

Ferda ALTAYTAŞ

MASTER OF SCIENCE THESIS

2025-ANTALYA

T.C.
AKDENİZ UNIVERSITY
INSTITUTE OF HEALTH SCIENCES
DEPARTMENT OF GENE AND CELL THERAPY

**THE POSSIBLE ANTI-TUMORAL/ANTI-METASTATIC
EFFECTS OF BMP1 INHIBITOR ALONE OR IN
COMBINATION WITH DOXORUBICIN ON
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ADVISOR

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This thesis was supported by The Scientific and Technological Research Council of
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2025-ANTALYA

To the Directorate of the Institute of Health Sciences;

This study has been accepted by our jury as the thesis of Master of Science Thesis in the Department of Gene and Cell Therapy, Gene and Cell Therapy Master of Science program. 10/07/2025

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I hereby declare that this thesis is my own work, that I have not committed any unethical acts at any stage from the planning to the writing of the thesis, that I have obtained all the information in this thesis within academic and ethical rules, that I have cited all the information and comments not obtained through this thesis, and that I have included these sources in the list of sources.

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ACKNOWLEDGEMENTS

First and foremost, I would like to express my deepest gratitude to my advisor, Prof. Dr. Nuray ERIN, for her continuous guidance and encouragement throughout my thesis.

I am also sincerely thankful to Prof. Dr. Ahter Dilşad ŞANLIOĞLU, whose humility and support have always been a source of inspiration, and to all the esteemed faculty members of our department for their valuable contributions to my academic journey.

I would like to thank Assoc. Prof. Dr. Derya KIVRAK SALIM, a member of my thesis defense committee, for her insightful comments and constructive feedback.

My heartfelt thanks go to my dear lab mates, especially to Dr. Esra TAVŞAN, for her invaluable guidance and for making the laboratory a lovely place of learning and encouragement throughout this journey.

I am deeply grateful to my family for their unwavering support, and to my sister, Kübra ALTAYTAŞ, who, despite the distance, has always made me feel her presence and encouragement.

My special thanks go to Mahir GUNEBAKAN for his readiness to help and for always bringing positive energy to me whenever I most needed.

I would like to thank all my professors and friends, specifically I am thankful to Nefsun DANIS and Almira UZUN, for sharing not only laughter but also tears with me throughout this journey, and for being the kind of partners in crime that made every moment more meaningful. Also, thanks to my beloved cat, Kedoş, for her silent companionship, warmth, and the comfort she brought into my life throughout this journey and beyond. She will always be remembered with love

Lastly, I would like to thank myself for not giving up, for showing resilience in the most difficult moments, and for staying in this journey until the very end.

For every question, there is a path, and for every path, there is a reason to keep going.

ABSTRACT

Objective: Preventing distant organ metastases is critical for reducing mortality in metastatic breast cancer. Current treatments are insufficient in preventing spread and are limited by resistance and side effects. Doxorubicin (DOX) suppresses tumor progression but causes cardiotoxicity and resistance. Bone morphogenetic protein-1 (BMP-1), a metalloproteinase involved in tumor microenvironment and extracellular matrix remodeling, supports metastasis and poor prognosis. BMP-1 inhibitors may reshape the tumor microenvironment, reduce metastasis, and enhance chemotherapy effectiveness. This thesis aimed to evaluate the effects of the BMP-1 inhibitor UK-383367, alone and in combination with DOX, on tumor progression, lung and liver metastasis, immune cell subpopulations, and cytokine responses in *in vivo* breast cancer models.

Method: *In vivo* models were established using 4TBM and 4TLM cell lines in BALB/c female mice, and tumor volume, lung and liver metastatic burden, distribution of immune cell subpopulations, and cytokine levels (TNF- α , IL-6, IL-10, IFN- γ) were evaluated.

Results: Treatment with UK alone was found to reduce the lung metastatic burden and support T cell-mediated immune responses; however, it showed limited effects on primary tumor volume. DOX alone resulted in reduced tumor volume and metastatic spread while activating the immune response. The combination treatment of UK and DOX was observed to most effectively reduce tumor volume and metastatic burden, enhance the cytotoxic T cell response, and decrease immunosuppressive cell populations. Cytokine analyses revealed that IL-6 and IFN- γ levels increased in the treatment groups, while IL-10 levels exhibited context-dependent variability.

Conclusion: The results of our study demonstrated that targeting BMP-1 represents a promising approach in the treatment of metastatic breast cancer, and that the combination of UK-383367 and DOX has the potential to reduce metastatic spread and support the immune response.

Key words: BMP-1, UK-383367, Doxorubicin, Metastatic Breast Cancer, Immune response, Tumor microenvironment

ÖZET

Amaç: Metastatik meme kanserinde mortaliteyi azaltmada uzak organ metastazlarının önlenmesi kritik bir hedeftir. Mevcut tedaviler metastatik yayılımı engellemede yetersiz kalmakta, tedaviye direnç ve yan etkilerle sınırlanmaktadır. Meme kanseri tedavisinde yaygın kullanılan doksorubisin (DOX), tümör progresyonunu baskılasa da kardiyotoksisite ve ilaç direnci ile ilişkilidir. Metalloproteinaz olan BMP-1, tümör mikroçevresi ve ekstraselüler matriks yeniden modellemesinde rol alır ve yüksek ekspresyonu metastatik yayılımı destekleyerek kötü prognoza katkıda bulunur. Bu nedenle BMP-1 inhibitörlerinin, tümör mikroçevresini yeniden şekillendirerek metastatik yükü azaltma ve kemoterapi etkinliğini artırma potansiyeli umut vericidir. Bu çalışmada BMP-1 inhibitörü UK-383367'nin tek başına ve DOX ile kombinasyonunun farklı *in vivo* meme kanseri modellerinde tümör progresyonu, akciğer ve karaciğer metastaz yükü, immün hücre alt popülasyonları ve sitokin yanıtları üzerindeki etkilerinin kapsamlı şekilde değerlendirilmesi hedeflenmiştir.

Yöntem: BALB/c dişi farelerde 4TBM ve 4TLM hücre hatları kullanılarak oluşturulan *in vivo* modellerde tümör hacmi, akciğer ve karaciğer metastaz yükü, immün hücre alt popülasyonlarının dağılımı ve sitokin düzeyleri (TNF- α , IL-6, IL-10, IFN- γ) değerlendirilmiştir.

Bulgular: Tek başına UK tedavisi, akciğer metastaz yükünü azaltmakta, T hücre aracılı immün yanıtı desteklemektedir. Ancak primer tümör hacmi üzerinde sınırlı etkisi olduğu gözlenmiştir. DOX'un tek başına uygulamasında tümör hacmi ve metastatik yayılım azalırken, immün yanıtın aktive olduğu belirlenmiştir. UK ve DOX kombinasyon tedavisinin tümör hacmi ve metastatik yükü en etkin şekilde azalttığı, sitotoksik T hücre yanıtını güçlendirdiği ve immünosupresif hücre popülasyonlarını azalttığı saptanmıştır. Sitokin analizlerinde, tedavi gruplarında IL-6 ve IFN- γ seviyelerinin arttığı ve IL-10 düzeylerinin bağlama bağlı değişkenlik gösterdiği belirlenmiştir.

Sonuç: Çalışmamızın sonuçları BMP-1'in hedeflenmesinin metastatik meme kanseri tedavisinde umut verici bir yaklaşım olduğu, UK-383367 ve DOX kombinasyonunun metastatik yayılımı azaltma ve immün yanıtı destekleme potansiyeline sahip olduğu göstermiştir.

Anahtar Kelimeler: BMP-1, UK-383367, Doksorubisin, Metastatik Meme Kanseri, İmmün yanıt, Tümör Mikroçevresi

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ICONS & ABBREVIATIONS

BMP-1	:	Bone Morphogenetic Protein-1
UK	:	UK-383367 (BMP-1 inhibitor)
DOX	:	Doxorubicin
TGF- β	:	Transforming Growth Factor Beta
ECM	:	Extracellular Matrix
TME	:	Tumor Microenvironment
TNF- α	:	Tumor Necrosis Factor Alpha
IL-6	:	Interleukin-6
IL-10	:	Interleukin-10
IFN- γ	:	Interferon-gamma
DDR	:	DNA Damage Response
MMPs	:	Matrix Metalloproteinases
MDSC	:	Myeloid-Derived Suppressor Cells
NK	:	Natural Killer
CTLs	:	Cytotoxic T Lymphocytes
MHC	:	Major Histocompatibility Complex
EMT	:	Epithelial-Mesenchymal Transition
MET	:	Mesenchymal-Epithelial Transition
RPMI	:	Roswell Park Memorial Institute (medium)
FBS	:	Fetal Bovine Serum
LPS	:	Lipopolysaccharide
ConA	:	Concanavalin A
H&E	:	Hematoxylin and Eosin

1. INTRODUCTION

Breast cancer is the most prevalent malignancy among women and a primary cause of cancer-related mortality globally. Metastasis, in particular to remote organs, is the primary determinant that increases mortality rates. The metastasis includes the stages of invasion, intravasation, survival in circulation, extravasation, and colonization, including the dynamic interaction between tumor cells and the tumor microenvironment significantly impacts this process (Datta et al., 2021). Breast cancer typically metastasizes to the brain, liver, lungs, and bone. Although effective treatments can be applied to localized cancers, there is no effective treatment method for metastatic cancers yet.

Metastatic niche formation and invasion are facilitated by the remodeling of the extracellular matrix (ECM) and tumor microenvironment (TME). Bone Morphogenetic Protein-1 (BMP-1), a proteolytic member of the TGF- β superfamily, is a key player in the metastatic process (Davis et al. 2016). It converts procollagens into mature collagens, thereby contributing to the regulation of the ECM and the latent activation of TGF- β . A poor prognosis has been linked to high BMP-1 expression, which has been demonstrated to facilitate mechanisms such as angiogenesis, migration, and invasion (Ye et al. 2009).

Doxorubicin (DOX) is widely used in chemotherapeutic treatments because of its versatile antitumoral effects, including DNA intercalation, topoisomerase-II inhibition, reactive oxygen species (ROS) production, and apoptosis induction (Kciuk et al., 2023). Nevertheless, it has substantial side effects, including cardiotoxicity and the development of drug resistance, which restricts the efficacy of the treatment and necessitates the development of novel combinational therapies.

We aimed to examine the impact of the BMP-1 inhibitor UK-383367, alone and in combination with DOX, on tumor development, metastatic burden, and immune response across various metastatic breast cancer *in vivo* models. Through the utilization of BALB/c female mouse models, a comprehensive evaluation was conducted to assess the tumor volume, lung and liver metastasis burden, distribution of immune cell subpopulations,

TNF- α , IL-6, IL-10, and IFN- γ levels. Additionally, we focused on the impact of BMP-1 inhibition and combination therapy on the dynamics of the tumor microenvironment, immune response, and metastatic process control. In addition, we compared low and high dose applications in terms of treatment efficacy and toxicity profiles, thus aiming to increase sensitivity to chemotherapy and reduce treatment-related side effects. The obtained findings were aimed at contributing to the development of new targeted, highly effective, and low-side-effect strategies for the use of BMP-1 inhibitors in combination with chemotherapy in the treatment of metastatic breast cancer.



2. LITERATURE REVIEW

2.1. Human Breast Cancer: A General Overview

Breast cancer is the most prevalent form of cancer worldwide. It accounts for one out of every eight instances of cancer in both sexes and 25% of all cancers in women, with 70% of deaths taking place in environments with little resources. The most prevalent malignancy in women is breast cancer, which is also one of the three most prevalent cancers worldwide, along with lung and colon cancer. In 2022, breast cancer was diagnosed in 2.3 million women worldwide, resulting in 670,000 fatalities (*WHO Global Breast Cancer Initiative: Breast Cancer Awareness Month*, 2024). Breast cancer is a disease that affects women of all ages after puberty, but the incidence rate increases in later life in every country.

Breast cancer shows a heterogeneous structure. For this reason, different prognoses and treatments are needed for the different histopathology and molecular subtypes of breast cancer. Especially, metastatic breast cancer that can be easily spreadable to distant organs is challenging because of resistance in treatment. The metastasis has mainly 6 steps, which are invasion, intravasation, survival in circulation, extravasation, and colonization in distant areas (Figure1).

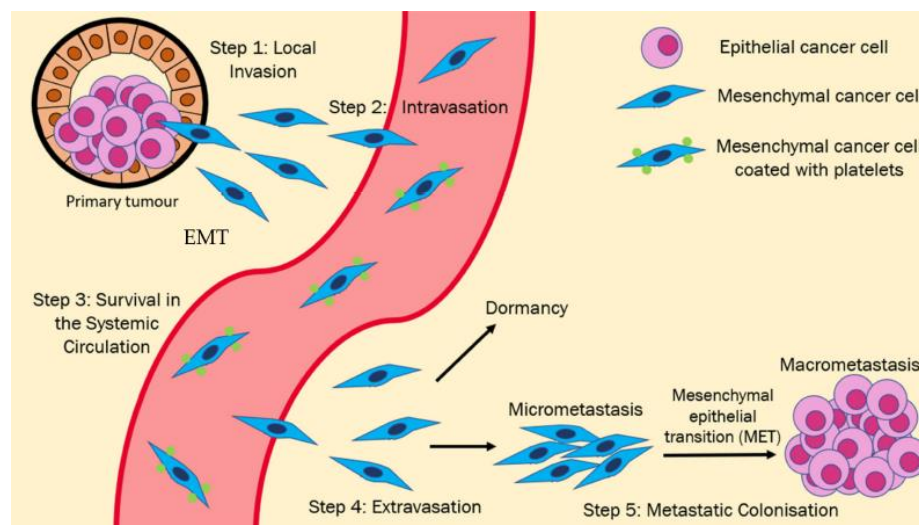


Figure1: The metastatic cascade. In the metastatic cascade, cancer cells first undergo EMT to gain invasiveness, enter the circulation, extravasate into target tissues, and form dormant cells or micrometastases. MET activation is required for clinically evident macrometastases to develop (Datta et al., 2021).

In all these steps, many different molecular pathways and mechanisms take roles, which means there are many different potential treatment strategies. Using chemotherapeutic agents is the most common approach for cancer treatment. However, high toxicity to chemotherapeutic agents and the possibility of resistance push researchers to find new approaches for cancer treatment. Especially, target molecules that focus on tumor microenvironment, extracellular matrix (ECM) and epithelial-mesenchymal transition (EMT) are promising as anti-metastatic targets.

Breast cancer has a heterogeneous structure. It has many different molecular subtypes and clinical behaviors. Also, metastatic potential of different subtypes varies. The subtypes are divided based on gene expression profiles and immunohistochemical markers. ER+/PR+, HER2-positive and triple negative breast cancer (TNBC) are the main subtypes by immunohistochemically while the PAM50 gene signature that is prediction analysis of microarray50, classifies 5 more subtypes of Breast cancer as Luminal A, Luminal B, Normal-like TNBC, Basal-TNBC and Her2-enriched based on gene expression profiling for molecular characterization of the tumor as stated in the Figure 2 (Turner et al. 2021). TNBC has the highest potential for metastasis that is followed by Her2-enriched. Lehmann et al. (2016) made more advanced molecular classification with 4 different subtypes for TNBC by using gene expression profiles from 21 BC data sets from 587 cases. All subtypes show different heterogeneity of breast cancer. Breast cancer also shows intra-tumoral heterogeneity that causes drug resistance and increases the metastatic potential of the tumor due to the existence of different molecular subtypes in a single tumor. This situation is supported by the microenvironment and clonal evolution of the tumor and causes one of the biggest obstacles to personalized medicine.

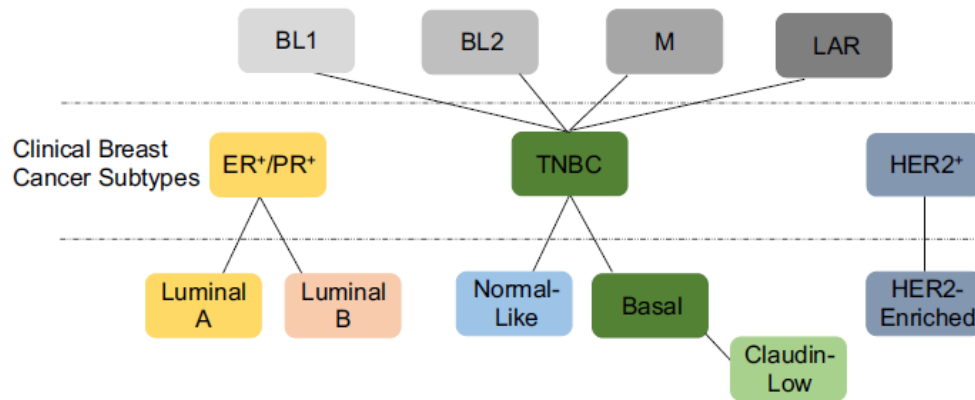


Figure2: Molecular classification of Breast Cancer. The clinical subtypes has mainly 3 groups ER+/PR+, TNBC, HER2+. The 5 different subtypes of Breast cancer as Luminal A, Luminal B, Normal-like TNBC, Basal-TNBC and Her2- Enriched Breast Cancer by PAM50. Basal-like 1 (BL1), basal-like 2 (BL2), mesenchymal (M) and luminal androgen receptor (LAR) are the molecular subtypes of TNBC (Turner et al. 2021).

2.2. BMP-1 in Tumor: Mechanisms, Findings, and Therapeutic Potential

Bone morphogenetic proteins(BMPs) are secreted cytokines and growth factors that are belong to the TGF- β super family and they play a role in cellular differentiation, apoptosis, proliferation, and mobility, especially in embryonic development and homeostatic balance of tissue (Ye et al. 2009, Davis et al. 2016). The wide spectrum of BMPs indicates that BMPs could be important agents in tumor biology. Also, the breast cancer studies on BMPs showed that disruption in the signaling pathway of BMPs affects tumorigenesis and metastatic processes (Zabkiewicz et al. 2017). In human breast cancers, BMP ligands are overexpressed (Pickup et al. 2015). Unlike other BMPs, Bone morphogenetic protein 1 (BMP-1) is not a growth factor, it is a zinc-dependent metalloproteinase that mainly takes a role in extracellular matrix (ECM) and collagen maturation. BMP-1 is one of the pro-collagen C-proteinase(PCP) enzymes and PCPs are primarily considered for their promoting ability of collagen remodeling and their pro-collagen processing abilities in some biological processes such as fibrosis and wound healing (Turtle & Ho, 2004). The developmental biology researches show that some proteinases do not only take role in destruction of structure but also re-establishment of structure and the regulation of signaling processes. Thus, the role of BMP-1 in tumor progression is not only limited to

remodeling of ECM. BMP-1 has also multidimensional effects such as growth factor activation and stromal dynamics reorganization. As a metalloproteinase in astacin family, BMP-1 has enzymatic feature to activate latent TGF- β by degrading ECM proteins such as thrombospondin-1 (TSP-1) and activation of signal pathways via Smad2/3 phosphorylation (Naba et al., 2014). As the ECM re-enriches, accessibility of substrates for BMP-1 has increased and the process creates a positive feedback mechanism as stated in Figure 3. This positive feedback mechanism facilitates invasion, EMT and establishment of the metastatic niche especially in TME. It also supports some other processes such as cancer stem cell phenotype, fibroblast activation, and immune escape. This model shows that BMP-1 is not only a ECM regulator but also it is a catalyst of tumor-promoting signaling networks (Sarras, 1996). For this reason, BMP-1 is an important actor as a therapeutic target in aggressive and metastatic type tumors.

