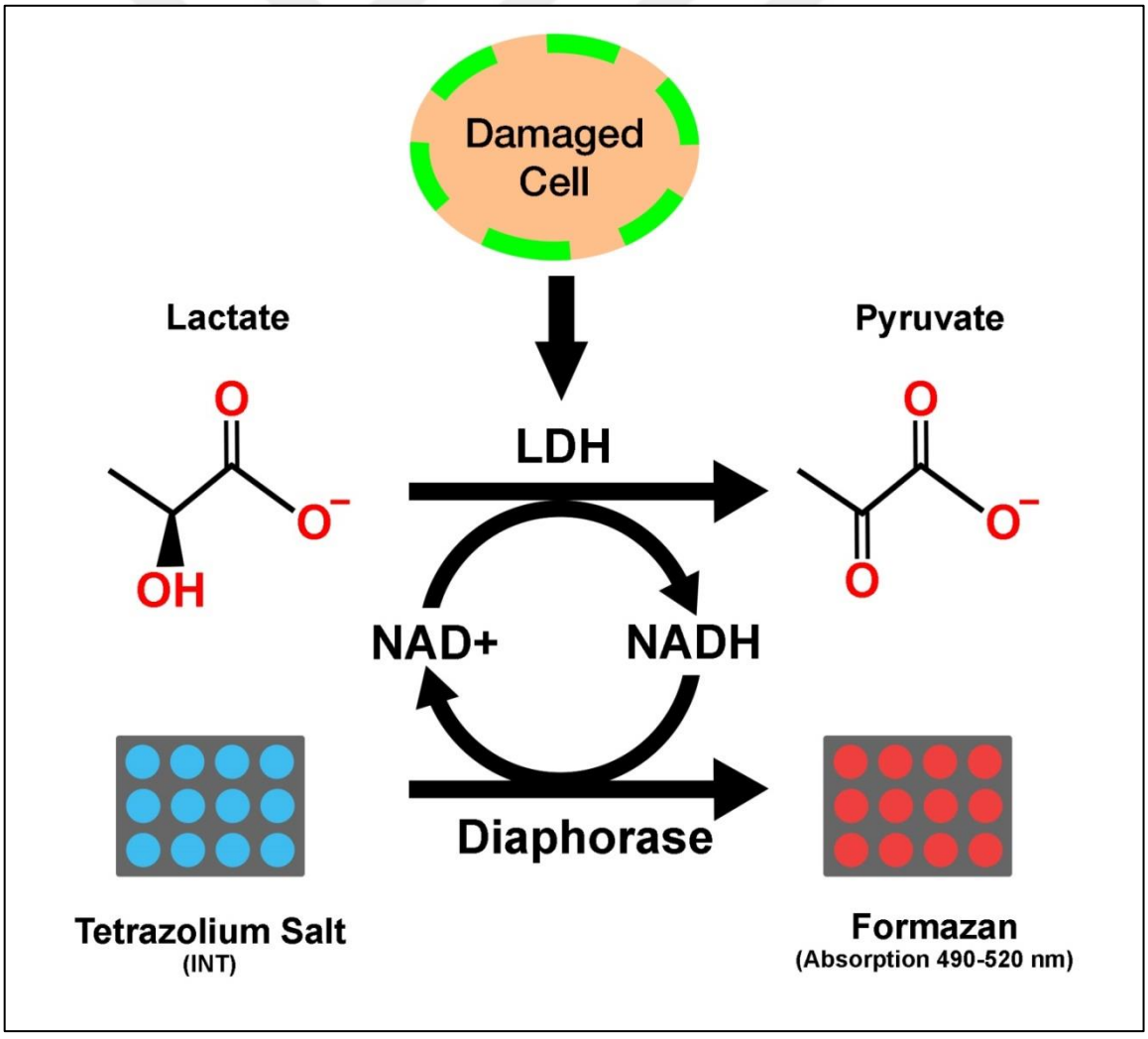

Figure 2.3. LDH Cytotoxicity Principle.

2.13. CYTOKINE ANALYSIS BY LEGENDPLEX

LEGENDplex is a type of multiplex bead-based assay that enables the detection of multiple cytokines, chemokines, and other soluble proteins in a single sample simultaneously. The assay uses fluorescently labeled beads, each coated with a capture antibody specific for a particular protein of interest.

During the assay, the beads are incubated with the sample containing the proteins to be analyzed. The proteins in the sample bind specifically to their corresponding capture antibodies on the beads. Then, a mixture of biotinylated detection antibodies, each specific for a different protein, is added to the beads. The detection antibodies bind specifically to the captured proteins, forming a sandwich complex.

Finally, streptavidin-phycoerythrin (SA-PE) is added to the sample. SA-PE binds specifically to the biotinylated detection antibodies, releasing a fluorescent signal that is corresponding to the amount of protein captured on each bead. The fluorescent signals from the different beads are then assessed via a flow cytometer, and the amounts of each protein in the sample are quantified based on the intensity of the fluorescence.

The LEGENDplex assay is a high-throughput, sensitive, and precise method for analyzing cytokine and protein expression in biological samples. It is particularly useful for studying complex biological systems, such as immune responses, where multiple cytokines and chemokines are involved.

The target of the experiment was to observe the impact of breast cancer cells and the combination of SPP/SPT on effector T cells' cytokine production ability. To achieve this, supernatants from T cells that were incubated alone or with cancer cells with or without 500/50 µg/ml SPP/SPT were gathered. A multiplex assay that can detect 13 cytokines was used to identify the cytokine concentrations in the collected samples, including interleukin-2, interleukin -10, interleukin -4, interleukin -6, interleukin -17A, TNF-α, granzyme A, granzyme B, IFN-γ, perforin, sFasL, sFas, and Granulysin. The LEGENDplex v8.0 software and a flow cytometer were utilized to analyze the samples based on the manufacturer's protocol.

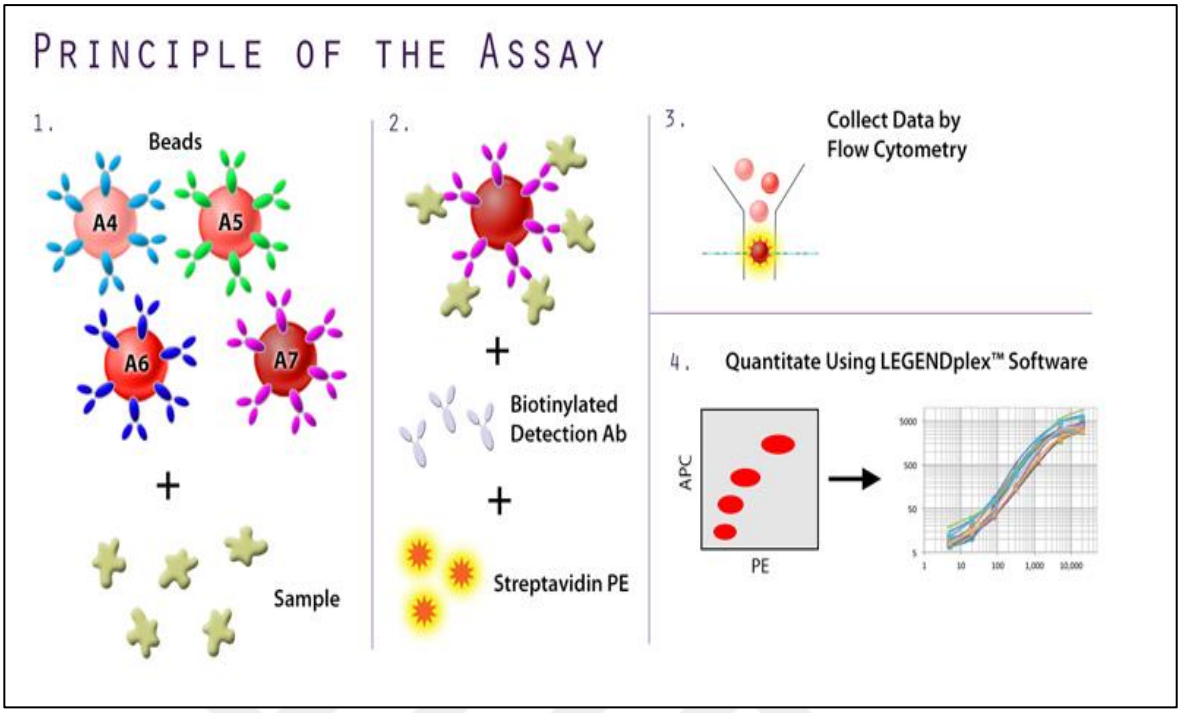

Figure 2.4. LEGENDplex cytokines analysis assay principle.

2.14. STATISTICAL ANALYSIS

In this research, statistical assessments were done via GraphPad Prism version 9.1.2 software. The data were assessed using unpaired Student's t-test, one-way analysis of variance (ANOVA), and two-way ANOVA. The results were reported in the format of the mean value along with its corresponding standard deviation (SD), and statistical significance was marked by asterisks with varying degrees of significance, such as *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. If the data was not significant, values near to the p-value of 0.05 were reported.

3. RESULTS

3.1. EFFECTS OF SPP, SPT, AND THEIR COMBINATION ON THE VIABILITY OF BREAST CANCER CELLS

Assessment of viability by MTS assay showed that SPP, SPT, and their combination could prevent the growth of breast cancer cells (MDA-MB-231and MCF-7) in time- and dose-dependent manners. After 72 h of SPP treatment, the effect on viability at different concentrations (500, 1000, 2000, 3000, 4000, and 5000 µg/ml) was approximately 65 ± 0.92%, 57.24 ± 6%, 30.42 ± 5%, 19.87 ± 3.5%, 14.14 ± 0.78%, and 9.99 ± 1.4%, respectively for the MCF-7 cells and approximately 69.34 ± 9.4%, 55.9 ± 11.15%, 44.07 ± 7%, 42.19 ± 2.5%, 30.87 ± 2.4%, and 19.27 ± 5%, respectively for the MDA-MB231 cells compared to the negative controls **(Figure 3.1. A, B) (Figure 3.2. A, B)**. After treating the cells with SPP for 72 h, the IC_{50} (with a 50% decrease in viable cells) was 1081 µg/ml for MCF-7 cells and 1320 µg/ml for MDA-MB231 cells **(Figure 3.2. D, E)**. In contrast, for the healthy MCF 10A cells **(Figure3.1. C) (Figure 3.2. C)**, the IC_{50} was 2249 µg/ml, which was approximately two-fold the IC50 for cancer cells **(Figure 3.2. F)**. For the SPT molecule, we discovered that after treatment with SPT for 72 h, the inhibitory effect on proliferation was approximately the same in cancer cells (MDA-MB-231and MCF-7) and healthy cells MCF 10A **(Figure 3.1. D-F) (Figure 3.2. G-I)**. The IC_{50} of SPT was as follows; 153.4 µg/ml for MDA-MB-231cells and 142.4 µg/ml for MCF-7 cells, which was toxic to the healthy cell line MCF 10A **(Figure 3.2. J-L)**. These results suggested that treatment with 50 µg/ml SPT showed a non-significant change in the viability of cancer cells and only a 25% decrease in viability upon treatment with 500 µg/ml SPP **(Figure 3.1. G, H)**. Meanwhile, the combination of SPP and SPT (500/50 µg/ml SPP/SPT) had low toxicity for the healthy cell line, which showed about 84% viability in healthy cells **(Figure 3.1. I)**. However, the viability of the cancer cell lines was 45-55% after treatment with this combination **(Figure 3.1. G, H)**. Therefore, we

performed subsequent experiments to confirm the antiproliferative effects of SPP, SPT, and their combination.

