

T.R.
GEBZE TECHNICAL UNIVERSITY
GRADUATE SCHOOL

**THE INVESTIGATION OF POTENT c-MET INHIBITORS PYRIDAZINE
TRIAZOLE DERIVATIVES ON ESTROGEN RECEPTOR POSITIVE AND
NEGATIVE BREAST CANCER CELL LINES**

BUSE AKTÜRK

A THESIS OF MASTER OF SCIENCE
DEPARTMENT OF BIOTECHNOLOGY

ADVISOR: PROF. DR. ELİF DAMLA ARISAN

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LİSANSÜSTÜ EĞİTİM ENSTİTÜSÜ

ÖSTROJEN RESEPTÖRÜ POZİTİF VE NEGATİF MEME KANSERİ
HÜCRE HATLARINDA POTANSİYEL c-MET İNHİBİTÖRLERİ
PİRİDAZİN TRİAZOL TÜREVLERİNİN ARAŞTIRILMASI

BUSE AKTÜRK

YÜKSEK LİSANS TEZİ
BİYOTEKNOLOJİ ANABİLİM DALI

DANIŞMAN: PROF. DR. ELİF DAMLA ARISAN

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To my Family

ABSTRACT

Breast cancer (BC) remains a leading cause of cancer-related morbidity and mortality worldwide, with triple-negative and hormone receptor-positive subtypes presenting significant therapeutic challenges due to their aggressive nature and limited treatment options. Receptor tyrosine kinases (RTKs) are single pass transmembrane receptors that bind to growth factors as ligands. They have key roles in cellular metabolism, differentiation, growth, and motility. The c-Met receptor tyrosine kinase has emerged as a promising therapeutic target, given its role in tumor progression, metastasis, and resistance to therapy. This thesis aims to systematically investigate the anticancer efficacy and underlying molecular mechanisms of newly synthesized compounds designed to inhibit c-Met signaling in BC cell lines. This is a study to evaluate pyridazine triazole derivatives as c-Met inhibitors in BC models. The effects of newly synthesized pyridazine triazole derivatives, including C1, C2, C3, C4, and C5, were evaluated in both MDA-MB-231 and MCF-7 BC models. Cellular assays, including MTT and colony formation, were employed to assess cytotoxicity and effects on cell proliferation. Mitochondrial membrane potential, reactive oxygen species (ROS) generation, nuclear morphology, and lipid droplet dynamics were evaluated using fluorescent staining. Western blotting was performed to determine the impact of the compounds on key signaling proteins such as EGFR, Akt, and c-Met. The results demonstrated that compounds exerted potent, dose-dependent cytotoxic effects in both cell lines, significantly reducing cell viability and colony formation. These agents induced mitochondrial dysfunction, modulated ROS levels, and disrupted lipid metabolism. Western blot analysis revealed marked downregulation of EGFR and Akt, indicating effective inhibition of critical oncogenic pathways. Notably, the most active compounds showed comparable or superior effects to cabozantinib, a clinically approved multi-kinase inhibitor. In conclusion, this study provides compelling evidence that novel c-Met-targeted compounds can effectively impair breast cancer cell survival and key signaling pathways. These findings highlight the therapeutic potential of dual pathway inhibition and support further development of these agents as promising candidates for BC treatment.

Keywords: Breast cancer, c-Met inhibitor, RTKs, Pyridazine triazole derivatives, c-Met/HGF signaling pathway.

ÖZET

Meme kanseri (MK), agresif yapıları ve sınırlı tedavi seçenekleri nedeniyle üçlü negatif ve hormon reseptörü pozitif alt tiplerinin önemli terapötik zorluklar sunduğu dünya çapında kanserle ilişkili morbidite ve mortalitenin önde gelen nedenlerinden biri olmaya devam etmektedir. Reseptör tirozin kinazlar, büyüme faktörlerine ligandlar olarak bağlanan tek geçişli transmembran reseptörlerdir. Hücrel metabolizma, farklılaşma, büyüme ve hareketlilikte önemli rolleri vardır. c-Met reseptör tirozin kinazı, tümör ilerlemesi, metastaz ve tedaviye dirençteki rolü göz önüne alındığında, umut verici bir terapötik hedef olarak ortaya çıkmıştır. Bu tez çalışmasında, MK hücre hatlarında c-Met sinyallemesini inhibe etmek üzere tasarlanmış yeni sentezlenen bileşiklerin antikanser etkinliğini ve altta yatan moleküler mekanizmalarını sistematik olarak araştırmayı amaçlamaktadır. Bu, MK modellerinde c-Met inhibitörleri olarak piridazin triazol türevlerini değerlendirmek için bir çalışmadır. C1, C2, C3, C4 ve C5 dahil olmak üzere yeni sentezlenen piridazin triazol türevlerinin etkileri hem MDA-MB-231 hem de MCF-7 BC modellerinde değerlendirildi. MTT ve koloni oluşumu dahil hücrel analizler, sitotoksiteyi ve hücre çoğalması üzerindeki etkileri değerlendirmek için kullanıldı. Mitokondriyal membran potansiyeli, reaktif oksijen türleri (ROS) üretimi, nükleer morfoloji ve lipid damlacık dinamikleri floresan boyama kullanılarak değerlendirildi. Bileşiklerin EGFR, Akt ve c-Met gibi temel sinyal proteinleri üzerindeki etkisini belirlemek için Western blot yapıldı. Sonuçlar, bileşiklerin her iki hücre hattında da güçlü, doza bağlı sitotoksik etkiler uyguladığını, hücre canlılığını ve koloni oluşumunu önemli ölçüde azalttığını gösterdi. Bu ajanlar mitokondriyal işlev bozukluğunu indükledi, ROS seviyelerini düzenledi ve lipid metabolizmasını bozdu. Western blot analizi, EGFR ve Akt'nin belirgin şekilde aşağı regülasyonunu ortaya koydu ve bu da kritik onkogenik yolların etkili bir şekilde inhibe edildiğini gösterdi. Özellikle, en aktif bileşikler, klinik olarak onaylanmış bir çoklu kinaz inhibitörü olan Kabozantinib ile karşılaştırılabilir veya daha üstün etkiler gösterdi. Sonuç olarak, bu çalışma, yeni c-Met hedefli bileşiklerin meme kanseri hücrelerinin hayatta kalmasını ve temel sinyal yollarını etkili bir şekilde bozabileceğine dair ikna edici kanıtlar sunmaktadır. Bu bulgular, çift yol inhibisyonunun terapötik potansiyelini vurgular ve bu ajanların meme kanseri tedavisi için umut verici adaylar olarak daha fazla geliştirilmesini destekler.

Anahtar Kelimeler: Meme kanseri, c-Met inhibitör, RTKs, Pridazin triazol türevleri, c-Met/HGF sinyal yolağı.

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LIST OF SYMBOLS AND ABBREVIATIONS

AC	: Adjuvant Chemotherapy
ADCs	: Antibody-drug conjugates
AI	: Aromatase Inhibitor
Akt	: Protein Kinase B
ANOVA	: Analysis of Variance
APS	: Ammonium Persulfate
AR	: Androgen Receptor
AXL	: AXL receptor tyrosine kinase
ABCG2	: ATP Binding Cassette Subfamily G Member 2
BC	: Breast Cancer
BCRP	: Breast Cancer Resistance Protein
BCS	: Breast-Conserving Surgery
BodiPy	: Boron-dipyrromethene
BRCA1	: Breast Cancer Gene 1
BRCA2	: Breast Cancer Gene 2
BSA	: Bovine Serum Albumin
cAMP	: Cyclic Adenosine Monophosphate
CDK4/6	: Cyclin-Dependent Kinase 4 and 6
c-Met	: Mesenchymal-epithelial transition factor
CNS	: Central nervous system
CO ₂	: Carbon Dioxide
C1	: Compound 1
C2	: Compound 2
C3	: Compound 3
C4	: Compound 4
C5	: Compound 5
c-Src	: Cellular Sarcoma Tyrosine Kinase
CTLA4	: Cytotoxic T-Lymphocyte Associated Protein 4
DAPI	: 4',6-Diamidino-2-Phenylindole
DBD	: DNA Binding Domain
DFG-out	: Aspartate-Phenylalanine-Glycine motif out
DMEM	: Dulbecco's Modified Eagle Medium
DMSO	: Dimethyl Sulfoxide
DOX	: Doxorubicin
DPBS	: Dulbecco's Phosphate-Buffered Saline
ddH ₂ O	: Double Distilled Water (Deionized Water)
dH ₂ O	: Deionized Water (Distilled Water)
E1	: Estrone
E2	: 17 β -Estradiol
E3	: Estriol
E4	: Estetrol
ECL	: Enhanced Chemiluminescence
EDTA	: Ethylenediaminetetraacetic Acid
EGFR	: Epidermal Growth Factor Receptor
Em	: Excitation
ER	: Estrogen Receptor
ER α	: Estrogen Receptor alpha

ERBB1	: Epidermal Growth Factor Receptor 1 (EGFR)
ERE	: Estrogen Response Element
ERK	: Extracellular Signal-Regulated Kinase
Erβ	: Estrogen Receptor beta
ESR1	: Estrogen Receptor 1 Gene
ESR2	: Estrogen Receptor 2 Gene
Em	: Emission
Ex	: Excitation
FBS	: Fetal Bovine Serum
FDA	: Food and Drug Administration
FLT3	: FMS-like tyrosine kinase 3
FOX1	: Forkhead Box Protein 1
GAPDH	: Glyceraldehyde 3-Phosphate Dehydrogenase
GATA3	: GATA Binding Protein 3
GPCRs	: G Protein-Coupled Receptors
GPOR	: G Protein-Coupled Estrogen Receptor
GPR30	: G Protein-Coupled Receptor 30
GR	: Glucocorticoid Receptor
H2DCFDA	: 2',7'-Dichlorodihydrofluorescein Diacetate
H ₂ O ₂	: Hydrogen Peroxide
HAT	: Histone Acetyltransferase
HB-EGF	: Heparin-Binding EGF-Like Growth Factor
HER2	: Human Epidermal Growth Factor Receptor 2
HGF	: Hepatocyte Growth Factor
HR	: Hormone Receptor
HRP	: Horseradish Peroxidase
HSP90	: Heat Shock Protein 90
IARC	: International Agency for Research on Cancer
ICI	: Immune Checkpoint Inhibitor
INPP4B	: Inositol Polyphosphate-4-Phosphatase Type II B
IPT	: Immunoglobulin-plexin-transcription domain
JAK	: Janus Kinase
KIT	: KIT proto-oncogene receptor tyrosine kinase
Ki-67	: Antigen Kiel 67
kDa	: Kilodalton
LAG3	: Lymphocyte Activation Gene 3
LBD	: Ligand Binding Domain
MDR1	: Multidrug Resistance Protein 1
MAPK	: Mitogen-Activated Protein Kinase
mbER	: Membrane-Bound Estrogen Receptor
miRNA	: microRNA
mTOR	: Mechanistic Target of Rapamycin
mTORC2	: Mechanistic Target of Rapamycin Complex 2
MTT	: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NaCl	: Sodium Chloride
NF-κB	: Nuclear Factor kappa-light-chain-enhancer of activated B cells
NLS	: Nuclear Localization Signal
NK1	: N-terminal and first kringle domain
NAC	: Neoadjuvant Chemotherapy
ns	: Not Significant

Nm	: Nanometer
nER	: Nuclear Estrogen Receptor
OS	: Overall survival
p300/CBP	: p300 / CREB-Binding Protein
PARP	: Poly (ADP-ribose) polymerase
PBS	: Phosphate-Buffered Saline
PCAF	: P300/CBP-Associated Factor
PD-1	: Programmed Death-1
PD-L1	: Programmed Death-Ligand 1
PDGFR β	: Platelet-Derived Growth Factor Receptor Beta
PFS	: Progression-free survival
PI	: Propidium Iodide
PI3K	: Phosphatidylinositol 3-Kinase
PIK3CA	: Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Alpha
PIP2	: Phosphatidylinositol 4,5-Bisphosphate
PIP3	: Phosphatidylinositol (3,4,5)-Trisphosphate
PGR	: Progesterone Receptor Gene
PR	: Progesterone Receptor
PSI	: Plexin-Semaphorin-Integrin domain
PTEN	: Phosphatase and Tensin Homolog
PVDF	: Polyvinylidene Fluoride
RET	: Rearranged during Transfection
RIPA	: Radioimmunoprecipitation assay buffer
ROS	: Reactive Oxygen Species
RPM	: Revolutions Per Minute
RTKs	: Receptor Tyrosine Kinases
SDS	: Sodium Dodecyl Sulfate
SEMA	: Semaphorin domain
SKBR-3	: A breast cancer cell line overexpressing HER2
Sp1	: Specificity Protein 1
SPH	: Serine protease homolog domain
STAT	: Signal Transducer and Activator of Transcription
TAMRAD	: Tamoxifen and Everolimus (mTOR inhibitor) trial name
TBT	: Tris-Buffered Saline
TBS-T	: Tris-Buffered Saline with Tween 20
TFs	: Transcription Factors
TIGIT	: T cell Immunoreceptor with Ig and ITIM domains
TIM-3	: T-cell Immunoglobulin and Mucin-domain containing-3
TK	: Tyrosine kinase
TM	: Transmembrane helix
TNBC	: Triple-Negative Breast Cancer
TP53	: Tumor Protein p53
Tris-HCl	: Tris(hydroxymethyl)aminomethane Hydrochloride
Tween 20	: Polyoxyethylene Sorbitan Monolaurate
V	: Volt
VEGFR	: Vascular Endothelial Growth Factor Receptor
WHO	: World Health Organization
XBP	: X-box Binding Protein 1
°C	: Degrees Celsius

μg	: Microgram
μL	: Microliter
μm	: Micrometer
mL	: Milliliter



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1. INTRODUCTION

Breast cancer (BC) is more frequently seen in workers worldwide and is at the top of the list of cancer-related deaths. Recent research indicates that BC ranks among the most frequently diagnosed cancers in women globally, with over 2 million new cases reported in 2020 (Duggan et al., 2020). The incidence and mortality rate of BC have increased over the last three decades, and according to estimates, the annual rate of new cases worldwide is expected to be approximately 2.7 million by 2030 (Ferlay et al., 2021). BC risk factors can be categorized into two main groups modifiable and non-modifiable. Modifiable factors include hormone therapy, physical activity, alcohol and cigarette consumption, inadequate vitamin supplements, consumption of processed foods, and exposure to chemicals. Non-modifiable factors are infertility, old age, genetic mutations, ethnicity, menstrual period, pregnancy and breastfeeding, family history of breast and ovarian cancer, and density of breast tissue (Łukasiewicz et al., 2021a). The risk factors of BC are shown in Figure 1.1.

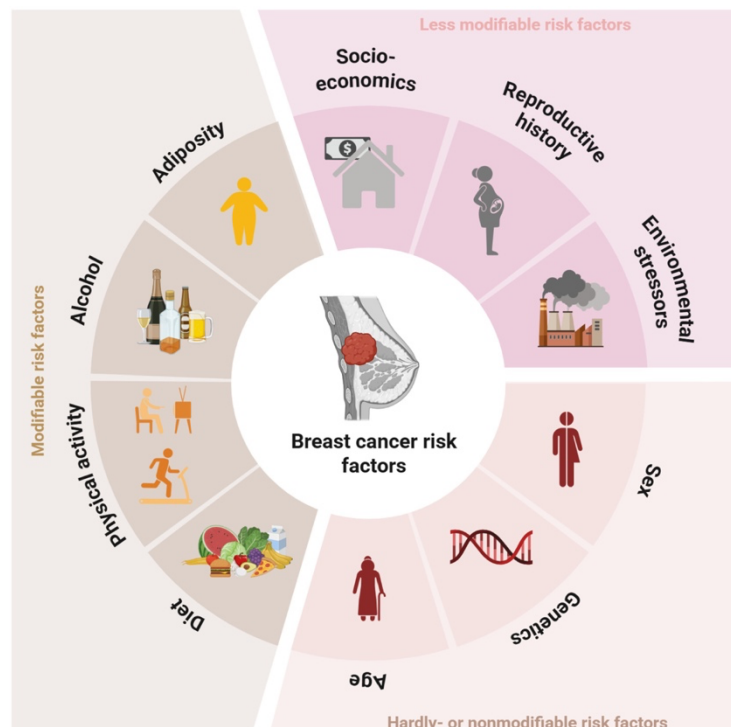


Figure 1.1: Risk factors of BC (Füreder et al., 2025).

1.1 Subtypes of Breast Cancer

BC is classified immunohistochemically according to the expression status of estrogen (ER), progesterone (PR), and human epidermal growth factor receptor 2 (HER2). It is divided into four subclasses, like luminal A, luminal B, HER2+ (also known as HER2-enriched), and triple-negative breast cancer (TNBC), depending on the combination of these biomarkers (Key, 2013). ER expression is relatively high in approximately 75% of invasive BC (Baumgarten & Frasor, 2012). ER regulates PR expression. Since the PR is present in about half of ER+ BC cases and is rarely found in ER-tumors, PR status serves as an indicator of ER pathway activity (Nicolini et al., 2018). Since ER and PR are highly expressed in BC cells, they are considered biomarkers in the diagnosis of BC (Colomer et al., 2018). Higher PR levels are positively associated with overall survival, resistance to treatment, and the time to recurrence. Lower PR levels are generally associated with a more aggressive disease process and less recurrence potential (Purdie et al., 2014). Since HER2 expression in BC accounts for approximately 25% of cancer, it plays an important role in choosing the appropriate treatment for the disease (Vaz-Luis et al., 2013). Overexpression of HER2 is one of the earliest events in the carcinogenesis of BC; therefore, it has increased the success rate of diagnosing metastatic BC by 80%; that is, it is a biomarker used to detect the presence of tumors and recurrence (Freudenberg et al., 2009). Luminal A-type BC cells are a subtype characterized by ER or PR+ and HER2-. They have a low expression level of Ki67, one of the cell proliferation markers. The Ki-67 antigen provides information about cell proliferation, as well as the aggressiveness of the cancer cell and the response to treatment (Davey et al., 2021). It expresses luminal genes such as ESR1, GATA3, XBP1, and FOXA1. Luminal A-type BC cells grow more slowly and have a higher survival rate, a higher response rate to hormone therapy, and a more limited response to chemotherapy (Matro et al., 2015). These cells have longer survival times when they relapse. MCF-7 BC cells are a cell type belonging to this class. They are ER+ PR+, and HER2-. In other words, they are hormone-dependent, have a lower proliferation rate, and are more sensitive to endocrine treatments. Luminal B-type BC cells have a higher prognosis than luminal A-type cells and lower expression of luminal genes such as PGR and FOX1. Their Ki-67 antigen expression level is high, indicating a higher proliferation rate and a worse prognosis (Inic et al., 2014). They can be ER+ but PR-. Luminal B-type cells can benefit from

hormonal treatment together with chemotherapy. HER2+ BC cells have higher HER2 expression in the absence of ER and PR. They grow much faster than luminal-type BC cells (**Figueroa-Magalhães et al., 2014**). They are a more aggressive cell type and have a worse prognosis. They require combined treatments with drugs targeting receptor tyrosine kinase inhibitors, such as lapatinib and neratinib, for treatment (**Segovia-Mendoza et al., 2015**). TNBC is a type of BC characterized by ER-, PR-, and HER2- BC cells. They constitute approximately 20% of all BC cells. TNBC cells exhibit a more aggressive behavior, relapse earlier, and can be diagnosed in more advanced stages of the disease (**Mayrovitz, 2022**). Their proliferation rate is high, and they are genomically unstable due to changes in DNA repair genes (**Collignon et al., 2016; Mayrovitz, 2022**). The MDA-MB-231 BC cell line belongs to the TNBC group. It is both more aggressive and has more metastatic properties, although it does not respond to hormone therapy. Due to this, treatment options are more limited (**Kumar & Aggarwal, 2016**). In women, breast cells are sensitive to the effects of estrogen and progesterone hormones, as well as disruptions in the balance of these hormones. Increased hormonal stimulation has been one of the main factors that increase the risk of BC (**Key, 2013**). Changes in the physiological level of hormones cause an increase in the risk of BC in women before and after menopause (**Lukasiewicz et al., 2021b**). The frequency of BC seen in men is much rarer than in women, and its detection tends to be later than in women. In men, advanced age, increased estrogen hormone, family history of BC, and gene mutations (BRCA1/BRCA2) (**Giordano, 2018**). The risk of developing BC in individuals begins at the age of 40. Today, almost 80% of BC cases recorded are in individuals aged 50 and over. However, it has been noted that TNBC is more commonly seen in women younger than 40 (**McGuire et al., 2015**). If there is a family history of BC, this is a factor that significantly increases the risk of BC. Approximately 20% of patients diagnosed with BC reported a previous history of BC in their family (**Mukama et al., 2020**). Genetic mutations are directly related to an increased risk of BC. For example, mutations in the BRCA1 genes on chromosome 17 and BRCA2 genes on chromosome 13 are directly associated with an increased risk of BC (**Shiovitz & Korde, 2015**). Not only the BRCA gene, but also mutations in the PTEN and TP53 genes, are associated with BC (**Shahbandi et al., 2020**). Conditions that cause hormonal imbalances, such as menstruation, pregnancy, and menopause, are important in the incidence of BC. Subtypes of BC are shown in **Figure 1.2**.