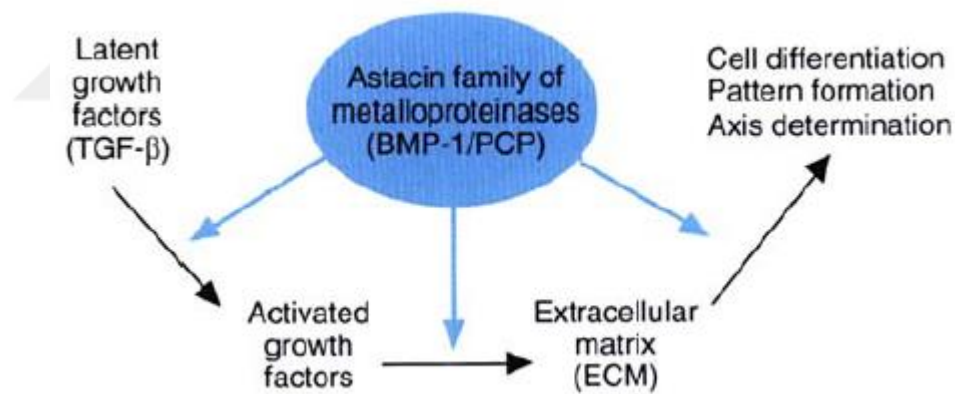


Figure3: The relationship between BMP-1 as astacin metalloproteinases, growth factors and developmental processes (Sarras, 1996).

Some studies have showed that BMP-1 expression is increased in various types of cancer and this increase is associated with poor prognosis including breast cancer (Zhou et al., 2022). For example, high expression of BMP-1 has been shown to be associated with tumor stroma remodeling and metastasis (Naba et al., 2014). In a recent study, Erin et al. (2025) showed that BMP-1 does not only activate TGF- β signaling but also supports proliferation of tumor, spheroid formation and chemoresistance in metastatic mammary carcinoma. Although *in vivo* studies that directly demonstrate the functional effects of BMP1 are limited, all these outcomes show that BMP-1 is potential biomarker for

prognostic prediction and a promising therapeutic target for oncotherapy. Unlike direct TGF- β inhibition, which may cause systemic side effects, targeting BMP-1 offers a more selective approach to modulate tumor-specific microenvironmental signaling.

BMP-1 promotes the formation of mature fibrillar collagens and ECM degradation by cleaving the C-terminal propeptides of procollagens. Early studies showed that dysfunction of BMP-1 activity is related to chronic kidney disease (Goff et al., 2015) and it causes pathogenicity in various cancers via ECM remodeling (Chen et al., 2024). BMP-1 activates the profibrotic cascade, but it does not directly regulate TGF β signaling. For this reason, selective inhibition of BMP-1 would be a promising strategy to moderate enormous fibrosis as well as inflammation in cancer.

2.3. UK-383367 molecule as a TGF- β Superfamily Inhibitor

UK-383367 is a small molecule that inhibits BMP-1 by chelating the catalytic zinc ion in the active site of BMP-1 selectively (Figure 4). UK gained attention due to its >10,000-fold selectivity over similar proteases such as matrix metalloproteinases (MMPs). The role of BMP-1 in collagen formation is suppressed, as well as the restructure of ECM, and fibrotic responses are limited (Fish et al., 2007). This inhibition indirectly modulates TGF β signaling because it prevents BMP-1 activity instead of directly targeting TGF β receptors so it offers a selective action mechanism (Bai et al., 2019).

UK was originally chosen for its dermal anti-scarring potential. Preclinical studies showed promising results on the evidence of the efficiency of UK-383367. Bai et al (2019) evaluated a decrease in profibrotic gene expression via TGF β stimulation, which was demonstrated in the *in vitro* experiment design with UK pre-treated renal tubular epithelial cells and fibroblast lines. In addition, they analyzed UK inhibition on BMP-1 and its direct effect on deposition of fibrotic markers in mouse models for chronic kidney disease. Also, some other studies showed that UK-383367 is also effective on metastatic cancer models *in vitro*. UK treatment reduces the expression of fibrotic markers and inhibits cell proliferation with colony formation while enhancing chemotherapeutic efficacy (Erin et al., 2025). In summary, UK-383367 is a potential selective BMP-1 inhibitor that disrupts

the enzymatic processing of procollagens and so diminishes the main profibrotic and tumorigenic signals. Also, the ability of the UK to indirectly modulate TGF β activation makes it a promising candidate for further therapeutic development targeting BMP-1 mediated diseases.

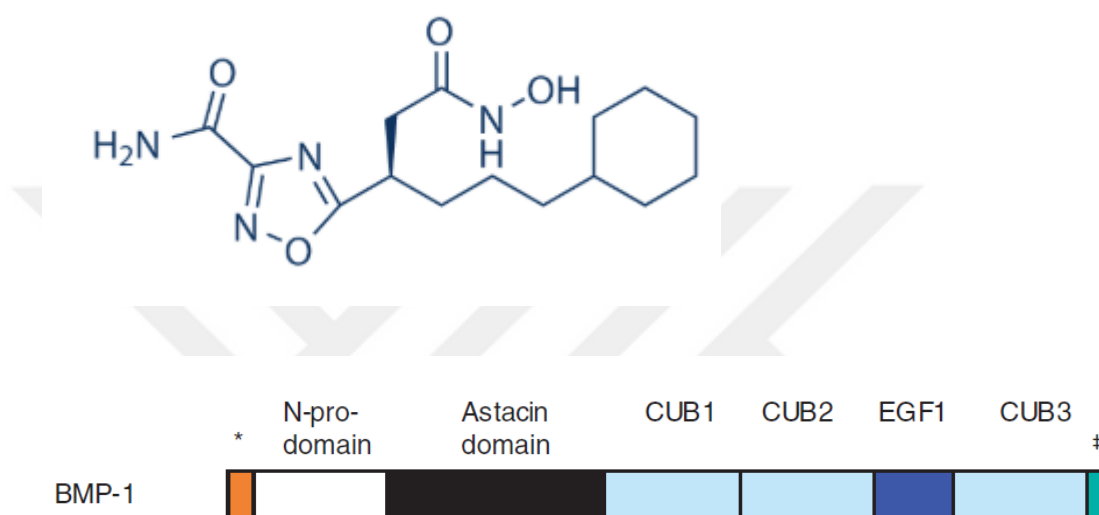


Figure 4: Molecular structures of UK-383367(A) and BMP-1 (B)

2.4. The Action Profile of Doxorubicin as a Common Chemotherapeutic Agent

Doxorubicin (DOX) is an anthracycline antibiotic that is originally made from the bacterium *Streptomyces peucetius* (Arcamone et al., 1969) and it is one of the most common chemotherapeutic agents in many different tumor types, including breast cancer. DOX is an important agent in adjuvant and neoadjuvant treatments because of its cytotoxic nature on proliferating tumor cells. Although DOX is a first-line drug in many cancers, cardiotoxicity and drug resistance are two main obstacles that are caused by DOX treatment (Al-Malky et al., 2019).

The antitumoral effects of DOX are supplied with cell-level multifunctional mechanisms. These mechanisms include DNA intercalation, topoisomerase-II inhibition, reactive oxygen species (ROS) production, triggering of apoptosis, senescence, autophagy, etc. (Kciuk et al., 2023).

The primary therapeutic effect of DOX is due to the intercalating ability of DOX into DNA, and so it prevents replication and transcription processes of rapidly dividing tumor cells. DOX can intercalate into the GC-rich region of the DNA double helix by forming hydrogen bonds, and so it disrupts the supercoil structure of DNA and mostly decreases the stability of the nucleosome in promoter regions. Thus, DNA replication and transcription processes are disrupted. Also, DNA-DOX covalent structure triggers the DNA damage response (DDR) pathway (Figure 5A).

Topoisomerases are important enzymes to keep DNA in the correct structure during replication and transcription (Kciuk et al., 2023). Topoisomerase-II is one of the main targets of DOX by causing topoisomerase trapping, which means topoisomerase-II cannot re-ligate of double-stranded breaks (DSBs) into DNA and causes lethal DNA damage in cancer cells. Thus, DSBs accumulate in the cell and cause they trigger DNA damage response pathways. Proteins associated with cell cycle arrest and apoptosis are involved in this process (Figure 5B & 5C).

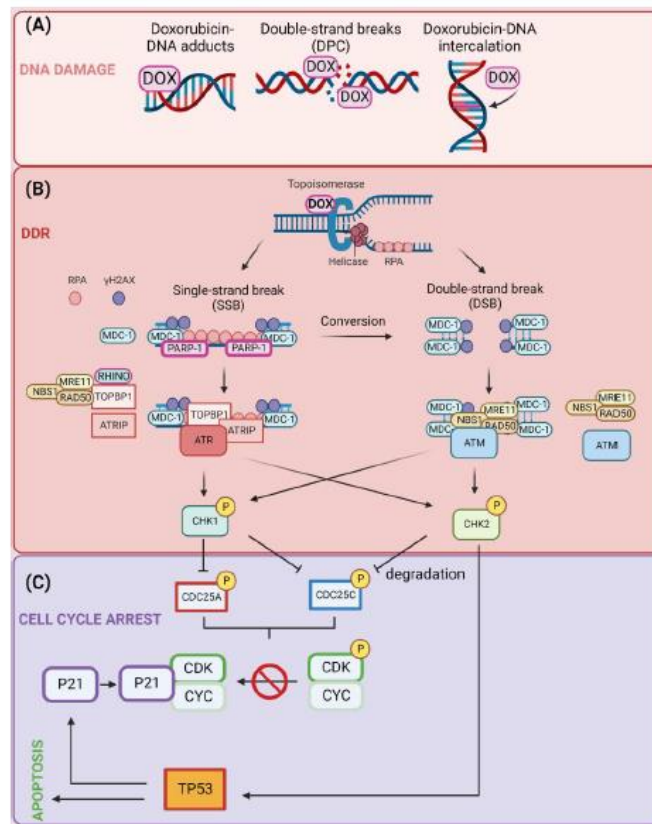


Figure 5: The summary of DNA damage mechanism and cellular response to Doxorubicin (DOX). Doxorubicin causes DNA damage by forming DNA adducts and double-strand breaks (A). In the damage response, ATR and ATM pathways are activated, the cell cycle is arrested via CHK1/CHK2 (B), and apoptosis is triggered via TP53 (C) (Kciuk et al., 2023).

DOX can also interact with cardiolipin in the inner mitochondrial membrane and then initiate reactive oxygen species (ROS) production (Kciuk et al., 2023). Excessive ROS production causes disruption of mitochondrial membrane via DNA and mitochondrial DNA damage and cytochrome c release into the cytosol, which causes apoptosis via caspase activation. This binding damages mitochondrial integrity and results in mitochondrial dysfunction. Also, excessive ROS causes oxidative damage to the macromolecules such as lipids, proteins, nucleic acids, and increases cytotoxic conditions. Additionally, DOX triggers immunogenic cell deaths due to the damage-associated molecular patterns that are released by dying tumor cells and activation of the immune system. This immune activation contributes to the antitumor activity of DOX through the recruitment of cytotoxic CD8⁺ T cells (Sritharan & Sivalingam, 2021).

In summary, DOX has multifaceted anticancer activity mechanisms, which are DNA intercalating, topoisomerase II inhibition, ROS-mediated oxidative stress, mitochondrial disruption, and immune system activation (Rawat et al., 2021). Although DOX has a wide range of anticancer activity, it also has significant limitations (Figure 6). One of which is dose-dependent cardiotoxicity that is caused by the generation of ROS and irreversible damage in mitochondria. The other side effect is myelosuppression that results in neutropenia and increases the risk of infection. Also, other dose-dependent side effects, such as gastrointestinal toxicities, affect the quality of patient life. DOX treatment can also develop chemoresistance that limits the long-term efficacy of DOX (Sritharan & Sivalingam, 2021). These mechanisms indicate that the efficiency of DOX decreases over time, and the need emerges for higher doses or alternative treatment strategies. It is also a critical clinical challenge to decide between effective tumor cell kill and severe systemic toxicity of DOX.

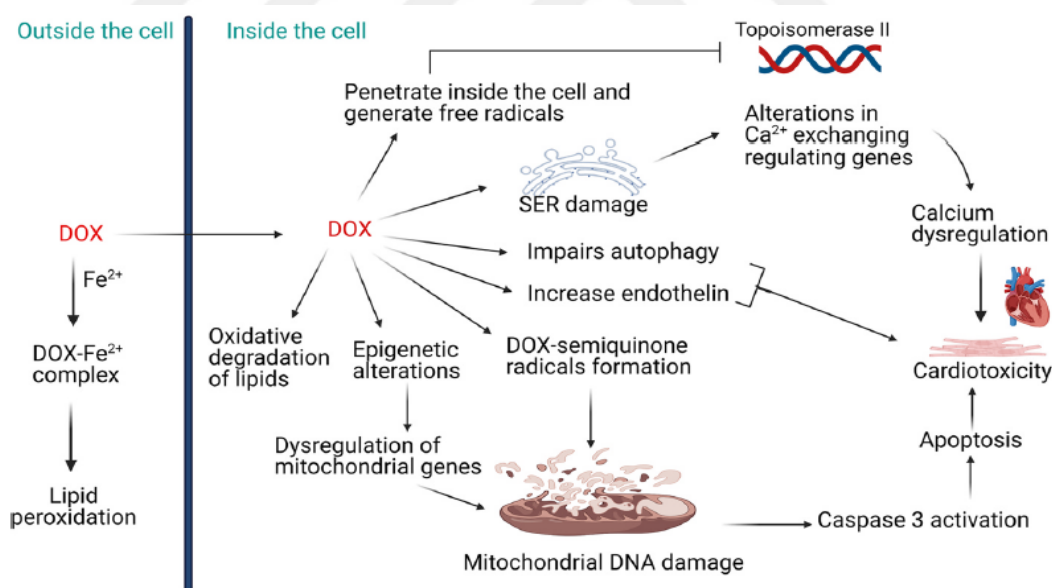


Figure6: The cardiotoxicity of Doxorubicin. This picture shows the different mechanisms of action of Dox-induced cardiotoxicity inside the cell and outside the cell (Rawat et al., 2021).

Combinational therapies by using lower doses of DOX and getting similar therapeutic efficacy are the focus of current oncological research due to the high toxicity of DOX. In this regard, flexible therapeutic applications with agents such as UK as an inhibitor of BMP-1 have high potential not only to control cytotoxic activity but also to prevent

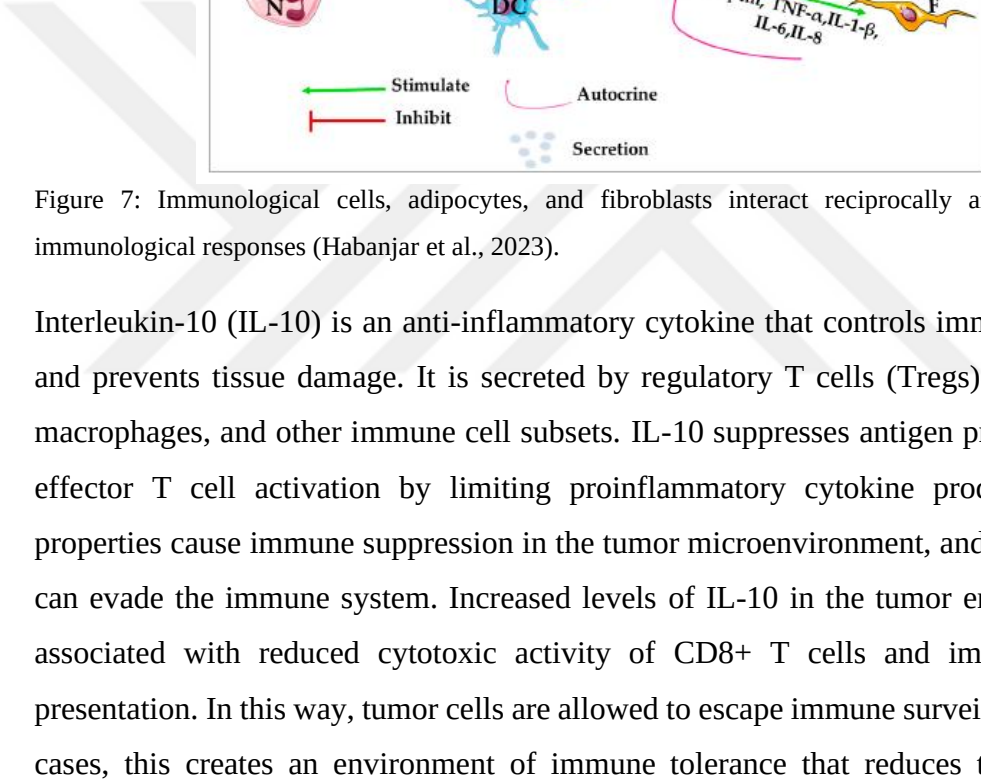
metastatic progression via remodeling the TME. As mentioned earlier, BMP-1 is critical for ECM remodeling and activation of TGF β signal activation and inhibition of BMP-1 contributes to the remodeling of tumor stroma. The selective inhibition of BMP-1 with UK treatment in combination with DOX may help suppress tumor invasion and fibrotic responses. These combinational approaches are promising strategies in terms of increasing therapeutic response with lower doses, and so decreasing toxicity.

2.5. Immunomodulators in Cancer

The tumor microenvironment is a dynamic structure consisting not only of tumor cells but also immune cells and the cytokines secreted by these cells. Various cytokines in this microenvironment play crucial roles in tumor growth, spread, and escape from the immune system. The IFN- γ , IL-6, IL-10, and TNF- α are the most investigated cytokines in cancer immunology, and they play critical roles in the tumor microenvironment. Each of these cytokines has specific biochemical features and expression patterns. Additionally, the effects of these cytokines can vary depending on the context and dose, and in some cases, they can exhibit both tumor-suppressive and tumor-promoting roles.

Interferon gamma (IFN- γ) is produced by natural killer (NK) cells, natural killer T (NKT) cells, CD4⁺ Th1 cells, and CD8⁺ cytotoxic T lymphocytes (CTLs); it activates macrophages, enhances antigen presentation by increasing major histocompatibility complex (MHC) expression, and facilitates recognition and destruction of tumor cells by supporting the Th1 response. However, long-term high IFN- γ expression may contribute to immune escape by increasing immunosuppressive molecules such as PD-L1 on tumor cells (Habanjar et al., 2023).

Interleukin-6 (IL-6) is a pleiotropic cytokine that has both pro-inflammatory and regenerative functions. IL-6 is secreted by monocytes, macrophages, stromal, and tumor cells, and promotes angiogenesis, EMT, and metastasis by increasing inflammation in the tumor microenvironment. IL-6 is particularly crucial for directing CD4⁺ T cells to become Th17 cells, and can also sustain chronic inflammation (Czajka-Francuz et al., 2021).