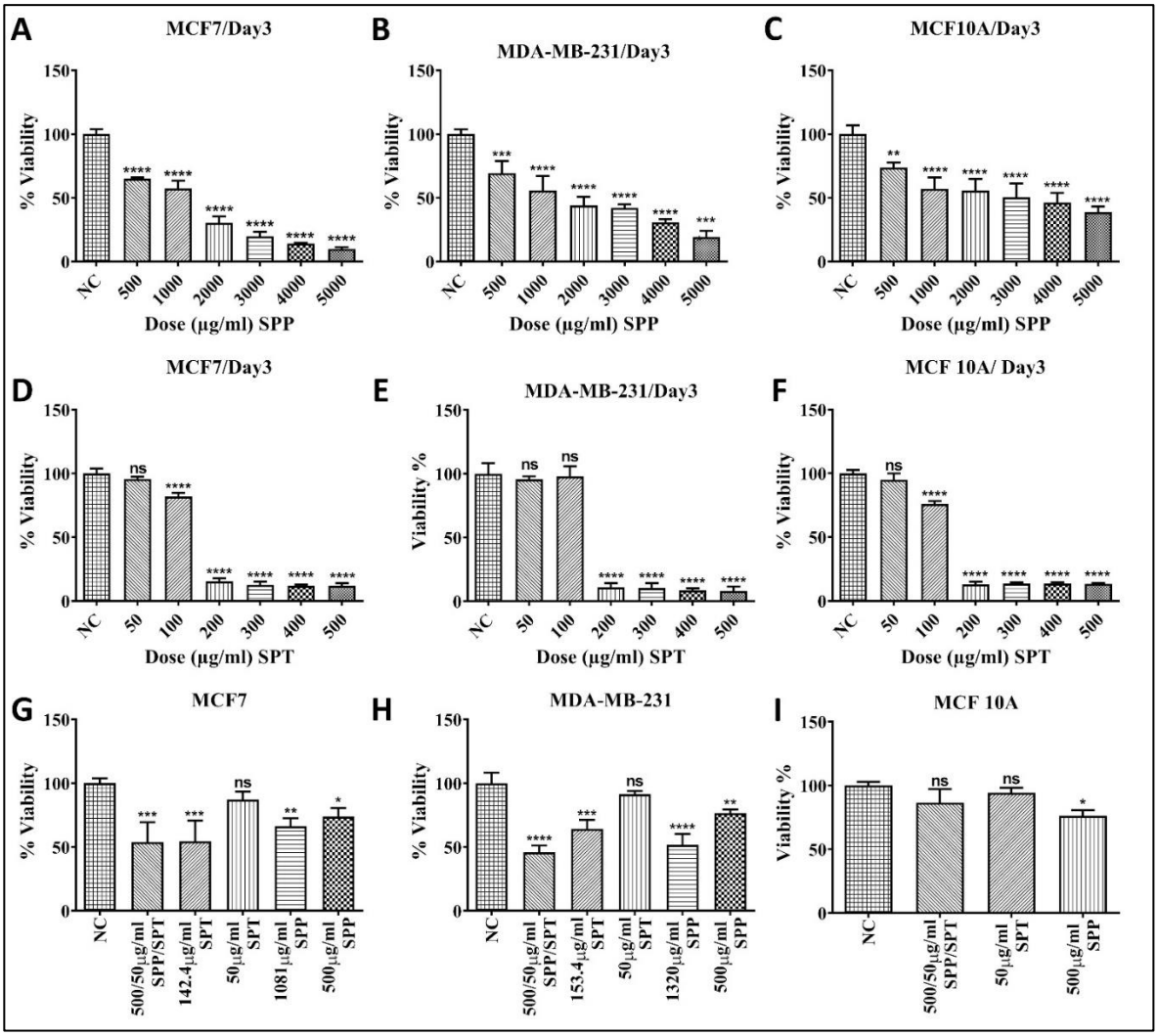

Figure 3.1. The effects of boron derivatives on breast cancer cells. **(A, B, C)** Percentage of viable MCF-7, MDA-MB-231, and MCF 10A cells after incubation with SPP for 72 h. **(D, E, F)** The percentage of viable MCF-7, MDA-MB-231, and MCF 10A cells after incubation with SPT for 72 h. **(G)** Percentage of viable MCF-7 cells incubated with 500 µg/ml SPP, IC50 of SPP, 50 µg/ml SPT, IC50 of SPT, and the combination (500/50 µg/ml SPP/SPT) for 72 h. **(H)** Percentage of viable MDA-MB231 cells treated with 500 µg/ml SPP, IC50 of SPP, 50 µg/ml SPT, IC50 of SPT, and the combination (500/50 µg/ml SPP/SPT) for 72 h. **(I)** Percentage of viable MCF 10A cells treated with 500 µg/ml SPP, 50 µg/ml SPT, and the combination (500/50 µg/ml SPP/SPT) for 72 h. Data are reported in the format of mean ± standard deviation (n=3) (*P < 0.05, **P < 0.01, ***P < 0.001,

****P < 0.0001, ns; non-significant > 0.05). Results obtained was evaluated in relation to the negative control using one-way ANOVA.

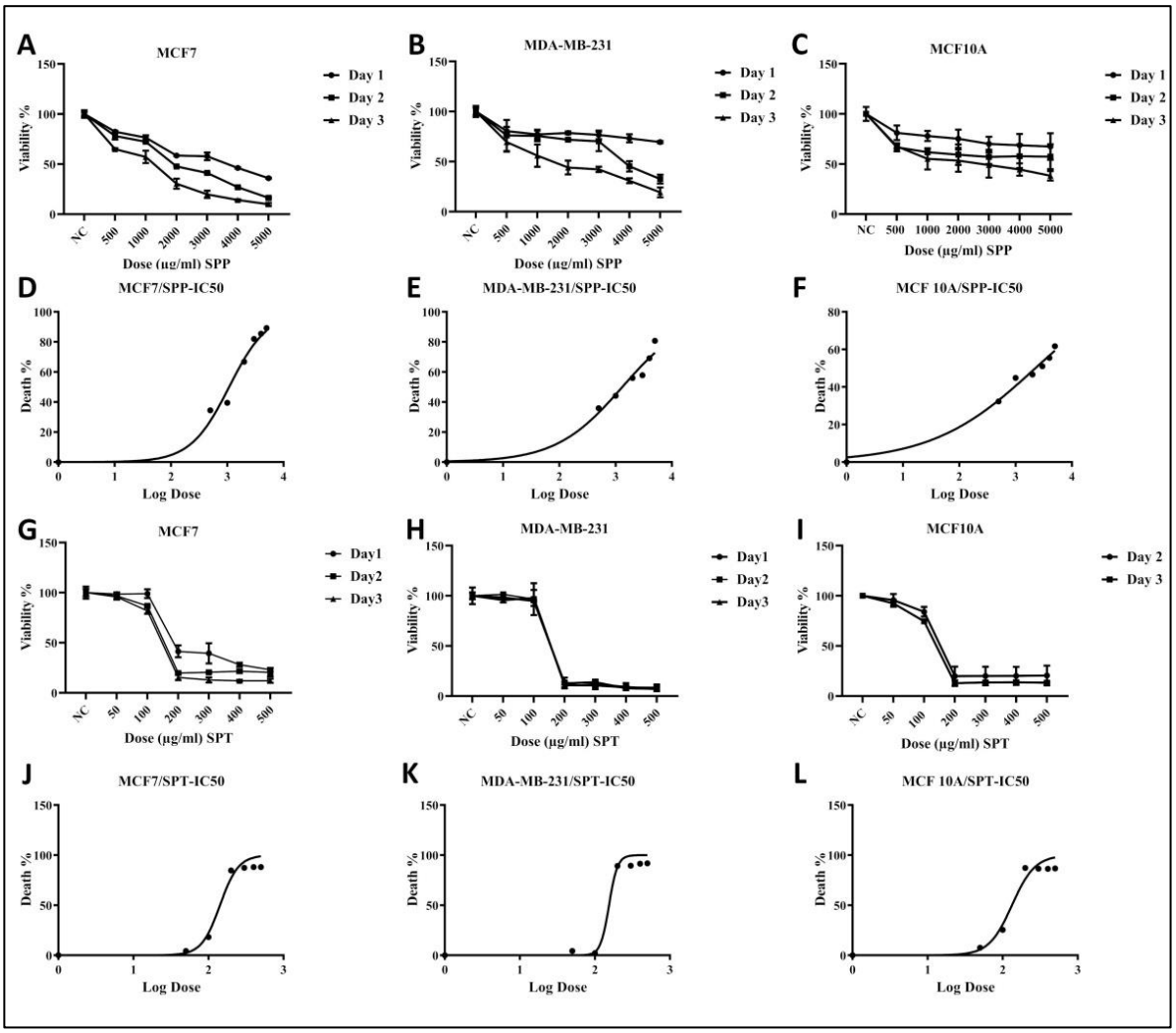

Figure 3.2. The growth inhibition effect of boron derivatives. **(A)** Viability percentage of MCF-7 cells treated with (SPP) for 24, 48 and 72 h, respectively; **(B)** Viability percentage of MDA-MB231 cells treated with (SPP) for 24, 48 and 72 h, respectively; **(C)** Viability percentage of MCF 10A cells treated with (SPP) for 24, 48 and 72 h, respectively; **(D)** Calculation of SPP IC_{50} on MCF-7 cells after 72 h treatment; **(E)** Calculation of SPP IC_{50} on MDA-MB231 cells after 72-h incubation; **(F)** Calculation of SPP IC_{50} on MCF 10A cells after 72 h treatment; (G) Percentage of viable MCF-7 cells incubated with (SPT) for 24, 48, and 72 h, respectively; **(H)** Percentage of viable MDA-MB231 cells incubated with (SPT) for 24, 48, and 72 h, respectively; **(I)** Percentage of viable MCF 10A cells treated with (SPT) for 48 and 72 h, respectively; **(J)** Calculation of SPT IC_{50} on MCF-7 cells after

72 h treatment; **(K)** Calculation of SPT IC_{50} on MDA-MB231 cells after 72 h incubation; **(L)** Calculation of SPT IC_{50} on MCF 10A cells after 72 h treatment.