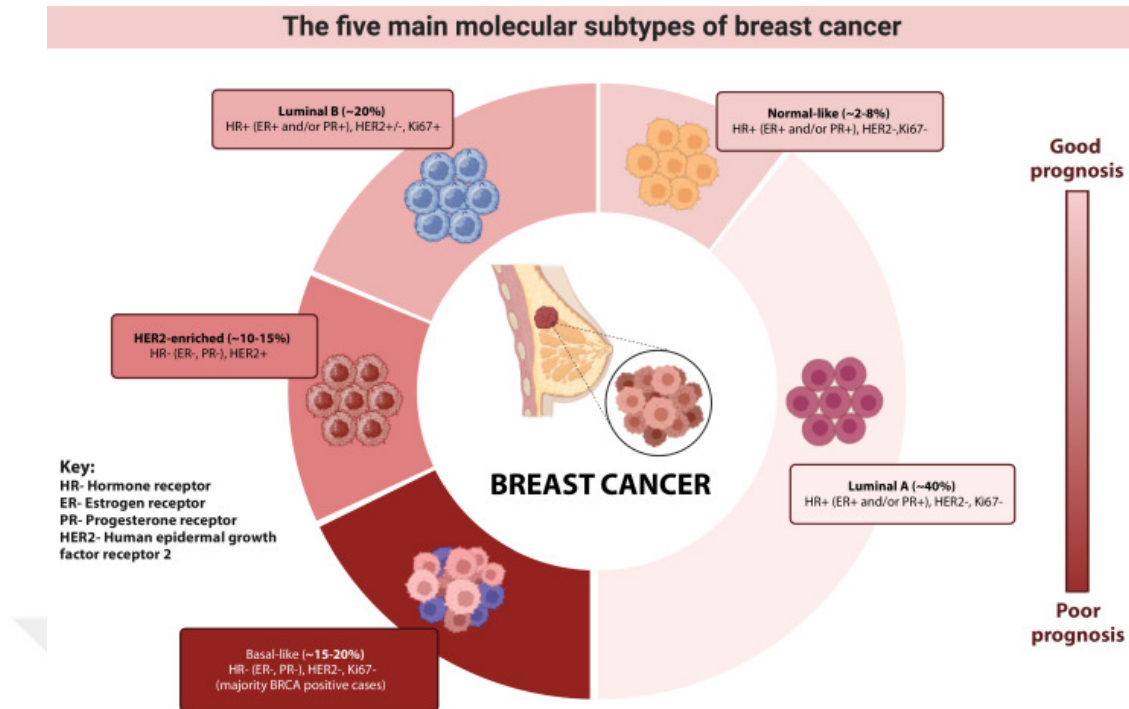


Figure 1.2: The molecular subtypes of BC (Sheva et al., 2024).

1.2 Estrogen Receptors

The ER family comprises the nuclear ER (nER) and GPER1 (G protein-coupled estrogen receptor), characterized by conserved domain structures, including the DNA binding domain (DBD) and the ligand binding domain (LBD) (Yoh et al., 2023). ER-alpha and ER-beta are the two main isoforms of nER, which are responsible for the regulation of the female reproductive system, the protection of the central nervous system, and the maintenance of bone mass, in addition to their functions in physiological processes. The ESR1 gene encodes the ER alpha gene, while the ER-beta gene is encoded by the ESR2 gene (Fuentes & Silveyra, 2019). The functions and effects of these two genes show both similarities and important differences. The primary function of the ESR 1 gene is to encode the ER alpha protein, which is predominantly found in breast tissue. In the presence of estrogen, ER alpha dimerizes and passes into the nucleus, binding to the ERE (Estrogen response element) regions in the DNA and initiating the transcription of target genes (Reinert et al., 2017). The C domain corresponds to the DBD domain with a zinc finger motif. The D domain is characterized by the presence of NLS (nuclear localization signaling) that is exposed after the conformational change caused by the interaction caused by the binding of the

ligand and is necessary for translocation to the nucleus (**Montano et al., 1995**). The E domain is the ligand binding domain containing the LBD. The F domain located at the C-terminus acts as a modulator of transcriptional activity (**Koide et al., 2007**). The structural and functional regions of ER are given in **Figure 1.3**.

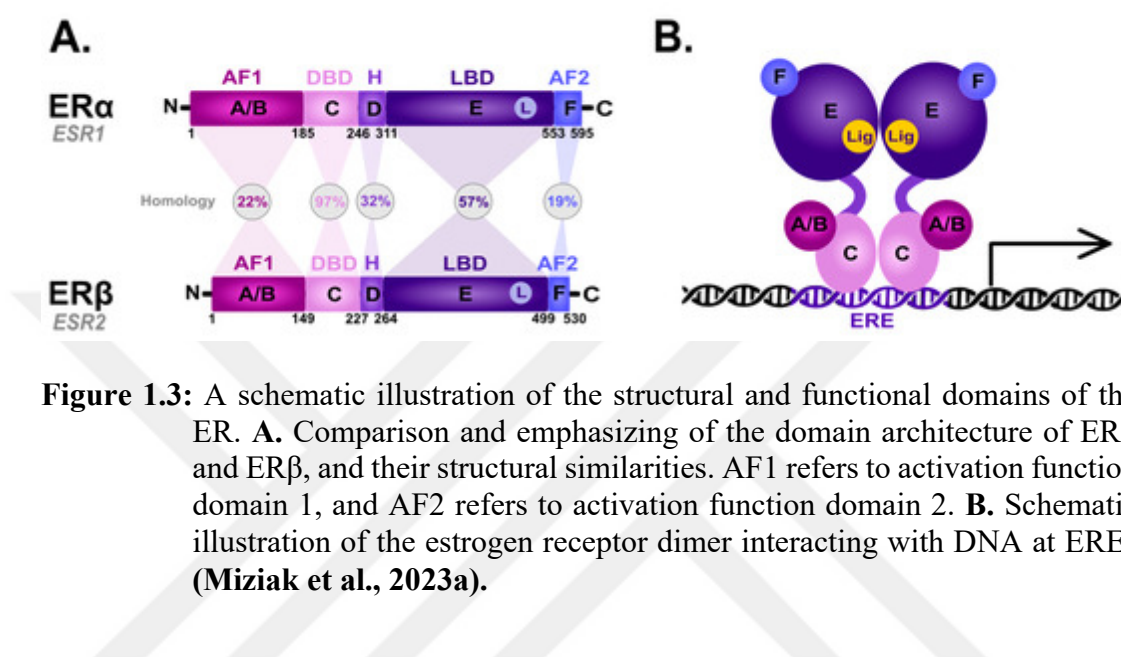


Figure 1.3: A schematic illustration of the structural and functional domains of the ER. **A.** Comparison and emphasizing of the domain architecture of ERα and ERβ, and their structural similarities. AF1 refers to activation function domain 1, and AF2 refers to activation function domain 2. **B.** Schematic illustration of the estrogen receptor dimer interacting with DNA at EREs (**Miziak et al., 2023a**).

ESR1 gene expression is present in approximately 70% of breast cancers and enables tumors to respond to hormonal treatments such as tamoxifen and aromatase inhibitors (**Li, McGinn, et al., 2022**). Mutations in the ESR1 gene allow the receptor to remain active without estrogen and to resist endocrine treatment. The primary function of the ESR2 gene is to encode the ER-beta protein. ER-beta is found in breast tissue, especially in myoepithelial cells, stroma, and endothelial cells, and may play a role in the regulation of target genes other than ER alpha (**Dalal et al., 2022**). Its expression is usually low in breast cancer but may be higher in TNBC (**Piperigkou et al., 2022**). High ESR2 expression is associated with better survival and better treatment response. ER beta has been described as a tumor suppressor in some studies and has shown suppressive effects on tumor proliferation, migration, and EMT (epithelial-mesenchymal transition) (**Dalal et al., 2022**). The structures of ER are given in **Figure 1.4**.

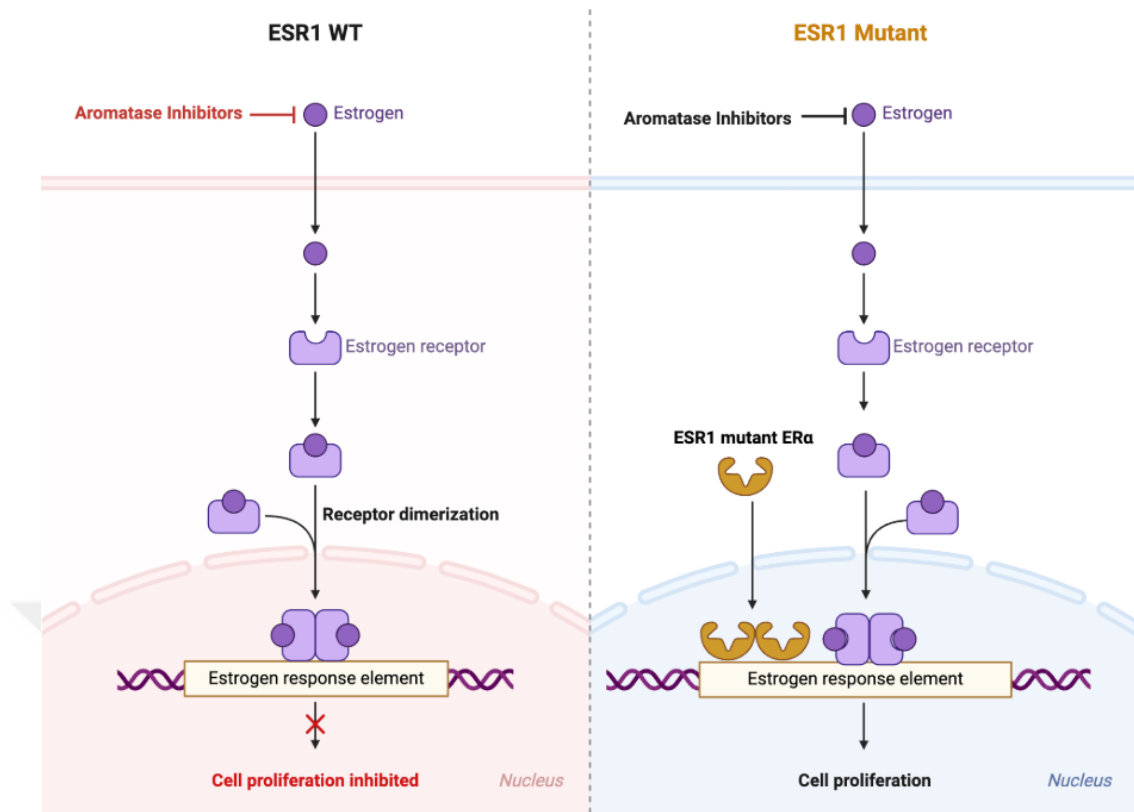


Figure 1.4: Schematic representation of ESR1 mutation in breast cancer (Created with BioRender.com).

1.3 Estrogen Receptor Signaling

ER signaling in BC refers to the cellular processes initiated by estrogens binding to their receptors, primarily ER α , ER β , and the membrane GPER, which regulate cell proliferation, differentiation, and survival in breast tissue (Clusan et al., 2023). In 70% of ER+ BC, estrogens are the principal signals that drive tumor growth and progression, mainly through the nuclear ER α , which is considered the most critical receptor for BC development and a pivotal therapeutic target. Dysregulation of ER signaling, resulting from altered expression, mutations, or changes in coregulator activity, leads to uncontrolled cell proliferation and cancer progression and can contribute to resistance to endocrine therapies (Girgert et al., 2019a). During tumorigenesis, ER signaling is reprogrammed, and differentiation-supporting functions are replaced by tumor-promoting gene expression patterns. This makes ER signaling a central focus for BC prevention and treatment. ER signaling can be divided into two subclasses as genomic and non-genomic signaling (Vrtačnik et al., 2014).

1.3.1 Non-genomic Signaling of Estrogen Receptor

Non-genomic ER signaling which is also called membrane-initiated pathway in which estrogen induces very rapid cellular responses via ER located on the plasma membrane or in the cytoplasm without initiating gene transcription in the cell nucleus (**Girgert et al., 2019b**). These responses occur within seconds to minutes and affect processes for example cell proliferation, migration, and survival by activating intracellular signaling cascades, second messengers, and kinases. These pathways play an essential role in BC biology, especially in aggressive and treatment-resistant tumors. Estrogen and ER complexes predominantly activate kinase pathways like Ras-Raf-MEK-MAPK and PI3K/AKT/mTOR (**Pupo et al., 2016a**). When estrogen binds to the receptor, Ras (GTPase) is activated, and MAPK phosphorylation and activation occur with Raf activation, which then phosphorylates the MEK protein (**Zivadinovic & Watson, 2005**). Therefore, the transcription of target genes is regulated by the activation of activating proteins belonging to the transcription factor family, such as c-Jun and c-Fos (**Rocca et al., 2022**). Alternatively, PI3K/AKT/mTOR activation is provided by mbER (membrane-bound ER). This activation occurs through the direct interaction of ER with different proteins. First, when estrogen is bound, mbER directly interacts with and activates a tyrosine kinase (TKs) called Src. Src plays a crucial role in initiating signal transmission within the cell. Second, the activated Src interacts with and activates phosphatidylinositol 3-kinase (PI3K). PI3K ensures the formation of a second messenger called PIP3 in the cell membrane (**Johnson et al., 2022**). Akt activation occurs with the transmission of the signal. PIP3 produced by PI3K ensures that the serine/threonine kinase called AKT is attracted to the cell membrane and activated by phosphorylation. Activated AKT activates mTOR, leading to its phosphorylation. mTOR plays a critical role in processes such as cell growth, metabolism, protein synthesis regulation, and cell survival (**Ciruelos Gil, 2014**). GPR30 is a membrane receptor that, unlike the classical nuclear ER (ER α and ER β), is localized to the plasma membrane and mediates the rapid, nongenomic effects of estrogen. GPER plays a vital role in hormone receptor-negative tumors, especially BC and TNBC (**Treeck et al., 2020**). When E2 estrogen binds to GPER, conformational changes occur in the receptor, and G protein activation occurs. This activation initiates rapid signaling within seconds to minutes within the cell, independent of classical nuclear ERs (**Kim et al., 2015a**). Activation of GPER activates c-Src TKs. This is a

critical step in the initiation of intracellular kinase cascades (Jiménez-Salazar et al., 2021). GPER triggers the release of heparin-bound EGF (HB-EGF) via metalloproteinases, which leads to the transactivation of EGFR (epidermal growth factor receptor). EGFR activation initiates MAPK/ERK and PI3K/AKT pathways (Lappano et al., 2014). GPER activates PI3K/AKT and MAPK/ERK signaling pathways directly or through EGFR, promoting cell proliferation, migration, and survival (Pupo et al., 2016b). GPER activation also leads to rapid changes in second messengers, such as increased cAMP and calcium mobilization within the cell (Kim et al., 2015b). The non-genomic pathway of ER is shown in Figure 1.5.

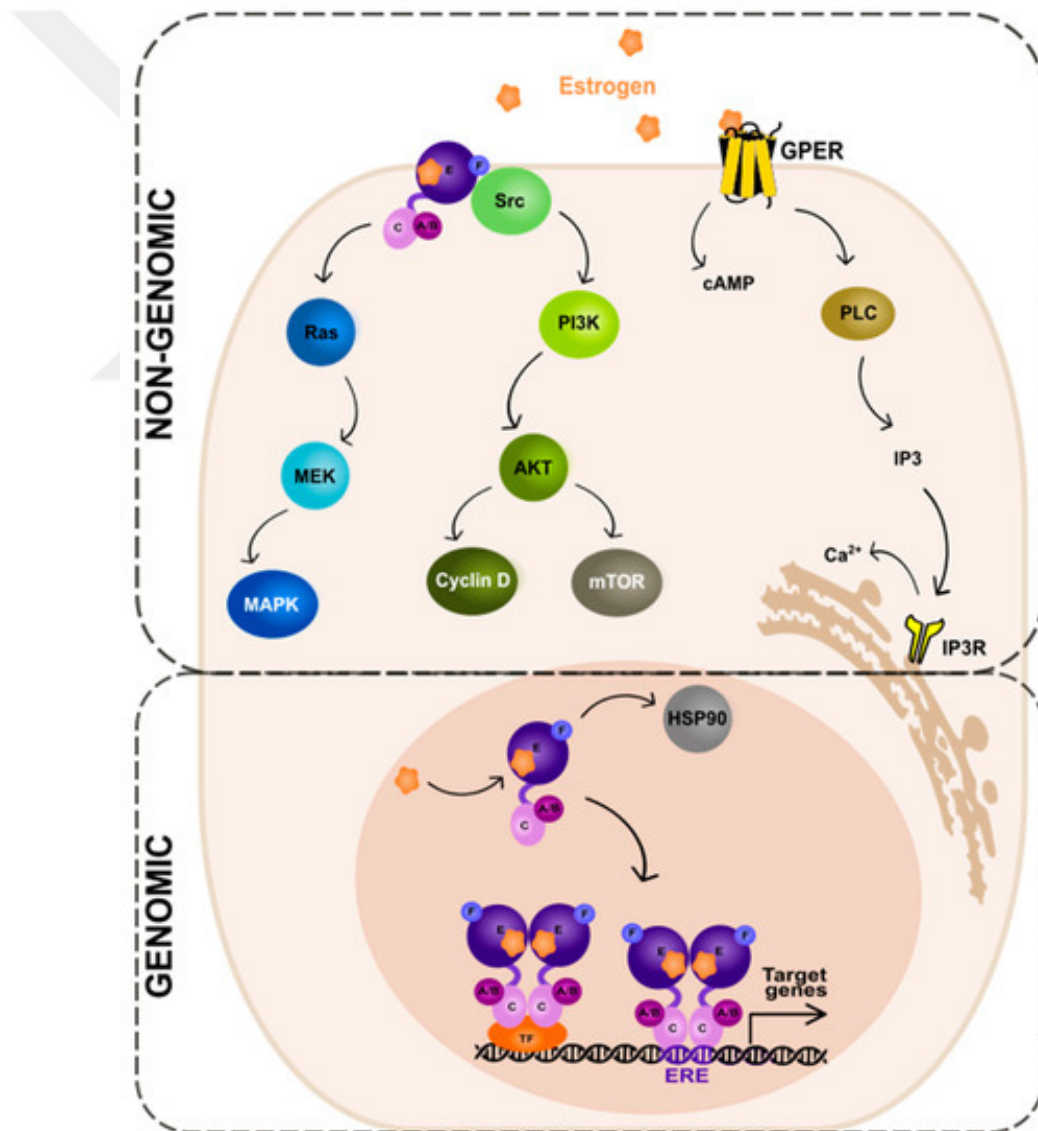


Figure 1.5: The genomic and non-genomic actions of the ER (Miziak et al., 2023a).

1.3.2 Genomic Signaling of Estrogen Receptor

In genomic ER signaling, estrogen and ER complexes are transported to the nucleus. In the nucleus, ER can regulate gene expression in two ways; indirectly and directly. In the indirect (non-classical) pathway, instead of binding directly to DNA, ER binds to DNA-binding transcription factors (TFs) through protein-protein interactions (**Safe & Kim, 2008**). For example, by interacting with TFs such as Sp1 and NF- κ B, it modulates the expression of genes that lack EREs in their promoters. In this mechanism, the ER controls the activation of TFs by interacting with coactivator proteins, thereby initiating or repressing the transcription of various genes (**Björnström & Sjöberg, 2004**). TFs such as Sp1 and NF- κ B bind to their specific DNA sequences. ER modulates the activity of these TFs by binding to them. Indirectly regulated genes do not have EREs in their promoter regions; ER controls the expression of these genes without EREs (**Safe & Kim, 2008**). If there is an ERE in the promoter of a gene, the ER binds directly to this region and changes the expression of the gene in the classical genomic way. When estrogen binds to the ER, the ER undergoes a conformational change, and this change leads to the release of the ER from HSP90 (heat shock protein complex). HSP90 is a chaperone protein that protects the unbound ER from destruction in the cell (**Powell et al., 2010**). In the studies, unliganded ER α , that is, unbound to estrogen, is a protein that is rapidly destroyed in the cell and has a half-life of 4-5 hours. The synthesis and destruction rates of ER α were measured in MCF-7 BC cells (**Wilson et al., 2010**). Activation of HAT (Histone acetyltransferase) enzymes is required for the ER to initiate gene transcription fully. HAT enzymes loosen the chromatin structure, change the location of nucleosomes, and thus facilitate access of RNA polymerase II to genes. HAT enzymes, such as p300/CBP, acetylate proteins of the transcription machinery, increasing gene expression, and interact with other HAT enzymes such as PCAF. ER cross-talks with other steroid hormone receptors such as PR, GR (glucocorticoid receptor), and AR (androgen receptor) in BC and some other cancer types, such as endometrial cancer (**Buxant et al., 2010; Pan et al., 2020**). These receptors can bind to similar DNA regions and regulate the recruitment of other TFs and chromatin regulatory proteins to the site of binding. The genomic effects of ER may be shaped not only by itself but also by the presence and action of other steroid receptors. In BC, PR is both a gene product induced by ER α and a protein that physically interacts with ER α and can alter

the binding sites of ER α to DNA. This interaction may result in distinct gene expression profiles in BC cells, potentially influencing patient prognosis. However, the mechanisms by which ER is modulated by PR to manage BC better are not fully understood. Studies have shown that AR also plays a role in ER binding to DNA. In addition, when GR is activated in BC, it suppresses gene expression regulated by ER α . The study has shown that GR activation reduces cell proliferation in ER⁺ BC cells and suppresses the association of ER with DNA (Tonsing-Carter et al., 2019). Figure 1.5 shows the genomic pathways of ER signaling.