Interleukin-10 (IL-10) is an anti-inflammatory cytokine that controls immune responses and prevents tissue damage. It is secreted by regulatory T cells (Tregs), M2-polarized macrophages, and other immune cell subsets. IL-10 suppresses antigen presentation and effector T cell activation by limiting proinflammatory cytokine production. These properties cause immune suppression in the tumor microenvironment, and so tumor cells can evade the immune system. Increased levels of IL-10 in the tumor environment are associated with reduced cytotoxic activity of CD8⁺ T cells and impaired antigen presentation. In this way, tumor cells are allowed to escape immune surveillance. In some cases, this creates an environment of immune tolerance that reduces the efficacy of immunotherapeutic interventions (Accogli et al., 2021).

Tumor necrosis factor alpha (TNF- α) is a pro-inflammatory cytokine produced by various cells, primarily macrophages and T cells. At high doses, it can promote tumor destruction by inducing apoptosis in tumor cells. However, at low doses, TNF- α can promote tumor growth and metastasis by maintaining chronic inflammation. Chronic inflammation can promote tumor growth by activating NF- κ B signaling, inducing survival and proliferation genes, enhancing angiogenesis, and boosting matrix metalloproteinase (MMP) formation.

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Thus, TNF- α can also help tumors evade the immune system by attracting immunosuppressive cells (Najafi et al., 2018).

The interactions of these major cytokines with the many immune cell populations that are present inside the microenvironment of the tumor are a dynamic ecosystem in which lymphoid and myeloid cells, as well as stromal and vascular components, are involved. Further, the cumulative influence of these cytokines is more complicated.

Cytokines and immune cell populations in the tumor microenvironment control the immunological status and the progression or regression of tumors. Activated CD8⁺ T cells secrete IFN- γ , which eliminates tumor cells and improves antigen processing and presentation, so enhancing the immune response. In contrast, continuous IL-6 secretion by tumor cells and stromal factors stimulates Th17 cell expansion and enhances malignant cell viability and proliferation, hence influencing the immune response in favor of tumorigenesis. IL-10's anti-inflammatory properties, although advantageous in acute inflammation, may facilitate immune evasion inside the tumor microenvironment by inhibiting effector T cell activation and diminishing dendritic cell functionality. TNF- α , capable of inducing apoptosis at elevated concentrations while fostering a low-grade inflammatory milieu conducive to tumor proliferation at reduced levels, underscores the complexity of cytokine-mediated regulation in cancer.

3. MATERIALS & METHODS

3.1 Cell culture

***In Vitro* Proliferation of Metastatic Breast Cancer Cell Lines**

The 4TBM and 4TLM cell lines used in this study are derived from the 4THM and 4T1 breast cancer models respectively. 4T1 cells are a highly aggressive metastatic breast cancer line that spontaneously occurs in Balb-C mice. 4THM cells are obtained from heart metastases in mice that have been injected with 4T1 tumor cells. The 4TBM is prone to brain metastasis, while the 4TLM is prone to liver metastasis. Although both cell lines have high metastatic potential, they differ in terms of metastasis pattern and aggressiveness levels. Cell culture studies were performed in a sterile laminar air flow work cabinet. Cells stored in liquid nitrogen were thawed in a 37°C water bath and seeded in 10 mm cell culture petri dishes.

DMEM/F12 (Dulbecco's modified Eagle's minimal essential medium) by adding 5% fetal bovine serum (fbs), 1 mM sodium pyruvate, 0.02 mM non-essential amino acids, and 40 mg/ml gentamicin to the medium was used to create an environment for maintaining normal activities of 4TBM or 4TLM cell lines. The cells were incubated in 37°C incubator that contains 5% CO₂ and required humidity. Cell lines were monitored daily under an inverted microscope for viability, proliferation, and the presence of contamination. When cell density exceeded 80% in petri dishes, cells were passaged to proliferate.

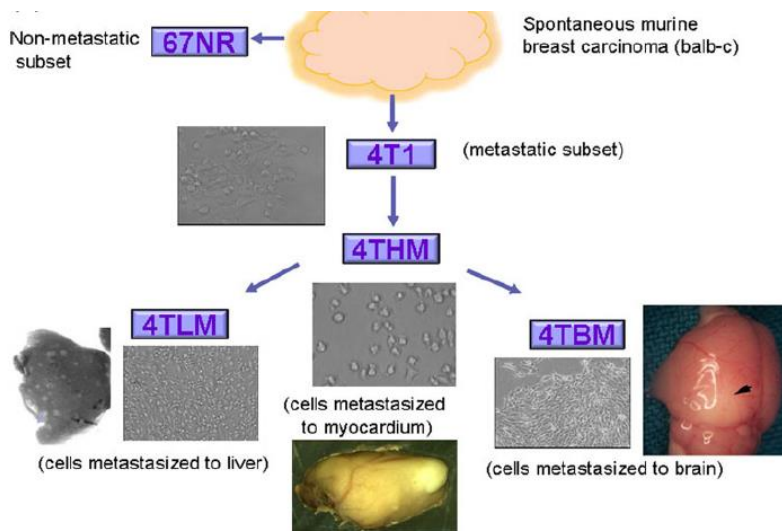


Figure 3.1: The 4TLM and 4TBM cell lines used in the study (Erin et al., 2013).

Cell Counting

The 4TLM or 4TBM cell lines are adherent to the petri dish surface. Cells are separated from the surface before counting. Firstly, the culture medium is removed, and then, 1ml of Trypsin-EDTA solution was added to the petri dish to remove cells from the surface. It is incubated for 3-5 minutes. Then, the fresh medium was added at a ratio of 1:3 to protect the cells from the enzymatic activity of Trypsin-EDTA. The suspension was transferred to 15 ml sterile centrifuge tubes, and it was centrifuged at 700 rpm for 3 minutes. The supernatant of the suspension was discarded and the resulting cell pellet was homogenized in 5 ml of fresh medium. The cell suspension was prepared by diluting it at a ratio of 1:10 and manual cell counting was performed using a Thoma slide (hemocytometer) under the microscope. After cell counting, the suspension was prepared with Hank's solution to contain 100.000 live cells for *in vivo* injection.

3.2 Metastatic Cancer Modeling with BALB/c Mouse Model and Treatment Protocol

Animal models

BALB/c female mice (8–10 weeks old), an inbred mouse strain with an albino phenotype and characterized immune responses, were used for the experimental tumor model. The BALB/c mouse model exhibits high compatibility with 4T1 breast cancer cells and is widely used for generating metastatic cancer models. Female Balb-c mice were obtained from Kobay company (Ankara) and brought to Akdeniz University Faculty of Medicine experimental animal unit until they were 8-10 weeks were used (8-10 weeks old).

Metastatic cancer modeling

Metastatic cancer cells (4TBM or 4TLM) were injected into the upper right breast tissue of the mice in 0.1 ml PBS at 100,000 cells/mouse. Palpable tumors measuring 2-3 mm appears 6 days after the injection. Similar experiments are frequently performed in Prof. Erin laboratory and the results of these experiments have been published (Erin et al. 2019; Erin et al. 2014).

After tumor injection, mice were randomly divided into four groups: untreated control group, BMP1 inhibitor UK-383367 only group, Doxorubicin only group, and UK-383367 & Doxorubicin combination group. By this grouping, the effects of each agent alone and in combination were examined, as well as to evaluate potential synergistic or antagonistic interactions (Figure 3.2.).

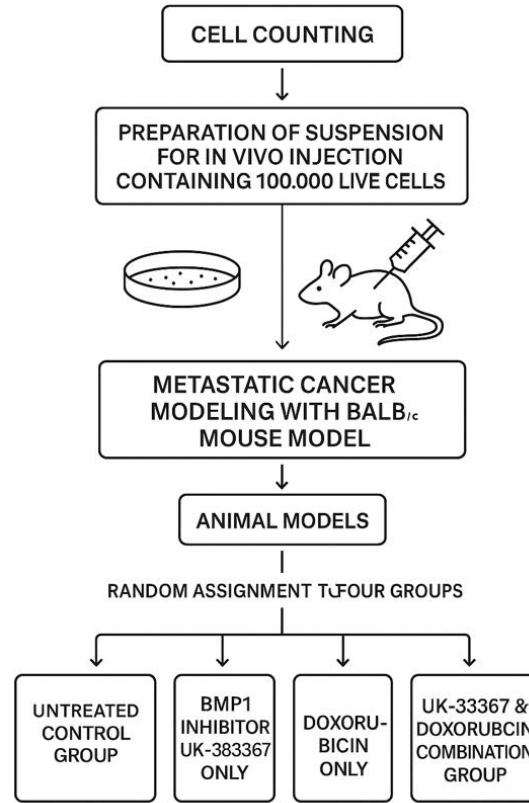


Figure 3.2: *In vivo* experiment plan for metastatic cancer modeling. First, cell counting was performed, then injection suspension containing 100,000 live cells was prepared. A metastatic cancer model was created using BALB/c mouse model. After injection, animals were randomly divided into four groups as control, UK-only, DOX and UK+DOX combination groups.

Monitoring of Tumor Progression

The animals were observed every day via the palpation method after tumor injection. Tumor formation was observed approximately 5-6 days after injection, and tumor progression was measured by using a caliper and recorded weekly. The tumor volume of the animals was calculated with the formula below. The health, activity, and general behavior patterns of animals were tracked daily.

$$\text{Tumor volume (mm}^3\text{)} = (\text{length} \times (\text{width})^2) / 2$$

Treatment Regimen and Drug Delivery

The treatment protocol was started after 5 days of tumor injection. UK-383367 and DOX in saline solution were applied via intraperitoneal administration to the groups in determined dosages. DMSO in saline solution was applied to the Control Group during the treatment protocol. Treatments are prepared freshly in a sterile environment. The different dosages of UK-383367 in different metastatic breast cancer models (4TBM & 4TLM) were analyzed in different experimental setups. To test the standalone and combination effects of UK-383367 treatment on tumor progression and metastatic development were aimed with this approach. The dosage determination of every experiment was decided with the outcome of the previous experiment. The chemotherapeutic agent Doxorubicin (DOX) was applied weekly via intraperitoneal administration to both DOX monotherapy and UK-DOX combination groups.

End-Point Sample Collection

Following tumor injection, on days 25–27, when the animals in the control group began to die, mice in all groups were sacrificed under anesthesia using ketamine by ethical termination criteria. After sacrifice, systematic dissection was performed to remove the tumor tissue, lungs, liver, spleen, and lymph nodes. For immunological analyses, spleen and regional lymph nodes were taken into 15 ml sterile Falcon tubes containing RPMI medium separately and transferred on ice. Cell suspensions obtained from these tissues were used in flow cytometry and mixed leukocyte culture analyses. For histopathological examination, the tumor, liver, and the rest of the spleen were fixed in 10% neutral formalin solution, and the lungs were fixed in Bouin's fixative solution for special staining procedures for metastases counting. All samples were stored under appropriate conditions to evaluate the effects of treatment groups on tumor progression and immune response. The weight of the tumors in every group was measured to compare tumor progression.

3.3. The Mixed Leukocyte Culture Analyses

The mixed leukocyte culture was prepared in a sterilized laminar cabinet. RPMI medium was removed from 15 ml falcon tubes that had the spleen and lymph nodes from animals. Then, the spleen, and lymph nodes were washed twice with double gentamicin PBS solution. Then, the spleen and lymph nodes were crushed separately in a medium containing 1x PBS double gentamycin, passed through a mesh, and turned into a cell

suspension and collected in 15 ml test tubes. Then, the washing step was started. For this, 1x PBS double gentamycin was added and after pipetting, the cells were sedimented by centrifugation at 1000 rpm for 3 minutes and the supernatant was discarded. This process was repeated 2 times. After washing, the groups were diluted with 5-13 ml RPMI medium according to the density of the cell pellet. Spleen and lymph cells from each group were counted with a Thoma slide. The counted cells were calculated to be a total of 4-5 million cells per well.

The suspended spleen and lymph cells of the mice in each group were combined among themselves in the determined ratios and planted in 48-well petri dishes as indicated in Figure 3.3.

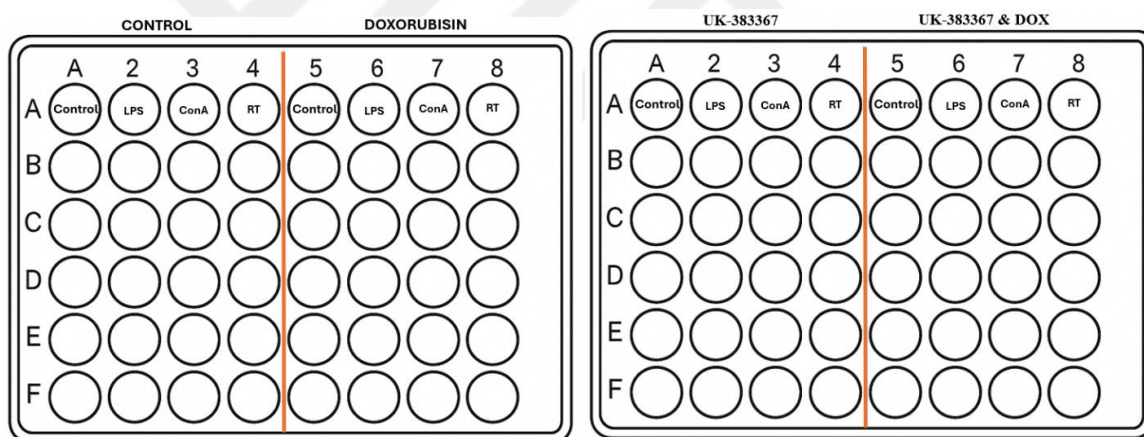


Figure 3.3.: The mixed leukocyte cell culture plate assay plan. The cell suspensions of lymph nodes and spleen of 4 groups were added to the wells that contain cells which are non-stimulated cells (control), LPS-stimulated cells (LPS), conA-stimulated cells (ConA) and irradiated 4TBM or 4TLM containing cells.

Immune cells were stimulated with Concanavalin-A (ConA), lipopolysaccharide (LPS), and 35 gray irradiated tumor cells (50,000 cells/well) (each group was replicated for 6 and the total volume was completed to 600 μ l with RPMI medium). TNF- α measurement was performed from supernatants collected 16 hours after stimulation (150 μ l). The supernatants (600 μ l) 40 hours after stimulation were collected for IL-6, IFN- γ , and IL-10 cytokines measurements.

3.4. ELISA

For quantitative analysis of cytokine levels in culture supernatants, ELISA kits from BioLegend were used in accordance with the manufacturer's instructions as described below. Samples were prepared in appropriate dilutions and added to microtiter plates, and absorbance values were measured at specific wavelengths and analyzed.

TNF- α Measurement

The required solutions are prepared as below:

Wash Buffer: %0,05 Tween20 in PBS

Blocking buffer: %1 BSA in 1XPBS

Assay Diluent: %1 BSA in 1xPBS

Coating Buffer: 8,4gr. NaHCO₃, 3,56GR. Na₂CO₃; to1L; pH 9,5.

Capture Ab: 1/500 dilution in coating buffer

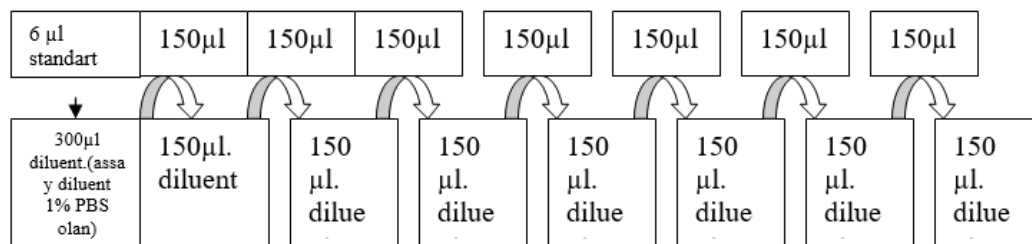
Detection Ab: 1/500 dilution in assay diluent

Enzyme, Avidin HRP: 1/15000 dilution in assay diluent

Substrate: TMB substrate A and TMB substrate B

Stop solution: 2N H₂SO₄

Standards: Dilute standard from the kit with 200 μ L assay diluent (std1=1000pg/ml).



According to the manufacturer's protocol, microtiter plates were first incubated at 4°C overnight by adding 100 μ L of capture antibody to each well. Then, plates were washed four times with wash buffer, and 200 μ L of blocking buffer was added to each well and

incubated for 1 hour at room temperature. Washing steps were repeated at each step to prevent the plates from drying out. Then, standards (serial dilution) and samples were added to each well as 100 µl, and plates were incubated at room temperature for 2 hours. Following this washing step, detection antibody (1:500) diluted in assay diluent was added as 100 µl to each well and incubated for 1.5 hours. After the fourth wash, HRP conjugate (1:15,000 dilution) was added as 100 µl and incubated for 30 minutes. After the fifth wash, TMB substrate (prepared by mixing equal volumes of A and B solutions) was added as 100 µl and color development was monitored for approximately 30 minutes at room temperature in the dark. The 100 µl of stop solution was added to each well to terminate, and the absorbance values were measured at 450–570 nm wavelengths.

IFN- γ Measurement

The required solutions are prepared as below for IFN- γ :

Wash Buffer: %0,05 Tween20 in PBS

Blocking buffer: %10 FBS in PBS

Assay Diluent: %1 BSA in PBS

Coating Buffer: 8,4gr. NaHCO₃, 3,56GR. Na₂CO₃; to1L; pH to9,5

Capture Ab: 1/300 dilution in coating buffer

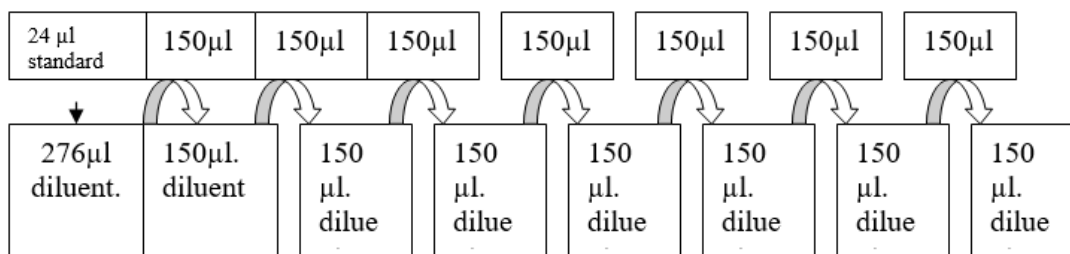
Detection Ab: 1/300 dilution in assay diluent

Enzyme, Avidin HRP: 1/5000 dilution in assay diluent.

Substrate: TMB substrate A and TMB substrate B

Stop solution: 2N H₂SO₄

Standard: Dilute the standard from the kit with 200 µL assay diluent. Std1=2000pg/ml



100 µl of capture antibody was added to each well of the microplate, and the plates were incubated at +4°C overnight. The next day, the plates were washed three times with wash buffer, and then 300 µl of blocking buffer was added to each well and incubated for 1 hour at room temperature. After blocking, the plates were washed two more times, standards and samples were added in 100 µl volumes, and the plates were incubated for 2 hours at room temperature. After four washing steps, 100 µl of tracer (detection) antibody was added to each well and incubated for 1.5 hours. After the following four washing steps, 100 µl of HRP conjugate was applied, and the plates were incubated for 30 minutes at room temperature. After the fifth washing step, 100 µl of TMB substrate was added, and color development was monitored for approximately 30 minutes at room temperature in the dark. The 100 µl of stop solution was added to each well to terminate, and the absorbance values were measured at 450–570 nm wavelengths.