3.2. SPP, SPT, AND THEIR COMBINATION PROMOTES APOPTOSIS IN BREAST CANCER CELLS

To confirm the results of the MTS assay on cancer cells, we performed annexin V and cell death assay. The annexin V assay allowed us to measure apoptotic events at 24, 48, and 72 h **(Figure 3.4.)**. The efficacy of SPP, SPT, and their combination in inducing apoptosis in MCF-7 / MDA-MB-231 cells was time-dependent, with statistically significant results. Over time, treating MCF-7 cells with SPP resulted in a total apoptosis of approximately 16% at 24 h, 23% at 48 h, and 25% at 72 h. SPT triggered cell death by approximately 15% at 24 h, 19% at 48 h, and 21% at 72 h. In addition, treatment with the combination caused cell death of approximately 14% at 24 h, 19% at 48 h, and 19% at 72 h of cell death **(Figure 3.3. A-C)**. In MDA-MB231 cells, treatment with SPP significantly increased the total apoptotic cells by approximately 11% at 24 h and 20% at 48 h, with another 20% observed at 72 h, whereas treatment with SPT induced apoptosis by approximately 20% at 24 h and 30% at both 48 h and 72 h. Contrarily, treatment of MDA-MB-231cells with the combination of SPP and SPT (500/50 µg/ml SPP/SPT) resulted in total cell death by approximately 20% at 24 h, 30% at 48 h, and 25% at 72 h **(Figure 3.3 D-F)**.

3.3. CELL CYCLE DISTRIBUTION OF MDA-MB-231OR MCF-7 CELLS AFTER TREATMENT WITH SPP, SPT, AND COMBINATION

To combine the data on viability with those related to cellular apoptosis, we evaluated the cell cycle of MDA-MB-231/ MCF-7 cells **(Figure 3.3. G, H) (Figure 3.5.)**. We found that treatment with SPP substantially arrested MCF-7 cells in the G0/G1 phase and MDA-MB231 cells in the G2/M phase of the cell division, and treatment with SPT resulted in a significant arrest of both MDA-MB231 and MCF-7 cells in the G2/M phase of the cell division. Treatment with the combination (500/50 µg/ml SPP/SPT) resulted in a substantial arrest of MCF-7 cells in the G0/G1 phase and raised the percentage of MDA-MB231 cells arrested in the G2/M state by 30%, despite the statistically non-significant change.

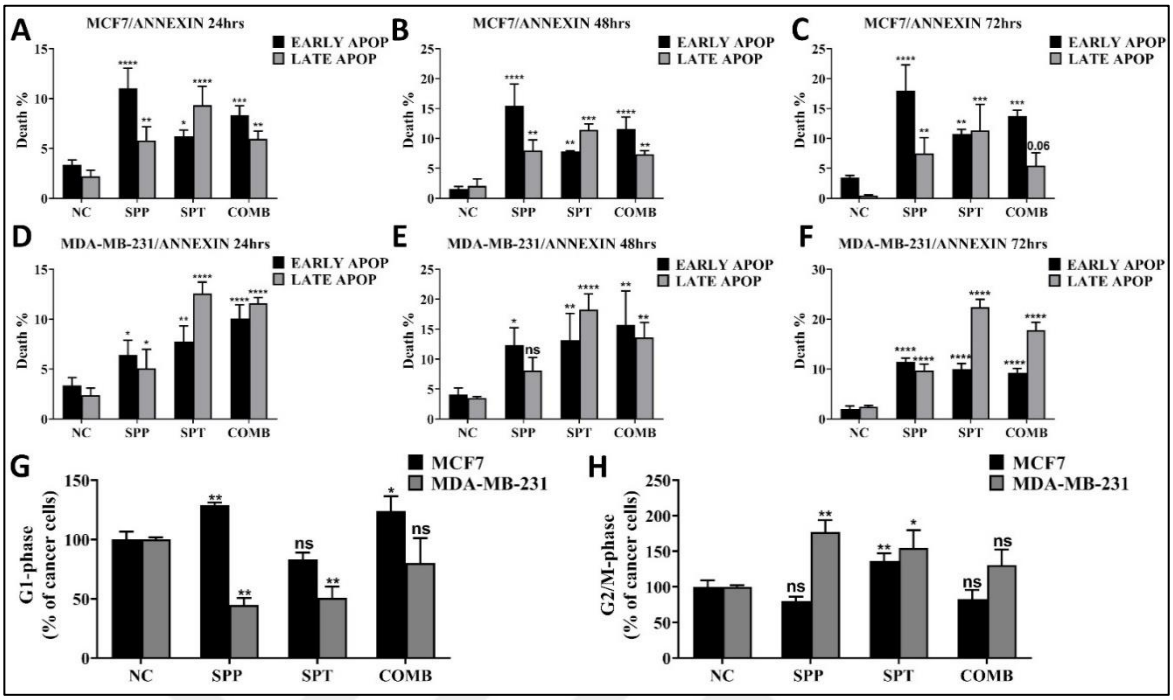

Figure 3.3 The effect of boron derivatives on apoptosis and the cell cycle status of breast cancer cells. (**A-C**) Apoptosis in MCF-7 cells treated with the IC_{50} of SPP, IC_{50} of SPT, and the combination (500/50 µg/ml SPP/SPT) for 24, 48, and 72 h, respectively. **(D-F)** Apoptosis in MDA-MB231 cells treated with the IC_{50} of SPP, IC_{50} of SPT, and the combination (500/50 µg/ml SPP/SPT) for 24, 48, and 72 h, respectively. **(G&H)** Cell cycle changes in MDA-MB-231/MCF-7 breast cancer cells upon treatment with the IC_{50} of SPP, IC_{50} of SPT, and the combination (500/50 µg/ml SPP/SPT) for 72 h, based on the cancer cell percentage arrested in G1 and G2/M-phase. Data are reported in the form of mean ± standard deviation (*P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, ns; non-significant > 0.05). The apoptosis experiment data were examined in relation the negative control by two-way ANOVA, and the cell cycle data were evaluated in relation to the untreated sample by one-way ANOVA. The experiments were independently repeated three times.

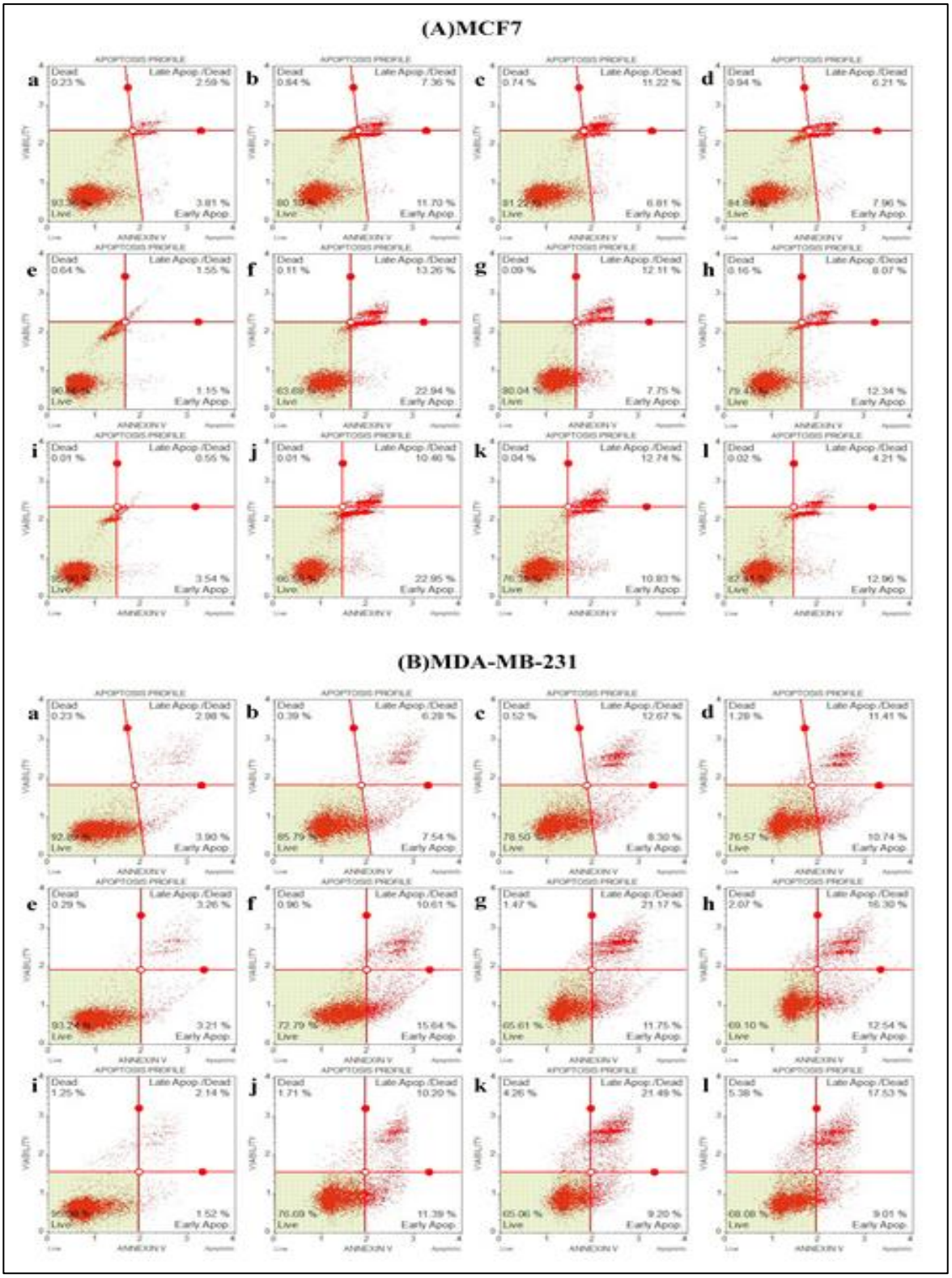

Figure 3.4. Flow cytometry assay of Boron derivatives-induced apoptosis in (A) MCF-7 and (B) MDA-MB231 cells. The cells were treated for 24, 48, and 72 h, respectively. (a) untreated cell for 24 h; (e) untreated cells for 48 h; (i) untreated cells for 72 h; (b) cells incubated with IC50 of SPP for 24 h; (f) cells incubated with IC50 of SPP for 48 h; (j) cells

incubated with IC50 of SPP for 72 h; (c) cells incubated with IC50 of SPT for 24 h; (g) cells incubated with IC50 of SPT for 48 h; (k) cells treated with IC50 of SPT for 72 h; (d) cells treated with combination (500/50 µg/ml SPP/SPT) for 24 h; (h) cells incubated with combination for 48 h (l) cells incubated with combination for 72 h.