1.4 Molecular Mechanism of Breast Cancer

Genetic mutations and alterations in signaling pathways play a crucial role in the onset and progression of BC (Ortega et al., 2020). As a result of mutations, it can disrupt fundamental regulatory processes for the cell, such as cell survival, proliferation, apoptosis, and DNA repair, leading to tumor formation. Changes that occur due to mutations and alterations in signaling pathways lead to the diversity observed in different types of BC (Carvalho et al., 2025a). In luminal BC estrogen is a steroid hormone associated with the reproductive system in women. It has four different forms with different functions, namely E1, E2, E3, and E4. The E1 form, called estrone, increases after menopause and has a pro-inflammatory effect on breast fat tissue (Qureshi et al., 2020). The E2 form is called estradiol and is found in large quantities in the bloodstream. The production of E3 and E4 forms occurs during pregnancy. While the placenta is responsible for the production of the E3 form, the fetus is responsible for the secretion of E4 (Miziak et al., 2023b). The E2 form plays a role in regulating biological processes. It performs its functions by binding to two nuclear TFs, ER α and ER β . Mutations in ER α and ER β receptors lead to the proliferation of cancer cells (Liu et al., 2020). ER α plays a vital role in regulating target genes related to proliferation, cell cycle, and apoptosis in cancer development (Miricescu et al., 2020a). When E2 binds to ERs in the plasma membrane, it can activate plasmatic ER α , which binds to caveolin-1. With the activation of ER α , it can activate the PI3K/AKT/mTOR downstream pathway. This pathway is responsible for various cellular processes, consisting cell proliferation, cell metabolism, apoptosis, and angiogenesis. When it is overactivated, it increases cell proliferation and prevents apoptosis, thereby contributing to the development of BC (Miricescu et al., 2020a). Almost 60% of many

types of cancer, including BC, overactivate the PI3K/AKT/mTOR pathway with different variations (Engelman, 2009). Activation of this pathway is one of the primary reasons for the development of treatment resistance (Martini et al., 2014). After ER α is activated, p85, one of the subunits of PI3K, interacts with E2, and the p110 catalytic subunit is activated. Thus, AKT comes into play. mTOR Complex 2 phosphorylates AKT and brings it into an active form. This active form promotes the phosphorylation of target proteins found in the nucleus, cytoplasm, and cell membrane (Miricescu et al., 2020b). However, PI3K changes due to genetic mutations and changes in BC. One of the subunits of PI3K (p110 α) is a catalytic unit regulated by the PI3KCA gene. When this catalytic unit is mutated, it causes hyperactivation of the PI3KCA gene, leading to the loss of PTEN and subsequent tumor growth (Carvalho et al., 2025b). PI3K are lipid kinases that transmit extracellular signals (growth factors, cytokines, etc.) into the cell. They are divided into three classes. The most studied and cancer-related class is PI3K. Class I is divided into two subgroups. PI3K IA, activated by RTKs, GPCRs, and oncogenes such as Ras. PI3K IB is activated only by GPCRs. PI3K IA is the subgroup most directly related to cancer (Rodgers et al., 2021). This complex includes a regulatory subunit (e.g., p85) and a catalytic subunit (p110 α). Activation results in the conversion of PIP2 to PIP3. PIP3 triggers cellular growth and proliferation. Important tumor suppressors that inhibit this pathway include PTEN, which converts PIP3 back to PIP2. INPP4B is tumor suppressor associated with TNBC (Guerrero-Zotano et al., 2016). Mutations in the PIK3CA gene (encoding p110 α) are common in BC (especially in the luminal A and HER2+ subtypes). These mutations overactivate the PI3K pathway, contributing to tumor development and treatment resistance (Maira et al., 2012). Progesterone is a hormone belonging to the same steroid hormone group as estrogen, which is responsible for pregnancy, the menstrual cycle, and breast epithelial cell proliferation in women (Li et al., 2022). **Figure 1.6** shows the PI3K/AKT/mTOR signaling pathway in BC.

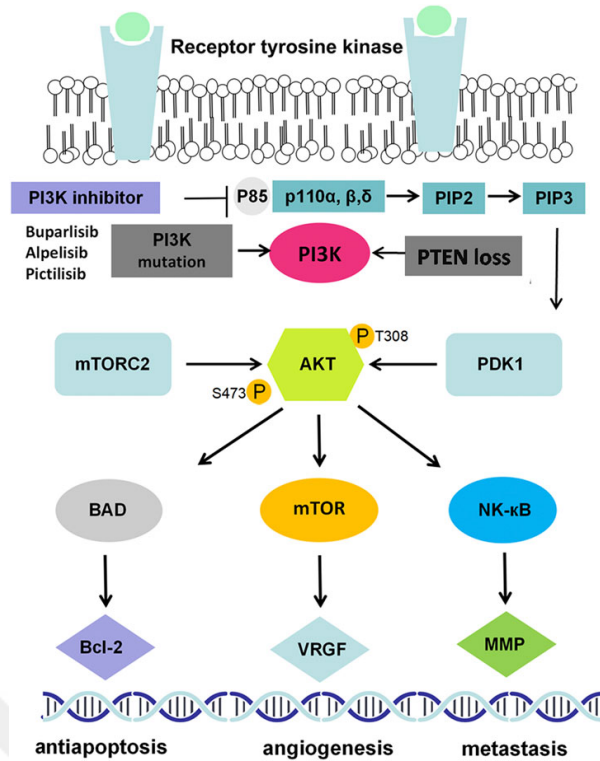


Figure 1.6: PI3K/AKT signaling pathway in BC (Dong et al., 2021).

Since PR signaling in BC triggers the transition from paracrine regulators to autocrine regulation, it is considered a clue to tumor development. However, the role of progesterone hormone in BC has not been fully explained (Azeez et al., 2021). TNBC is characterized as ER-, PR-, and HER2-, exhibiting a wide heterogeneity in terms of genomics due to the frequent occurrence of genetic mutations (Derakhshan & Reis-Filho, 2021). The genes BRCA1 and BRCA2 are crucial for cellular DNA repair. They are responsible for the activation of points in the cell cycle to detect the presence of errors in the cell cycle and to evaluate DNA damage. They participate in cytoplasmic division, inhibit the amplification of damaged oncogenes in DNA, and induce cell apoptosis if not repaired (Derakhshan & Reis-Filho, 2021). Mutations in the BRCA1 gene inhibit the homologous recombination pathway, causing alternative DNA damage repair mechanisms to be activated; mutations in the BRCA2 gene, on the other hand, compromise cytoplasmic division and cause an increase in the number of binuclear cells (Choi et al., 2023). Mutations in these two genes lead to genomic instability and progression to tumor initiation (Xie et al., 2022). Research has indicated that cancer patients with BRCA1 gene mutations also have TP53 gene alterations (Fu et al., 2022). Changes in the PI3K/AKT/mTOR signaling pathway lead to disruptions

in cellular processes, and as a result of mutations in the PIK3CA gene, tumor cells exhibit increased proliferation, migration, and resistance to apoptosis (**Marvalim et al., 2023**). In a study comparing TNBC mutations and PIK3CA gene mutations in hormone receptor-positive BC, it was stated that this gene mutation was less common in the TNBC type. While this rate is 10% in the TNBC type, this mutation is 28% in the hormone receptor-positive BC type (**Mosele et al., 2020**). In other words, it has been understood that TNBC is more affected by mutations occurring in DNA repair compared to the PIK3CA mutation. When a mutation occurs in the DNA repair mechanism, it causes the mutation to spread during cell division, thus making the tumor cell uncontrollable and causing it to exhibit aggressive behavior (**Mosele et al., 2020; Reinhardt et al., 2022**).

1.5 The Role of c-Met

c-Met (cellular-Mesenchymal epithelial transition factor) is a member of the RTKs regulates key cellular functions, including survival, growth, and motility (**Faiella et al., 2022**). When c-Met is overactivated, it can lead to tumor growth and metastasis of cancer cells. This has made c-Met a target for cancer treatment (**Sierra & Tsao, 2011**). HGF (Hepatocyte Growth Factor) acts as a ligand for c-Met and activates it (**Zhang et al., 2018**). HGF binds to c-Met, providing autophosphorylation of intracellular kinase domains and inducing c-Met dimerization (**Uchikawa et al., 2021**). Phosphorylated kinase domains activate downstream signaling pathways. C-Met consists of SEMA (Semaphorin), PSI (plexin-semaphorin integrin), IPT1-4 (4 consecutive immunoglobulin-plexin transcription factors), and TM (transmembrane helix) parts. The structure and functional domains of c-Met are shown in **Figure 1.7**.

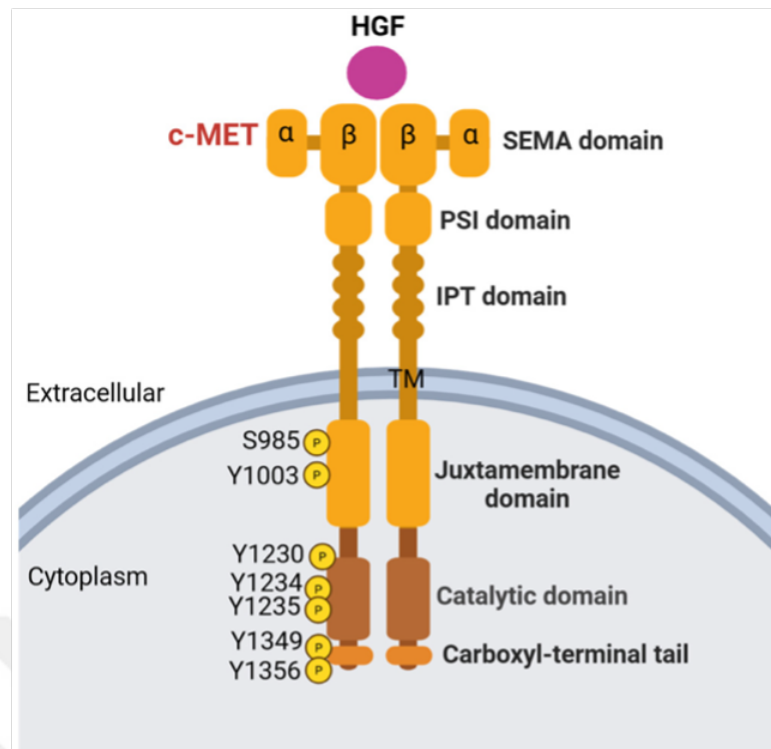


Figure 1.7: Structural diagram of the c-MET receptor showing its extracellular domains (SEMA, PSI, IPT), transmembrane region, and intracellular domains with key phosphorylation sites involved in signal transduction upon HGF binding (Created with BioRender.com).

The SEMA domain has key role in HGF binding and forms the basis of receptor dimerization triggered by the ligand (Linossi et al., 2021). The transmembrane domain plays a role in signal transmission through the cell membrane into the cell. The intracellular domain has TK activity. After ligand binding, this domain is auto-phosphorylated and carries out signal transmission (Raj et al., 2022a). HGF is a biological growth factor secreted by mesenchymal stem cells with mitogenic and morphogenic properties. HGF is synthesized as an inactive single-chain polypeptide and is cleaved by serine proteases into alpha (69 kDa) and beta (34 kDa) chains; a disulfide bond connects these two chains and forms the active heterodimeric form (Mi et al., 2022). HGF binds to the c-met receptor via two different binding sites. The first is the high-affinity NK1 domain (comprising the N-terminal and first kringle domain of HGF), which interacts strongly with c-met. The SPH domain, found on the beta chain, contributes to receptor activation by interacting with c-Met's SEMA domain. A single HGF molecule can bind to two c-Met receptors at distinct sites, facilitating their dimerization and subsequent activation (Raj et al., 2022).

1.5.1 c-Met/HGF Signaling in Breast Cancer

Binding of HGF to c-Met triggers receptor dimerization and initiates autophosphorylation of the intracellular tyrosine kinase domain. This phosphorylation occurs particularly at tyrosine residues Y1230, Y1234, and Y1235. These phosphorylations increase the kinase activity of the receptor and create multiple phosphotyrosine binding sites for the binding of signal transduction complexes **(Arnold et al., 2017)**. Various downstream signaling pathways are activated by phosphorylating Y1249 and Y1256 in the C-terminal region, which enables the binding of adaptor proteins in signal transduction **(Raj et al., 2022b)**. PI3K/AKT/mTOR, RAS/RAF/ERK (MAPK), STAT3, and other TFs are the main signaling pathways that are activated. In the PI3K/AKT/mTOR signaling pathway, phosphotyrosines such as phospho-Y1313 interact with the p85 regulatory subunit of PI3K, supporting cell proliferation, survival, and metabolic activity. It also contributes to treatment resistance in cancer cells **(Hervieu & Kermorgant, 2018)**. The RAS/RAF/ERK (MAPK) pathway is responsible for cell growth and differentiation. When c-Met is activated, it activates the RAS protein and triggers this cascade. TFs such as STAT3 are phosphorylated by c-Met activation and regulate gene expression in the nucleus. This increases cell proliferation, migration, and invasion **(Jiang et al., 2024)**. c-Met expression is particularly seen at a high rate in TNBC and basal-like BC types. Since these types are hormone receptor-like ER, PR and HER2-, targeted treatment options are limited, and they exhibit more aggressive behavior. In HER2+ BC types, excessive c-Met expression leads to resistance to anti-HER2 treatment such as trastuzumab (Herceptin). This resistance is associated with c-Met, which supports cancer stem cell properties and confers resistance to cell apoptosis. HGF binding to c-Met and interaction with other pathways are shown in **Figure 1.8**.

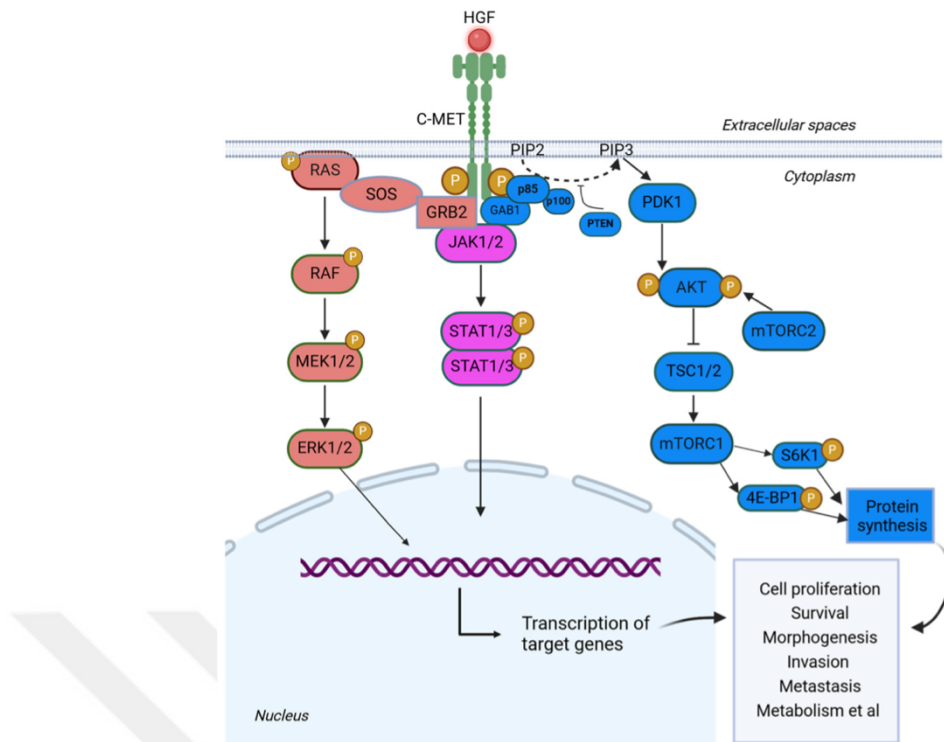


Figure 1.8: Schematic overview of c-MET signaling pathways activated by HGF, illustrating downstream cascades (RAS/MAPK, JAK/STAT, PI3K/AKT/mTOR) that regulate gene transcription and promote cell proliferation, survival, invasion, and metastasis (Created with BioRender.com).

The basis for the abnormal c-Met function in BC involves various mechanisms, including genetic mutations, excessive protein production, the cell's output of HGF, continuous c-Met activation (autocrine signaling), and an increase in the number of MET gene copies (Faiella et al., 2022b; Wood et al., 2021). Dysregulated c-Met activity promotes tumor cell growth, survival, and chemotherapy resistance while inducing the epithelial-mesenchymal transition (EMT), thereby enhancing cellular migration and invasion capabilities (Park & Richardson, 2020). Since excessive production of c-Met in TNBC facilitates the spread of the tumor to surrounding tissues and metastasis to distant organs, c-met has a vital place in TNBC treatment. Excessive c-met expression in BC patients causes the disease to survive and progress more rapidly. c-Met expression is excessive in TNBC patients. c-Met expression is low in normal breast tissue, begins to increase in precancerous lesions, and reaches its highest level in invasive BC (Iovino et al., 2022). Approximately 14-54% of invasive BC have very high c-Met expression and have lower survival rates (Abouelfadl et al., 2022; Iovino et al., 2022).

1.5.2 Chemoresistance of c-Met in Breast Cancer

As BC progresses, cancer cells become more resistant to chemotherapy. During this process, the cell's growth and survival signaling pathways change, and more than one pathway assumes the same function, thereby facilitating cell survival **(Prihantono & Faruk, 2021)**. Resistant cancer cells can continue to grow and proliferate even under challenging conditions (e.g., under drug treatment). Recent research indicates that increased expression of the c-Met protein or amplification of its gene plays a role in the development of drug resistance in BC. **(Wang et al., 2019)**. c-Met increases the production of proteins that expel drugs from the cell membrane; this prevents chemotherapy drugs from accumulating in the cell, creating resistance. Overproduction of c-Met in doxorubicin-resistant cells also causes a drug transporter protein called BCRP/ABCG2 to become more active. c-Met triggers the PI3K/AKT signaling pathway in the cell, enabling cancer cells to grow, move, and survive **(Kryczka & Boncela, 2018)**. The amount of c-Met mRNA and protein is significantly higher in resistant cells; a subunit of PI3K is also increased. These cells were found to have high levels of active AKT protein, indicating that c-Met activates the PI3K/AKT pathway. A separate study found that c-Met and another drug transporter, MDR1, were higher in cancer cells resistant to multiple drugs **(Zhong et al., 2024)**. Overproduction of c-Met contributes to cancer cells gaining stem cell properties. In pancreatic cancer cells, those with high c-Met acted like stem cells and formed tumor spheres, while those with low levels did not have this ability. When the c-Met inhibitor XL184 (Cabozantinib) was used, tumorsphere formation was reduced, indicating that c-Met played a crucial role in the presence of stem cell-like cancer cells **(Guo et al., 2024)**. A recent study revealed that c-Met was also elevated in cells with high HER2 protein levels, which contributed to resistance to trastuzumab treatment. Therefore, drugs targeting c-Met may be an effective treatment alternative for patients resistant to trastuzumab **(Elshazly & Gewirtz, 2022)**. Another study has shown that c-Met activates STAT3, and STAT3, in turn, increases c-Met production, creating a continuous activation cycle in resistant cancers **(Zhu et al., 2021)**. The c-Met inhibitor capmatinib reduced tumor growth and increased drug sensitivity in BC cells that were resistant to treatment. Excess c-Met creates resistance to drugs that target EGFR; when c-Met inhibitors are used, cells develop an alternative resistance mechanism by increasing EGFR expression **(Rastogi et al., 2016)**. A compound that concurrently

inhibits both c-Met and EGFR (doxazosin) has been shown to markedly suppress tumor progression and metastasis in TNBC cells. The c-Met and EGFR/HER2 signaling pathways are crucial drivers of metastasis across various cancers, with a particularly significant role in BC. (S. Kim et al., 2023). While EGFR (ERBB1) is elevated in both brain-metastatic and trastuzumab-resistant BC, c-Met upregulation is specifically pronounced in brain-metastatic cells. c-Met and EGFR pathways are promising targets for treatment. When used together, neratinib (EGFR/HER2 inhibitor) and cabozantinib (c-Met inhibitor) inhibited tumor growth in BC cells that had metastasized to the brain (Uribe et al., 2021). As a result, excess c-Met increases resistance to chemotherapy, especially in TNBC; however, this resistance can be overcome with drugs or combination therapies that target c-Met.