IL-6 Measurement

The required solutions are prepared as below for IL-6:

Wash Buffer: %0,05 Tween20 in PBS

Blocking buffer: %10 FBS in PBS

Assay Diluent: %10 FBS in PBS

Coating Buffer: 8,4gr. NaHCO₃, 3,56GR. Na₂CO₃ to1L; pH to9,5

Capture Ab: 1/1000 dilution in coating buffer

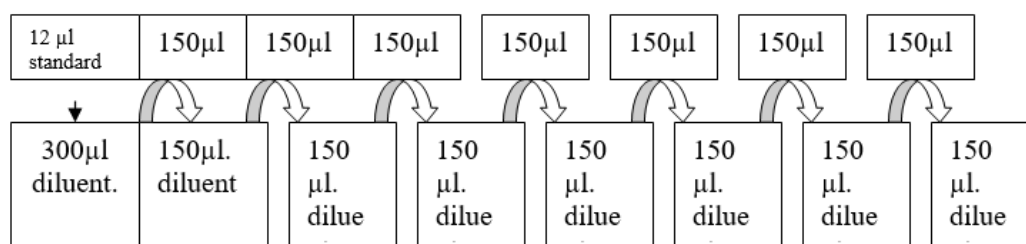
Detection Ab: 1/1000 dilution in assay diluent

Enzyme, Avidin HRP: 1/5000 dilution in assay diluent

Substrate: TMB substrate A and TMB substrate B

Stop solution: 2N H₂SO₄

Standard: Dilute the standard from the kit with 200 µL assay diluent. std1=2000pg/ml



The 100 µl of capture antibody of IL-6 was added to each well of the microplate, and the plates were incubated at +4°C overnight. The next day, the plates were washed three times with wash buffer, then 200 µl of blocking buffer was added to each well and incubated for 1 hour at room temperature. After blocking, the plates were washed two more times, standards and samples were added in 100 µl volumes, and incubated for 2 hours at room temperature. The plates were then washed three times, 100 µl of tracer (detection) antibody was applied, and incubated for 1.5 hours. After the next three washing steps, 100 µl of HRP solution was added to each well, and the plates were incubated for 30 minutes at room temperature. After the final four washes, 100 µl of TMB substrate was added, and the reaction was left at room temperature in the dark for approximately 30 minutes. The 100 µl of stop solution was added to each well to terminate, and the absorbance values were measured at 450–570 nm wavelengths.

IL-10 Measurement

The required solutions are prepared as below for IL-10:

Wash Buffer: %0,05 Tween20 in PBS

Blocking buffer: %10 FBS in PBS

Assay Diluent: %10 FBS in PBS

Coating Buffer: PBS

Capture Ab: 1/500 dilution in coating buffer

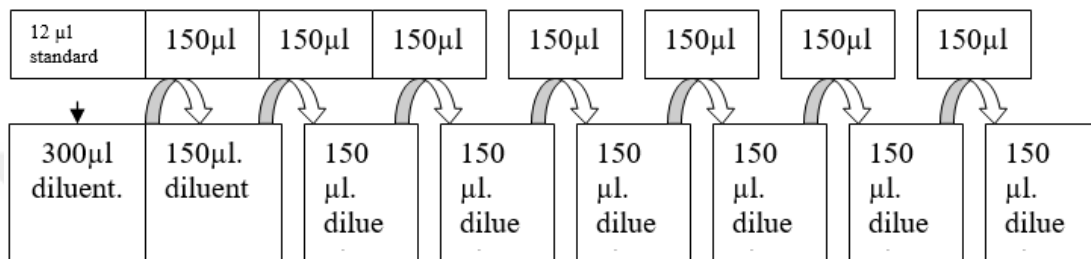
Detection Ab: 1/500 dilution in assay diluent

Enzyme, Avidin HRP: 1/5000 dilution in assay diluent

Substrate: TMB substrate A and TMB substrate B

Stop solution: 2N H₂SO₄

Standard: Dilute the standard from the kit with 200 μ L assay diluent. std1=2000pg/ml



The 100 μ l of capture antibody was added to each well of the microplate, and the plates were incubated at +4°C overnight. The next day, the plates were washed four times with wash buffer, and then 200 μ l of blocking buffer was added to each well and incubated for 2 hours at room temperature. After blocking, the plates were washed four times again, standards and samples were added in 100 μ l volumes, and incubated for 2 hours at room temperature. Following this, after four washing steps, 100 μ l of tracer (detection) antibody was applied, and the plates were incubated for 1.5 hours at room temperature. After the fourth wash, 100 μ l of HRP conjugate was added and incubated for 30 minutes. After the final five washes, 100 μ l of TMB substrate was added to each well, and color development was observed for approximately 30 minutes at room temperature in the dark. The 100 μ l of stop solution was added to each well to terminate, and the absorbance values were measured at 450–570 nm wavelengths.

3.5. Flow Cytometry

For flow cytometry, 50 μ l of cell suspension was taken from spleen samples, and 100 μ l of cell suspension was taken from lymph nodes, and transferred to analysis tubes.

In the flow cytometry analysis, two separate surface staining panels were applied to characterize immune cell populations. In the first panel, T cell subpopulations were targeted, and CD3 (FITC), CD4 (PerCP), and CD8 (PE) antibodies were used for this purpose. CD3⁺CD4⁺ cells were defined as helper T cells, and CD3⁺CD8⁺ cells were

defined as cytotoxic T cells. In the second panel, suppressor myeloid cells (MDSC) were analyzed; in this panel, CD11b (PerCP) and GR-1 (PE) antibodies were used together, and CD11b⁺GR-1⁺ cells were evaluated as MDSC. 3 µl of each antibody was added to the cell suspensions, and the samples were incubated at room temperature in the dark for 30 minutes. After incubation, the samples were subjected to washing steps and fixed and prepared for analysis. Fluorochrome combinations specific to target cell populations were used in the selection of antibodies; channel planning was performed in line with FITC (green), PE (red) and PerCP (yellow) filters.

All analyses were performed using the Cube6 flow cytometry device. FL1 (FITC) for CD3, FL2 (PE) for CD8 or GR-1 and FL3 (PerCP) for CD4 or CD11b channels are used. Using forward scatter (FSC) and side scatter (SSC) parameters, debris and dead cells were excluded, and gating was applied for the total cell population that contains only live cells. Subpopulation analyses were performed within this gate. All data were analyzed using VenturiOne software, and percentage distributions, histograms, and scatter plots were obtained through this software.

3.6. Tissue Processing and Histopathological Analysis for Metastases Counting

The Macroscopic Evaluation of Lung Tissues

In the first step of histopathological analysis, the lung tissues fixed in Bouin's solution were evaluated. The Bouin's solution provided a yellow contrast, and made tumor nodules on the surface more visible. The number of metastatic nodules in each lung lobe was counted and recorded. The macroscopic evaluation was used to compare the lung metastasis burden between the groups.

The Tissue Processing of Tumor, Lung, and Liver

The lung tissues were washed several times after tumor counting to remove the Bouin's solution. After that, the tumor, lung, spleen, and liver tissues were fixed in 10% neutral buffered formalin for 24–48 hours at room temperature. Following fixation, tissues were examined with alcohol washing steps to remove water inside the tissues for long-term storage. Briefly, samples were dehydrated through a graded ethanol series (70%, 80%, 96%, and 100%). Then, the samples were cleared in xylene and subsequently infiltrated with melted paraffin wax. After embedding in paraffin blocks, liver tissues were sectioned

at a thickness of 4–5 μm using a microtome. Sections were mounted on glass slides for subsequent histological staining and microscopic analysis.

Hematoxylin–Eosin (H&E) Staining Protocol

Tissue sections with a microtome of 4–5 μm thickness were taken from the liver paraffin blocks, placed on a slide, and incubated in an oven (37°C) overnight. Staining was performed based on the standard H&E protocol with the following steps:

Deparaffinization and Dehydration

The slides were kept in xylene for 15 minutes to remove paraffin. They were passed through alcohol series 100%, 90%, 80%, 70% ethanol solutions for 5 minutes each to remove xylene from the tissues. Then, rehydration was completed by keeping in distilled water for 5 minutes.

Staining

The sections were kept in a diluted hematoxylin with 50% distilled water solution for 30–45 seconds using a basket. After staining, the sections were washed under tap water for 2–3 minutes. Then, they were dipped into 1% Eosin solution 5 times using the dip-and-remove method. They were washed briefly in tap water again, then kept in distilled water for 1 minute.

Dehydration and Clarification

The sections were passed through 70%, 80%, 90% and 100% ethanol series for one minute each for dehydration. 100% ethanol for 3 minutes, and finally xylene for 10 minutes, were applied to ensure clarification.

Mounting

The mounting (closing) solution was dropped onto the slide, and the coverslip was carefully placed to prevent air bubbles.

Histological Evaluation and Quantitative Analysis

The 4–5 different sections were taken from the liver tissues. Tumor areas were randomly selected from each of these sections, and a total of 20 different areas were evaluated for the presence of metastatic lesions. 10x resolution scanned slide images were analyzed by using the ImageJ analysis program. During the evaluation process, lesions were marked according to morphological criteria as described in our previous studies (Erin et al., 2014).

The number of metastatic foci in each area was recorded, and comparisons between groups were statistically evaluated.

3.5. Statistical Analysis

In the comparison of data belonging to four groups (Control, UK, DOX, UK+DOX), one-way analysis of variance (One-Way ANOVA) was used to determine the general group effect. In cases where a significant difference was detected as a result of ANOVA, post-hoc tests (Tukey or Games-Howell) were applied for pairwise comparisons between groups. In cases that did not conform to normal distribution or where group variances were not equal, ANOVA with Welch correction was preferred. In pairwise group comparisons, an independent sample t-test or, when necessary, a t-test with Welch correction was used. The obtained data were expressed as mean standard error, and $p < 0.05$ was considered statistically significant.

4. RESULTS

4.1. Preliminary Evaluation of Dose-Response and Potential Toxic Effects of UK-383367 in an *In vivo* Experimental Model:

This experimental setup was planned to determine the applicable dose of UK *in vivo* and preliminarily evaluate its dose-dependent toxic effects. Three groups, Control, UK 1 mg/kg, and UK 5 mg/kg were created with 4TLM injections. The treatment was given daily and intraperitoneally. Tumor volumes were measured weekly, and the tumor weight was recorded at the end of the experiment. The mixed leukocyte cultures were prepared from spleens and lymph nodes, and subsequently, cytokine levels were measured using the ELISA method; lung and liver metastasis were calculated as described in the Methods section.

Tumor volumes and final tumor weights did not differ significantly among the Control, UK 1 mg/kg, and UK 5 mg/kg groups. However, both UK-treated groups showed a significant reduction in the number of macroscopic lung metastases. In contrast, the UK 5 mg/kg group showed a significant increase in liver metastasis area as determined by H&E staining. ELISA results from mixed leukocyte cultures showed that the UK 1 mg/kg group exhibited a significant increase in IFN- γ secretion both in LPS- and RT-stimulated cells. A detailed interpretation of these findings is presented in the following section.

Evaluation of Tumor Volume, Tumor Weight, Together with Lung and Liver Metastasis

The effects of different doses of UK-383367 on *in vivo* tumor progression, tumor volume, tumor weight, and lung and liver metastases were examined. Applications were performed intraperitoneally in the control, 1 mg/kg, and 5 mg/kg UK-383367 groups.

As seen in Figure 4.1, a significant decrease in tumor volume was not observed in the treatment groups. When tumor weights were examined (Figure 4.1. B), although there was no significant difference between the groups, a slight decrease was noted in the high-dose UK (5 mg/kg) group. When evaluated in terms of metastatic spread, the number of nodules in the lungs decreased significantly in both the low- and high-dose UK groups compared

to the control group (Figure 4.1. C). Liver metastasis was significantly increased in the UK 5 mg/kg group (Figure 4.1. D); H&E staining images of liver tissues are presented in Figure 4.2.



Figure 4.1. Evaluation of Tumor Volume, Tumor Weight, Together with Lung and Liver Metastasis. The effects on tumor volume (A), tumor weight (B), lung (C), and liver metastasis(D) in the control and UK-383367 treatment groups are shown in the Figure. *p < 0.05 vs Control, data are mean ± SE; Welch’s t-test

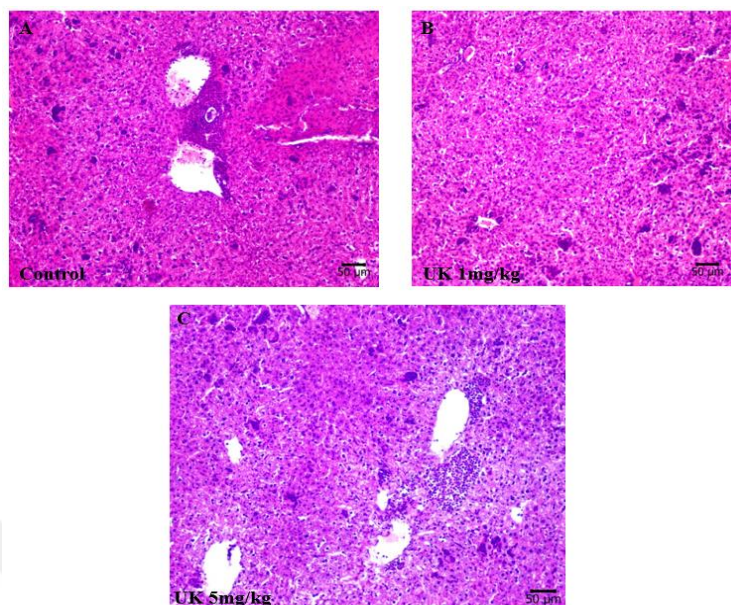


Figure 4.2. H&E staining of liver tissues from the control (A), UK 1 mg/kg (B) and UK 5 mg/kg (C) groups. A decrease in tumor cell density is observed in the 1 mg/kg UK applied group. (Magnification: 10x, scale bar: 50 μ m).

Effect of Different Doses of UK-383367 on IFN- γ Release in ConA-Stimulated Cells

IFN- γ cytokine levels were measured in mixed leukocyte cultures stimulated with ConA. A significant increase in IFN- γ secretion was observed in both UK treatment groups compared to the control group. The highest mean IFN- γ level was detected in the UK 1 mg/kg group. A significant increase was also found in the UK 5 mg/kg group compared to the control group, but it remained at a lower level compared to the UK 1 mg/kg level.

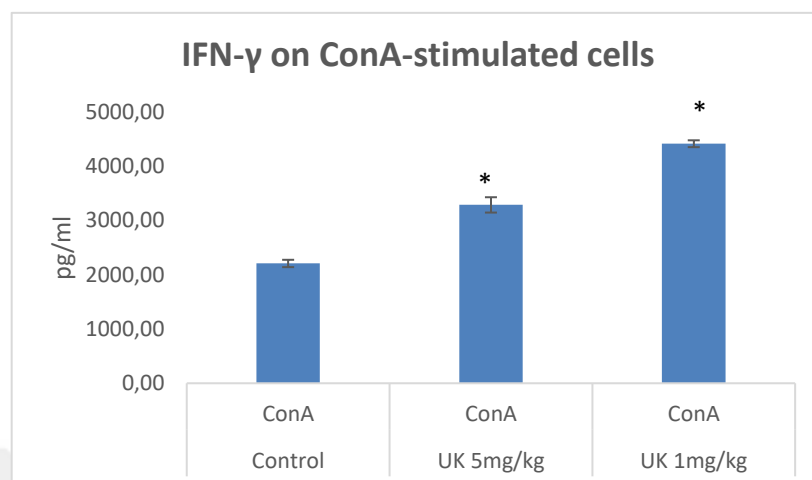


Figure 4.3. The effect of different doses of UK-383367 on IFN- γ release in ConA-stimulated cells. * $p < 0.05$ vs Control, data are mean $\pm$ SE; Welch's t-test

Effect of Different Doses of UK-383367 on IFN- γ and IL-6 Release in LPS-Stimulated Cells

The effects of proinflammatory cytokine response in LPS-stimulated cells for the 3 groups were evaluated. In Figure 4.4, the highest IFN- γ release was detected in the 1 mg/kg UK group, and this increase was found to be statistically significant ($p < 0.05$). In the 5 mg/kg UK group, IFN- γ levels slightly decreased compared to the control group, but did not reach the level of statistical significance.

When the IL-6 results were evaluated in Figure 4.5, it was observed that both doses of UK administration significantly increased IL-6 release ($p < 0.05$); however, the most significant increase was detected in the 1 mg/kg UK group. These findings suggest that UK-383367 may induce a more potent proinflammatory cytokine response in LPS-stimulated cells when administered at low doses.

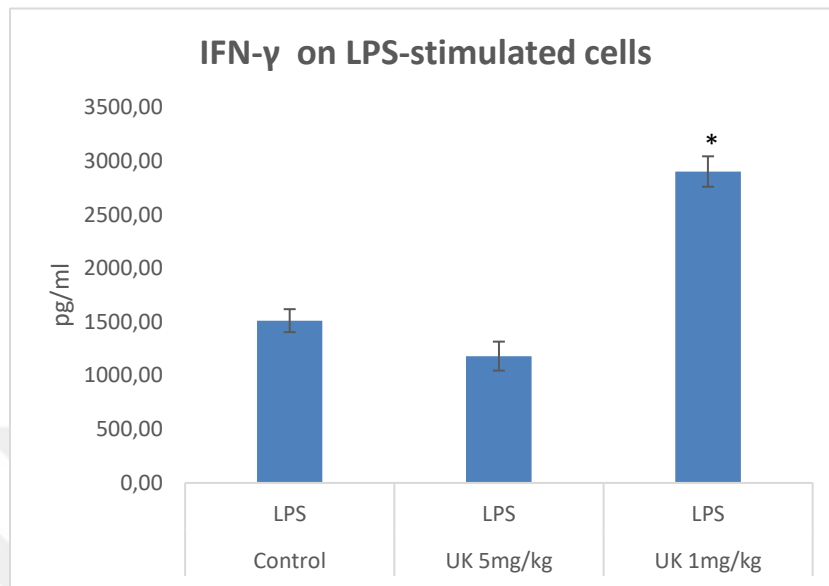


Figure 4.4. The effect of different doses of UK-383367 on IFN- γ release in LPS-stimulated cells. * $p < 0.05$ vs Control, data are mean $\pm$ SE; Welch's t-test

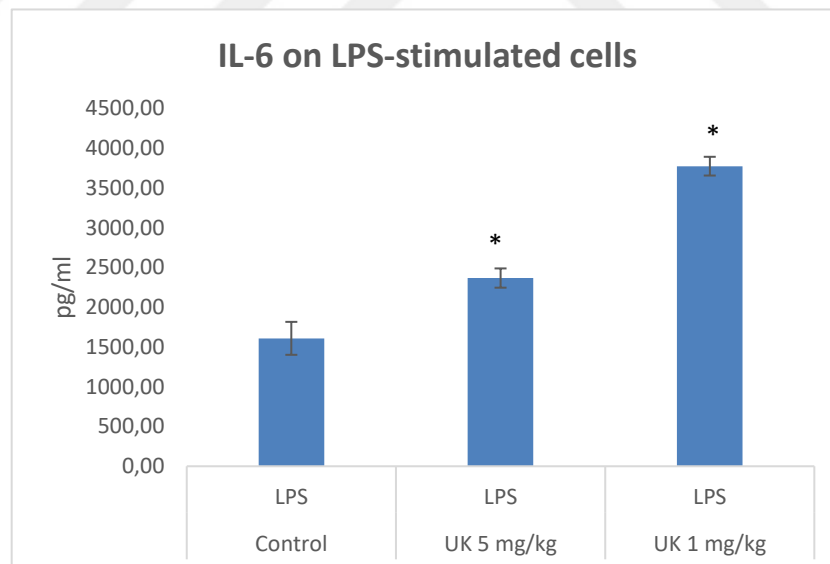


Figure 4.5. The effect of different doses of UK-383367 on IL-6 release in LPS-stimulated cells. * $p < 0.05$ vs Control, data are mean $\pm$ SE; Welch's t-test

Effect of Different Doses of UK-383367 on IFN- γ Release in Radiotherapy (RT)-Stimulated Cells

The UK 1mg/kg group gave a significant increase in IFN- γ in cells stimulated with radiotherapy. The low-dose UK administration is suggested to have a potential synergistic effect with radiotherapy and supporting the T cell-mediated immune response.