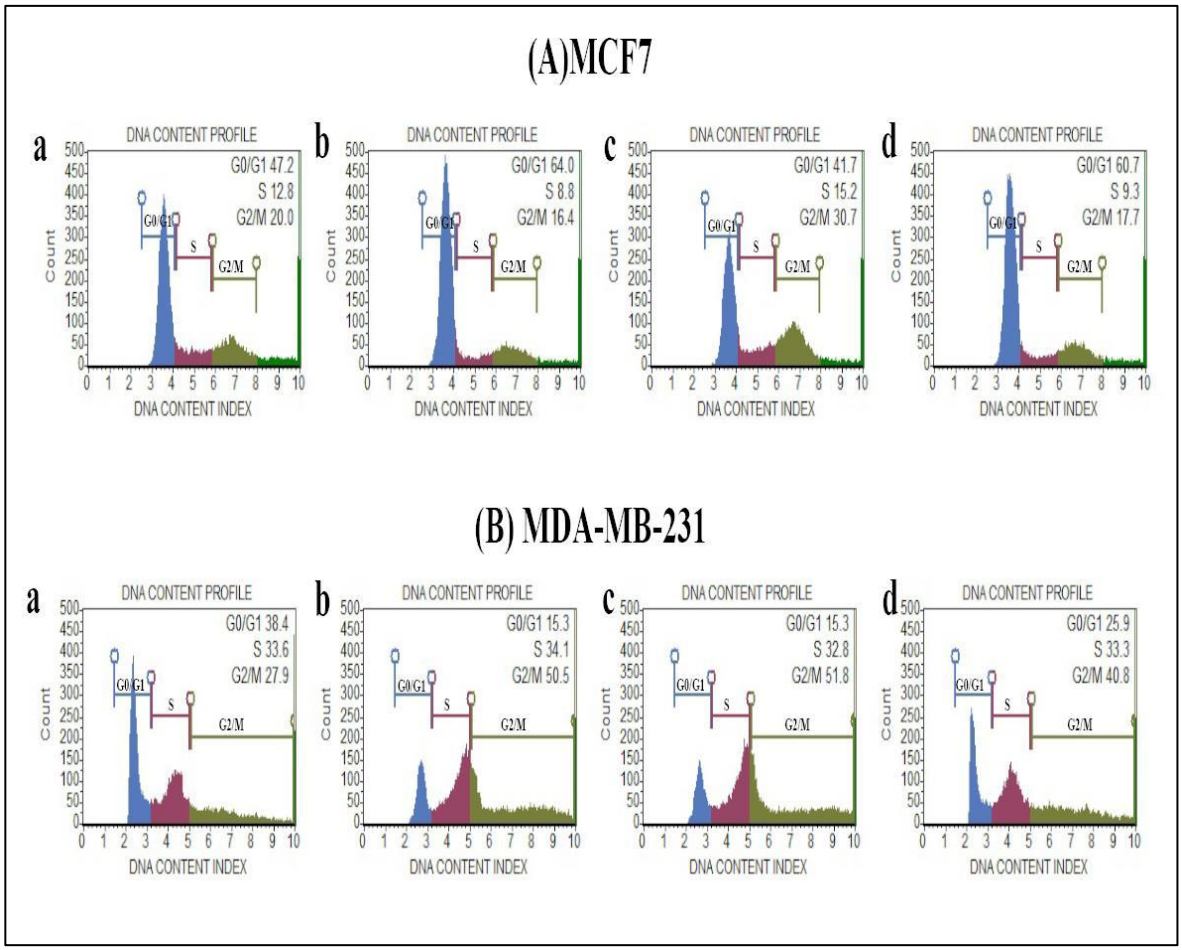

Figure 3.5. The effect of Boron derivatives on cell cycle distribution in (A) MCF-7 and (B) MDA-MB231 cells. The cells were treated for 72 h. (a) untreated cells; (b) cells incubated with IC50 of SPP; (c) cells incubated with IC50 of SPT; (d) cells incubated with combination (500/50 µg/ml SPP/SPT).

3.4. EFFECTS OF SPP, SPT, AND THEIR COMBINATION ON THE PD-L1 LEVEL IN MDA-MB-231/ MCF-7 CELLS

MDA-MB231 and MCF-7 Cells were treated with the IC_{50} of SPP, IC_{50} of SPT, or their combination (500/50 µg/ml SPP/SPT) for 72 h. Flow cytometry was utilized to detect the expression of CD274 on the surface of the cells **(Figure 3.6. A-D)**. The expression of CD274 surface marker on MDA-MB231 cells increased about two-fold when treated with SPP and SPT and 1.7 fold when treated with the combination compared to the control. Meanwhile, the expression of CD274 surface marker in MCF-7 cells increased by approximately 1.4 to 1.6 fold when treated with SPP, SPT, and the combination when evaluated in relation to the untreated sample.

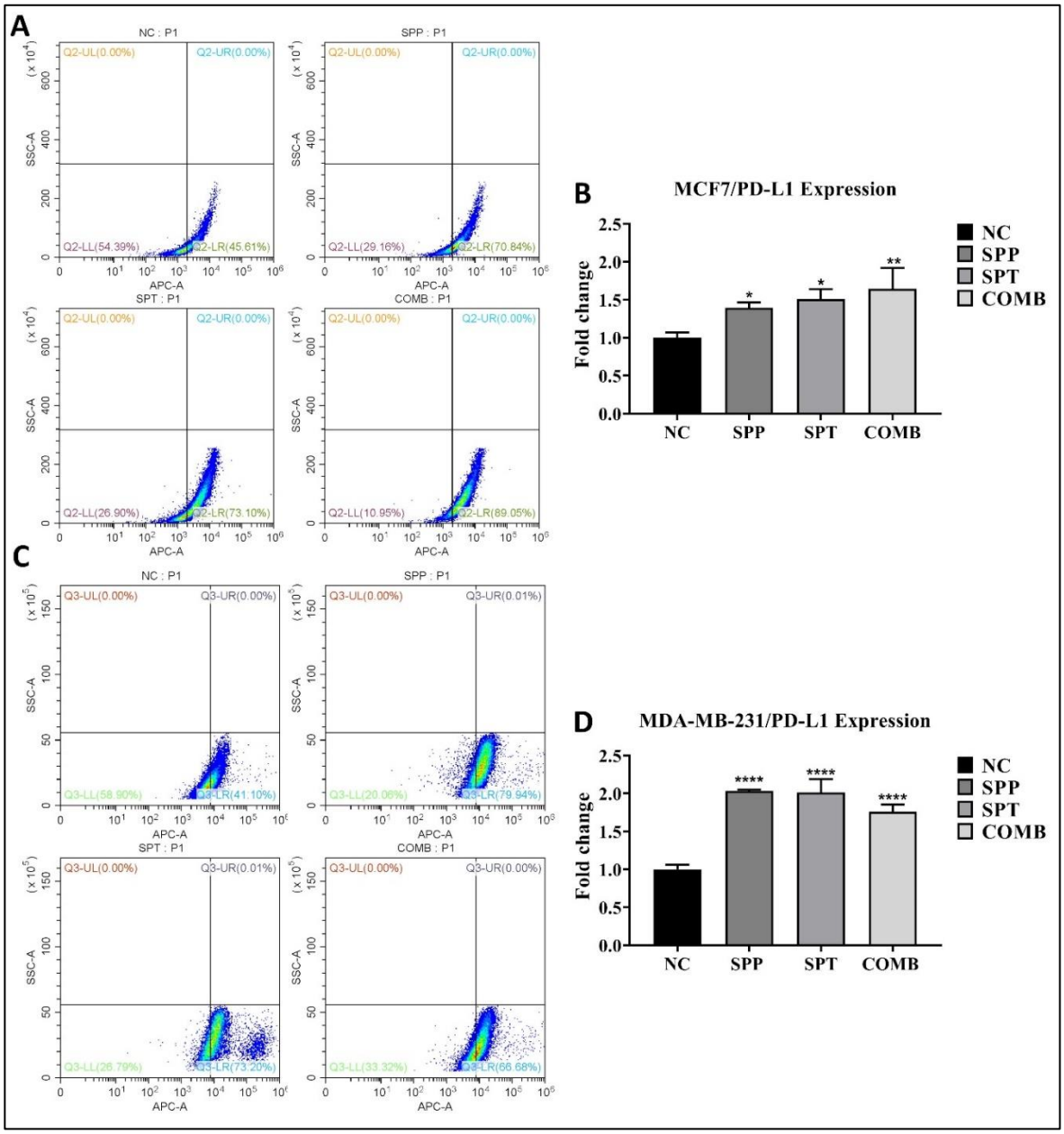

Figure 3.6. The effect of boron derivatives on the CD274 expression. **(A, B)** The expression of CD274 in MCF-7 cells after treatment with the IC_{50} of SPP, IC_{50} of SPT, and the combination (500/50 µg/ml SPP/SPT) for 72 h. **(C, D)** The expression of CD274 in MDA-MB231 cells after treatment with the IC50 of SPP and IC_{50} of SPT and the combination (500/50 µg/ml SPP/SPT) for 72 h. Data are reported in the format of the average ± SD (n=3) (*P < 0.05, **P < 0.01, ****P < 0.0001, ns; non-significant > 0.05). Data obtained were evaluated in comparison to the negative control using one-way ANOVA.

3.5. EFFECT OF SPP, SPT, AND THEIR COMBINATION ON THE LEVEL OF GENE EXPRESSION OF APOPTOTIC GENES

Aiming to detect the possible molecular mechanism behind the effect of SPP, SPT and their combination, gene expression screening was performed. In MDA-MB-231 cells, the results showed higher levels of the pro-apoptotic mediator (caspase-3) and decrease in Tp53 gene expression, as well downregulation of Bcl-2 and Bax family. In Mcf-7 cells, we observed higher levels in (caspases, Bax) and reduction in Bcl-2. As shown in Figure 3.7.

3.6. EFFECTS OF SPP, SPT, AND THEIR COMBINATION ON THE LEVEL OF APOPTOTIC PROTEIN EXPRESSION

Finally, as shown in Figure 3.7 the results of the gene expression were proved by protein analysis, demonstrating upregulation of pro-apoptotic mediators (caspase 3, Bax (in Mcf-7 cells), and Bak (in Mda-mb-231 cells)) and higher levels of tumor suppressor protein (Tp53) only in Mcf-7 cells.