1.5.3 c-Met Inhibitors

A key strategy in cancer treatment is to inhibit the abnormal activity of c-Met tyrosine kinase. In recent years, numerous small-molecule compounds have been designed to inhibit the kinase domain of c-Met at its active site. Most of these inhibitors competitively bind to ATP, inhibiting tyrosine phosphorylation and thus blocking c-Met signaling (Puccini et al., 2019a). Class I and Class II inhibitors are the two primary types of small-molecule c-Met inhibitors that are distinguished by their structure and ability to bind to the c-Met kinase domain. Class I inhibitors (e.g., tivantinib, lapatinib, crizotinib) are generally highly selective and bind to the ATP binding site of c-Met in a U-shaped manner and target the activation loop. These inhibitors typically bind to the active conformation of c-Met, exhibiting more specific effects. Triazolotriazine, quinazoline, and similar structures are frequently seen in this group (Zhao et al., 2021). Class II inhibitors (e.g., foretinib, cabozantinib) target the inactive (DFG-out) conformation of c-Met while competitively binding to ATP and can generally inhibit multiple kinases¹²³. These inhibitors have cyclic or acyclic urea groups and can more effectively control tumor growth, angiogenesis, and metastasis by blocking other kinases such as VEGFR, AXL, and RET (Nazari et al., 2024). Therefore, they have broad antitumor effects, especially in aggressive and resistant tumors. Foretinib and cabozantinib also affect the tumor microenvironment by inhibiting targets other than MET, such as VEGFR2, PDGFR β , and AXL. Both types of inhibitors have been thoroughly investigated in clinical trials to assess their

pharmacodynamic and pharmacokinetic profiles, effectiveness, safety, and the likelihood of developing drug resistance (Song et al., 2015). Crizotinib and cabozantinib are the first small-molecule c-Met inhibitors to receive FDA approval and have been used in various solid tumors, primarily lung, thyroid, and kidney cancers. Cabozantinib is promising by suppressing tumor growth, migration, and angiogenesis, especially in BC subtypes where c-Met is highly expressed, such as TNBC (Abdelaziz & Vaishampayan, 2017). Foretinib is also a multi-kinase inhibitor and is being investigated for its efficacy in TNBC and other cancers. More recently, highly selective c-Met inhibitors like lapatinib have been developed and granted FDA approval, especially for lung cancer patients harboring MET exon 14 skipping mutations. However, these agents have not yet progressed to clinical trials for BC (Liang & Wang, 2020). Crizotinib has demonstrated moderate antiproliferative effects in BC cell lines, and synergistic or mixed responses have been observed when used in combination with HER2 inhibitors. Additionally, crizotinib has been demonstrated to impact DNA replication and apoptosis by modulating the cell cycle in BC cells. Considering the high c-Met expression in BC and the resistance to anti-EGFR therapy, it has been shown that combination therapies targeting both EGFR and c-Met, especially in TNBC cell lines, may have synergistic antitumor effects. For example, the combination of EGFR inhibitors, such as gefitinib or cetuximab, with tepotinib has provided a potent anti-proliferative impact in TNBC cells (You et al., 2021). In conclusion, c-Met tyrosine kinase inhibitors have significant potential in cancer treatment, both in monotherapy and combination therapies. The new generation inhibitors being developed have the potential to provide clinical benefits, especially in resistant and aggressive BC subtypes, by aiming to be more selective and less toxic (Jain et al., 2024).

1.5.4 Off-Target and Cytotoxic Effects of c-Met

c-Met inhibitors can arrest the function of c-Met not only against tumor cells but also in normal tissues. In this case, unwanted adverse effects may occur, potentially affecting the patient's quality of life (Bansal et al., 2023). Many medications that target c-met have been developed for various cancers, particularly BC. Most developed drugs have serious side effects and insufficient efficacy, but only a few have yielded effective results in clinical trials. The FDA revoked approval of Bevacizumab for BC treatment

due to its association with severe adverse effects, including tumor necrosis and bleeding when combined with paclitaxel, ultimately concluding that the risks outweighed the benefits **(Z. Zhang et al., 2022)**. When tivantinib, a c-Met inhibitor, was tested for liver cancer treatment, it led to significant adverse effects, including hematological toxicity and neutropenia (a reduction in blood cell count). The cabozantinib c-met inhibitor recommended for the treatment of BC caused side effects such as fatigue, increased liver enzymes, high blood pressure, stomach discomfort, shortness of breath, and loss of appetite **(Bruchbacher et al., 2024)**.

1.5.5 Approaches to Overcome the Difficulties in c-Met Targeting

Various approaches are combined to address the obstacles associated with c-Met-targeted therapies in BC, with the goal of enhancing treatment effectiveness while minimizing adverse effects. Simply examining the amount of c-Met may not be sufficient; therefore, research is ongoing to identify more reliable biomarkers that will enable patient-specific treatment selection **(El-Sayes et al., 2021)**. In complex tumors with multiple genetic alterations, the use of different drugs in combination may increase the effectiveness of treatment. Combining c-Met drugs with treatments targeting other signaling pathways can both improve efficacy and prevent the development of resistance **(Kucinska et al., 2024)**. Combination treatments, particularly those involving chemotherapy and immunotherapy, have yielded positive results in both laboratory and clinical studies. In a separate study, c-Met was found to be very active in TNBC cells resistant to PARP inhibitors **(Singh & Yadav, 2021)**. When talazoparib and crizotinib were used together, the growth of resistant TNBC cells was significantly inhibited. However, targeting c-Met alone did not increase the efficacy of talazoparib in these resistant cells to the expected extent. Researchers observed that EGFR was also very active in these cells and interacted with c-Met. When the EGFR inhibitor and PARP inhibitor were used together, growth was suppressed more effectively in cells with low c-Met levels **(Chu et al., 2020)**. When c-Met and EGFR were suppressed at the same time, TNBC cells resistant to talazoparib became sensitive to the drug again. In other words, targeting both c-Met and EGFR at the same time in TNBC can break the resistance that develops against PARP inhibitors **(Ou et al., 2024)**. Next-generation c-Met inhibitors are under development to overcome the limitations of current therapies. These advanced inhibitors are

specifically engineered to selectively target c-Met and to be more effective against resistance-causing mutations, thereby improving efficacy and minimizing side effects. Design processes are constantly being developed to make these new drugs safer and have fewer side effects (**H. Zhang et al., 2024**). New drug delivery technologies are being developed to ensure that c-Met drugs reach only tumor cells and do not harm healthy tissues. New technologies, such as nanoparticles or antibody-drug conjugates (ADCs), enable the drug to be concentrated in tumor tissue while reducing the risk of side effects in other parts of the body (**Fong et al., 2025**).

1.5.6 Cabozantinib (XL184)

Cabozantinib is an orally available and efficiently absorbed medication capable of concurrently blocking several receptor tyrosine kinases, such as c-Met, VEGFR2, RET, FLT3, and KIT (**Iweala et al., 2024; O’Sullivan Coyne et al., 2022**). In both *in vitro* and *in vivo* studies, cabozantinib inhibits the c-Met and VEGFR2 signaling pathways, thereby stopping tumor growth and causing tumors to shrink (**Hagege et al., 2022**). Cabozantinib is a drug that can reach brain tissue and is approved by the US FDA for use in advanced stages of thyroid, kidney, and liver cancer (**Choy et al., 2022**). Several clinical studies have been conducted in BC using cabozantinib alone or in combination with other drugs. In the first Phase II study, a response rate of 13.6% and a disease control rate of 46.7% were achieved with cabozantinib in patients with advanced BC (**Tolaney et al., 2017**). Later, in another Phase II study in patients with TNBC, the response rate of cabozantinib was found to be 9%. In a study in which cabozantinib and the immunotherapy agent nivolumab were used together, the response rate was 6%, and the median progression-free survival was 3.6 months; no unexpected serious side effects were observed (**Barroso-Sousa et al., 2021**). In HER2+ BC patients with bone metastases, cabozantinib demonstrated a 38.5% response rate on bone scans, with median progression-free survival (PFS) of 4.3 months and median overall survival (OS) of 19.6 months. Notably, no complete or partial tumor responses were observed. In a separate study of BC patients with brain metastases, cabozantinib showed central nervous system (CNS) response rates of 5% in HER2+ patients, 14% in hormone receptor-positive (HR+) patients, and 0% in TNBC patients, with an overall median PFS of 3 months (**Leone et al., 2020**).

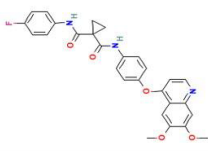
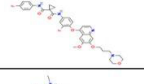
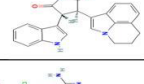
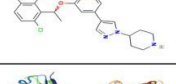
1.6 Current Therapeutic Strategies for Breast Cancer

There are various treatment methods for BC, including surgery, endocrine therapy, chemotherapy, immunotherapy, and targeted therapy. Mastectomy and breast-conserving surgery (BCS) are local therapeutic approaches utilized in the management of early-stage invasive BC. Administering chemotherapy in early-stage BC can lower the likelihood of cancer recurrence by about 30%. **(Mano & Awada, 2004)**. NAC, AC (Adjuvant chemotherapy), and salvage chemotherapy are among the chemotherapy methods found in BC treatment. NAC is a method recommended for large tumors, especially TNBC and HER2+ BC **(Moo et al., 2018)**. The NAC enables prediction of treatment response and prognosis in aggressive BC subtypes, informing subsequent therapeutic decisions. For patients with ER- or PR+ tumors, endocrine therapy remains the standard adjuvant treatment, with efficacy directly correlating to hormone receptor expression levels **(Walsh et al., 2020)**. In postmenopausal patients, either tamoxifen or aromatase inhibitor (AI) monotherapy serves as an alternative therapeutic option. Studies have shown that tamoxifen can be used as an alternative because it causes side effects such as fractures and osteoporosis in patients receiving AI treatment **(Knauer et al., 2014)**. A study has shown that when chemotherapy and neoadjuvant endocrine therapy are applied in combination in early ER+ BC patients, it can increase the BCS rate in postmenopausal patients with luminal BC **(Alba et al., 2012)**. The efficacy of CDK4/6 and aromatase inhibitors has been evaluated in clinical trials for endocrine treatment of advanced HER+ BC **(Mayer et al., 2022)**. Previous clinical studies with CDK4/6 inhibitors have been inconsistent in the intensity and duration of these inhibitors and are insufficient in terms of side effects **(D Johnston et al., 2020)**. Integrating endocrine treatments and CDK4/6 inhibitors has increased the options in endocrine treatments. Clinical evidence indicates that combining aromatase inhibitors with CDK4/6 inhibitors achieves comparable long-term survival outcomes to neoadjuvant chemotherapy, while demonstrating moderately improved breast conservation rates and reduced treatment-related toxicity **(Cantini et al., 2024)**. In HR+ and HER2- BC, clinical trials prioritize monitoring Ki67 reduction and complete cell cycle arrest when evaluating CDK4/6 inhibitor combinations, as pathological complete response (pCR) is not a clinically relevant endpoint for this subtype in neoadjuvant endocrine therapy **(Hurvitz et al., 2020)**. This therapeutic approach, advised for HR+ and HER2- BC, is not as advanced as neoadjuvant chemotherapy or

targeted therapies used for early-stage TNBC or HER2+ BC. The introduction of anti-HER2 targeted treatments has markedly improved the prognosis for patients with HER2+ BC. Trastuzumab is the first anti-HER2 targeted drug used for HER2+ BC (**J. Wang & Wu, 2023a**). The effectiveness of anti-HER2 agents such as pertuzumab and lapatinib has been assessed in clinical studies involving patients with HER2+ BC. Adding docetaxel and pertuzumab to trastuzumab in neoadjuvant treatment affected pCR by 45%, while adding docetaxel alone to trastuzumab affected pCR by 29%; therefore, the pertuzumab and trastuzumab neoadjuvant pair can also be used as an alternative treatment for HER2+ BC in stage II-III (**Reina Sofi et al., 2012**). Although significant advances have been made in targeted therapies for HER2+ BC, resistance to anti-HER2 agents remains a major obstacle to effective treatment, highlighting the need for novel strategies to overcome this challenge. Additionally, BC impairs the cytotoxic activity of activated T cells against tumor cells through inhibitory factors such as PD-1, TIM-3, LAG3, TIGIT, and CTLA4, along with the interactions of their corresponding ligands expressed on cancer cells. This inhibits the response to treatment by promoting the exhaustion of T cells. Immune checkpoint blockade demonstrates antitumor activity as monotherapy in advanced BC (**Schumacher & Schreiber, 2015**). Monotherapeutic immune checkpoint inhibitors demonstrate limited efficacy in metastatic TNBC. Clinical trial data reveal an 18.5% overall response rate for pembrolizumab in previously treated, PD-L1-positive metastatic TNBC (**Adams et al., 2019**). In early-stage BC, the efficacy of ICIs has been investigated in combination with chemotherapy (**Santa-Maria et al., 2022**). The combination of pembrolizumab with chemotherapeutic agents such as paclitaxel, doxorubicin, and cyclophosphamide was linked to a higher incidence of progression to Stage II and III disease. In the neoadjuvant treatment of BC, the addition of pembrolizumab resulted in higher pCR rates compared to chemotherapy alone: 44% versus 17% in HER2- BC, 30% versus 13% in HR+/HER2- cases, and 60% versus 22% in TNBC (**Bauml et al., 2019**). Gene therapy is a therapeutic approach that involves introducing genetic material into target cells using a vector, in order to modify the expression of a gene product by directly editing the gene. This strategy offers significant potential for cancer treatment (**Youssef et al., 2024**). In a phase 1 clinical trial, the reliability of a genetic prodrug activation therapy targeting the HER2 gene promoter was tested in 12 BC patients, and according to the results obtained, it was understood that the targeted gene expression in this approach was detected in 90% of

the patients and this approach was reliable (Tokura et al., 2024). In an anti-breast cancer treatment approach using microRNA, promising results were shown in preventing the proliferation and development of BC cells. miR-34a is one of the first miRNA agents to enter clinical trials and is believed to be effective in the treatment of BC (J. Wang & Wu, 2023b). Activation of the PI3K/AKT/mTOR pathway critically drives endocrine therapy resistance and represents a validated therapeutic target (Liu et al., 2023). In a clinical trial conducted in phase 2 (TAMRAD), including HR+ metastatic BC patients and postmenopausal patients, aromatase inhibitor treatment failed. However, when the results of the tamoxifen and mTOR inhibitor combination and tamoxifen monotherapy were compared, it was shown that the combination therapy yielded better results than tamoxifen monotherapy (El Sayed et al., 2019). c-Met inhibitors and their structural properties, as well as clinical trials on BC, are shown in Table 1.1.

Table 1.1: c-Met inhibitors, structural characteristics, clinical trials on BC. (Iweala et al., 2024).

c-Met inhibitors and mechanism	Structural formula	Molecular Formula	Clinical trial	Phase	No of patients	Breast cancer subtypes	Design	Status
Cabozantinib Multi- TKI		$C_{28}H_{32}FN_3O_5$	NCT01739438	2	35	TNBC	Cabozantinib	Completed
			NCT03916586	2	18	TNBC	Cabozantinib + nivolumab	Completed
			NCT01441947	2	68	HR+	Cabozantinib + fulvestrant	Active not recruiting
			NCT02260531	2	36	HER2+ with brain metastasis	Cabozantinib + trastuzumab	Completed
Foretinib Multi- TKI		$C_{34}H_{34}F_2N_4O_6$	NCT01147484	2	47	TNBC	Foretinib	Completed
			NCT01138384	1	19	HER2+	Foretinib + lapatinib	Completed
Capmatinib Selective- TKI		$C_{22}H_{17}FN_6O$	NCT05243641	1b/2	56	Metastatic	Capmatinib + neratinib	Recruiting
			NCT03742349	1	64	TNBC	Capmatinib + partalizumab + LAG525	Terminated
Tivantinib Selective-TKI		$C_{23}H_{18}N_4O_2$	NCT01575522	2	22	TNBC	Tivantinib	Completed
			NCT00827177	1	87	Solid tumors, including Breast cancer	Tivantinib + sorafenib	Completed
Crizotinib Multi- TKI		$C_{21}H_{22}Cl_2FN_3O$	NCT03620643	2	58	Lobular breast cancer, Lobular TNBC	Crizotinib + fulvestrant	Active not recruiting
			NCT02074878	1	3	Metastatic	Crizotinib + sunitinib	Terminated
Onartuzumab		$C_{4422}H_{6120}N_{1160}O_{1362}S_{21}$	NCT01186991	2	185	TNBC	Onartuzumab + bevacizumab + paclitaxel	Completed

1.7 Future Perspectives

A better understanding of the c-Met function in BC biology has key role in developing patient-specific and more effective treatment methods. Current research has revealed how c-Met regulates complex signaling networks within the cell and its impact on tumor growth, metastasis, and resistance to treatment. Studies have demonstrated that c-Met interacts with multiple signaling pathways and promotes BC cell metastasis, making it a potentially effective therapeutic target **(Ho-Yen et al., 2015)**. Novel and advanced strategies are currently under development to improve the efficacy of therapies aimed at the c-Met protein. These approaches involve creating new classes of c-Met inhibitors that offer more uniform distribution within the body and are associated with reduced side effects. Combination treatments that target other pathways, in addition to c-Met, are being tested to overcome the problem of developing resistance to treatment **(Dulak et al., 2011)**. Incorporating c-Met-targeting treatments into personalized treatment plans has the potential to improve patient survival. By analyzing the c-Met level and other genetic features in each patient's tumor, the most appropriate and effective treatment can be selected **(Hassan et al., 2016)**. Numerous scientific studies have confirmed the role of molecular profiling in personalized treatment. Biomarkers are essential for selecting the most suitable therapy for each patient, forecasting treatment outcomes, and reducing the risk of avoidable side effects. With biomarkers, patients who will benefit most from c-Met treatment can be selected, and excessive drug use can be prevented. MET gene amplification has been identified as a key biomarker in determining patients for whom c-Met inhibitors may be effective **(Ji et al., 2025)**.

1.8 Pyridazine Triazole Derivatives

The heterocyclic compound class contains at least one atom other than carbon in the ring structure. It has an important place in drug development. It can be used in the design of drugs such as antibiotics, antivirals, antifungals, antioxidants, and antitumors **(Guan et al., 2024)**. The triazole structure is the bioisostere of the amide bond, and this structure has critical importance in drug-target interactions due to its high solubility potential in water and metabolic stability **(Al-Wahaibi et al., 2023)**. Pyrazoline derivative chemical compounds are considered bioactive and are being

investigated as potential antitumor agents. Fluorine atoms are added to heterocyclic drug structures to increase the activity of bioactive molecules. The increase in the number of newly synthesized and FDA-approved drugs containing fluorine in their structures also proves that they have various biological activities (**Rizzo et al., 2023**). Today, approximately 20% of antibiotic and anticancer drugs contain fluorine atoms. *In vitro* and *in vivo* studies have investigated the anticancer and antimicrobial effects, yielding promising results in non-cancerous cell lines (**Abbas et al., 2024**).



2. PURPOSE OF THE STUDY

This thesis study aims to systematically investigate the antitumor properties and mechanistic actions of novel c-Met pathway inhibitors in breast cancer cell lines. The study will characterize how these compounds influence critical cellular functions including survival, growth, death pathways in both triple-negative and hormone receptor-positive breast cancer subtypes. Further objectives include delineating compound-mediated alterations in EGFR/PI3K/Akt signaling networks and assessing their impact on mitochondrial integrity, ROS balance, and lipid homeostasis. Through integrated cellular, molecular, and biochemical profiling, this work seeks to advance understanding of c-Met-targeted therapeutics and facilitate development of enhanced breast cancer treatment.

3. MATERIALS AND METHODS

3.1 Materials

Cell lines, cell culture consumables, and reagents were given in **Table 3.1.**; chemical reagents that were used during this thesis study were given in **Table 2.2.** Laboratory equipment and devices were given in **Table 3.3.**

Table 3.1: Cell lines, cell culture consumables, and reagents.

Material Name	Company
Dimethyl sulfoxide (DMSO)	BioFroxx
Dulbecco's Phosphate Buffer Saline 1X BioFroxx P04-36503 (DPBS) pH: 7.4	PAN Biotech
Gibco™ DMEM	Life Technologies
Gibco™ Fetal Bovine Serum (FBS)	Life Technologies
Penicillin, Streptomycin (10000 U/mL) Penicillin, 10 mg/mL Streptomycin	Life Technologies
Trypsin 0.05%/EDTA 0.02% in DPBS	PAN Biotech
MDA-MB-231 (HTB-26)	ATCC
MCF-7 (HTB-22)	ATCC

Table 3.2: List of chemicals.