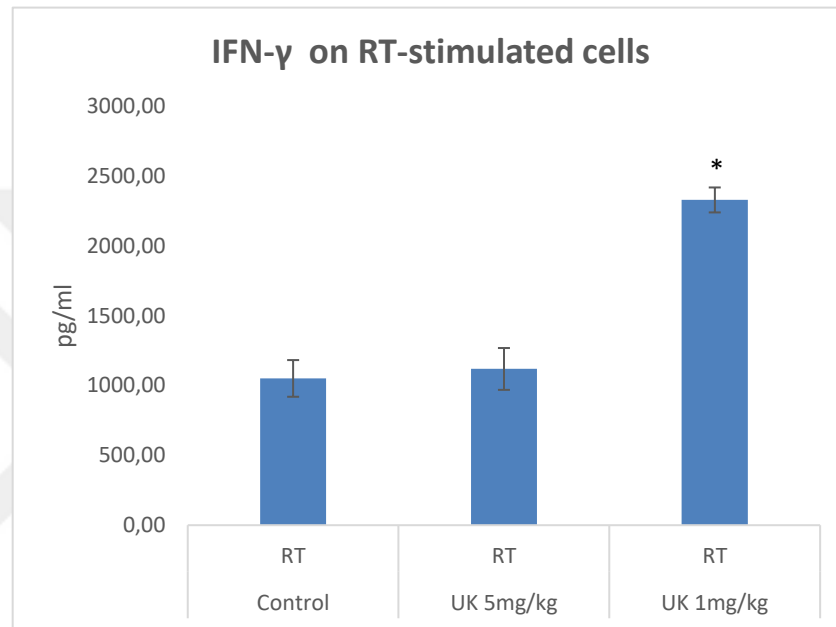


Figure 4.6. The effect of different doses of UK-383367 on IFN- γ release in radiotherapy-stimulated cells. * $p < 0.05$ vs Control, data are mean $\pm$ SE; Welch's t-test

4.2. Dose-Dependent Effects of UK-383367 alone and in combination with DOX on Cytokine Release with Different Stimuli on 4TBM injected mice model:

This experimental setup was planned to determine the applicable dose of UK-383367 treatment *in vivo* and to preliminary evaluate possible dose-dependent toxic effects in an less aggressive tumor cell line. Four experimental groups, each containing 7 mice, were formed: Control, UK, DOX and UK+DOX combination group. In order to create a breast cancer model, tumor injections were performed using the 4TBM cell line. The treatments were administered at a dose of 2.5 mg/kg intraperitoneally, every other day. In the DOX and combination groups, DOX was administered weekly at decreasing doses of 5 mg/kg, 2.5 mg/kg and 1.25 mg/kg, respectively. In the control group, physiological serum containing DMSO was administered intraperitoneally. Tumor volumes were measured weekly in all groups and tumor weights were recorded at the end of the experiment. At

the end of the experiment, mixed leukocyte cultures were prepared with cell suspensions obtained from spleen and lymph nodes and cytokine levels were analyzed by ELISA method. Lung metastases were evaluated by macroscopic observation and liver metastases were detected and counted by H&E staining method.

Evaluation of Tumor Volume, Tumor Weight, Together with Lung and Liver Metastasis on 4TBM mice models

In the *in vivo* tumor model created with the 4TBM cell line, the effects of the applied treatment groups on tumor development and metastasis were evaluated in Figure 4.7. While tumor volume increased significantly in the control group over the weeks, especially the UK-383367 + DOX combination group provided a significant level of suppression in tumor volume (Figure 4.7.A). Tumor weight was detected at the lowest level in the UK+DOX combination group, followed by the DOX and UK-only groups (Figure 4.7.B). Lung metastasis was significantly reduced with DOX and UK treatments, and especially the metastatic burden was at the lowest level in the DOX group (* $p < 0.05$) (Figure 4.7.C). Liver metastasis was significantly suppressed in all treatment groups compared to the control group (* $p < 0.05$) (Figure 4.7.D). H&E staining of liver tissue sections showed the minimum metastatic spread in UK-only group. All treatment groups showed a decrease when compared to control group (* $p < 0.05$) (Figure 4.8).

In the 4TBM model, complete tumor regression was not observed in any of the control group animals (0/7). In the UK-383367 and DOX monotherapy groups, one animal in each group (1/7) exhibited complete tumor disappearance. In the combination treatment group (UK+DOX), complete regression was observed in one animal, and four additional mice showed substantial tumor shrinkage, indicating enhanced therapeutic efficacy in the combination group.

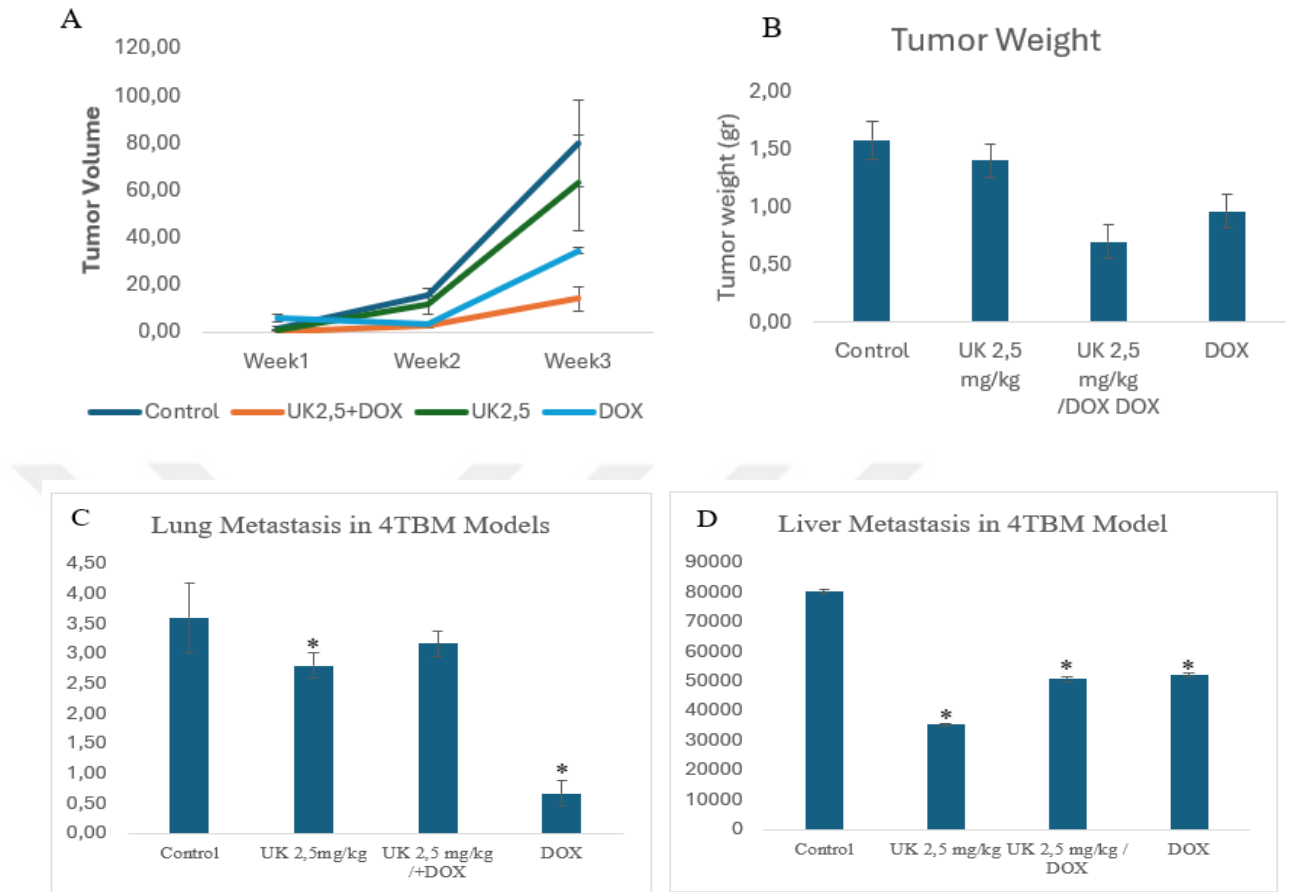


Figure 4.7. Effects of UK-383367 and/or Doxorubicin (DOX) treatment on 4TBM tumor model. (A) Tumor volume was measured weekly and a significant decrease was observed in the combined treatment group. (B) Tumor weights measured at the end of the experiment were found to be the lowest in the UK+DOX combination group. (C) Lung metastasis was evaluated macroscopically and a significant decrease was observed especially in the DOX-only group. (D) Liver metastasis was counted by H&E staining and a significant decrease was detected in the treatment groups compared to the control group. Data are presented as mean $\pm$ SD. * $p < 0.05$, significant difference compared to the control group. (N=7)

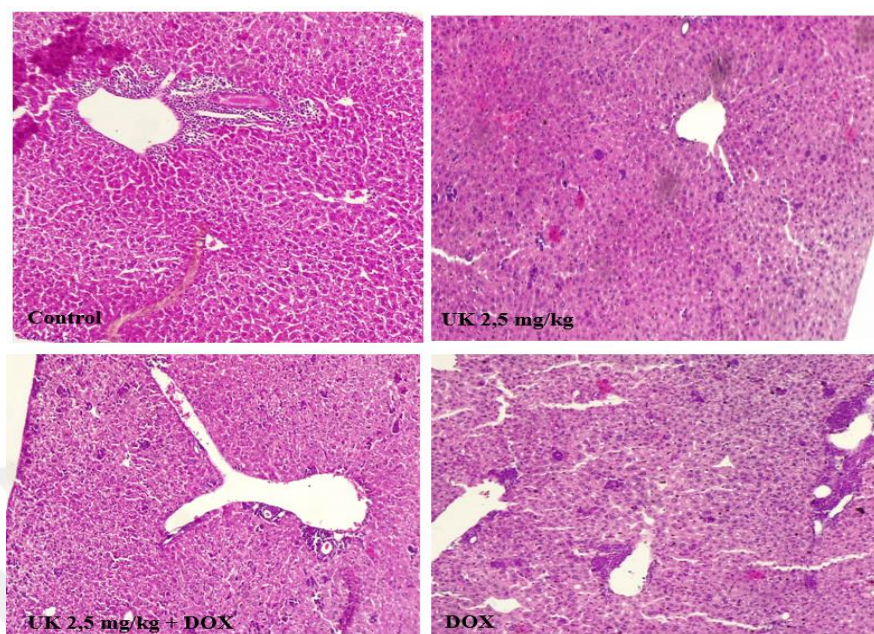


Figure 4.8. H&E staining of tumor tissues from the control, UK 2,5 mg/kg, UK 2,5mg/kg+ DOX, and DOX groups. A decrease in tumor cell density is observed in the all treatment groups while the most significant decrease was on UK 2,5 mg/kg group. (Magnification: 10x, scale bar: 50 μ m).

Evaluation of the Effects of UK-383367 alone and in combination with DOX Treatment on TNF- α Release Under Different Stimulations on 4TBM mice models

TNF- α release was evaluated under different stimulation conditions. In ConA-stimulated cells, the highest TNF- α level was observed in the DOX group. Upon LPS stimulation, a significant increase in TNF- α levels was detected in all treatment groups compared to the control (* p <0.05). In unstimulated cells, only the DOX group showed a significant increase (* p <0.05). No significant differences were observed among the treatment groups after stimulation with irradiated 4TBM cells.

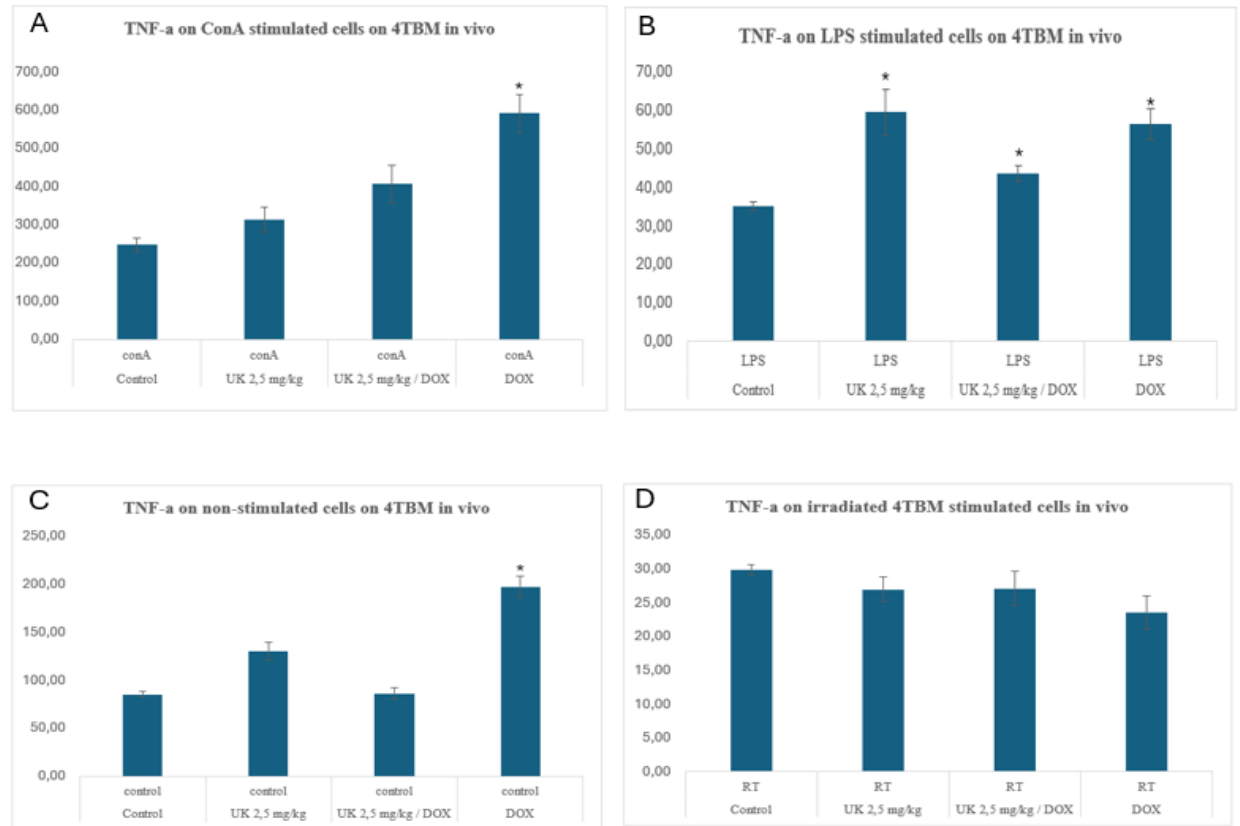


Figure 4.9. Effect of UK-383367 and/or Doxorubicin (DOX) treatments on TNF- α release in cells obtained from 4TBM tumor mice. (A) In cells stimulated with ConA, the highest TNF- α levels were observed in the DOX group. (B) After stimulation with LPS, a significant increase in TNF- α release was detected in the UK, UK+DOX and DOX groups compared to the control group. (C) In unstimulated cells, a significant increase in TNF- α levels was observed only in the DOX group (* $p < 0.05$). (D) In cells stimulated with irradiated 4TBM, no significant difference was observed between the treatment groups. Data are presented as mean $\pm$ SD. * $p < 0.05$ indicates a significant difference compared to the control group. (N=7).

Evaluation of the Effects of UK-383367 alone and in combination with DOX Treatment on IFN- γ Release Under Different Stimulations on 4TBM mice models

IFN- γ release varied depending on the stimulation condition. Following LPS stimulation, IFN- γ levels significantly decreased in the DOX and UK+DOX groups, while an increase was observed in the UK group. ConA stimulation led to elevated IFN- γ levels in all treatment groups. In both unstimulated and irradiated 4TBM-stimulated cells, all treatment groups showed a significant decrease in IFN- γ release compared to the control (* $p < 0.05$).