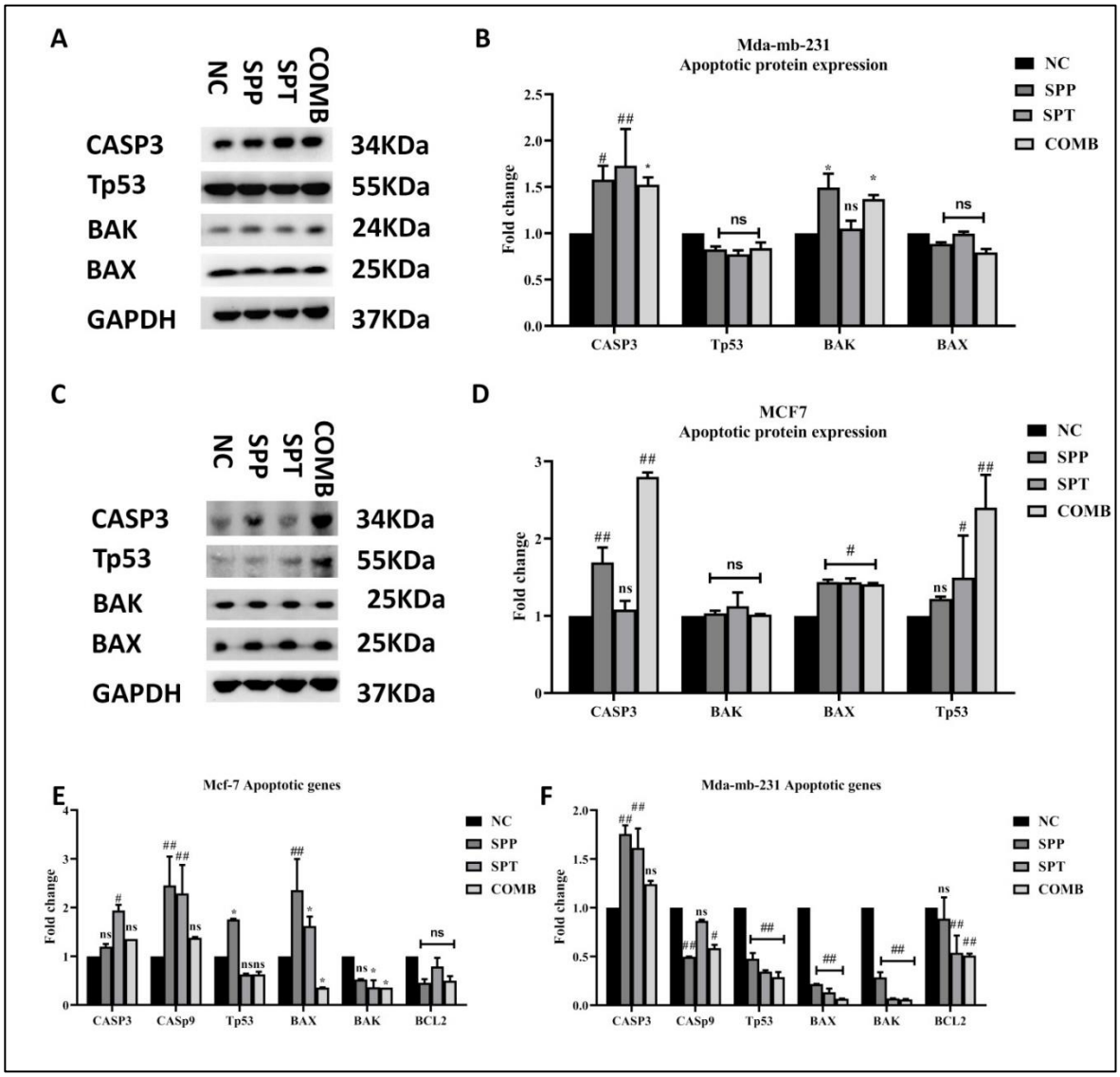

Figure 3.7. Effect of boron derivatives on apoptotic mediators expression on protein and gene levels. (A-D) The expression of apoptotic mediators proteins in MCF-7 and MDA-MB231 cells incubated with the IC50 of SPP and IC50 of SPT and their combination (500/50 µg/ml SPP/SPT) for 72 h were analyzed by western blotting. (E, F) The changes in the levels of the apoptotic mediators genes in MCF-7 and MDA-MB231 cells upon incubation with the IC50 of SPP and IC50 of SPT and their combination (500/50 µg/ml SPP/SPT) for 72 h were analyzed by RT-qPCR assays. Data are reported in the format of the average ± SD (n=3) (*P < 0.05, **P < 0.01, ***P < 0.001, #P < 0.0001, ns; non-significant > 0.05). One-way ANOVA performed the statistical analysis in comparison to the negative control. (Note: #: **; ##: ***)

3.7. EFFECTS OF SPP, SPT, AND THEIR COMBINATION ON THE LEVEL OF HIPPO PROTEIN EXPRESSION

The aforementioned experiments demonstrated that SPP, SPT, and their combination could inhibit the proliferation of breast cancer cell lines on one hand and support their resistance by inducing the expression of CD274 surface protein, which is one of the immune checkpoints involved in the immune evasion mechanisms, on the other hand. Therefore, aiming to check out the potential molecular machinery behind the effects of SPP, SPT, and their combination, we reviewed the literature and found that several studies showed that the Hippo pathway has a vital function in controlling cell multiplication, and that there is a significant correlation between YAP, which is one of the Hippo pathway members, and CD274 expression. Therefore, we aimed to investigate the effects of SPP, SPT, and their combination on Hippo protein expression (Kibra, Sav1, Mob1, Phospho-Mob1, and Phospho-YAP) using western blot analysis. Treatment of MDA-MB231 cells with SPP significantly decreased the expression of Mob-1 and Phospho-YAP, whereas treatment with SPT and the combination resulted in significant downregulation in the expression of Mob-1 with no change in the expression of Phospho-YAP, despite the slight downregulation caused by treatment with the combination of SPP and SPT **(Figure 3.8. A, B)**. Treatment with SPP, SPT, and their combination caused a downregulation in the expression of Mob-1 and Phospho-YAP (YAP-p), with non-significant changes in the other protein levels in MCF-7 cells **(Figure 3.8. C, D)**.

3.8. EFFECT OF SPP, SPT, AND THEIR COMBINATION ON THE LEVEL OF GENE EXPRESSION OF ONCOGENES

To further explore what are the mechanisms that cause the effects of SPP and SPT, the expression of downstream target oncogenes (*CYR61*, *CTGF*, *GADD45*, and *BIRC5*), which play a crucial role in cell duplication ((133)), and transcriptional co-activator (TAZ) of the Hippo pathway was investigated using RT-qPCR. In MCF-7 cells, SPP reduced the level of *CYR61* and *BIRC5*, with a significant increase in the level of *GADD45* and *TAZ*. Simultaneously, SPT significantly downregulated the expression of *CTGF* and *BIRC5*, with significant increase in *GADD45* gene expression. Furthermore, the treatment of MCF-7 cells

with combination led to significant downregulation of *CYR61*, *CTGF*, *TAZ*, and *BIRC-5* genes, with non-significant changes in *GADD45* **(Figure 3.8. E)** On the other hand, in MDA-MB-231cells, Both SPP and SPT, as well as their combination induced a down-regulated expression of *CYR61*, *CTGF*, *GADD45*, *TAZ*, and *BIRC-5* genes **(Figure 3.8. F)**.