Product Name	Company
Acetic acid	Sigma-Aldrich
Bradford Reagent	Clear Band
BODIPY 493/503	Sigma-Aldrich
MitoSpy™ Red	Biolegend
PI (Propidium Iodide)	BioLegend
H2DCFDA (General Oxidative Stress Indicator)	MedChem
DAPI	BioShop
Ethanol	Sigma-Aldrich
Luminol	PanReac AppliChem
Methanol	Sigma-Aldrich
10% APS (Ammonium Persulfate)	BioShop
10% SDS	AFG
Acrylamide (30%)	NeoFroxx
p-Coumaric Acid	Sigma Aldrich

Table 3.2: Continued.

PageRuler™ Plus Prestained Protein Ladder	Thermo Scientific
Propidium iodide	Invitrogen
Skim Milk powder	BioFroxx
Glycine	NeoFroxx
NaCl	KimyaLab
H ₂ O ₂ (30%)	Sigma Aldrich
MTT (Thiazolyl blue tetrazolium bromide)	BioFroxx
Tris-Base	BioShop
Tris-HCl	BioShop
Tween-20	BioShop

Table 3.3: List of laboratory equipment and devices.

Product Name	Company
+4C refrigerator	Arçelik
0.22 µm filter	GVS
0.45 µm filter	GVS
10 µL pipette	Oxford
10 µL tips	CAPP
1000 µL pipette	Oxford
1000 µL tips	CAPP
15 mL centrifuge tubes	CAPP
100 µL pipette	DLAB
100 µL tips	CAPP
-20C freezer	Flavel
24-well plate	TPP
3D orbital shaker	BIOMETRA
50 mL centrifuge tubes	CAPP
6-well plate	TPP
-80C freezer	Nuve
96-well plate	TPP
Cryotube	CAPP
Autoclave machine	Nuve
Heat Block	DLAB
Magnetic stirrer	DLAB
Microcentrifuge tubes	CAPP
Mini Blot Transfer Module	Invitrogen
pH meter	HANNA
PowerEase™ Touch Power Supply	Invitrogen
Spin	DLAB
Tissue Culture Flasks 25	TPP
Varioskan™ LUX multimode microplate reader	Thermo Fisher Scientific

Table 3.3: Continued.

Vortex	DLAB
Waterbath	Electromag
QuantaCell device	Optofil
Microplate Reader	BioTek
ZOE fluorescent cell imager	Bio-Rad Laboratories
Varioskan TM LUX multimode microplate reader	Thermo Fisher Scientific

3.2 Methods

3.2.1 Cell Thawing

Frozen MDA-MB-231 (HTB-26) and MCF-7 (HTB-22) BC cell lines in the freezing medium containing 90% FBS (Fetal Bovine Serum) and 10% DMSO were taken from a -80°C freezer. The cells in cryotubes were quickly thawed and transferred into the centrifuge tubes with a 1:5 ratio of growth medium to reduce the adverse effects of DMSO. Cells were centrifuged at 5000 rpm for 5 minutes. After centrifugation, the supernatant was removed, and the pellet was dissolved in the growth medium. Cells were transferred into 3 mL of complete growth medium in a T25 flask and placed in a 37°C incubator with 5% CO₂.

3.2.2 Cell Passaging

MDA-MB-231 and MCF-7 cells were cultured in DMEM (Dulbecco's Modified Eagle's Medium) supplemented with 10% FBS and 1% penicillin/streptomycin. Cells were incubated in an incubator at 37°C with 5% CO₂. When the cells reached confluency, the medium in the T25 flask was removed, and the flask was washed with 1X PBS to discard any residual medium. 500 µL of Trypsin-EDTA was added to detach the cells, and the flask was placed in the incubator for 5 minutes to activate the trypsin and detach the cells from the surfaces. Cells were collected with 1.5 mL of medium to inhibit the activity of Trypsin-EDTA in centrifuge tubes. Cells were centrifuged at 5000 rpm for 5 minutes. After the centrifugation, the supernatant was removed, and the pellet was dissolved in medium. The cell suspension was transferred into a 3 mL medium in a T25 flask, and then the flask was placed in the incubator.

3.2.3 Cell Freezing

MDA-MB-231 and MCF-7 cells were cultured in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin. Cells were incubated in an incubator at 37°C with 5% CO₂. When the cells reached confluency, the medium in the T25 flask was removed, and the flask was washed with 1X PBS to discard any remaining medium. 500 µL of Trypsin-EDTA was added to detach the cells, and the flask was placed in an incubator for 5 minutes to activate the trypsin and detach the cells from the surfaces. Cells were collected with 1.5 mL of medium to inhibit the activity of Trypsin-EDTA in centrifuge tubes. Cells were centrifuged at 5000 rpm for 5 minutes. After the centrifugation, the supernatant was removed and the pellet was dissolved with freezing medium containing 90% FBS and 10% DMSO, and transferred into a cryotube, and placed in -80°C.

3.2.4 MTT Cell Viability Assay

MDA-MB-231 and MCF-7 cells were cultured in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin. Cells were incubated in an incubator at 37°C with 5% CO₂. When the cells reached confluency, the medium in the T25 flask was removed, and the flask was washed with 1X PBS to discard any remaining medium. 500 µL of Trypsin-EDTA was added to the cell detaching, and the flask was placed in an incubator for 5 minutes to activate the trypsin and detach the cells from the surfaces. Cells were collected with 1.5 mL of medium to inhibit the activity of Trypsin-EDTA in centrifuge tubes. Cells were centrifuged at 5000 rpm for 5 minutes. After the centrifugation, the supernatant was removed, and pellet was dissolved with 1 mL of medium. Cells were placed in a Thoma glass and counted with the QuantaCell Device. The cytotoxic effect of the C1, C2, C3, C4, C5, and Cabozantinib molecules on the MDA-MB-231 and MCF-7 cells was determined by the MTT cell viability assay. This assay measures the color change based on absorbance values by forming formazan salt through the cleavage of the MTT spacer's tetrazolium ring with an enhanced dehydrogenase enzyme in living cells. Cells were seeded at a density of 5x10³ cells per well in a 96-well plate and incubated overnight for their adherence. After adherence, the cells were treated with 1, 5, 10, 25, 50, and 100 µM doses of 6 molecules for 24 hours. Then, 10 µL of MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-

Diphenyltetrazolium Bromide) reagent (5mg/mL) was added to the medium and incubated for 4h. After incubation, the medium containing the MTT reagent was removed, and 100 μ L of DMSO was added to each well to dissolve the formazan crystals for 15 minutes in the dark and a shaken environment. After the incubation, the absorbance values were measured at 570 nm with a microplate reader (**Kumar et al., 2018**). The experimental setup of MTT cell viability is given in **Figure 3.1**.

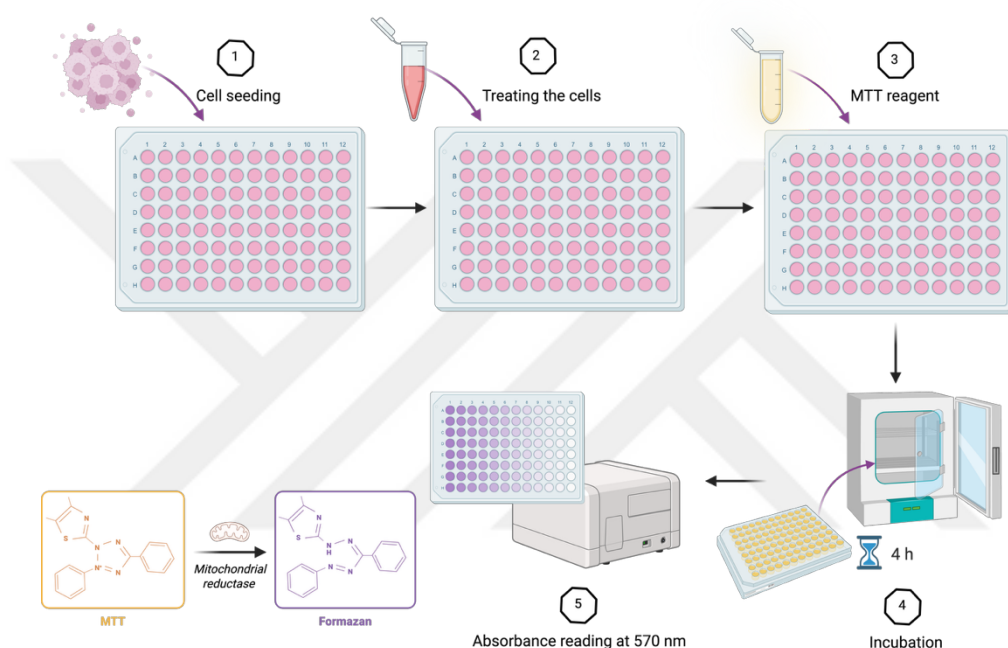


Figure 3.1: Schematic representation of MTT cell viability assay (Created with BioRender.com).

3.2.5 Colony Formation Assay

MDA-MB-231 and MCF-7 cells were seeded at a density of 2.5×10^3 cells per well in a 6-well plate and placed in an incubator to facilitate adherence. After the attachment, the cells were treated with the 1, 5, 10, 25, 50, and 100 μ M doses of C1, C2, C3, C4, C5, and Cabozantinib for 24h. After 24h, the medium was removed, and fresh medium was added. Then, the cells were allowed to form colonies for 10 to 14 days. After the colonies were formed, the medium was discarded, and the plate was washed twice with 1X PBS. 1 mL of fixation solution (1:3 acetic acid/methanol) was then added to fix

the colonies for 5 minutes. Then, fixation solution was discarded, and cells were stained with 500 μL of 0.5% crystal violet for 15 minutes. After the incubation, the dye was removed and washed with dH_2O to remove excess dye (Franken et al., 2006). The images of visible colonies were taken with a camera. The experimental setup of the colony formation assay is given in **Figure 3.2**.

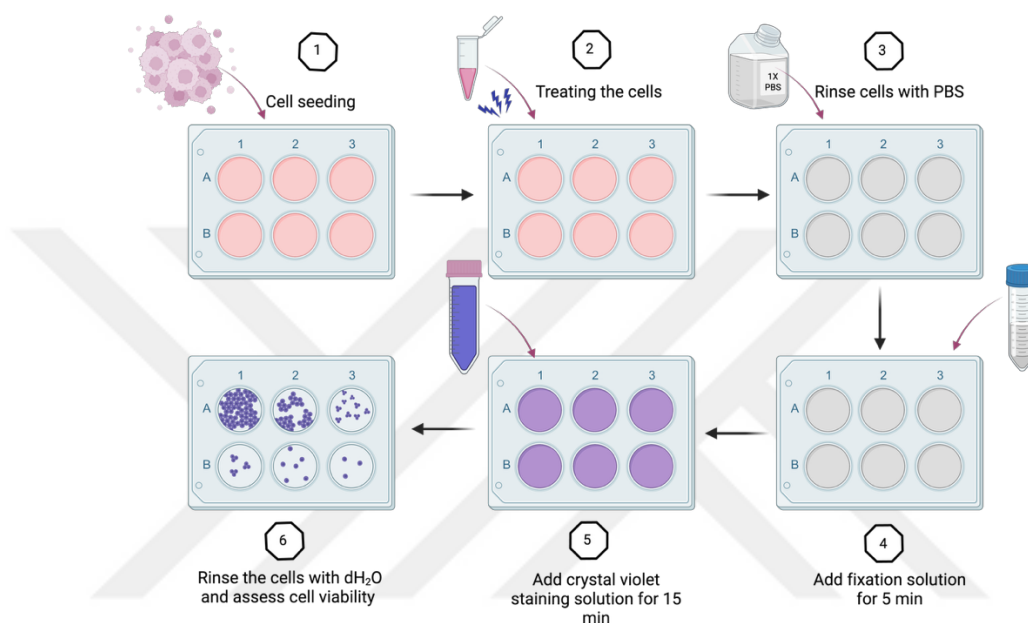


Figure 3.2: Schematic representation of colony formation assay (Created with BioRender.com).

3.2.6 Cell Proliferation Assay

MDA-MB-231 and MCF-7 cells were seeded at a density of 1×10^5 cells per well in a 24-well plate and incubated for adherence. After the attachment, the cells were treated with the 10, 25 μM doses of C1, C2, C3, C4, C5, and Cabozantinib for 24, 48, and 72 hours. For the 24h of treatment, the medium was discarded, and the cells were washed with 100 μL of 1X PBS. Then, 100 μL Trypsin-EDTA was added to the wells to detach the cells from the surface. Then, 400 μL of complete medium was added to each well, and the samples were collected in 1.5 mL microcentrifuge tubes. The cells were counted while 10 μL of each sample was placed on the Thoma lam, and the lam was inserted into the QuantaCell Device. The experimental setup of the cell survival assay was given in **Figure 3.3**.

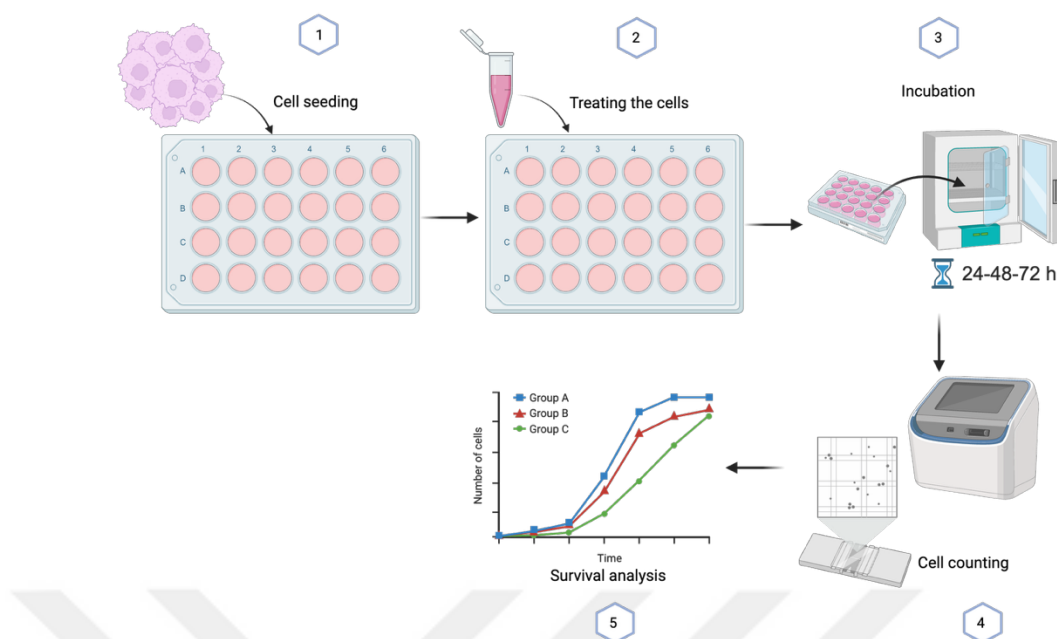


Figure 3.3: Schematic representation of cell proliferation assay (Created with BioRender.com).

3.2.7 Fluorescent Staining Assay

MDA-MB-231 and MCF-7 cells were seeded at a density of 3×10^4 cells per well in a 24-well plate and incubated for adherence. After the attachment, the cells were treated with the 1, 5, 10, 25, 50 μM doses of C1, C2, C3, C4, C5, and Cabozantinib for 24h. After 24h, cells were washed with 1X PBS, and cells were stained with MitoSpy, DAPI, H2DCFDA, BodiPy, and PI at a 1:1000 ratio in serum-free medium for 15 minutes at 37°C with a 5% CO_2 incubator. After incubation, mitochondrial changes, nuclear fragments, ROS (reactive oxygen species), neutral lipids, and apoptotic cells were visualized, and images of the cells were taken with a ZOE Fluorescent Microscope at 45 and 100 μm scale bars, respectively. The experimental setup of the cell survival assay was given in **Figure 3.4**.

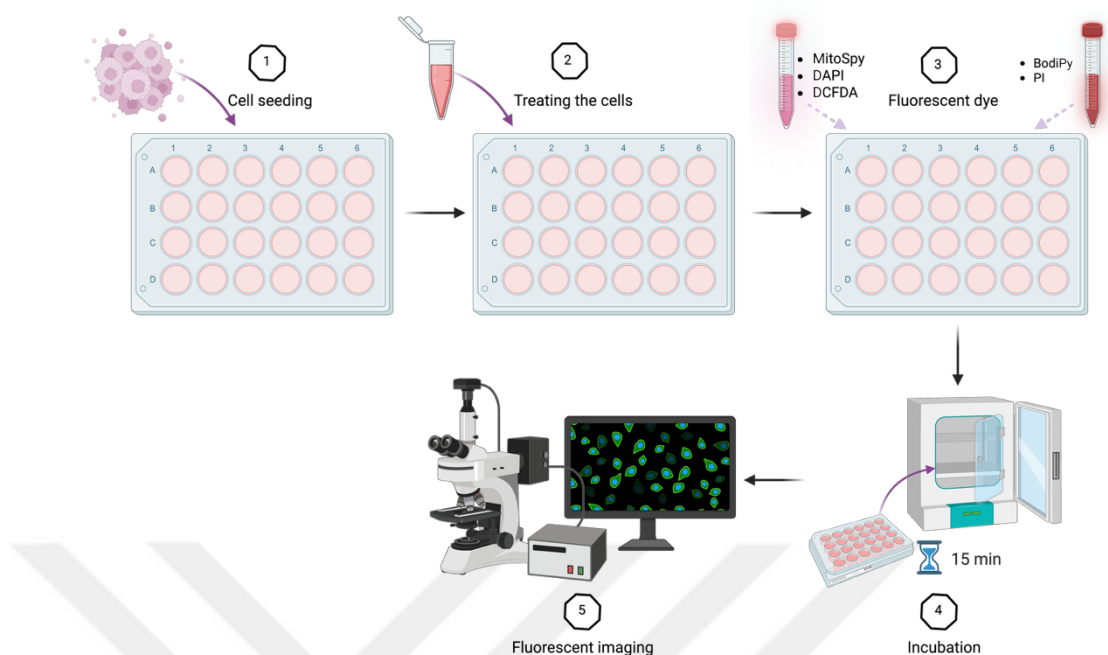


Figure 3.4: Schematic representation of fluorescent staining assay (Created with BioRender.com).

3.2.8 Fluorometric Measurement of ROS (Reactive Oxygen Species), and Mitochondrial Membran Potential

MDA-MB-231 and MCF-7 cells were seeded at a density of 3×10^4 cells per well in a 24-well plate and incubated for adherence. After the attachment, the cells were treated with the selected doses (10 μ M and 25 μ M) of C1, C2, C3, C4, C5, and Cabozantinib for 24h. After 24h, cells were washed with 1X PBS and cells were stained with MitoSpy, H2DCFDA at a 1:1000 ratio in serum-free medium for 30 minutes at 37°C with a 5% CO₂ incubator. After incubation, fluorometric measurements of the ROS (Ex/Em: 488/525 nm), and mitochondrial membran potential by MitoSpy™ Red (Ex/Em: 577/598 nm) were analysed by Varioskan™ LUX multimode microplate reader.

3.3 Detection of Protein Expression Level

3.3.1 Total Protein Isolation

MDA-MB-231 and MCF-7 BC cells were seeded at a density of 1×10^5 cells per well in a T25 flask and incubated overnight at 37°C in a 5% CO₂ incubator to promote adhesion. Cells were treated with doses of 10 µM and 25µM of C2, C3, C4, C5 and Cabozantinib for 24h. After 24 hours of treatment, the culture medium in the T25 flask was removed and washed with 1X PBS to discard any remaining medium residue. 500 µL of Trypsin-EDTA was added to the cell detaching, and the flask was placed in an incubator for 5 minutes to activate the trypsin and detach the cells from the surfaces. Cells were collected with 1.5 mL of medium to inhibit the activity of Trypsin-EDTA in centrifuge tubes. Cells were centrifuged at 5000 rpm for 5 minutes. After the centrifugation, the supernatant was removed, and the pellet was dissolved with 300 µL of ice-cold non-sterile 1X PBS and centrifuged at 13200 rpm for 3 minutes. After centrifugation, the supernatant was removed, and according to the amount of the pellet formed, RIPA (Radio-Immunoprecipitation Assay) Lysis Buffer supplemented with 10X phosphatase and 10X protease inhibitors was added to resuspend the pellet and incubated on ice for 10 minutes. After, they were centrifuged at 13200 rpm for 10 min at +4°C. Finally, total proteins were collected in the supernatant and kept at -20°C.

3.3.2 Bradford Assay

The Bradford Assay was used to determine protein concentration. The standard curve was created using BSA (Bovine Serum Albumin). 2 µL of each isolated protein sample was put into the 96-well plate with two technical repeats. 100 µL of Bradford Reagent was added to each well and incubated in a dark environment for 15 minutes at room temperature (Kielkopf et al., 2020). Following the incubation, the absorbance measurement was performed at 595 nm by a microplate reader. By using absorbance values obtained from the BSA standard curve, the amount of protein in the samples was calculated.