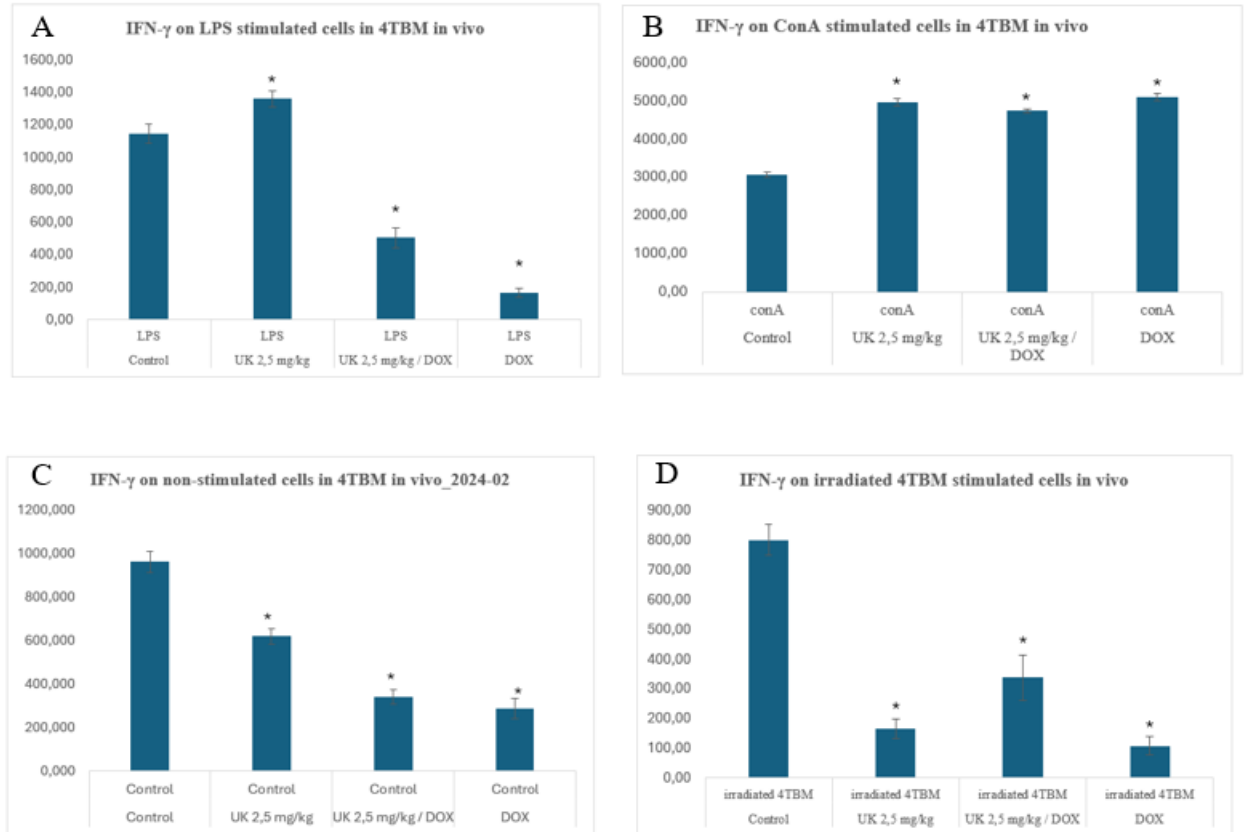


Figure 4.10. Effect of UK-383367 and/or Doxorubicin (DOX) treatments on IFN- γ release in cells obtained from 4TBM tumor mice. (A) After stimulation with LPS, a significant decrease in IFN- γ release was detected in the UK+DOX and DOX groups compared to the control group. However, IFN- γ release increased in UK group. (B) In cells stimulated with ConA, an increase in the IFN- γ levels was observed in all treatment groups. (C) In unstimulated cells, a significant decrease in IFN- γ levels was observed in all treatment groups. (D) In cells stimulated with irradiated 4TBM, a significant decrease in IFN- γ levels was observed in all treatment groups. Data are presented as mean $\pm$ SD. * $p < 0.05$ indicates a significant difference compared to the control group. (N=7).

Evaluation of the Effects of UK-383367 alone and in combination with DOX Treatment on IL-6 and IL-10 Release Under Different Stimulations on 4TBM mice models

IL-10 release consistently decreased across all treatment groups under all stimulation conditions. Significant reductions were observed following ConA, LPS, and irradiated 4TBM stimulation, as well as in unstimulated cells, compared to the control group (* $p < 0.05$).

ConA stimulation led to a significant increase in IL-6 release in all treatment groups compared to the control (* $p<0.05$). No significant changes were observed in IL-6 levels following LPS or irradiated 4TBM stimulation, or in unstimulated conditions.

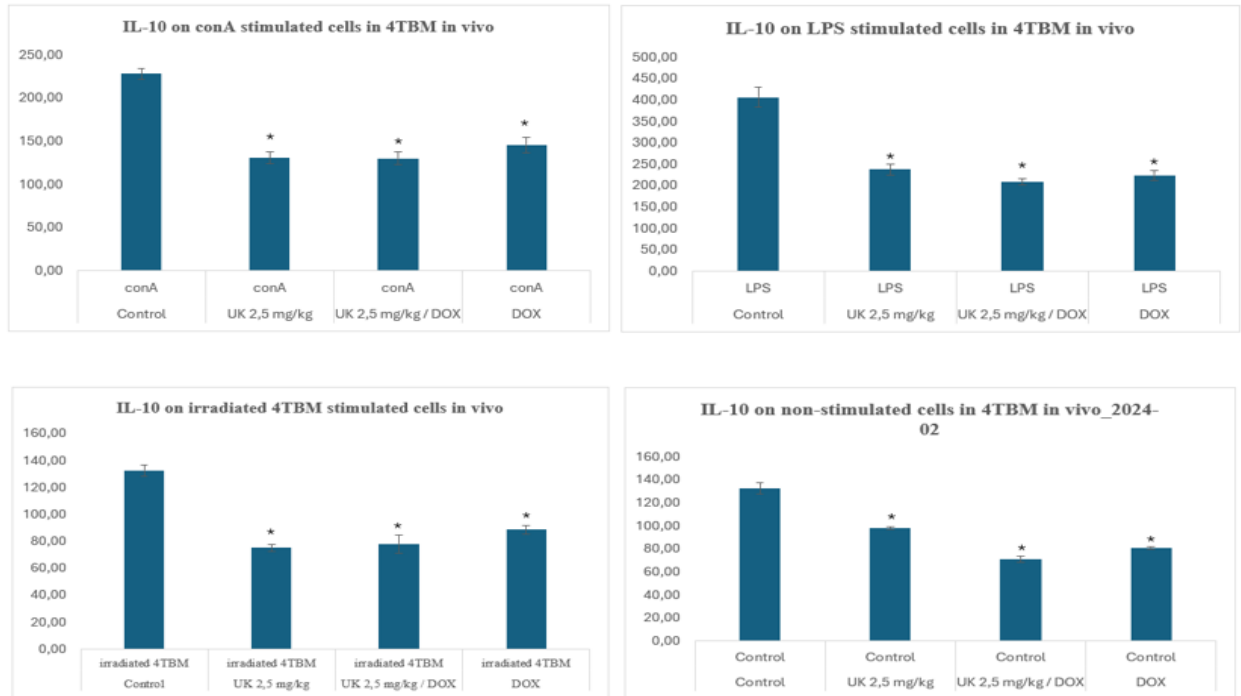


Figure 4.11. Effect of UK-383367 and/or Doxorubicin (DOX) treatments on IL-10 release in cells obtained from 4TBM tumor mice. (A) After stimulation with conA, a significant decrease in IL-10 release was detected in all treatment groups compared to the control group. (B) In cells stimulated with LPS, a decrease in the IL-10 levels was observed in all treatment groups. (C) In cells stimulated with irradiated 4TBM, a significant decrease in IL-10 levels was observed in all treatment groups. (D) In non-stimulated cells, a significant decrease in IL-10 levels was observed in all treatment groups. Data are presented as mean $\pm$ SD. * $p<0.05$ indicates a significant difference compared to the control group. (N=7).

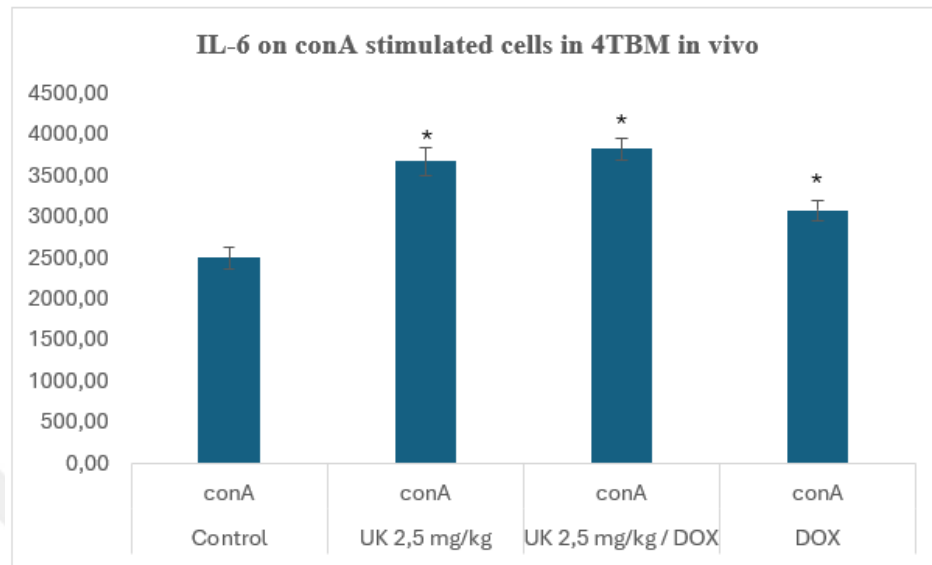


Figure 4.12. Effect of UK-383367 and/or Doxorubicin (DOX) treatments on IL-6 release in cells obtained from 4TBM tumor mice. After stimulation with conA, a significant increase in IL-6 release was detected in all treatment groups compared to the control group. There was no significant change in LPS, irradiated 4TBM, or control releases. Data are presented as mean $\pm$ SD. * $p < 0.05$ indicates a significant difference compared to the control group. (N=7).

4.3. Dose-Dependent Effects of UK-383367 alone and in combination with DOX on Cytokine Release with Different Stimuli on 4TLM injected mice model:

This experimental setup was planned to determine the applicable dose of UK-383367 treatment *in vivo* and to preliminary evaluate possible dose-dependent toxic effects in an aggressive tumor cell line. Four experimental groups were formed: Control (n=6), UK (n=6), DOX (n=6) and UK+DOX combination group (n=6). In order to create a breast cancer model, tumor injections were performed using the 4TLM cell line. The experiment was started with six animals in each group; however, in the first five days following intravenous injection, one animal was lost in all groups except the group in which UK and DOX were combined. No specific indication was observed regarding the possible causes of these early losses; however, stress due to tumor cell injection or individual animal differences may have been effective. The treatments were administered at a dose of 1.0 mg/kg intraperitoneally, every other day. In the DOX and combination groups, DOX was

administered weekly at decreasing doses of 5 mg/kg, 2.5 mg/kg and 1.25 mg/kg, respectively. In the control group, physiological serum containing DMSO was administered intraperitoneally. Tumor volumes were measured weekly in all groups and tumor weights were recorded at the end of the experiment. At the end of the experiment, mixed leukocyte cultures were prepared with cell suspensions obtained from spleen and lymph nodes and cytokine levels were analyzed by ELISA method. Cell suspensions from spleen and lymph nodes were analyzed using flow cytometry as detailly explained in the Method session. Lung metastases were evaluated by macroscopic observation and liver metastases were detected and counted by H&E staining method.

Evaluation of Tumor Volume, Tumor Weight, Together with Lung and Liver Metastasis on 4TLM mice models

In the *in vivo* tumor model established with the 4TLM cell line, the effects of the applied treatments on tumor development and metastasis were evaluated in Figure 4.13. While tumor volume increased significantly in the control group at week 3, tumor growth was suppressed in the UK-383367, DOX and UK+DOX combination groups (Figure 4.13.A). Tumor weight decreased in all treatment groups compared to the control group, with the most significant decrease observed in the UK+DOX combination group (Figure 4.13.B). Lung metastasis was significantly reduced in the all treatments groups especially in the UK+DOX combination group (* $p < 0.05$) (Figure 4.13.C). Liver metastasis was significantly suppressed in the UK+DOX combination groups (* $p < 0.05$), while limited effects were observed in UK-only group (Figure 4.13.D). H&E staining of liver tissue sections showed widespread metastatic infiltration in the control group, while more limited invasion areas were detected in the UK-383367 and DOX groups. The minimum tumor cell infiltration was observed in the DOX+UK combination group (Figure 4.14).

In the 4TLM model, no complete tumor regression was observed in the control group (0/6). UK alone and DOX alone resulted in complete tumor disappearance in one animal and marked shrinkage in another. The combination therapy group (UK+DOX) showed the complete regression observed in three out of six animals and substantial tumor shrinkage in another, supporting the enhanced efficacy of the combined treatment approach.

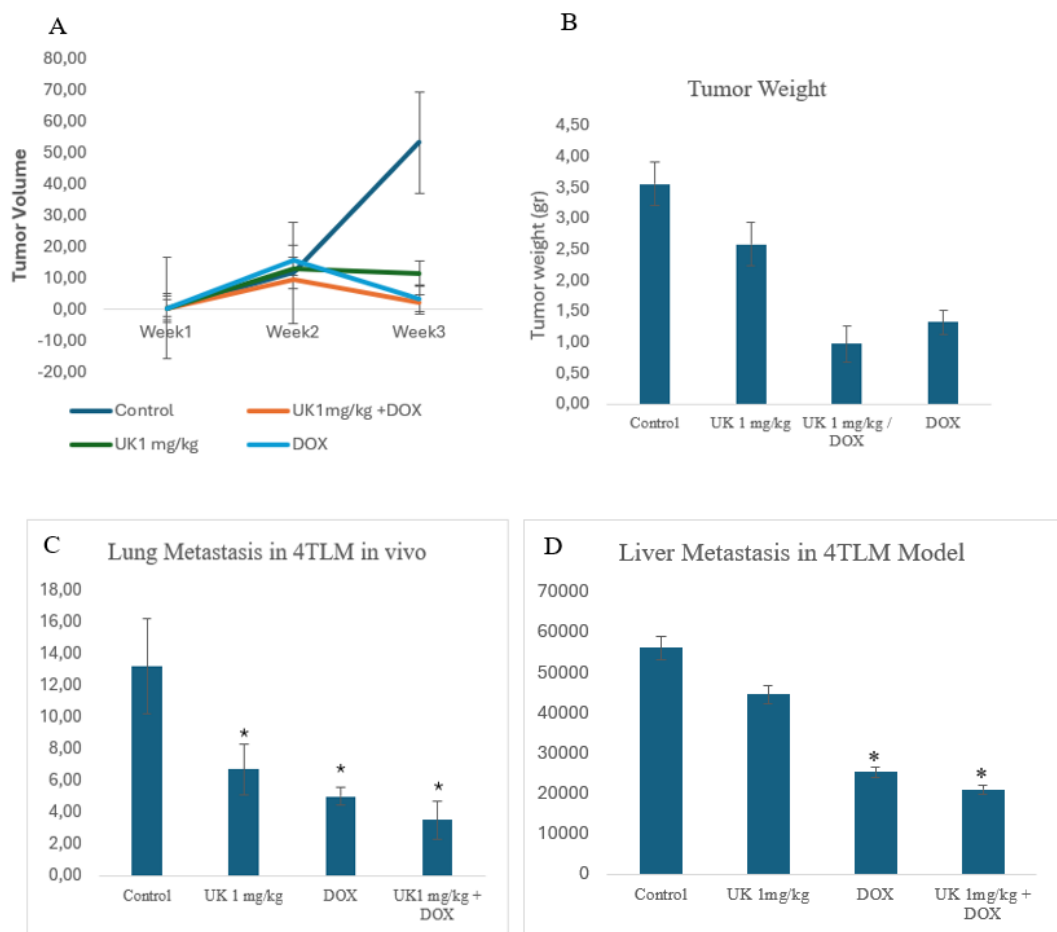


Figure 4.13. Effects of UK-383367 and/or Doxorubicin (DOX) treatment on 4TLM tumor model. (A) Tumor volume was measured weekly and a significant decrease was observed in the combined treatment group. (B) Tumor weights measured at the end of the experiment were found to be the lowest in the UK+DOX combination group. (C) Lung metastasis was evaluated macroscopically and a significant decrease was observed in all treatment groups. (D) Liver metastasis was counted by H&E staining and a significant decrease was detected in the DOX-only and UK+DOX groups compared to the control group (pixel). Data are presented as mean $\pm$ SD. * $p < 0.05$, significant difference compared to the control group. N=6

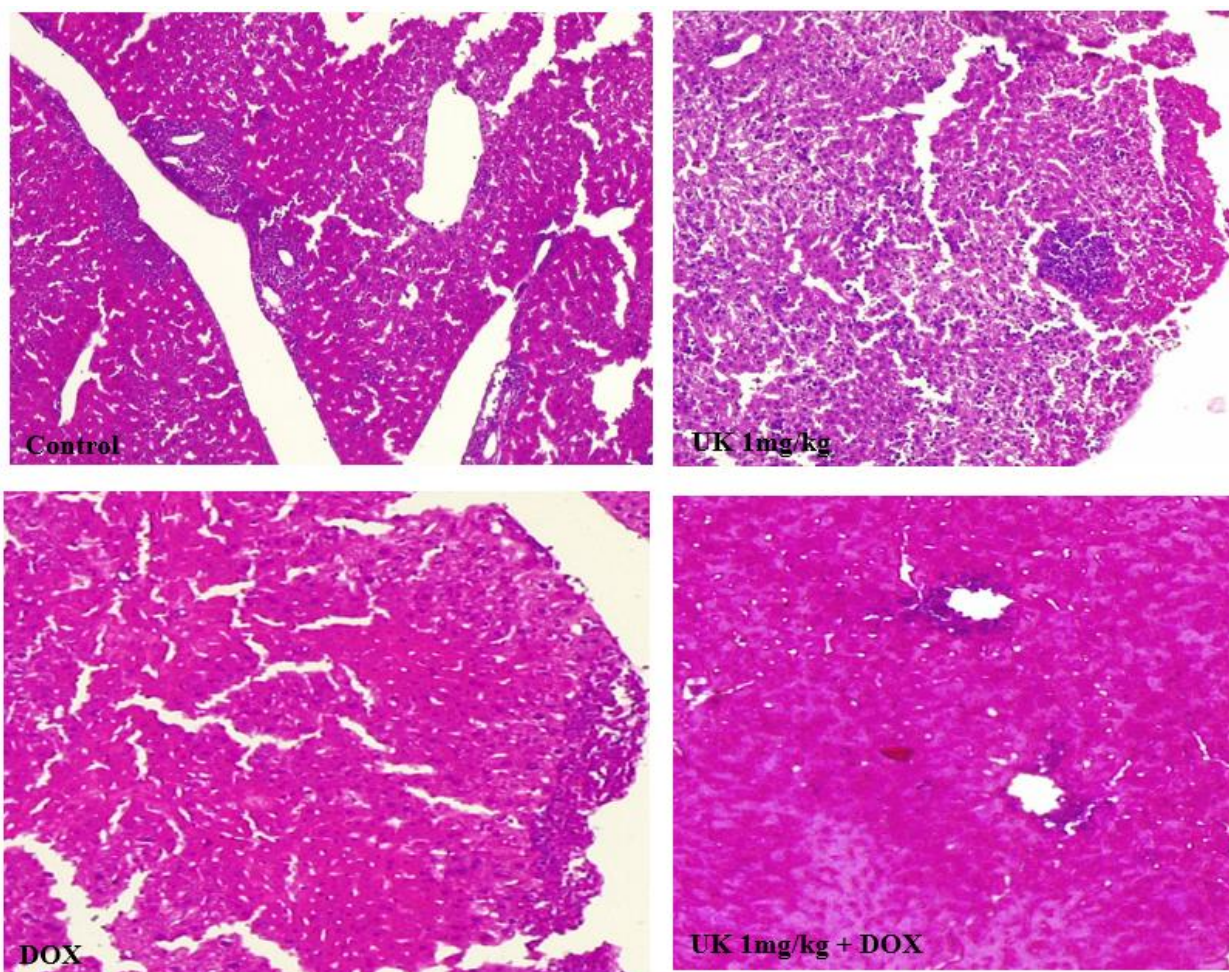


Figure 4.14. H&E staining of tumor tissues from the control, UK 1,0 mg/kg, DOX, and UK 1,0 mg/kg+ DOX groups. A decrease in tumor cell density is observed in all treatment groups, while the most significant decrease was on UK 1,0 mg/kg group. (Magnification: 10x, scale bar: 50 μ m).

Evaluation of the Effects of UK-383367 alone and in combination with DOX Treatment on IFN- γ Release Under Different Stimulations on 4TLM mice models

For the evaluation of IFN- γ release a significant response was observed only in ConA-stimulated cells in which the T cell response was dominant. No significant change in IFN- γ levels was detected in the LPS-stimulated or irradiated 4TLM cell-stimulated groups compared to the control groups.