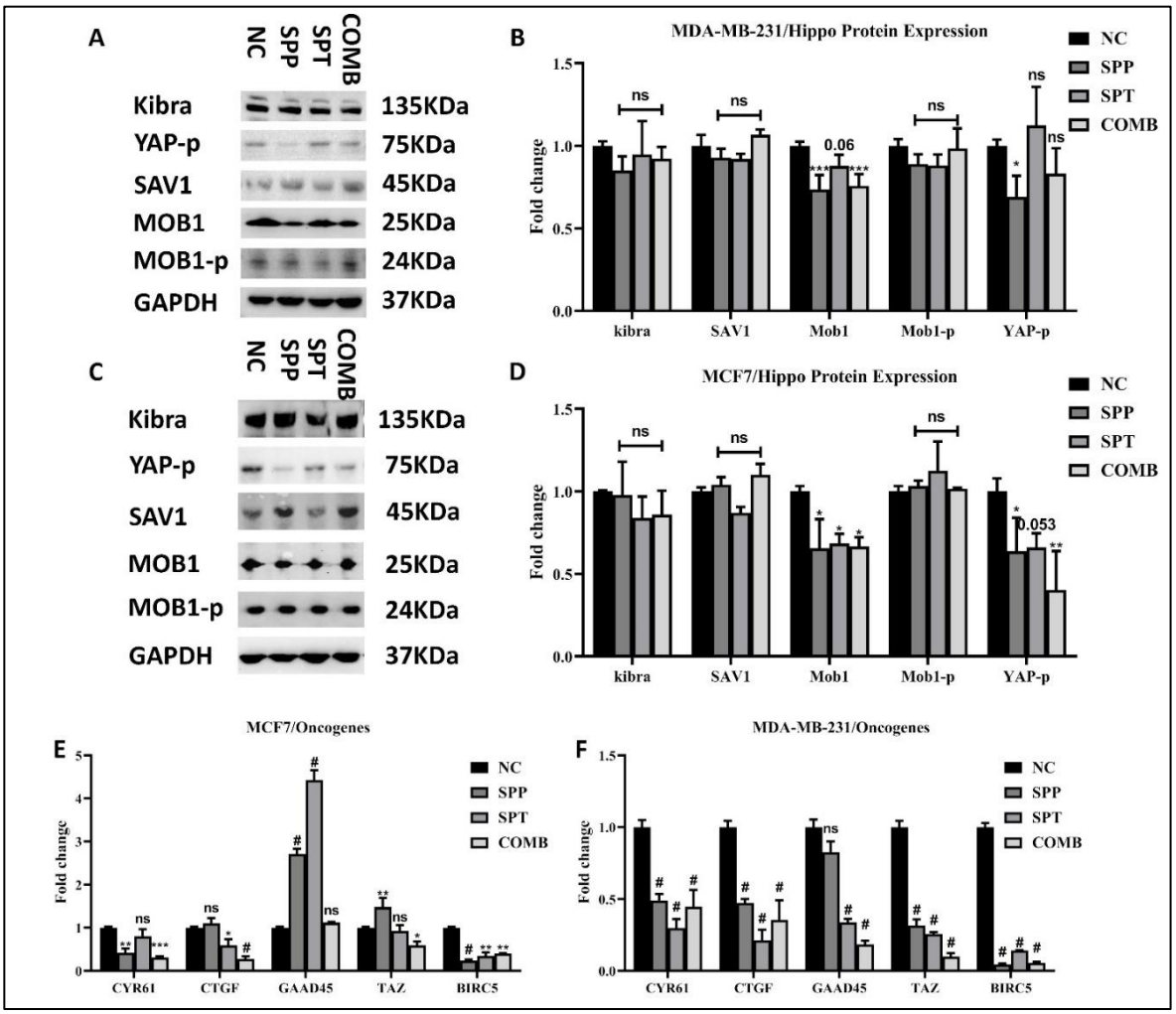

Figure 3.8. Effect of boron derivatives on Hippo protein expression and targeted oncogenes. **(A-D)** The expression of Hippo pathway-related proteins in MCF-7 and MDA-MB231 cells incubated with the IC_{50} of SPP and IC_{50} of SPT and their combination (500/50 µg/ml SPP/SPT) for 72 h were analyzed by western blotting. **(E, F)** The changes in the levels of the targeted oncogenes in MCF-7 and MDA-MB231 cells upon incubation with the IC_{50} of SPP and IC_{50} of SPT and their combination (500/50 µg/ml SPP/SPT) for 72 h were analyzed by RT-qPCR assays. Data are reported in the format of the average ± SD (n=3) (*P < 0.05, **P < 0.01, ***P < 0.001, $^{\#}$P < 0.0001, ns; non-significant > 0.05). One-way ANOVA performed the statistical analysis in comparison to the negative control.

3.9. CHARACTERIZATION OF T CELLS AND ALTERATIONS IN PD-1 LEVEL ON THE SURFACE OF T CELLS UPON INCUBATION WITH A COMBINATION OF SPP AND SPT ALONE AND IN CO-CULTURE WITH CANCER CELLS

We isolated T cells from the non-adherent fraction of PBMC, and the cell surface marker CD3 was investigated using flow cytometry. We found that approximately 75% of the population consisted of CD3+ T cells **(Figure 3.10. A)**. Afterwards, separated T cells were activated and expanded using T Cell TransAct^{TM} (αCD3/CD28 beads), and co-culturing with matured DCs was characterized using CD8 and CD4 surface markers. Approximately 70% of the cells were $CD8^+$ and 25% were $CD4^+$ **(Figure 3.10. B)**.

Furthermore, flow cytometry was used to evaluate the effect of breast cancer cells and the combination (500/50 µg/ml SPP/SPT) on the level of the immune checkpoint surface protein (CD279). We found that the expression level of CD279 was not affected upon co-culture with breast cancer cells. However, the expression of the CD279 marker significantly increased upon incubation of T cells with the combination, with a non-significant difference between incubation with the combination alone or in co-culture with breast cancer cells **(Figure 3.9. A-C)**.

3.10. CYTOTOXICITY OF EFFECTOR T CELLS ALONE AND IN THE PRESENCE OF COMBINATION MOLECULES AGAINST MDA-MB-231OR MCF-7 CELLS

The Lactate dehydrogenase activity in the culture supernatants of MDA-MB231 or MCF-7 breast cancer cells was evaluated showing substantial cytotoxic activity of specific effector T cells when co-cultured at a 30:1 ratio (effector/target), both alone and in the presence of the combination molecules. We observed no significant difference in the cytotoxicity of effector T cells against breast cancer cells, regardless of the presence or absence of the combination (500/50 µg/ml SPP/SPT), with approximately the same level of cytotoxicity **(Figure 3.9. D)**.

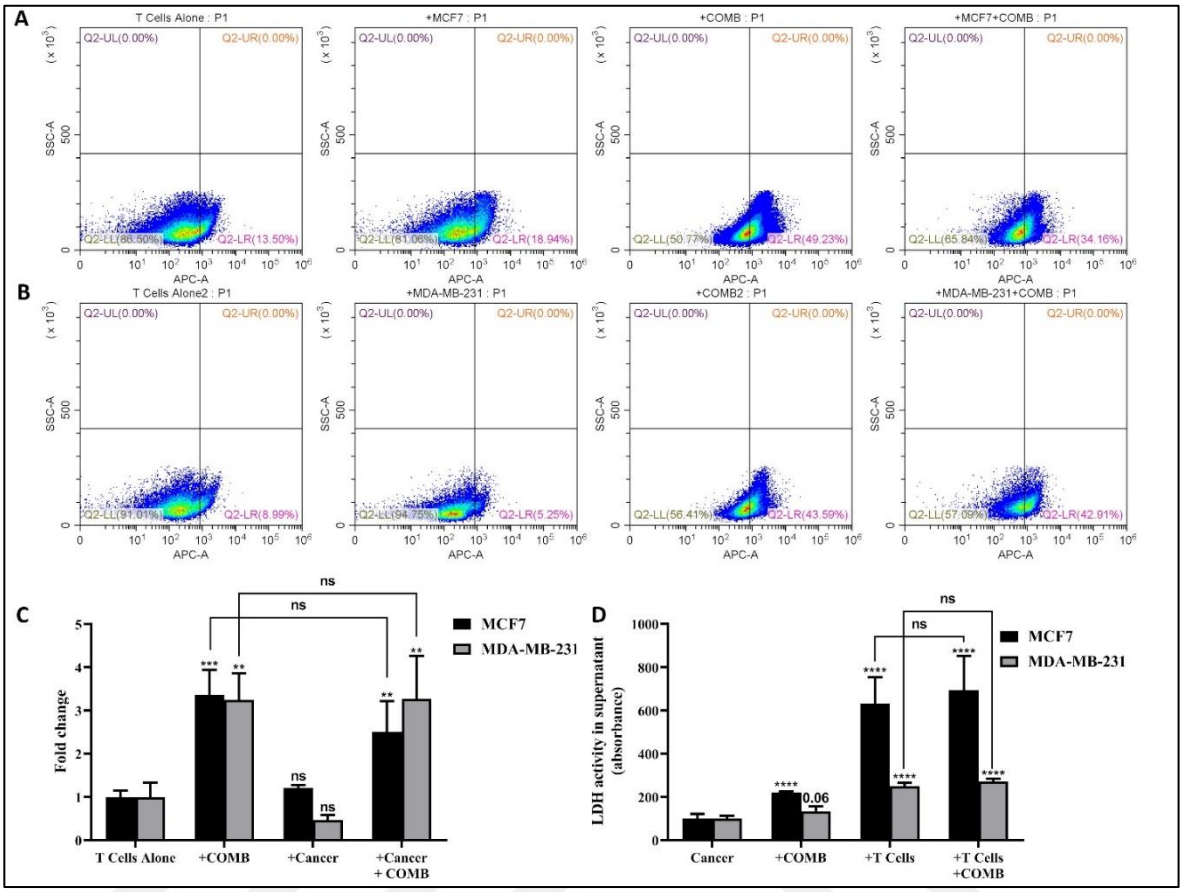

Figure 3.9. Alterations in CD279 surface marker expression and effector T cell cytotoxicity. **(A, B)** The Flow cytometric analysis representation of CD279 expression. **(C)** The effect of cancer cells and the combination molecules (500/50 µg/ml SPP/SPT) on the CD279 expression level in T cells. **(D)** The cytotoxic effect of effector T cells alone or together with the combination molecules against breast cancer cells was determined based on the activity of LDH in the supernatant. Data are reported in the format of average ± SD (n=3) (**P < 0.01, ***P < 0.001, ****P < 0.0001, ns; non-significant > 0.05). One-way ANOVA was used for the statistical analysis in comparison to the negative control, and the unpaired Student's t-test was used to compare the difference in cytotoxicity between the incubation of cancer cells with T cells alone and together with the combination and the difference in CD279 expression between incubation of the T cells with cancer cells alone and together with the combination.

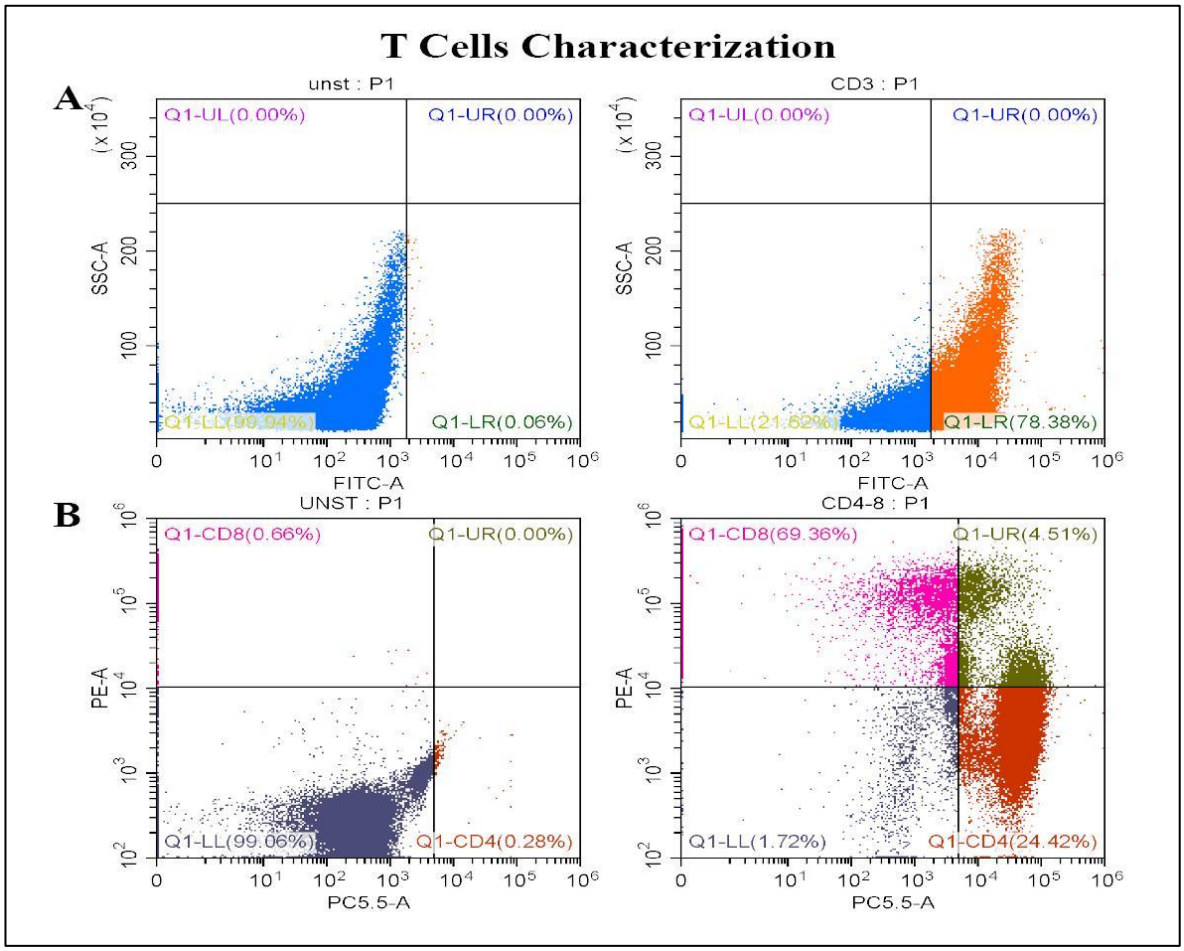

Figure 3.10. Characterization of the T cells isolated from the non-adherent fraction of PBMC using CD3, CD8, and CD4 surface markers. (A) Percentage of CD3+ T cells isolated from PBMC; (B) Percentage of CD4+ and CD8+ T cells.