3.3.3 Western Blotting Assay

Protein samples of 25 µg were prepared with 4X Laemmli Buffer according to the calculations obtained from the Bradford assay. Samples were heated at 95 °C for 5 minutes to denature proteins. After denaturation, 2.5 µL protein ladder and 25 µg protein samples were loaded in a 12% Tris-Glycine gel. The gel was placed in the tank, and 1X Running buffer was added to the tank. The running process was initiated with increased voltages of 90, 100, and 130 for 2.5-3 hours, respectively. So, the proteins were separated according to their molecular weight. After the running process was completed, proteins were transferred from the gel into a PVDF (polyvinyl fluoride) membrane. Firstly, the PVDF membrane was activated with methanol for 5 minutes and then washed with distilled deionized water (ddH₂O) for 5 minutes and 1X Transfer buffer for 10 minutes. The transfer process was achieved by a wet transfer system. The sandwich transfer model was created with sponges and filter papers. All of them were soaked in 1X transfer buffer. A sponge, two filter papers, a gel, a PVDF membrane, and two filter papers were placed from the anode to the cathode part of the cassette system. Then, the transfer module was put into a transfer system tank and filled with 1X transfer buffer. For the transfer of proteins whose molecular weight was higher than 100 kDa, the transfer was done for 135 minutes at a constant voltage of 20 V. After the transfer process, the membrane was incubated with 5% skim milk dissolved in 1X TBS-T (Tris-buffered saline containing 0.1% Tween 20) for 1.5 hours at room temperature on a shaker or rotator. The gel was stained with Coomassie G-250 blue to confirm that the running was completed appropriately for 1 hour at room temperature. Then, the gel was destained to remove excess dye and clear the background. After the blocking process, the membrane was incubated with the selected primary antibodies (Akt1/2/3, EGFR, Met, GAPDH) at a 1:1000 ratio overnight at 4°C on a rotator. After the primary antibody incubation, the membrane was washed with 1X TBS-T for 5, 15, and twice for 5 minutes, respectively. Then, the membrane was incubated with the 1:1000 HRP-Linked anti-mouse/rabbit secondary antibody for 4 hours at room temperature. After the secondary antibody incubation, the membrane was rinsed with 1X TBS-T or 5, 15, twice for 5 minutes, respectively. The next step was to visualize the bands. Enhanced chemiluminescence (ECL) was prepared as A and B solutions, which were prepared in a 15 mL Falcon tube. Then, they were mixed in a box. The membrane was submerged in this solution for 1 minute in a dark

environment (Mahmood & Yang, 2012). Following incubation, the membrane was placed in the iBright device (Thermo Scientific), and visualized for 30 sec, 45 sec, 1 min, and 2 min, respectively. The experimental setup of western blotting was given in Figure 3.5.

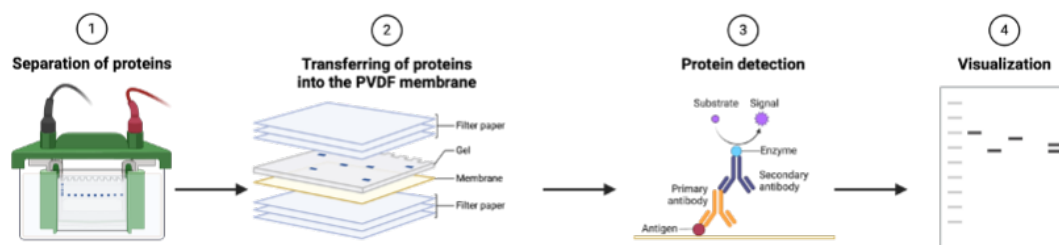


Figure 3.5: Schematic representation of immunoblotting assay (Created with Bioender.com).

3.4 Statistical Analysis

Statistical analysis was performed by GraphPad Prism 10 software (<https://www.graphpad.com>). A two-way ANOVA was used to analyze MTT cell viability and quantify the ROS data. Image J software (<https://imagej.net/ij/>) was used to arrange fluorescent staining images and analyse western blot quantification.

4. RESULTS

4.1 MTT Cell Viability Assay

The cytotoxic effects of newly synthesized pyridazine triazole derivatives, potent c-Met inhibitors, were determined by a colorimetric MTT cell viability assay on MDA-MB-231 and MCF-7 BC cells. Cells were exposed to dosages (1, 5, 7.5, 10, 25, 50, 100 μ M) of C1 (Compound 1), C2 (Compound 2), C3 (Compound 3), C4 (Compound 4), C5 (Compound 5), and the commercial drug Cabozantinib for 24 h. C1 demonstrated minimal cytotoxic activity against both cell lines across all tested concentrations (1-100 μ M), with cell viability remaining above 60% even at the highest dose. No statistically significant differences were observed between the two cell lines. C2 exhibited dose-dependent cytotoxicity with differential selectivity between cell lines. MDA-MB-231 cells showed greater sensitivity, with cell viability decreasing to approximately 45% at 100 μ M. MCF-7 cells demonstrated moderate resistance, maintaining ~65% viability at the highest concentration. C3 showed potent dose-dependent cytotoxic effects against both cell lines. Cell viability dropped significantly, starting from 25 μ M in MDA-MB-231 cells and 50 μ M in MCF-7 cells. At 100 μ M, both cell lines exhibited viability below 30%, indicating vigorous anti-proliferative activity. C4 demonstrated concentration-dependent cytotoxicity with preferential activity against MDA-MB-231 cells. At 50 μ M, MDA-MB-231 cell viability decreased to ~45%, while MCF-7 cells maintained ~65% viability. C5 exhibited robust cytotoxic activity against both cell lines. Cell viability decreased to approximately 20% in MDA-MB-231 and 30% in MCF-7 at the highest concentration. Cabozantinib used as a positive control, showed potent dose-dependent cytotoxicity against both cell lines, with cell viability decreasing to approximately 50% at 25 μ M in both cell lines. The MTT cell viability results are shown in **Figure 4.1**.

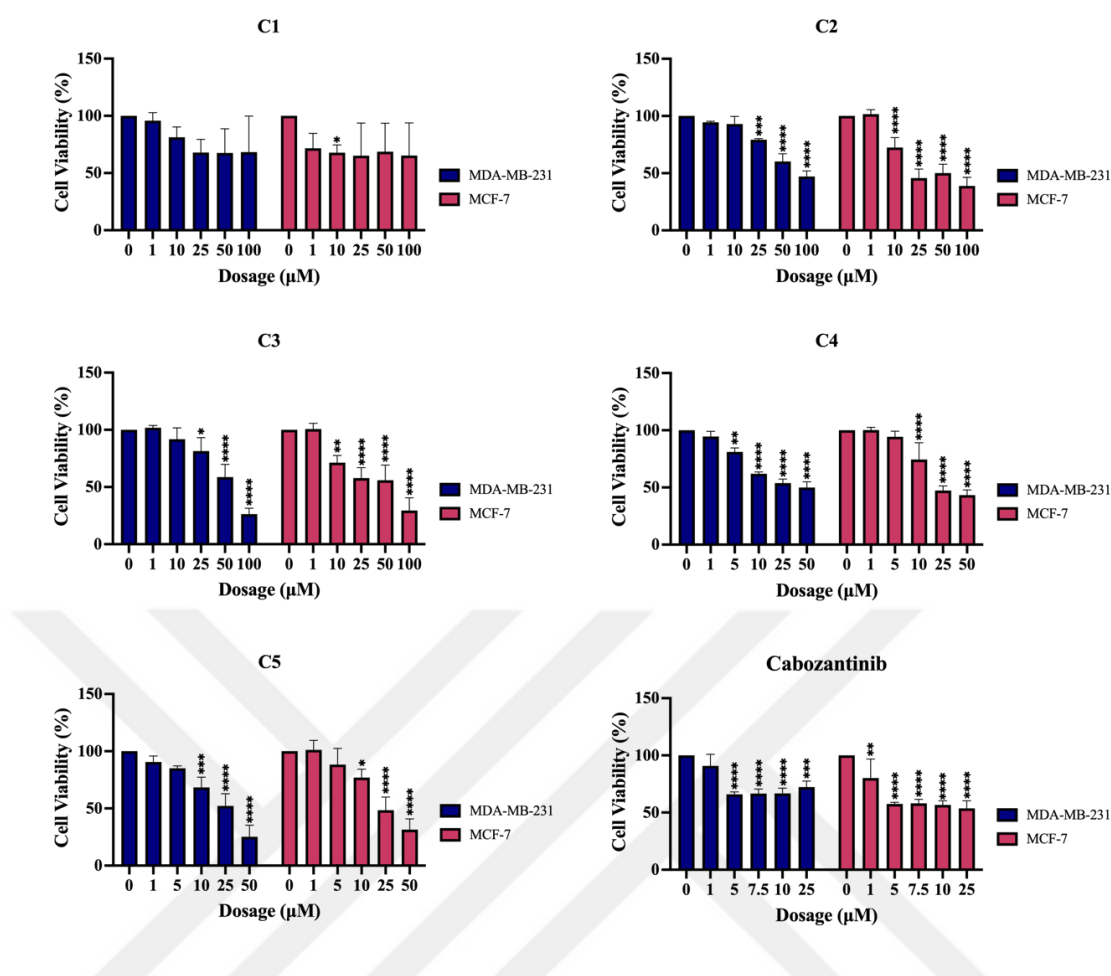


Figure 4.1: MDA-MB-231 and MCF-7 cells were seeded at a density of 5×10^3 cells per well, and treated with 1, 10, 25, 50, 100 μM doses of C1, C2, C3, C4, C5 and Cabozantinib c-Met inhibitors for 24 hours to see the effects on cell viability. Columns represent the mean $\pm$ standard deviation of three biological replicates with four technical replicates. Statistical analysis was performed with two-way ANOVA, followed by Dunnett's multiple comparisons test. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, ns: not significant.

4.2 Colony Formation Assay

The long-term proliferative capacity of MDA-MB-231 BC cells was assessed by colony formation assay after 24-hour treatment with increasing concentrations (1, 10, 25, 50, and 100 μM) of C1, C2, and C3 followed by staining and visualization of colonies. In C1-treated MDA-MB-231 cells, the control and 1 μM groups, numerous large and distinct colonies were observed. At 10 μM, a significant decrease in colony number and size was observed, with only a few small colonies remaining. At concentrations of 25 μM and above (50 and 100 μM), colony formation was almost

completely suppressed. It was concluded that colony formation capacity was inhibited as the dose increased in the treatment groups. In C2-treated cells, there were abundant colonies in the control and 1 μM groups. At 10 μM , a significant decrease in colony number and size was observed; however, a small number of colonies were still observed. At concentrations of 25 μM and above, colony formation was almost completely suppressed, leaving only a few small colonies. In C3-treated cells, a large number of colonies were observed in the Control and 1 μM groups. At 10 μM , there was a significant decrease in the number and size of colonies. At 25 μM , 50 μM and 100 μM concentrations, colony formation was completely eliminated; the layers were completely clear. The colony formation results are shown in **Figure 4.2**.

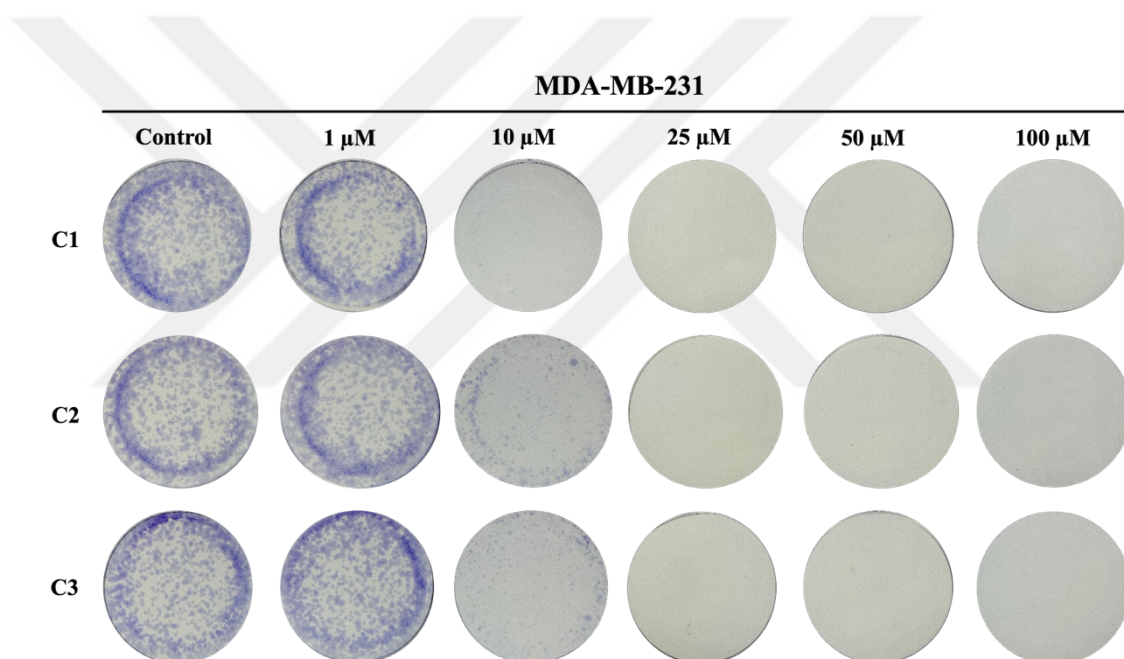


Figure 4.2: MDA-MB-231 cells were seeded at the density of 2.5×10^3 cells per well, and treated with the five different concentrations (1, 10, 25, 50, and 100 μM) concentrations of C1, C2, and C3 c-Met inhibitors for 24 hours. The colony formation of cells was conducted for 12 days. Formed colonies were stained with crystal violet.

MDA-MB-231 BC cells were evaluated for their long-term proliferative capacity following treatment with C4 and C5 at concentrations ranging from 1 to 50 μM . Cells were seeded at 2.5×10^3 cells per well, treated for 24 hours, and allowed to form colonies over 12 days before crystal violet staining. In C4-treated cells, at 0 and 1 μM concentrations, numerous and distinct colonies are seen in the plate; colony density

and size are quite close to the control group. At 5 μM , a slight decrease in colony number and size occurred. At 10 μM , a more distinct decrease in colony number and size is observed. At 25 μM and 50 μM , the decrease in colony formation continues, and there are still visible colonies; however, the colonies are smaller and sparse. In C5-treated cells, colony formation at 0 and 1 μM is similar to the control and is dense and large. Starting from 5 μM , a very significant decrease in colony number and size begins. At 10 μM , colonies are considerably reduced and small in size. At 25 μM and 50 μM , the layers are almost completely clear and colony formation is almost completely suppressed. Both compounds suppressed colony formation in MDA-MB-231 cells in a dose-dependent manner. However, C5 showed a much stronger clonogenic inhibitory effect than C4. These results suggest that C5 is more effective in suppressing the long-term proliferation capacity of MDA-MB-231 cells. The colony formation results are shown in **Figure 4.3**.

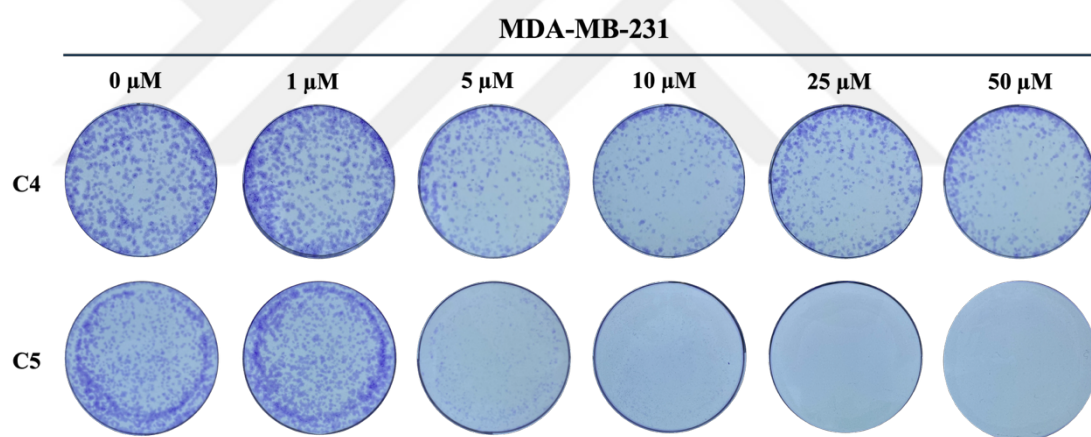


Figure 4.3: MDA-MB-231 cells were seeded at the density of 2.5×10^3 cells per well, and treated with the five different concentrations (1, 5, 10, 25, and 50 μM) concentrations of C4, and C5 c-Met inhibitors for 24 hours. The colony formation of cells was conducted for 12 days. Formed colonies were stained with crystal violet.

The effect of C1, C2, and C3 on the clonogenic potential of MCF-7 BC cells was evaluated using a colony formation assay. Cells were treated with increasing concentrations (1, 10, 25, 50, and 100 μM) of each compound for 24 hours, followed by a 12-day incubation period to allow colony growth, after which colonies were stained with crystal violet. In C1-treated cells, numerous and distinct colonies are

observed in the control (0 μM) and 1 μM groups. At concentrations of 10 μM and above (25, 50, 100 μM), colony formation was almost completely suppressed and no colony formation was observed. Colony formation was observed in the control and 1 μM groups in C2-treated cells. A dramatic decrease in colony number and size was observed starting from 10 μM . At concentrations of 25 μM and above, colony formation was completely suppressed. In C3-treated cells, the highest colony numbers were observed in the control and 1 μM groups. Colony formation was almost completely inhibited from 10 μM onwards, and no colonies were observed at concentrations of 25 μM and above. C1, C2 and C3 compounds strongly and dose-dependently suppressed colony formation in MCF-7 cells. C3 almost completely abolished colony formation even at the lowest concentration (10 μM), while complete inhibition was achieved with C1 and C2 at doses of 25 μM and above. These results indicate that all molecules effectively reduced the long-term proliferation capacity of MCF-7 cells, with C3 having the highest effect. The colony formation results of MCF-7 cells are shown in **Figure 4.4**.

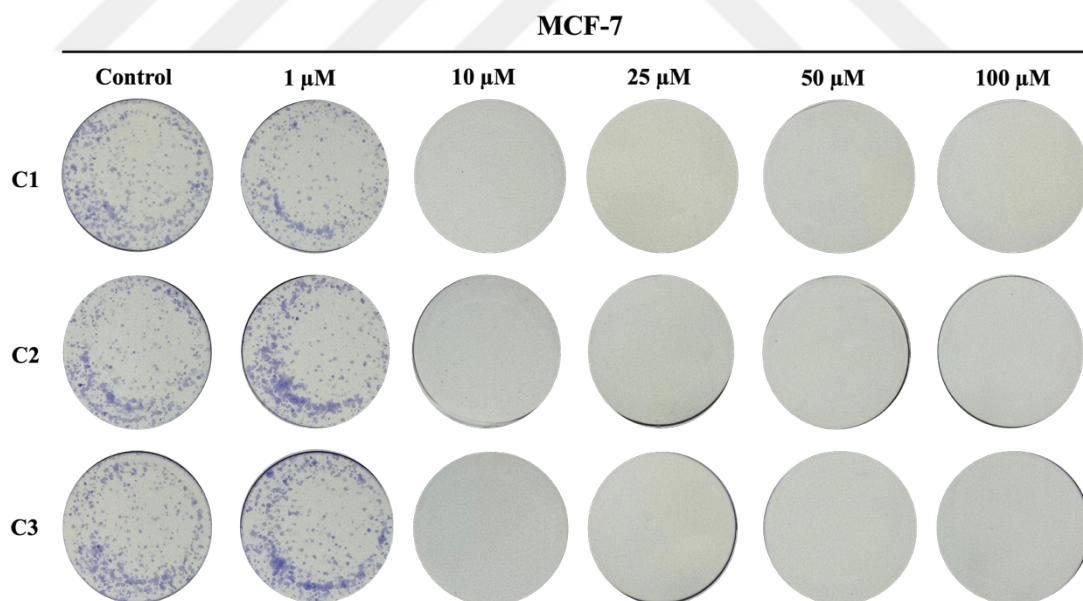


Figure 4.4: MCF-7 cells were seeded at the density of 2.5×10^3 cells per well, and treated with the five different concentrations (1, 10, 25, 50, and 100 μM) concentrations of C1, C2, and C3 c-Met inhibitors for 24 hours. The colony formation of cells was conducted for 12 days. Formed colonies were stained with crystal violet.

The long-term proliferation effect of C4 and C5, colony formation decreases markedly as the concentration increases in MCF-7 cells. While a high number of colonies is observed in the control and at low concentrations (1 μ M, 5 μ M), the number of colonies is significantly reduced at concentrations of 10 μ M and above. At 25 μ M and 50 μ M, almost no colonies are formed. As a result, C4 and C5 strongly inhibit colony formation in MCF-7 cells in a dose-dependent manner. The colony formation results of the C4 and C5 are given in **Figure 4.5**.