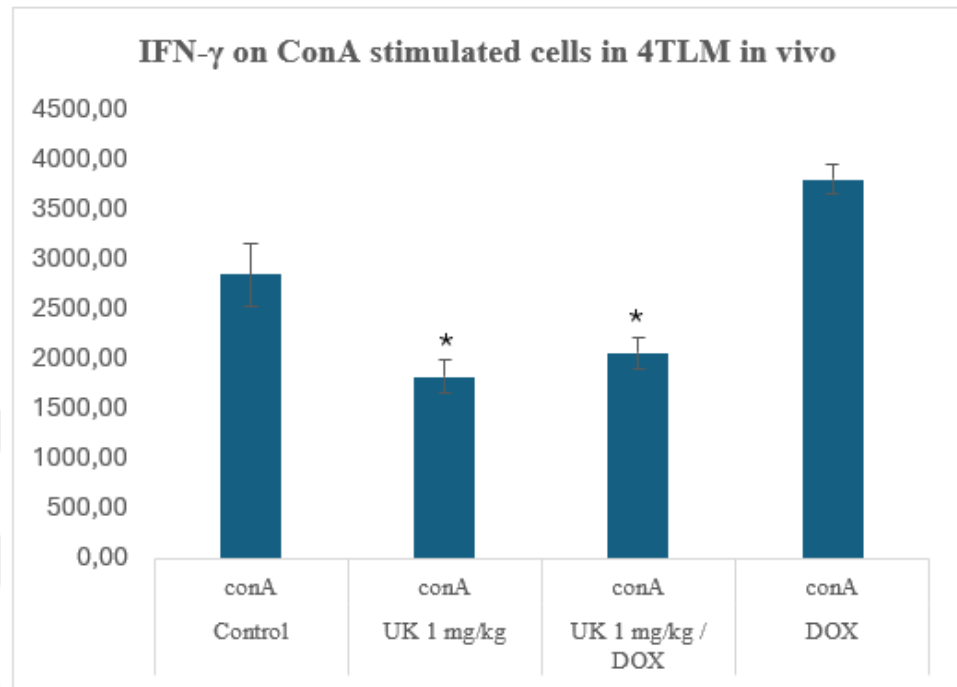


Figure 4.15. Effect of UK-383367 and/or Doxorubicin (DOX) treatments on IFN- γ release in cells obtained from 4TLM tumor mice. In cells stimulated with ConA, a significant decrease in the IFN- γ levels was observed in UK-alone and UK+DOX combination groups. Data are presented as mean $\pm$ SD. * $p < 0.05$ indicates a significant difference compared to the control group. N=6

Evaluation of the Effects of UK-383367 alone and in combination with DOX Treatment on IL-6 Release Under Different Stimulations on 4TLM mice models

In 4TLM-derived cells, IL-6 release increased in the UK and UK+DOX groups after LPS stimulation. ConA stimulation led to a significant increase only in the DOX group. All treatment groups showed elevated IL-6 levels after stimulation with irradiated 4TLM cells, while in unstimulated conditions, a significant increase was observed only in the DOX group (* $p < 0.05$).

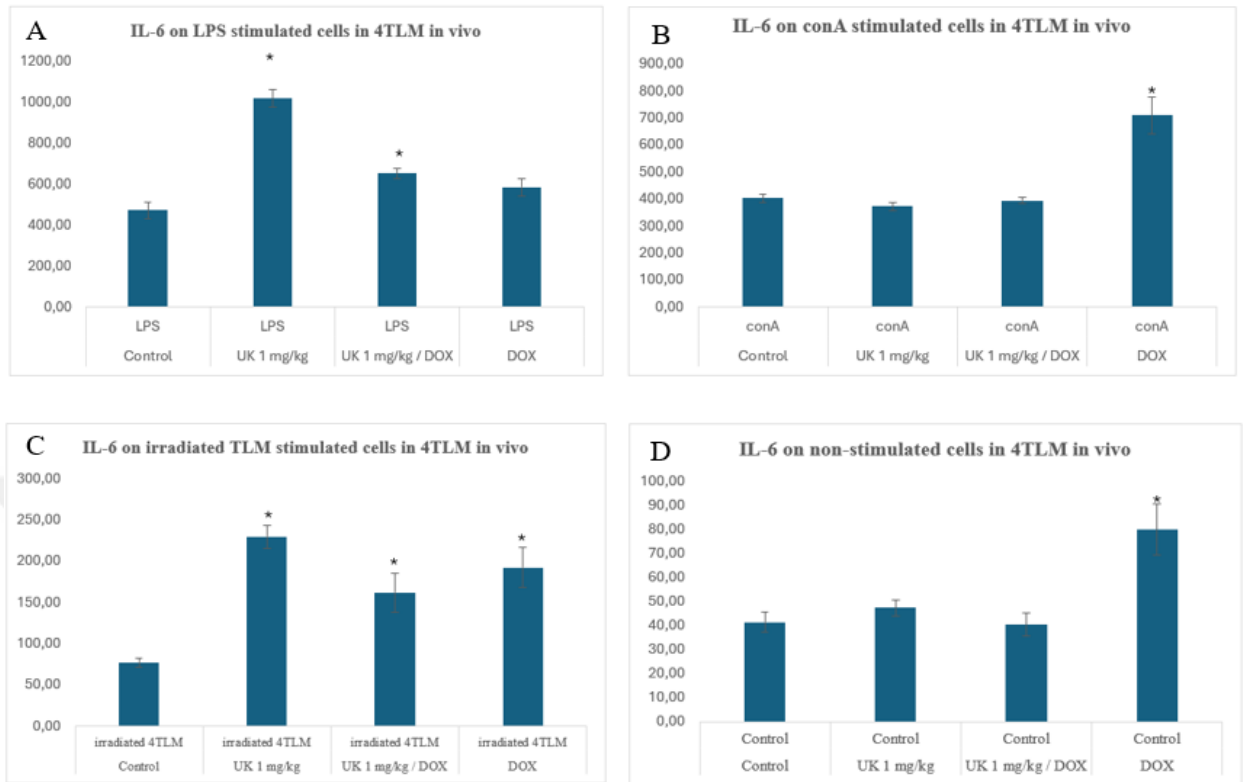


Figure 4.16. Effect of UK-383367 and/or Doxorubicin (DOX) treatments on IL-6 release in cells obtained from 4TLM tumor mice. (A) In cells stimulated with LPS, an increase in the IL-6 levels was observed in the UK alone and UK+DOX combination groups. (B) After stimulation with conA, a significant increase in IL-6 release was detected only in the DOX group compared to the control group. (C) In cells stimulated with irradiated 4TLM, a significant increase in IL-6 levels was observed in all treatment groups. (D) In non-stimulated cells, a significant increase in IL-6 levels was observed only in the DOX-only group. Data are presented as mean $\pm$ SD. * $p < 0.05$ indicates a significant difference compared to the control group. (N=6)

Evaluation of the Effects of UK-383367 alone and in combination with DOX Treatment on IL-10 Release Under Different Stimulations on 4TLM mice models

In 4TLM-derived cells, ConA stimulation led to a significant increase in IL-10 release only in the DOX group. LPS stimulation resulted in decreased IL-10 levels in all treatment groups. Upon stimulation with irradiated 4TLM cells, IL-10 levels significantly increased in the UK and UK+DOX groups. In unstimulated conditions, a significant decrease was observed in the UK+DOX and DOX groups (* $p < 0.05$).

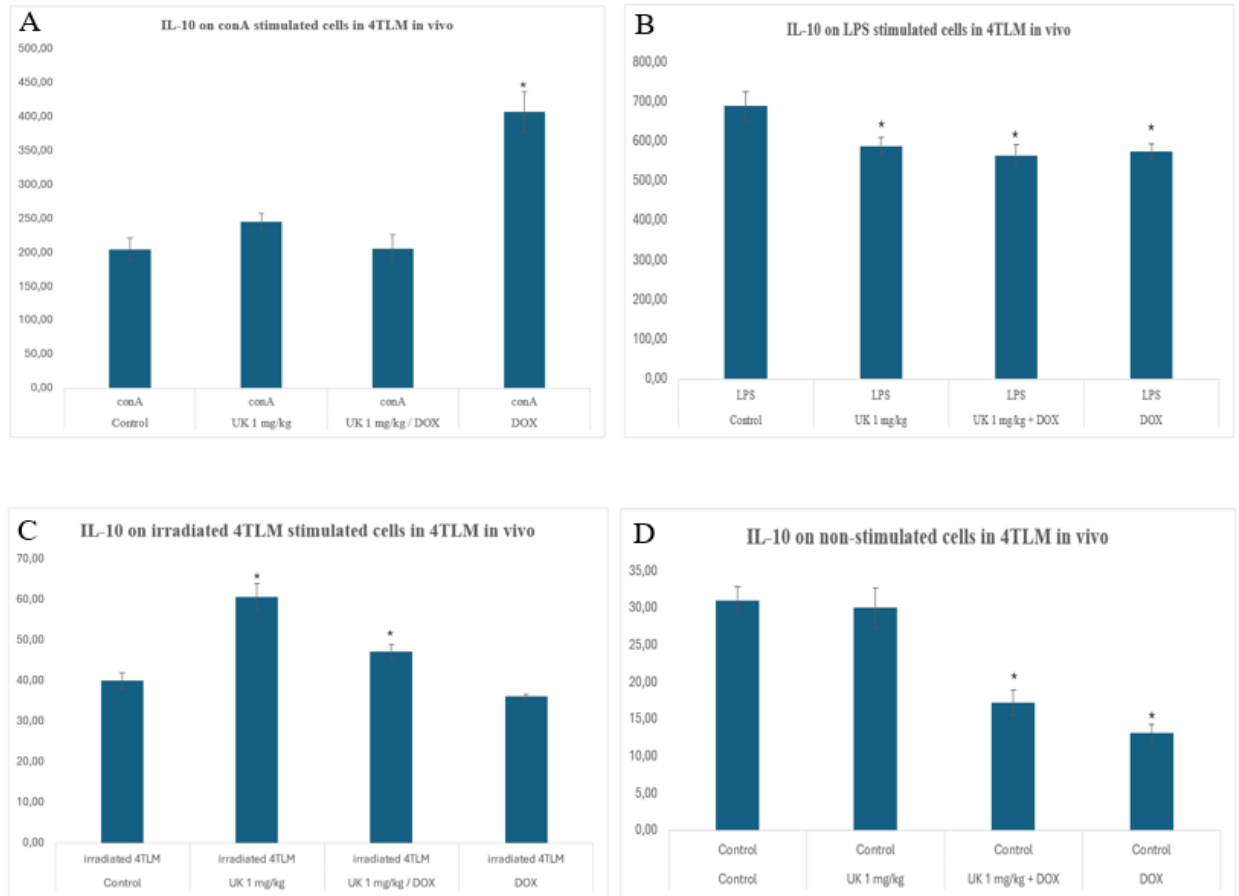


Figure 4.17. Effect of UK-383367 and/or Doxorubicin (DOX) treatments on IL-10 release in cells obtained from 4TLM tumor mice. (A) After stimulation with conA, a significant increase in IL-6 release was detected only in the DOX group compared to the control group. (B) In cells stimulated with LPS, a decrease in the IL-10 levels were observed in all treatment groups. (C) In cells stimulated with irradiated 4TLM, a significant increase in IL-10 levels was observed in UK-only and UK-DOX combination groups. (D) In non-stimulated cells, a significant decrease in IL-10 levels was observed in the UK+DOX combination and DOX-only group. Data are presented as mean $\pm$ SD. * $p < 0.05$ indicates a significant difference compared to the control group. (N=6)

Evaluation of T Cell Subtypes and Myeloid Cell Populations by Flow Cytometry on 4TLM mice model

The flow cytometry analyses on lymph node and spleen cell suspensions revealed the effects of treatment groups on immune cell populations. According to the myeloid-derived suppressor cell (MDSC; GR1⁺CD11b⁺) analysis in Figure 4.18, the MDSC rate was high in the control group, UK and DOX monotherapy applications significantly decreased

MDSC percentages compared to control group (* $p<0.05$). When the analysis of T cell subtypes in Figure 4.19 was examined the helper T cells (CD3⁺CD4⁺) ratio increased significantly especially in the DOX group (* $p<0.05$). This increase was more limited in UK-383367 monotherapy. Also, the cytotoxic T cells (CD3⁺CD8⁺) ratio increased significantly in the DOX and UK+DOX combination groups, while a significant decrease was observed when UK was applied alone (* $p<0.05$). CD4/CD8 ratio reached the highest level in the UK-383367 group and increased significantly compared to the control group (* $p<0.05$). This ratio decreased again in the combination group.

Flow cytometry analysis revealed a reduction in the GR-1⁺CD11b⁺ cell population in all treatment groups (UK, DOX, UK+DOX) compared to the control, with the most prominent decrease observed in the UK-treated group.

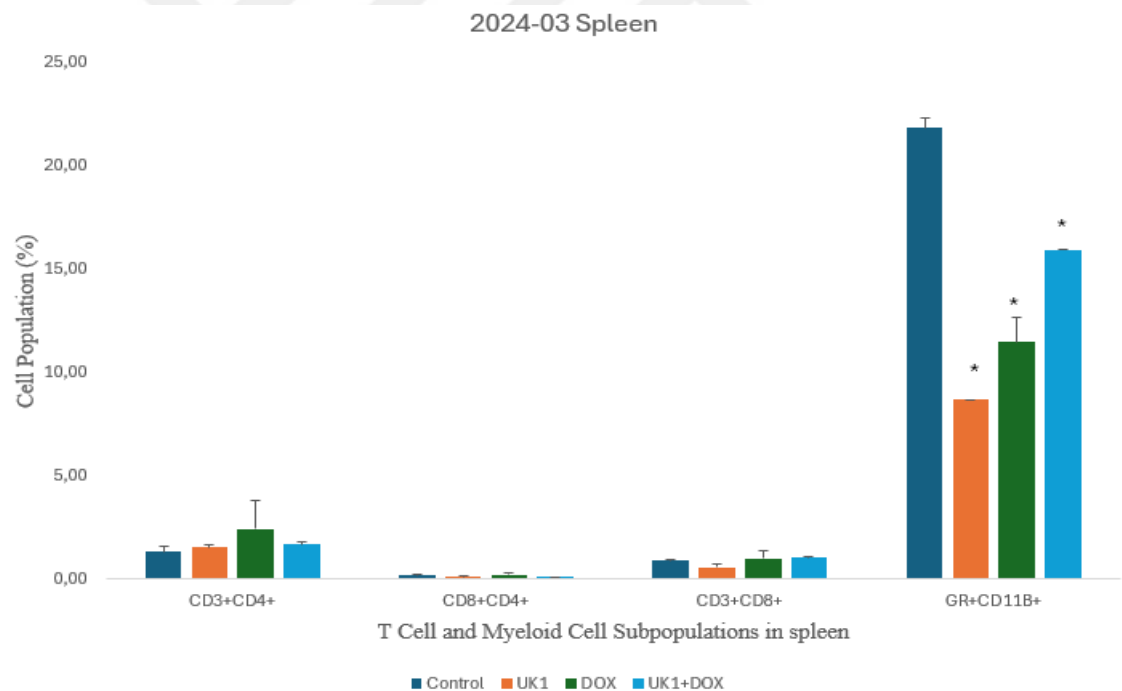


Figure 4.18. *Effect of UK-383367 and/or Doxorubicin (DOX) treatments on immune cell populations in the spleen of 4TLM mice models.* Flow cytometric analysis of splenic immune cell populations revealed no significant differences in CD3⁺CD4⁺, CD8⁺CD4⁺, or CD3⁺CD8⁺ T cell subsets among the treatment groups. However, the frequency of GR⁺CD11b⁺ myeloid cells was significantly reduced in all treatment groups (UK, DOX, and UK+DOX) compared to the control group (* $p<0.05$). Data are presented as mean $\pm$ SD (N=7). * $p<0.05$ compared to the control group. (N=6)

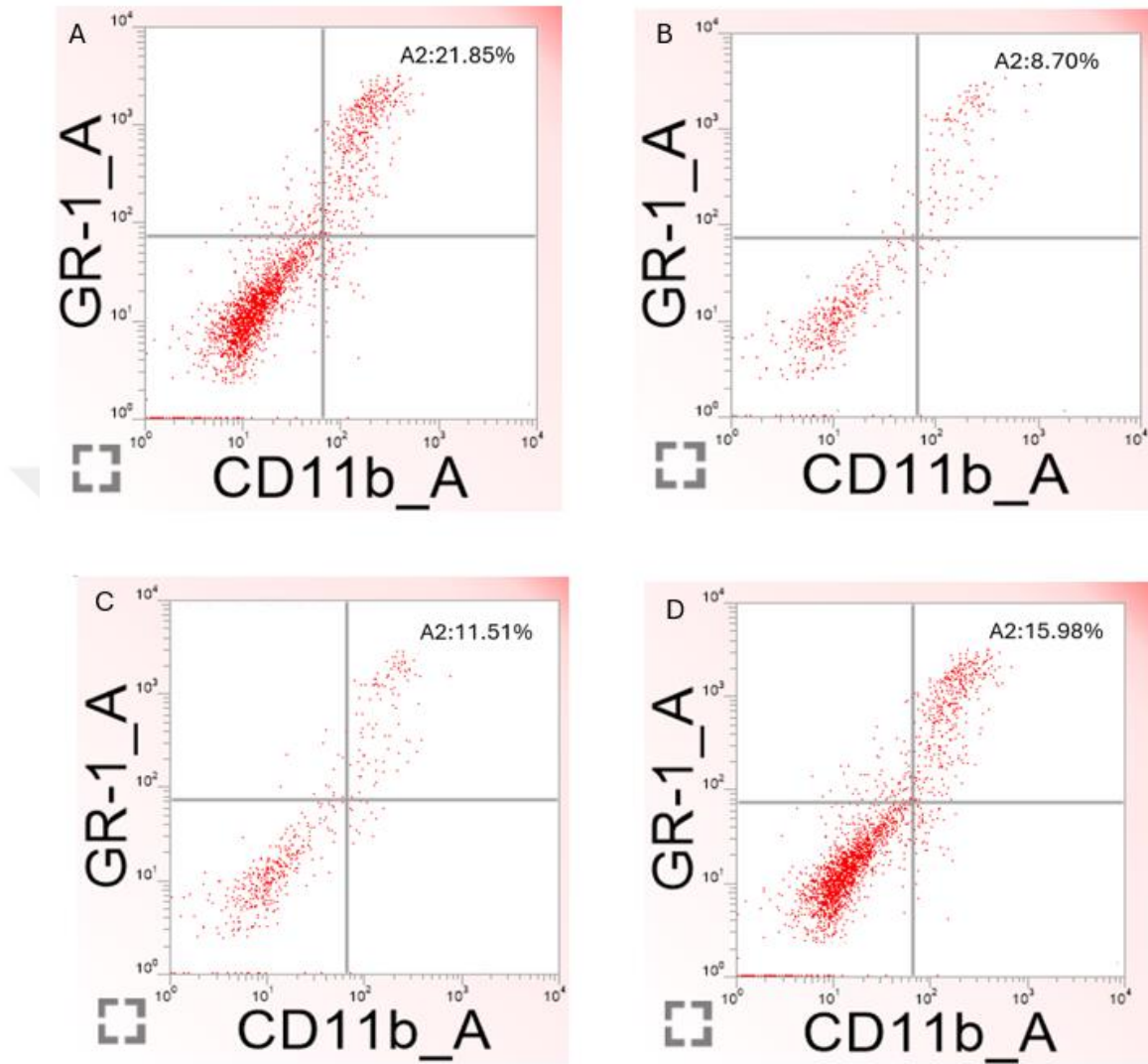


Figure 4.19. Flow cytometric analysis of GR-1⁺CD11b⁺ myeloid-derived suppressor cells (MDSCs) in spleens of 4TBM tumor-bearing mice following different treatments. Dot plots show representative GR-1 and CD11b staining profiles of splenocytes from each experimental group: (A) Control, (B) UK-383367 (UK), (C) Doxorubicin (DOX), and (D) UK+DOX combination treatment. The upper right quadrant (A2) represents the GR-1⁺CD11b⁺ double-positive population. A noticeable decrease in this population was observed in all treatment groups compared to the control. Percentages indicate the proportion of GR-1⁺CD11b⁺ cells within the total live cell population.