3.11. CHANGES IN CYTOKINES RELEASE BY EFFECTOR T CELLS IN CO-CULTURE WITH MDA-MB-231/MCF-7 ALONE AND ADDITIONAL APPLICATION OF COMBINATION MOLECULES

By investigating the level of cytokine release by effector T cells**,** we observed a significant increase in sFasL production (**Figure 3.11. A)**, Granzyme A (**Figure 3.11. C)**, Granzyme B (**Figure 3.11. D)** and perforin (**Figure 3.11. F),** with a slight increase in IFN-γ and Granulysin ((**Figure 3.11. B, E)** in reference to co-culture with MCF-7 breast cancer cells and a slight tendency for higher levels of sFasL, Granzyme B, and perforin ($P < 0.07$) in co-culture with MDA-MB231 cells in comparison to the concentration of these cytokines produced by T cells alone. In addition, we found a significant decline in sFasL, IFN-γ, Granzyme B, Granulysin, and perforin upon incubation with the combination (500/50 µg/ml

SPP/SPT) alone or in co-culture with MDA-MB-231/MCF-7 cells (**Figure 3.11. B, D, E, F)**. Moreover, There were no noteworthy or substantial distinctions observed in the levels of cytokines released from T cells upon incubation with the combination (500/50 µg/ml SPP/SPT) alone compared to the level of cytokines production from T cells incubated with cancer cells and the combination (500/50 µg/ml SPP/SPT) (**Figure 3.11. B, D, E, F)**.

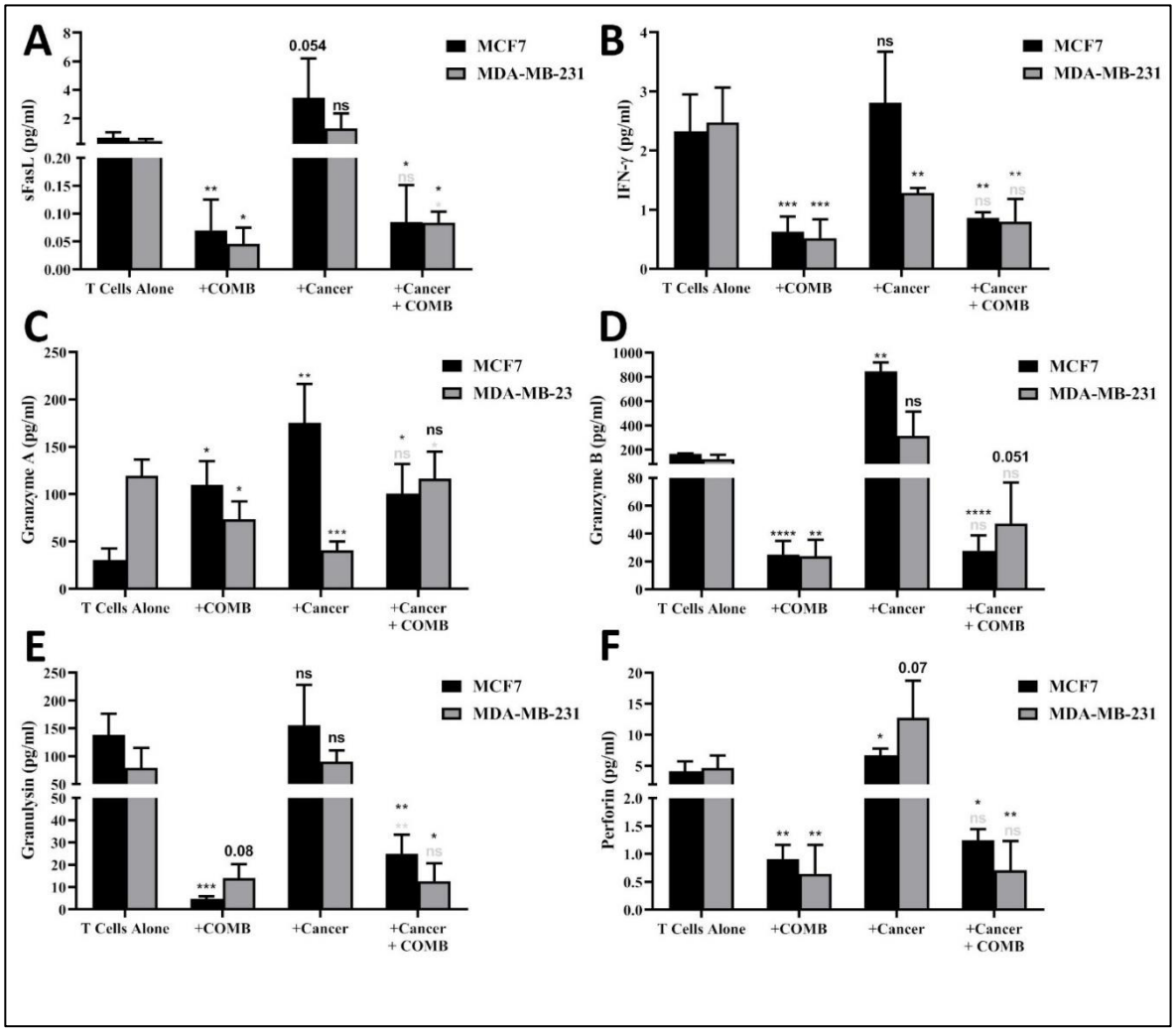

Figure 3.11. Changes in cytokine release by effector T cells. **(A-F)** Simultaneous detection of human sFasL, perforin, IFN-γ, Granzyme A, Granzyme B, and Granulysin concentrations was determined after 72-hrs co-culture experiments with specific active human T cells with the indicated target cells, MCF-7 and MDA-MB-231cells, in a 30:1 E/T ratio. The statistical assessment was done via unpaired Student's t-test. (*P < 0.05, **P < 0.01, ***P < 0.001, ns; non-significant > 0.05). The secondary statistical significance marks shown in pale color show the level of significance of the cytokine concentration of the T cells incubated with the combination (500/50 µg/ml SPP/SPT)

compared to the cytokine concentration of the T cells incubated with cancer cells and the combination (500/50 µg/ml SPP/SPT).

4. DISCUSSION

The outcomes of this study provide supporting evidence that boron derivatives SPP and SPT, and the combination of both molecules, with low toxicity in healthy cells, have the potential to exert anti-carcinogenic effects against both triple-negative and ER+ breast cancer cells. The derivatives were found to induce apoptosis by affecting the MOB1 protein and oncogenes involved in growth regulation. However, they also exert immunosuppressive effects by increasing CD279/CD274 expression and suppressing the production of cytolytic cytokines, thereby affecting T cell cytotoxicity. These findings open an exciting new avenue of study focused on boron derivatives in breast cancer research and pave the way for a further line of research to examine the anti-carcinogenic ability and mechanism of activity of these derivatives in *in vivo* studies, as well as to determine the synergistic effect of these boron derivatives at an optimal dose and the modified T cells activated against breast cancer cells to develop more effective cancer treatment strategies.

SPP and SPT showed a decrease in the viability of cancer cells that was dependent on the dose used. The effect was confirmed by performing an MTS assay on MCF-7 and MDA-MB231 breast cancer cell lines. In addition, we observed that the concentration of SPP causing 50% growth inhibition (IC_{50}) in the healthy cell line was two-fold higher than that in the cancer cell lines, as a first indication of the low side-effect potential of SPP in healthy cells. There is a correlation between the cytotoxic activity that varies with the dosage and the differences in the concentrations of IC50, which is consistent with a previous study (134). Meanwhile, the IC_{50} of SPT was the same for both cancer and healthy cells which is one of the limitations of this study is to use SPT alone. However, combining both molecules with the highest doses of low toxicity for healthy cells (500/50 µg/ml SPP/SPT) resulted in approximately 45-55% viability of cancer cell lines, while healthy cells remained 85% viable with the same treatment. This highlights the potential efficacy of this treatment in selectively targeting cancer cells, while preserving healthy cells.

The Annexin-V assay clearly showed that there was a time-dependent increase in both early and late stage apoptotic markers in MCF-7 and MDA-MB231 cells. In agreement with the cell viability results, annexin V and cell death assay indicated that the total apoptotic markers increased in MCF-7 cells by approximately 16% at 24 h, 23% at 48 h, and 25% at 72 h after treatment with the IC_{50} of SPP, and by approximately 15% at 24 h, 19% at 48 h, and 21% at

72 h with the IC_{50} of SPT; the combination of both molecules induced apoptosis by approximately 14% at 24 h, 19% at 48 h, and 19% at 72 h. Treatment of MDA-MB231 cells with SPP caused a noticeable increase in apoptotic markers by 11% after 24 h and by 20% after both 48 and 72 h. Additionally, treatment with SPT led to an apoptosis rate of 20% after 24 h, and 30% after both 48 and 72 h. The combination of SPP and SPT resulted in a total apoptosis of 20% after 24 h, 30% after 48 h, and 25% after 72 h. These results suggested that boron derivatives (SPP, SPT, and their combination) could exert antitumor effects against the (MCF-7) and (MDA-MB-231) cancer lines (135), and these data are also in line with the antitumor effect of these boron derivatives reported by our group against small-cell lung cancer cells (113).