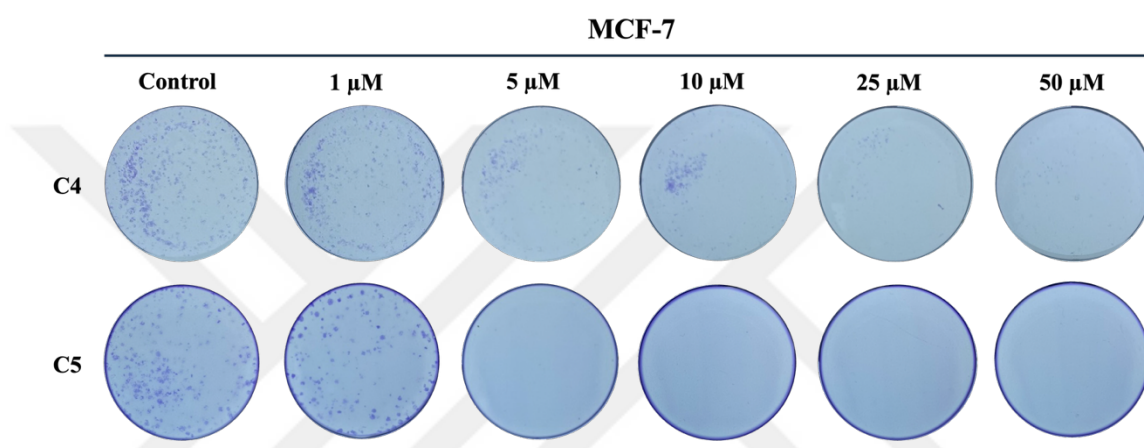


Figure 4.5: MCF-7 cells were seeded at the density of 2.5×10^3 cells per well, and treated with the five different concentrations (1, 5, 10, 25, 50 μ M) concentrations of C4 and C5 c-Met inhibitors for 24 hours. The colony formation of cells was conducted for 12 days. Formed colonies were stained with crystal violet.

In MDA-MB-231 cells, while a high number of colonies are observed in the control group, there is a slight decrease in colony number with 1 μ M Cabozantinib treatment. At concentrations of 5 μ M and above, colony formation is almost completely suppressed; in the 7.5, 10, and 25 μ M groups, colony formation is nearly absent. In MCF-7 cells, a high number of colonies are observed in both the control group and with 1 μ M Cabozantinib treatment. At concentrations of 5 μ M, 7.5 μ M, 10 μ M, and 25 μ M, there is a marked decrease in colony formation; however, this reduction is less pronounced compared to MDA-MB-231 cells, and colony formation persists at low levels. Cabozantinib inhibits colony formation in both cell lines in a dose-dependent manner. Inhibition occurs earlier and more strongly in MDA-MB-231 cells (with colony formation almost completely blocked at concentrations of 5 μ M and above). In

contrast, in MCF-7 cells, inhibition is observed at higher concentrations, and colony formation is not eliminated. These results indicate that Cabozantinib suppresses colony formation particularly effectively in MDA-MB-231 cells. The colony formation results of Cabozantinib are given in **Figure 4.6**.

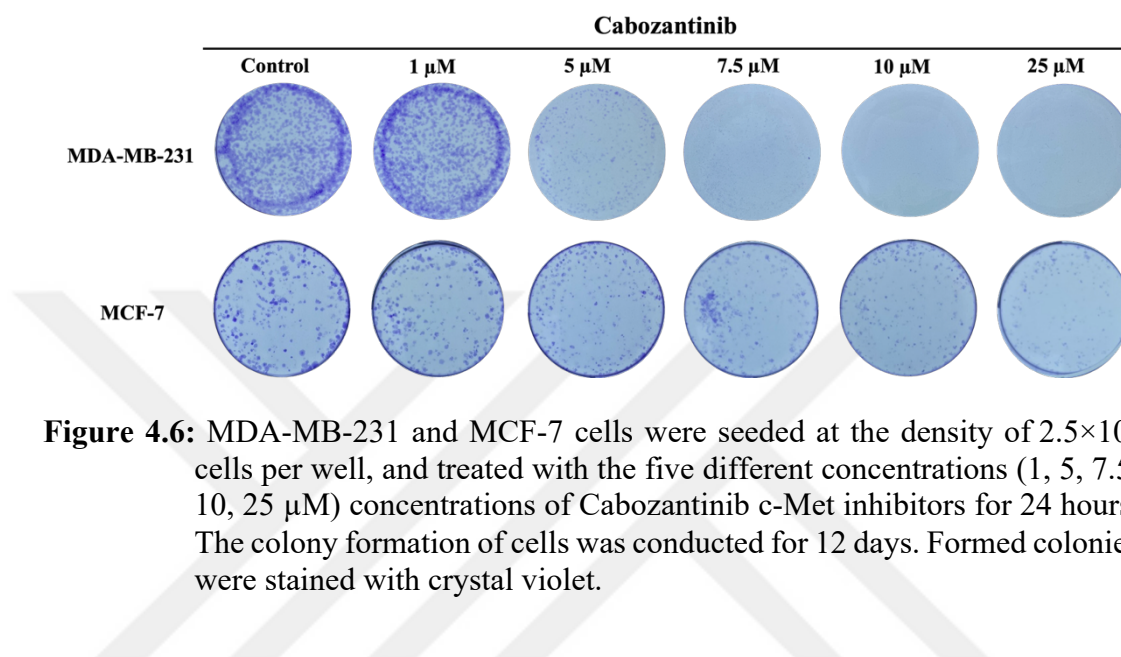


Figure 4.6: MDA-MB-231 and MCF-7 cells were seeded at the density of 2.5×10^3 cells per well, and treated with the five different concentrations (1, 5, 7.5, 10, 25 μ M) concentrations of Cabozantinib c-Met inhibitors for 24 hours. The colony formation of cells was conducted for 12 days. Formed colonies were stained with crystal violet.

4.3 Cell Survival Assay

The effects of pyridazine triazole derivatives, along with cabozantinib, on the proliferation of MDA-MB-231 cells were assessed over 72 hours using a cell survival assay. Cells were treated with 10 μ M and 25 μ M concentrations of each compound, and cell numbers were counted at 0, 24, 48, and 72 hours. Both 10 μ M and 25 μ M treatments of C1 had minimal impact on cell proliferation. Cell numbers in treated groups remained close to the initial seeding density and were not significantly different from controls at any time point. C2 exhibited dose-dependent anti-proliferative effects. At 10 μ M, cell growth was moderately inhibited, while at 25 μ M, a significant reduction in cell number was observed at 48 and 72 hours compared to the control. Significant differences between 10 μ M and 25 μ M were also noted at later time points. Both concentrations of C3 resulted in strong and sustained inhibition of cell proliferation. Cell numbers remained at or below the initial seeding density from 24 to 72 hours, with highly significant differences compared to the control. Additionally, a

significant difference between the two doses was observed at 48 and 72 hours. C4 showed marked, dose-dependent inhibition of cell growth. Both 10 μ M and 25 μ M treatments significantly reduced cell numbers at 48 and 72 hours, with a significant difference between the two concentrations at later time points. C5 demonstrated potent anti-proliferative activity at both concentrations, with cell numbers remaining near or below baseline throughout the experiment. Significant reductions were observed at 48 and 72 hours compared to the control, and a marked difference between the two doses was evident at later time points. Cabozantinib Used as a positive control, cabozantinib produced potent, dose-dependent inhibition of cell proliferation at both concentrations, with significant decreases in cell numbers at 48 and 72 hours. Differences between the two doses were also statistically significant at later time points. The cell survival results for MDA-MB-231 cells are shown in **Figure 4.7**.

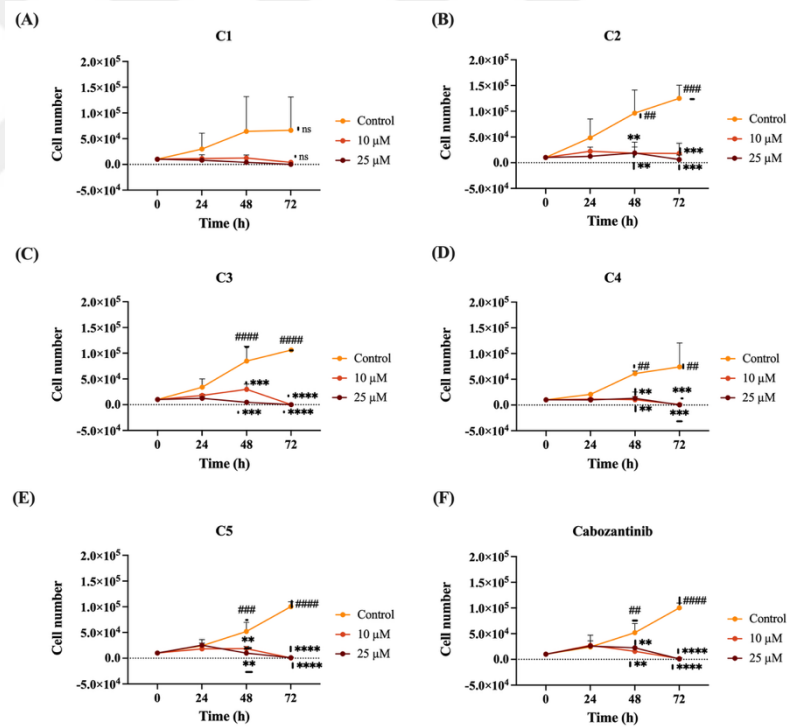


Figure 4.7: MDA-MB-231 cells were seeded at a density of 1×10^4 cells per well in a 24-well plate, and treated with 10 and 25 μ M doses of C1, C2, C3, C4, C5, and Cabozantinib c-Met inhibitors for 72 hours to see the effects on cell viability. Columns represent the mean $\pm$ standard deviation of two biological replicates with four technical replicates. Statistical analysis was performed with two-way ANOVA, followed by Dunnett's multiple comparison test against the initial control (##p<0.01, ###p<0.001, ####p<0.0001), and against the each time control (*p<0.01, **p<0.001, ***p<0.0001, ns: not significant).

The proliferative capacity of MCF-7 cells was evaluated for 72 h after treatment with pyridazine triazole derivatives at 10 μ M and 25 μ M concentrations. C1 showed minimal anti-proliferative activity against MCF-7 cells. While control cells exhibited normal, exponential growth at 72 h, both 10 μ M and 25 μ M treatments resulted in only modest growth inhibition, with no statistically significant difference compared to the control. Cell numbers in treated groups remained relatively close to control levels throughout the experimental period. C2 showed moderate dose-dependent anti-proliferative effects. 10 μ M treatment showed mild growth inhibition, while 25 μ M treatment resulted in more pronounced growth suppression at 48 and 72 h, which was statistically significant. C3 showed potent anti-proliferative activity at both concentrations. Control cells exhibited normal growth kinetics; both 10 μ M and 25 μ M treatments resulted in significant growth inhibition, with cell numbers remaining significantly below control levels. The 25 μ M concentration appeared to be more effective than the 10 μ M concentration, with significant differences between doses observed at 48 and 72 hours. C4 showed potent dose-dependent anti-proliferative effects. A 10 μ M treatment caused moderate growth inhibition, while a 25 μ M treatment caused significant growth suppression, and cell numbers remained significantly below the control at 48 and 72 h. C5 showed potent anti-proliferative activity comparable to the most active compounds. Both concentrations caused significant growth inhibition, and 25 μ M showed superior efficacy. Cabozantinib served as a positive control and showed potent dose-dependent anti-proliferative effects. The cell survival results for MCF-7 cells are shown in **Figure 4.8**.

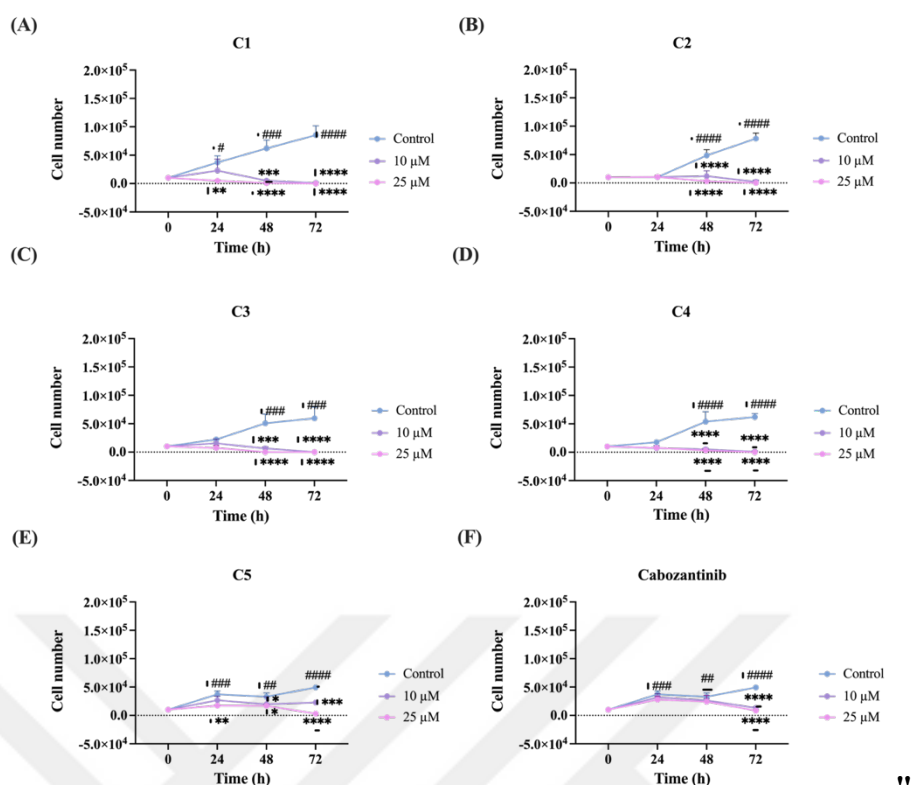


Figure 4.8: MCF-7 cells were seeded at a density of 1×10^4 cells per well in a 24-well plate, and treated with 10 and 25 μ M doses of C1, C2, C3, C4, C5, and Cabozantinib c-Met inhibitors for 72 hours to see the effects on cell viability. Columns represent the mean $\pm$ standard deviation of two biological replicates with four technical replicates. Statistical analysis was performed with two-way ANOVA, followed by Dunnett's multiple comparisons test against the initial control (#p<0.05, ##p<0.01, ###p<0.001, ####p<0.0001), and against the each time control (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, ns: not significant).

4.4 Fluorescent Staining Assay

Fluorescent staining was performed to assess mitochondrial membrane potential (MitoSpy, red), reactive oxygen species (ROS) generation (H2DCFDA, green), and nuclear morphology (DAPI, blue) in MDA-MB-231 and MCF-7 cells treated with C1, C2, C3, C4, C5, and cabozantinib at 10 μ M and 25 μ M for 24 hours. The C1 potent c-Met inhibitor molecule showed a similar red fluorescence signal in both the control group and the treatment group. In other words, it was concluded that the mitochondrial membrane potential was preserved. As a result of DAPI staining, it was observed that the nuclear integrity was preserved, and no apoptosis-specific nuclear fragmentation structure was observed. For the C2 potent c-met inhibitor molecule, a similar red

fluorescence signal was observed between the control group and the treatment groups. The ROS level gave a similar luminescence signal in all groups. The nuclear integrity was preserved, and no nuclear fragmentation formation was observed. A decrease in mitochondrial membrane potential was observed between 10 μ M and 25 μ M in the cells treated with C3 compared to the control group. An increase in the ROS level of the 25 μ M treatment group was observed compared to the control group. Nuclear fragmentation formation, which is a marker of apoptosis, was observed. A decrease in mitochondrial membrane potential was observed at high doses compared to the control group in the cells treated with C4. At high doses, an increase in ROS production and nuclear fragmentation formation was observed compared to the control group. For C5, as the dose increased, a deterioration in mitochondrial membrane potential, an increase in ROS production, nuclear fragmentation, and a decrease in cell number were observed. In cabozantinib-treated cells, a loss in mitochondrial membrane potential, an increase in ROS production, and nuclear fragmentation were observed as the dose increased. Fluorescent images of MDA-MB-231 cells were shown in **Figure 4.9**.

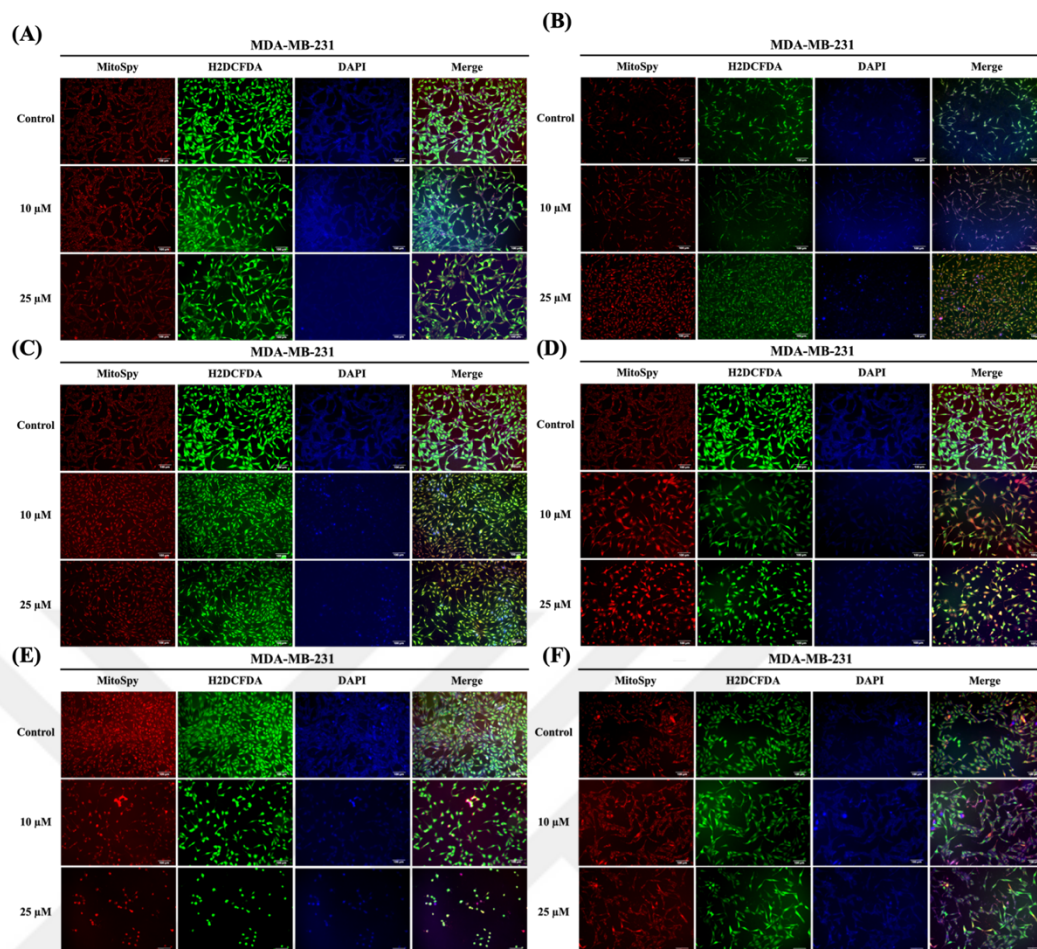


Figure 4.9: MDA-MB-231 cells were seeded at a density of 3×10^4 cells per well and exposed to 10 μ M and 25 μ M of potent c-Met inhibitors (A) C1, (B) C2, (C) C3, (D) C4, (E) C5, and (F) Cabozantinib for 24 hours. Fluorescent imaging of mitochondrial changes by MitoSpy (red), (ROS) Reactive Oxygen Species by H2DCFDA (green), nucleus by DAPI (blue). The merge represents the overlay of these three stains. —scale bar: 100 μ m.

In MDA-MB-231 cells, C1 showed decreasing in cell density and changes in morphology as the dose increased. In the control group, lipid droplets were denser and brighter. However, as the dose increased, there was a decrease in lipid formation, but no significant cell death occurred. C2, compared to the control group, showed a reduction in cell number and an increase in lipid formation, but it did not cause cell death. C3, compared to the control, showed a reduction in cell number and morphology, a decrease in lipid formation, and cell death. C4, compared to the control, showed a reduction in cell number, lipid droplet formation, and cell death. C5, compared to the control, showed a decrease in morphological changes, cell number, lipid droplet formation, and cell death. Cabozantinib, compared to the control group,

caused a change in cell morphology, a decrease in cell number and lipid droplets, and no significant cell deaths were observed. Fluorescent images for BodiPy and PI of MDA-MB-231 cells were shown in **Figure 4.10**.