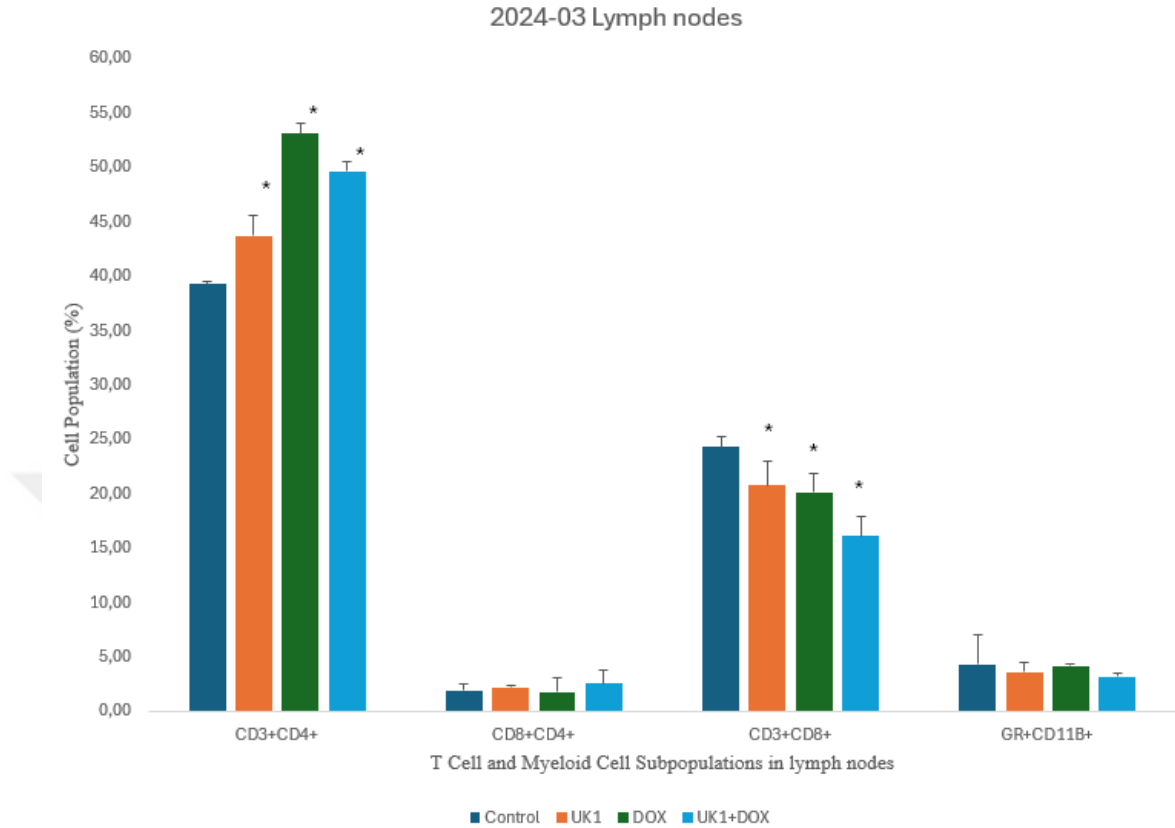


Figure 4.20. *Effect of UK-383367 and/or Doxorubicin (DOX) treatments on immune cell populations in lymph nodes of 4TLM mice models.* Flow cytometric analysis of lymph node-derived immune cell populations showed that the percentage of CD3⁺CD4⁺ T cells significantly increased in the UK, DOX, and UK+DOX groups compared to the control (* $p < 0.05$). The frequency of CD3⁺CD8⁺ T cells was significantly reduced in all treatment groups (* $p < 0.05$). No significant differences were observed in CD8⁺CD4⁺ and GR⁺CD11b⁺ myeloid cell populations across groups. Data are presented as mean $\pm$ SD (N=7). * $p < 0.05$ compared to the control group. (N=6)

5. DISCUSSION

The main cause of mortality due to breast cancer is metastatic spread, which occurs due to invasion of primary tumor cells to distant organs; in particular, the target organs are bone, liver, lung, and brain, to which breast cancer frequently metastasizes. In this case, the cancer is becoming resistant to treatment and seriously worsening the prognosis. Currently, there is no effective treatment option that can stop or target metastatic spread. One of the most important reasons for this issue is that the molecular mechanisms that drive the metastatic process and the interactions with the tumor microenvironment have not yet been fully elucidated.

Metastasis depends not only on the invasive capacity of tumor cells but also on the remodeling of the ECM, biochemical communication with stromal cells, and cellular transformation processes such as EMT. In this context, the focus of this thesis, BMP-1, is structurally different from the classical BMP family by having metalloproteinase function, and it is one of the rare members of the TGF- β superfamily with proteolytic activity. BMP-1 regulates the biomechanical structure of the ECM by targeting specific substrates that enable the conversion of pro-collagens to mature collagens. In addition, it can reshape signaling in the tumor microenvironment with its potential to activate latent TGF- β complexes.

Recent studies have shown that BMP-1 plays an active role in invasion, migration, and angiogenesis processes, and that high BMP-1 expression is associated with poor prognosis (Naba et al., 2014; Zhou et al., 2022). In this respect, BMP-1 should be considered not only as a matrix modulator but also as a microenvironment adaptation factor that increases the metastatic capacity of tumor cells. The findings obtained by suppressing BMP-1 with inhibitory agents within the scope of this thesis reveal the potential of targeting this protease as a therapeutic strategy in metastatic breast cancer.

This thesis evaluates the effects of the BMP-1 inhibitor UK-383367, alone and in combination with doxorubicin (DOX), on various metastatic breast cancer models *in vivo*. In investigations conducted using BALB/c female mice models, tumor volume, metastatic load in the lungs and liver, distribution of immune cell subpopulations, and levels of

proinflammatory cytokines (TNF- α , IL-6, IL-10, and IFN- γ) were assessed. The results derived from these studies demonstrate promising and novel outcomes in comparison to the existing literature.

This preliminary data formed the basis for subsequent experimental setups to define the optimal efficacy and dosage of UK *in vivo*. The findings revealed that the UK treatment could be more effective on metastatic potential and immune responses than on tumor progression. There was no statistical difference between the groups in terms of tumor volume and weight. The macroscopic lung metastasis counts decreased in both UK 1 mg/kg and UK 5 mg/kg groups, which were statistically significant in both groups compared to the Control group. This output suggested that the anti-metastatic effect of UK might have occurred independently of primary tumor suppression (Figure 4.1).

However, the result of histopathological evaluation of liver metastases with H&E stain indicated a statistically significant increase in the area of metastases of the UK 5 mg/kg group compared to the control group (Figure 4.1.C). This finding suggests that the high dose may be associated with a paradoxical effect or exceeding the toxic limit in some tissues (Figure 4.2).

The mixed leukocyte culture analysis showed that UK could produce dose-dependent immunomodulatory effects. The UK 1 mg/kg group showed the highest level of IFN- γ secretion in ConA-stimulated cells, followed by the UK 5 mg/kg and control groups, and results were statistically significant in both treatment groups compared to the control (Figure 4.3). In addition, the UK 1 mg/kg group had a significant increase in IFN- γ secretion both in LPS- (Figure 4.4) and RT-stimulated cells (Figure 4.6). These results indicate that the UK 1 mg/kg dose activates especially the IFN- γ response, and has a higher potential to support T cell-mediated immune responses.

The findings suggest that the 5 mg/kg dose of UK may suppress some immune responses, especially when evaluated together with the observed increase in liver metastases. The higher dose of UK could cause systemic or tissue-specific toxic effects. Therefore, the UK treatment in lower doses could offer a more balanced and effective profile in terms of

immunological responses and metastatic burden, which was preferred in subsequent experiments.

The second experimental model is planned to evaluate the effects of the UK and DOX combination with the 4TBM tumor model, which shows less aggressive characteristics compared to 4TLM. The findings indicated that the combination of UK and DOX was the most beneficial, particularly in diminishing tumor volume and mass (Figure 4.7 A-B). The combination group exhibited a substantial reduction in lung and liver metastatic burden (Figure 4.7 C-D). This indicates that the UK's ability to modify the tumor microenvironment (TME) and suppress TGF- β activation is complementary to the chemotherapeutic efficacy of DOX. As previously shown in the study by Erin et al. (2025), it is thought that BMP-1 inhibition has the potential to increase chemotherapy efficacy, and this combination holds promise in the treatment of metastatic breast cancer. This conclusion aligns with literature indicating that combined treatment techniques can enhance the efficacy of chemotherapeutic drugs while mitigating toxicity (Sritharan & Sivalingam, 2021; Rawat et al., 2021).

In vivo studies in the 4TBM model demonstrated that both UK-383367 and DOX monotherapies led to complete tumor regression in a small subset of animals (1/7 in each group), suggesting a modest therapeutic potential when used alone. Notably, the combination of UK+DOX resulted in complete tumor disappearance in one animal and substantial tumor shrinkage in more than half of the group (5/7), indicating a possible synergistic effect. This enhanced response may be attributed to the combined modulation of the tumor microenvironment by BMP-1 inhibition and the cytotoxic activity of DOX. While the overall rate of complete regression was limited, the frequency and extent of tumor shrinkage in the combination group highlight its potential as a more effective therapeutic strategy, warranting further investigation.

In cytokine analyses of the second model, an increase in IFN- γ (Figure 4.10 B) and TNF- α (Figure 4.9 B) levels of the combination group was observed. This result suggested that immune system activation could be strengthened with combination therapy. On the other hand, the decrease in IL-10 levels might be a factor of a reduction in immunological pressure and the strengthening of the anti-tumor response (Figure 4.11). An increase

observed in con-A stimulated IL-6 levels indicates that, in addition to immune activation, an inflammatory response may also be triggered. These results suggest that the dynamic structure of the tumor microenvironment plays a critical role in determining treatment responses, as it is previously shown by Habanjar et al. (2023).

In the last experimental model, the effects of UK alone and in combination with DOX were evaluated by using the 4TLM-derived *in vivo* models, which again showed high aggressiveness. This model is important in terms of simulating aggressive metastatic breast cancer because it shows faster progression and higher metastatic potential compared to the 4TBM model (Erin et al., 2013).

It is observed that the control group had an important increase in both the volume and weight of the tumor, while the applications of UK, DOX, and especially UK+DOX combination treatments were able to inhibit the progression of the tumor, with the combination group exhibiting the most significant decrease (Figure 4.13. A-B). Lung metastasis burden decreased significantly in all treatment groups (Figure 4.13. C), and liver metastases were significantly reduced, especially in the UK+DOX group (Figure 4.13. D). According to these findings, inhibiting BMP-1 has the potential to prevent the spread of metastatic breast cancer by modifying the microenvironment of the tumor. Furthermore, this impact generates a synergistic effect together with the chemotherapeutic action of DOX. Targeting BMP-1, as demonstrated in a recent study by Erin et al. (2025), not only improves the effectiveness of chemotherapy but also provides a beneficial strategy for preventing the progression of metastatic disease. In the 4TLM model, the UK and DOX alone groups led to complete tumor regression in only one animal each. In contrast, the UK+DOX combination resulted in complete regression in 3 out of 6 animals, with another showing marked shrinkage. These results suggest a synergistic effect and highlight the potential of combining BMP-1 inhibition with chemotherapy for treating aggressive tumor types such as 4TLM.

Cytokine analyses gave important data to understand the immune response and tumor microenvironment dynamics in the model. Significant changes in IFN- γ secretion were observed only with ConA stimulation, which indicates that the T cell response was dominant in this stimulus. No significant changes were observed in IFN- γ levels with LPS

or radiation stimulation, suggesting that these stimulation pathways may have limited effects on IFN- γ secretion (Figure 4.15). Under LPS stimulation, all treatment groups showed a decrease in IL-10 secretion (Figure 4.17 B), whereas the UK and UK+DOX groups showed an increase in IL-10 secretion under radiation stimulation (Figure 4.17 C). This situation demonstrates that IL-10, an immunosuppressive cytokine, demonstrates context-dependent variability in response to treatment (Accogli et al., 2021). The immunosuppressive environment may have been reduced by the decrease in IL-10 levels observed in the combination and DOX groups under non-stimulated conditions (Figure 4.17 D).

An increase in IL-6 levels was observed in the UK and UK+DOX combination groups in LPS-stimulated cells, while a significant increase was only observed in the DOX group in ConA stimulation. Irradiated TLM stimulated cells increased IL-6 levels in all treatment groups, and the DOX group was the only one to observe an increase in IL-6 levels in non-stimulated cells. These results demonstrate that the immune system can be activated in various ways and that treatment applications can enhance the inflammatory response (Czajka-Francuz et al., 2021). The inflammatory response is also triggered by these increases in IL-6, which indicate that the response might differ depending on the type of stimulation administered, in addition to treatment-induced immune activation.

Flow cytometry analyses revealed differences in immune cell subpopulations based on the treatment groups. MDSC (GR1⁺CD11b⁺) ratios decreased significantly in all treatment groups compared to the control group. This result indicates that the anti-tumoral immune response was supported by the decrease in immunosuppressive cells (Najafi et al., 2018). The DOX group exhibited the highest increase in CD3⁺CD4⁺ (helper T cell) ratios, whereas the UK-alone group exhibited a limited increase. CD3⁺CD8⁺ (cytotoxic T cell) ratios increased significantly in the DOX and combination groups, while it decreased in the UK-alone group. The CD4/CD8 ratio was measured highest in the UK-alone group, and at its lowest in the UK+DOX group. This demonstrates that combination therapy can preserve the immune equilibrium while enhancing the cytotoxic T cell response, whereas UK monotherapy might generate a more helper T cell-dominant response.

All these findings indicate that the UK as BMP-1 inhibitor is effective in reducing metastatic burden, immune suppressor cell populations, and supporting T cell-mediated immunity even in aggressive tumor models. In addition, its combination with DOX provides a synergistic effect in terms of reducing metastatic spread, reducing tumor volume, and supporting immune activation. As stated in different studies by Sritharan & Sivalingam (2021) and Rawat et al. (2021), combinational treatment strategies can increase the efficacy of chemotherapeutic agents and reduce toxicity, and so UK+DOX combinational therapy has significant potential in the treatment of metastatic breast cancer.

To sum up, the combination of the BMP-1 inhibitor UK and DOX is a promising approach for the treatment of metastatic breast cancer. This approach has the potential to reshape the tumor microenvironment, reduce metastatic spread, and activate the immune system. This also brings insight into the need for further research.

6. CONCLUSION & SUGGESTIONS

Bone morphogenetic protein 1 (BMP-1) is a protease that is crucial for the progression of tumors and the process of metastasis. Its functions include the activation of latent TGF- β and the remodeling of the extracellular matrix. The development of new treatment strategies to enhance the efficacy of chemotherapeutic agents and reduce metastatic burden may be facilitated by the studies on BMP-1 inhibitors. In this context, the use of BMP-1 inhibitor UK-383367 alone or in combination with doxorubicin (DOX) in metastatic breast cancer models seems promising for controlling metastatic spread and remodeling the tumor microenvironment.

In this thesis, the anti-tumoral and anti-metastatic effects of the BMP-1 inhibitor UK-383367 alone and in combination with doxorubicin (DOX) were evaluated in metastatic breast cancer models. The *in vivo* models, which are generated with the 4TBM and 4TLM cell lines, allowed for a comprehensive analysis of treatment efficacy by representing different levels of aggressiveness of metastatic breast cancer.

The experiment models showed that UK-383367 alone therapy has the potential to decrease the burden of lung metastases. Furthermore, UK-alone enhances the T cell-mediated immune response (increased IFN- γ) but does not have a significant impact on the volume of primary tumors when compared to UK+DOX combinational therapy. In addition, data showed that the metastatic burden in certain tissues (liver) may be increased by high-dose UK administration, whereas low-dose application provides a more balanced and safe profile.

The DOX alone therapy was found to be effective in reducing tumor volume and metastatic spread, as well as increasing the rates of helper and cytotoxic T cells via immune system activation.

The study showed that the UK-383367 and DOX combinational therapy is the most effective approach in suppressing tumor volume and metastatic spread while it strengthens the cytotoxic T cell response, reduces immune suppressor cell populations, and creates a synergistic effect.

The cytokine analyses revealed that the treatment groups increased the levels of IL-6 and IFN- γ and modulated the levels of IL-10 in a context-dependent manner. The findings indicate that treatments could support immune activation, but they might also affect the inflammatory response.

In conclusion, the targeting of BMP-1 could represent a promising approach in the treatment of metastatic breast cancer. Furthermore, the combination of UK-383367 with DOX appears to have significant potential in diminishing metastatic spread while enhancing treatment efficacy through the activation of the immune system.

It will be essential to observe cytokine responses at intermediate intervals throughout the treatment process, such as during week 1, week 2, and week 3, with a larger *in vivo* group to understand the specific stages at which the treatment influences the immune microenvironment and the mechanisms involved in this modulation in future studies. Also, using different metastatic organotropism breast cancer models will be an important step for the clinical translation of the treatment approach. In addition, it will be important to look closely at the molecular mechanisms that make BMP-1 inhibition and combination therapies work on metastatic breast cancer in future studies. This can be done by looking at changes after given combinational therapy in signaling pathways, mRNAs, and proteins, as well as finding biomarkers of response to treatment and gathering the mechanistic data needed for the clinical transition.

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RESUME

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BMP-1 İnhibisyonunun Metastatik Meme Kanserinde Tek Başına Ve Doksorubisin İle Birlikte Olası Anti-tümöral/Anti-metastatik Etkilerinin Araştırılması: Epitelyal-mezenkimal Geçiş, Ekstraselüler Matriks Ve İmmün Yanıttaki Değişiklikler	TUBITAK	2024-2025
The <i>In vivo</i> Effects of Combined Use of Gibberellic Acid and Doxorubicin in Metastatic Breast Cancer	Akdeniz Üniversitesi BAP	2022-2024
Aging alters the molecular dynamics of synapses in a sexually dimorphic pattern in zebrafish (<i>Danio rerio</i>)	TUBITAK	2012-2014

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