The cell cycle is a complicated procedure that takes place within a cell, resulting in the creation of two offspring cells (136). The cell cycle comprises 4 phases, where after cell split, the newly divided cells undergo a phase of growth (G1) during which they produce most of their cellular RNA, membranes, proteins, and other macromolecules. Subsequently, after the first growth phase (G1), DNA synthesis (S) takes place before the next growth phase (G2). Following G2, during the mitotic phase (M), chromosomal condensation, nuclear envelope breakdown, creation of mitotic spindles, chromosome binding to the spindles, and separation of sister chromatids occur (137). Cell cycle assay of the cancer cell lines revealed arrested phases after treatment. Cell cycle analysis showed that approximately 25-30% of MCF-7 cells were arrested significantly in the G1-phase after treatment with the IC_{50} of SPP and the combination (500/50 µg/ml SPP/SPT) similar to Panduratin A inhibited cell growth in breast cancer cells by inducing G0/G1-phase cell cycle arrest (138). The IC_{50} of SPT arrested 37% of MCF-7 cells in the G2/M state. At the same time, 77% of MDA-MB231 cells with IC_{50} of SPP, 55% with IC50 of SPT, and 30% with the combination were arrested in the mitotic phase, indicating that cells had undergone mitotic catastrophe. Our data are in line with the results of a published study showing that bortezomib, a boron-containing chemotherapy drug currently used in clinics, induces cell death via mitotic catastrophe (139, 140). These outcomes may explain the reduction in cell division in MCF-7 and MDA-MB231 cells after treatment with these boron derivatives.

Our data showing up-regulation of caspase-3 and BAX at the gene and protein levels in Mcf-7 cells and a slight increase in BAK protein level are consistent with a published study showing that BAK & BAX solely might stimulate the caspase cascade leading to triggering

the pyroptosis pathways (141). Moreover, decrease in the Tp53 protein level in Mda-mb-231 cells indicated that the growth inhibition occurs via a p53-independent pathway (142). Our data are consistent with the findings of a published study showing that bortezomib, which is a boron containing chemotherapy drug currently used in clinics, induced cell death via mitotic crisis as triggering of apoptosis was substantially impaired owing to a significant delay in turnover of Bcl-2 family members which is also observed in our RT qPCR results as decrease in Bcl-2 gene level (139, 140).

The results of the gene expression screening suggest that SPP, SPT, and their combination may induce apoptosis in both MDA-MB-231 and Mcf-7 breast cancer cell lines through different molecular mechanisms.

In MDA-MB-231 cells, SPP, SPT, and their combination led to the upregulation of the pro-apoptotic mediator caspase-3. This suggests that the treatment may cause caspase-3 to become active and causing apoptosis. These data are in line with the previously published results demonsterating that Doxorubicin triggers apoptosis through the caspase 3 activation (143).

In Mcf-7 cells, the increase in the levels of caspases and Bax, and the reduction in the Bcl-2 levels, suggest that SPP, SPT, and their combination may trigger apoptosis via the intrinsic apoptotic pathway.

The confirmation of the gene expression data by Western blot analysis provides further support for the potential pro-apoptotic effects of SPP, SPT, and their combination in breast cancer cells.

In Mcf-7 cells, the upregulation of pro-apoptotic mediators, such as caspase 3, Bax, and Bak, suggests that SPP, SPT, and their combination may activate the intrinsic apoptotic pathway, which involves the activation of Bax and Bak, the release of cytochrome c, and the subsequent activation of caspases and apoptosis. The higher level of Tp53 further supports the notion that the treatment may suppress the growth of Mcf-7 cells by triggering apoptosis.

In Mda-mb-231 cells, the upregulation of Bak suggests that SPP, SPT, and their combination may stimulate apoptosis through a mechanism that involves the subsequent release of cytochrome c and initiation of caspases. However, the downregulation of Bax family at the

gene expression level in MDA-MB-231 cells suggests that the impacts of SPP, SPT, and their combination on the apoptotic pathway in these cells may be more complex.

In this project, we have observed that the treatment of breast cancer cells MCF-7/MDA-MB231 with SPP, SPT, or their combination cause a substantial reduction in the expression of MOB1 protein. This finding is noteworthy since earlier researches have concluded that MOB1A has a significant role in apoptosis signaling and acts as a tumor suppressor (144, 145) (146). Contrarily, according to Yang Li et al.'s research, the expression of MOB1A was higher in gall bladder cancer compared to healthy tissues. Additionally, the gene levels of MOB1A were greater in pancreatic cancer tissues than healthy tissues (147). Consistently, it was demonstrated that it stimulates the survival of gallbladder cancer *in vitro* and *in vivo* (148). The current study proves the potential therapeutic use of boron derivatives in inhibiting the multiplication of breast cancer cells and promoting apoptosis by reducing MOB1 level. Therefore, these findings suggest that these compounds may have significant benefits as a treatment option.

Earlier researches have shown that CYR61, which belongs to the CCN (CTGF/Cyr61/NOV) family of growth controllers, is a secreted factor that contains cysteine and promotes angiogenesis. It has been linked with the development of tumors (149). CYR61 is abundant in breast cancers and plays a role in the acquiring of an estrogen-independent phenotype in breast cancer (150, 151). Additionally, in vitro studies on MCF-7 breast adenocarcinoma cells have revealed that Cyr61 is necessary for hormone-dependent DNA synthesis and cell proliferation. It is also expressed significantly in the invasive breast cancer cell line MDA-MB-231, contributing to the advancement of breast cancer (152). The gene BIRC5, also referred to as survivin, contains baculoviral inhibitor of apoptosis repeats and is related to the mitotic spindle checkpoint. Studies have shown that BIRC5 has significant contribution in the cancer development, as it impacts cell division and proliferation and hinders apoptosis (153). BIRC5 is abundantly expressed in the subtype of breast cancer known as triple-negative. Inhibiting BIRC5 has been demonstrated to reduce breast cancer cell growth, suggesting that BIRC5 has impact in driving cancer growth (154). Furthermore, disrupting the expression of Survivin has the ability to hinder cell growth, travelling, and invasion, while promoting cell death in MCF-7 cells (155). The findings of our study provide evidence for the possible antiproliferative abilities of SPP, SPT, and their combination, as we noticed a substantial decrease in the level of genes related to tumorigenesis, including CYR61,

CTGF, and BIRC-5, in MDA-MB-231/MCF-7 cells treated with these compounds. However, the function of GADD45A in apoptosis is still a subject of debate (156). Some research has shown that exposure to genotoxic agents can induce the overexpression of GADD45A, which can then function as a protein that promotes apoptosis (157). On the other hand, some studies have suggested that GADD45A may actually promote cell survival (158). Our results showed that SPP and SPT induced upregulated expression of the *GADD45* gene with a slight upregulation caused by the combination in MCF-7 cells, which was consistent with the pro-apoptotic effect induced in human MCF-7 breast carcinoma cells upon treatment with troglitazone by inducing *GADD45*, p53-contolled growth arrest, and DNA-damage-inducible gene expression (159, 160). In contrast, in MDA-MB231 cells, SPP, SPT, and their combination induced downregulation of *GADD45* expression in line with the induction of apoptosis. These findings highlight the potential of SPP, SPT, and their combination in regulating or controlling the level or amount of expression of key genes involved in breast cancer progression.

The findings on the effect of boron derivatives on CD274 expression and its correlation with YAP are noteworthy and require further investigation. The impact of chemotherapy on the interaction between the defence system and tumors has been investigated in several studies, and some have suggested that it may have negative consequences due to an increase in the expression of CD274 (161). Yosi Gilad et al. conducted a study on ER+ and TNBC types and noticed that the majority of chemotherapeutic agents led to a substantial rise in the level of CD274 (161). We demonstrated that boron derivatives lead to CD274 overexpression in MDA-MB231 and MCF-7 human breast cancer cell lines. Studies conducted recently have revealed a noteworthy link between the level of YAP and CD274 in human cancer cells, with YAP promoting immune evasion by increasing CD274 expression in BRAFi-resistant melanoma cells (99). The transcription of CD274 in NSCLC is regulated by YAP (100). Additionally, the level of CD274 protein was induced in MCF10A cells by TAZ-S89A and YAP-S127A (98). We found that SPP, SPT, and the combination significantly decreased the phospho-YAP protein level in MCF-7 cells, whereas only SPP and the combination showed an effect in MDA-MB231 cells. Thus, we considered that these boron derivatives might induce the level of CD274 in breast cancer cells via the downregulation of phospho-YAP and an increase in the level of YAP translocation to the nucleus and inducing the expression of CD274. These outcomes help in comprehending of the impact of boron derivatives on the

tumor-immune system interaction and highlight the potential of boron derivatives to induce the expression of CD274 in breast cancer cells through the downregulation of phospho-YAP.

Nowadays, cellular immunotherapy is a promising therapy for cancer (162). Our study illustrated the promising potential of cellular immunotherapy for cancer treatment. By activating naive T cells through incubation with mature DCs stimulated with cancer cell lysates and RNA (163-166), we observed an increase in the cytotoxic effect of effector T cells against cancer cells. This was evidenced by an increase in lactate dehydrogenase (LDH) release and the concentration of cytolytic cytokines such as sFasL, Perforin, Granzyme A, Granzyme B, and Granulysin (167). These results highlight the potential of this protocol to activate T cells as a therapeutic option for breast cancer treatment.

The results of this study emphasize the immunosuppressive effects of boron derivatives on effector T cells. In this study, we investigated the effect of boron derivatives on the activity of effector T cells by detecting their effect on CD279 expression and cytotoxicity of effector T cells. Additionally, we assessed their impact on cytokine release. We observed that these derivatives increased CD279 expression. Thus, the interaction between the highly expressed CD274 on the surface of cancer cells and CD279 on effector T cells leads to the inhibition of T cell activation and proliferation, and suppression of cytokine secretion, thereby promoting regulatory T cell differentiation (80, 86). Additionally, we found no significant difference in LDH activity upon incubation of effector T cells with cancer cells alone or in the presence of the combination. Moreover, the boron derivatives resulted in a strong decrease in cytokine concentrations, with no significant change in cytokine release upon treatment of T cells with the combination alone or in co-culture with cancer cells. The aforementioned results indicate that the combination of SPP and SPT has a strong immunosuppressant effect on T cells, which may have implications for the use of boron derivatives together with cancer immunotherapy.

5. CONCLUSION

In conclusion, boron derivatives SPP, SPT, and the combination of both molecules could have a potential anti-carcinogenic effect against both triple-negative breast cancer cells represented by MDA-MB231 cells and ER+ cells represented by MCF-7 cells in our study. Furthermore, these derivatives might induce apoptosis through their effects on the MOB1 protein and the oncogenes involved in growth regulation. However, these compounds could have side effects by stimulating CD279/CD274 expression and suppressing the release of cytolytic cytokines, which affects T cells' cytotoxicity.

In light of our promising data, we recommend investigating the anti-carcinogenic effect of the boron derivatives used in our study *in vivo* and further exploring their mechanism of action in detail. Moreover, we recommend determining the synergistic effect of these boron derivatives using an optimal dose, together with modified T cells activated against breast cancer cells.

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