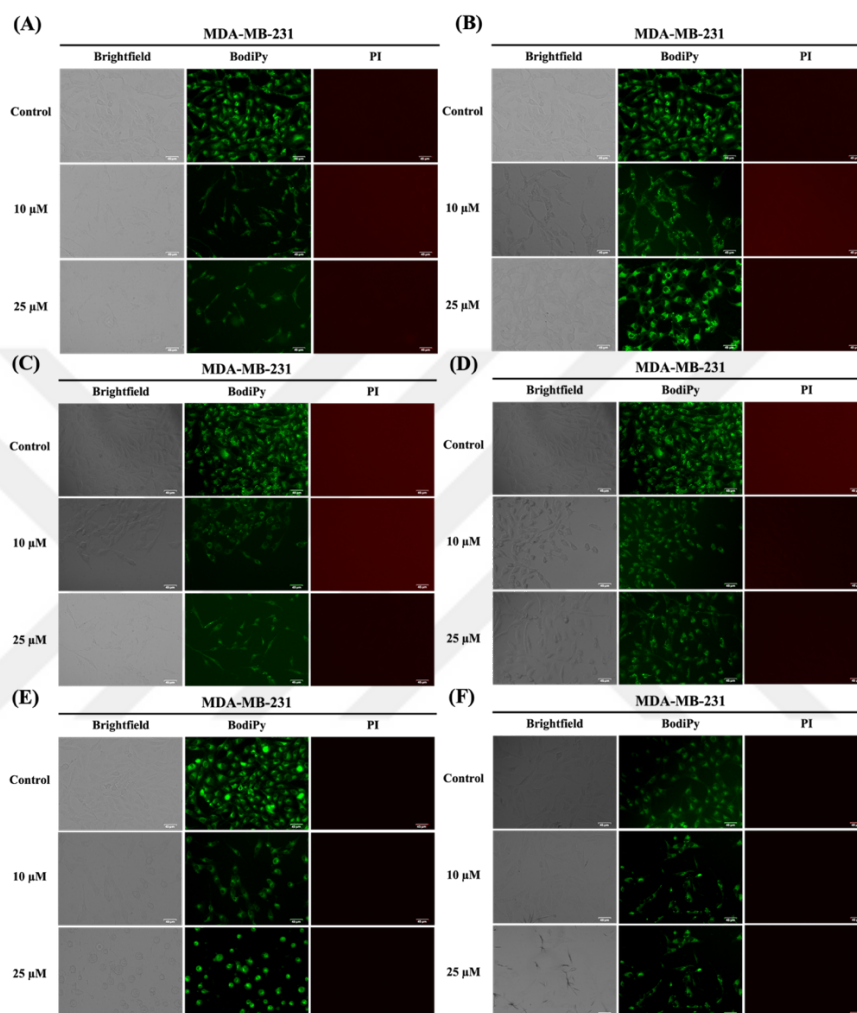


Figure 4.10: MDA-MB-231 cells were seeded at a density of 3×10^4 cells per well and exposed to 10 μ M and 25 μ M of potent c-Met inhibitors (A) C1, (B) C2, (C) C3, (D) C4, (E) C5, and (F) Cabozantinib for 24 hours. Fluorescent imaging of neutral lipids by BodiPy (green), dead cells by PI (red). — scale bar: 45 μ m.

In MCF-7 cells, C1 showed dose-dependent cytotoxic effects. Compared to the control group, a decrease in mitochondrial membrane potential, cell count, ROS production, and fragmentation formation in some nuclei was observed. C2 caused a dose-dependent decrease in cell count compared to the control group. At high doses, mitochondrial membrane potential was impaired, ROS production increased, and

nuclear fragmentation was observed. C3 was observed to disrupt mitochondrial membrane potential, an increase in ROS production, and an increase in nuclear fragmentation formation compared to the control group. C4 was observed to cause a decrease in cell count, loss of mitochondrial membrane potential, an increase in ROS production, and nuclear fragmentation formation compared to the control group. C5 was observed to maintain mitochondrial membrane potential, with no significant change in ROS production and no significant change in nuclear integrity compared to the control group. It was observed that cabozantinib caused a slight decrease in mitochondrial membrane potential, no substantial change in ROS production, and a reduction of nuclear density compared to the control group. Fluorescent images of MCF-7 cells were shown in **Figure 4.11**.

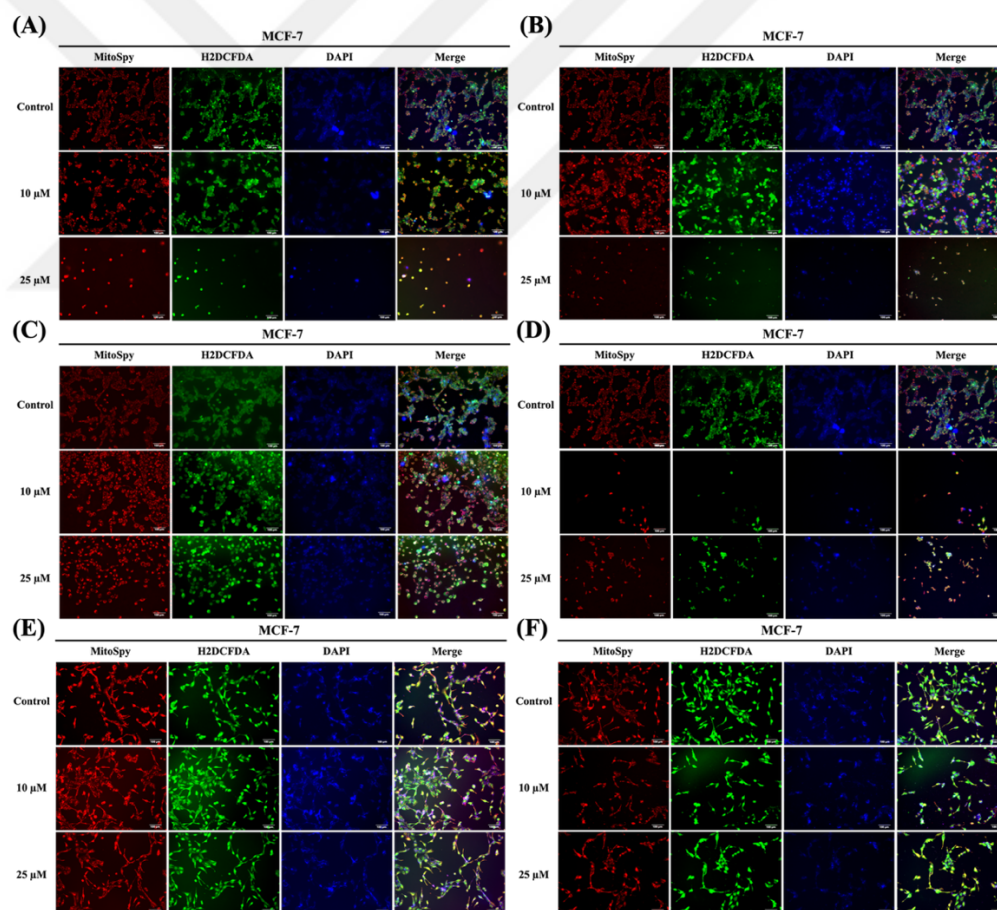


Figure 4.11: MCF-7 cells were seeded at a density of 3×10^4 cells per well and exposed to 10 μM and 25 μM of potent c-Met inhibitors (A) C1, (B) C2, (C) C3, (D) C4, (E) C5, and (F) Cabozantinib and for 24 hours. Fluorescent imaging of mitochondrial changes by MitoSpy (red), (ROS) Reactive Oxygen Species by H2DCFDA (green), nucleus by DAPI (blue). The merge represents the overlay of these three stains. —scale bar: 100 μm.

In MCF-7 cells, it was observed that C1 caused a decrease in cell density and changes in cell morphology compared to the control group. As the dose increased, a decrease in lipid droplets occurred. The number of dead cells was observed to be very low. C2 did not cause a significant change in cell morphology and cell density compared to the control group, did not cause a dramatic change in lipid droplets compared to the control, and no dead cells were observed. For C3, a slight decrease in cell density compared to the control group was observed. Lipid droplet formation was slightly decreased compared to the control. Cell death was not observed. In C4-treated cells, there was a slight decrease in cell number and lipid droplets compared to the control group, but no dead cells were observed. A slight decrease in cell number was observed in C5-treated cells compared to the control group. An increasing trend in lipid droplet formation was observed at 10 μ M, but no dead cells were observed. Decreased cell number and changes in cell morphology were observed in cabozantinib-treated cells compared to control. A trend towards decreased lipid droplet formation and no dead cells were observed. Fluorescent images for BodiPy and PI of MCF-7 cells were shown in **Figure 4.12**.

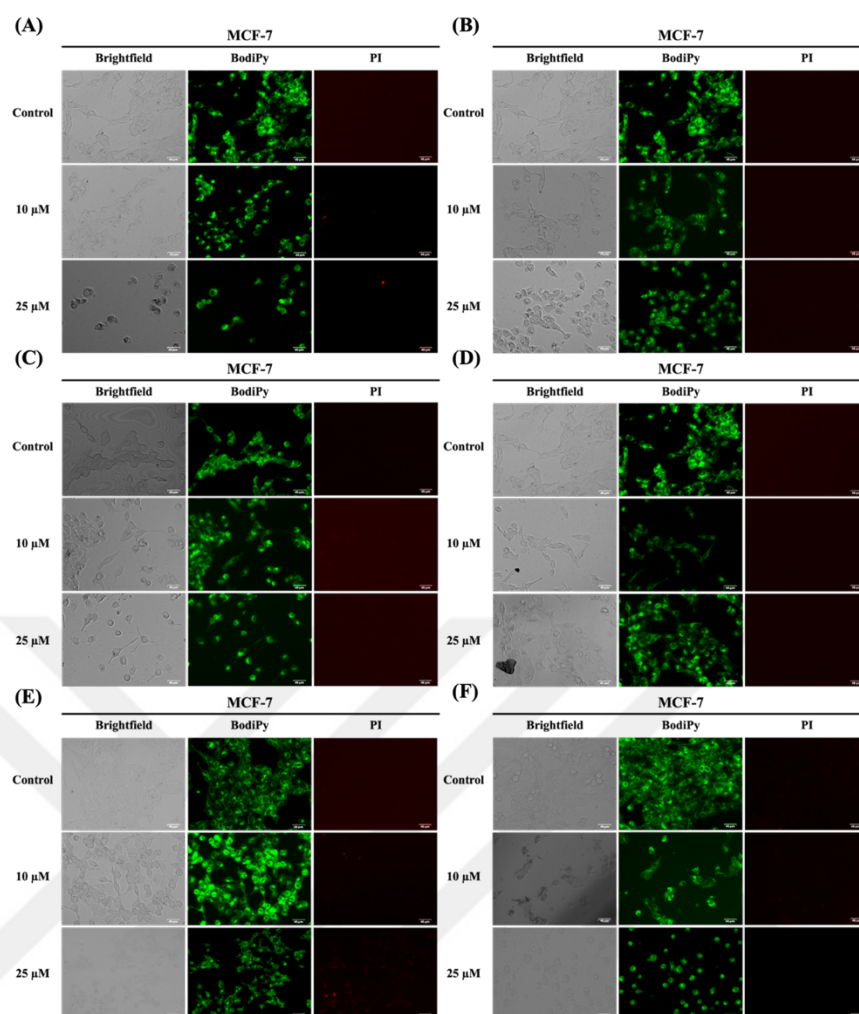


Figure 4.12: MCF-7 cells are seeded at a density of 3×10^4 cells per well and exposed to 10 μM and 25 μM of potent c-Met inhibitors (A) C1, (B) C2, (C) C3, (D) C4, (E) C5, and (F) Cabozantinib and (F) Cabozantinib for 24 hours. Fluorescent imaging of neutral lipids by BodiPy (green), dead cells by PI (red). —scale bar: 45 μm.

4.5 ROS Assay with Fluorometric Measurement

Intracellular reactive oxygen species (ROS) levels were quantified using the H2DCFDA fluorometric assay after 24 h of treatment with pyridazine triazole derivatives at concentrations of 10 μM and 25 μM. Both MDA-MB-231 and MCF-7 cell lines were analyzed to assess cell line-specific ROS responses. In MDA-MB-231 cells, C1 treatment caused a dose-dependent decrease in ROS levels, with fluorescence intensity decreasing from 100% (control) to approximately 75% at 10 μM and 70% at 25 μM. MCF-7 cells showed minimal response to C1 treatment and maintained ROS levels close to control values, with a slight increase observed at 25 μM. MDA-MB-

231 cells showed a significant dose-dependent decrease in ROS levels after C2 treatment. ROS intensity decreased to approximately 95% at 10 μ M and decreased to 60% at 25 μ M. MCF-7 cells showed minimal changes in ROS levels, remaining close to control values at both treatment concentrations, with only a slight decrease at 25 μ M. Both cell lines showed decreased ROS levels with C3 treatment. MDA-MB-231 cells showed decreased ROS intensity by approximately 50% at both 10 μ M and 25 μ M concentrations. MCF-7 cells also showed decreased ROS levels, decreasing to about 65% from control levels at both treatment concentrations. MDA-MB-231 cells showed a moderate decrease in ROS levels following C4 treatment, with fluorescence intensity decreasing to about 80–85% at both concentrations. MCF-7 cells maintained ROS levels similar to control values, with minimal changes observed in all treatment groups. MDA-MB-231 cells showed minimal changes in ROS levels, with a slight increase observed at 25 μ M. However, MCF-7 cells showed a significant decrease in ROS levels, with the intensity decreasing to approximately 55% at 10 μ M and 65% at 25 μ M. Both cell lines showed moderate decreases in ROS levels after Cabozantinib treatment. MDA-MB-231 cells showed ROS intensity decreasing to approximately 80% at both concentrations, while MCF-7 cells showed ROS levels decreasing about to 75–80%. The fluorometric measurement of ROS results were shown in **Figure 4.13**.

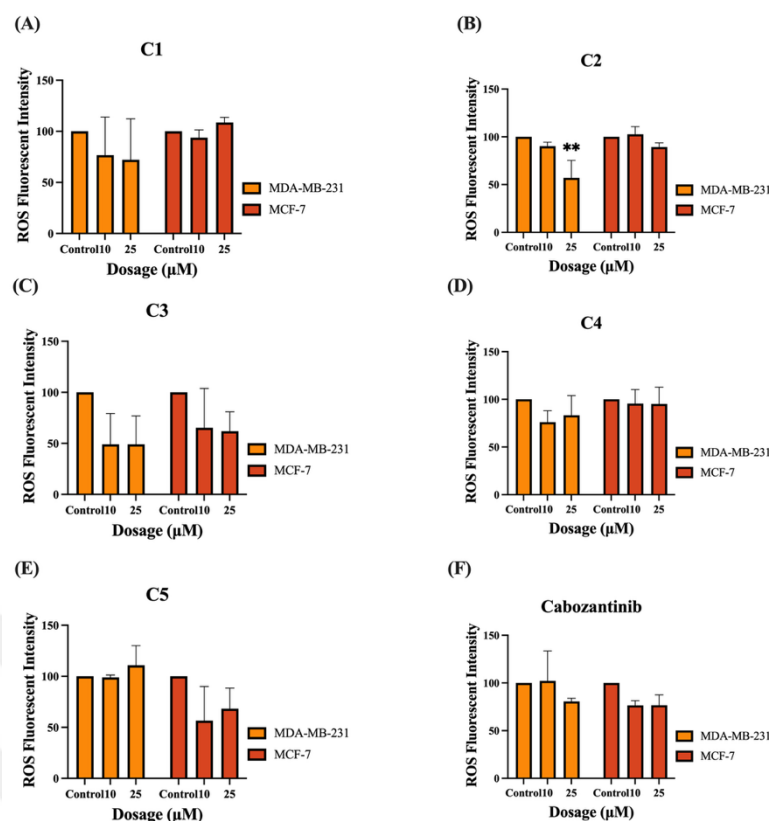


Figure 4.13: MDA-MB-231 and MCF-7 cells were seeded at a density of 3×10^4 cells per well and exposed to 10 μ M and 25 μ M of potent c-Met inhibitors (A) C1, (B) C2, (C) C3, (D) C4, (E) C5, and (F) Cabozantinib for 24 hours. Fluorometric measurement was detected with H2DCFDA with Ex/Em: 488/525 nm. Columns represent the mean $\pm$ standard deviation of two independent experiments with two technical replicates. Statistical analysis was performed with two-way ANOVA, followed by Dunnett's multiple comparisons test. ** $p < 0.01$. ns: not significant.

In MDA-MB-231 and MCF-7 BC cells, mitochondrial membrane potential was measured fluorometrically using a MitoSpy Red probe after 24 hours of treatment with C1, C2, C3, C4, C5, and Cabozantinib at 10 μ M and 25 μ M concentrations. Results are presented as the mean and standard deviation of two independent experiments. Significant change was not observed in mitochondrial membrane potential after C1 treatment both cell lines. The values remained at similar levels in all groups. While mitochondrial membrane potential remained constant with C2 treatment in MDA-MB-231 cells, a statistically significant increase was observed in MCF-7 cells at 25 μ M dose. This suggests that high doses of C2 may increase mitochondrial function in MCF-7 cells. No significant change was observed in mitochondrial membrane potential with C3 treatment in both cell lines. The values were close to the control

group at all doses. For the C4 treatment, a slight increase was observed in MDA-MB-231 cells at 10 μ M, and a value close to control levels was observed at 25 μ M. In MCF-7 cells, the membrane potential remained constant at all doses. A slight decrease was observed in MDA-MB-231 cells with C5 treatment depending on the dose increase, but these changes were not significant. In MCF-7 cells, a slight decrease was observed at 10 μ M, and a recovery approaching control levels was observed at 25 μ M. Cabozantinib, the positive control, kept the membrane potential constant in MDA-MB-231 cells, while it showed a slight decrease in MCF-7 cells at both doses. The fluorometric measurement of MitoSpy results were shown in **Figure 4.14**.

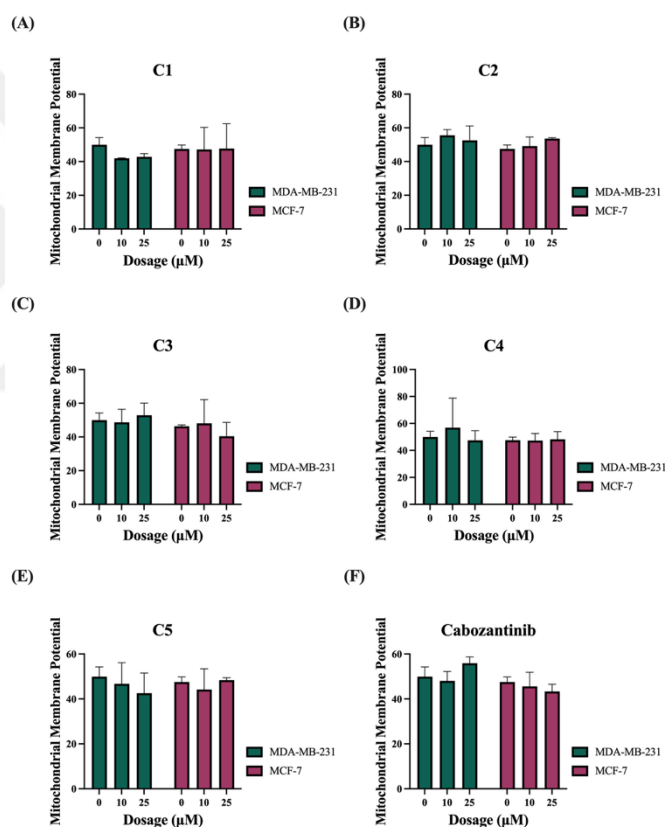


Figure 4.14: MDA-MB-231 and MCF-7 cells were seeded at a density of 3×10^4 cells per well and exposed to 10 μ M and 25 μ M of potent c-Met inhibitors (A) C1, (B) C2, (C) C3, (D) C4, (E) C5, and (F) Cabozantinib for 24 hours. Fluorometric measurement was detected with MitoSpy with Ex/Em: 577/598 nm. Columns represent the mean $\pm$ standard deviation of two independent experiments with two technical replicates. Statistical analysis was performed with two-way ANOVA, followed by Dunnett's multiple comparisons test. ns: not significant.

4.6 Detection of Protein Expression Level

Western blot analysis of MDA-MB-231 cells treated with C2, C3, C4, and cabozantinib at 10 μ M and 25 μ M concentrations demonstrates an apparent, dose-dependent reduction in the expression levels of both EGFR (175 kDa) and Akt1/2/3 (60 kDa) proteins compared to the untreated control. When C2 was applied at 10 μ M and 25 μ M, there was a reduction in EGFR and Akt1/2/3 expression. C3 downregulated EGFR and Akt1/2/3 expressions at 10 μ M compared to the control group. The effect of C4 on EGFR and Akt1/2/3 expression was seen more clearly at 25 μ M. Cabozantinib was used as a positive control and dose-dependently downregulated the expression of both proteins. The western blotting results of MDA-MB-231 are given in **Figure 4.15**.

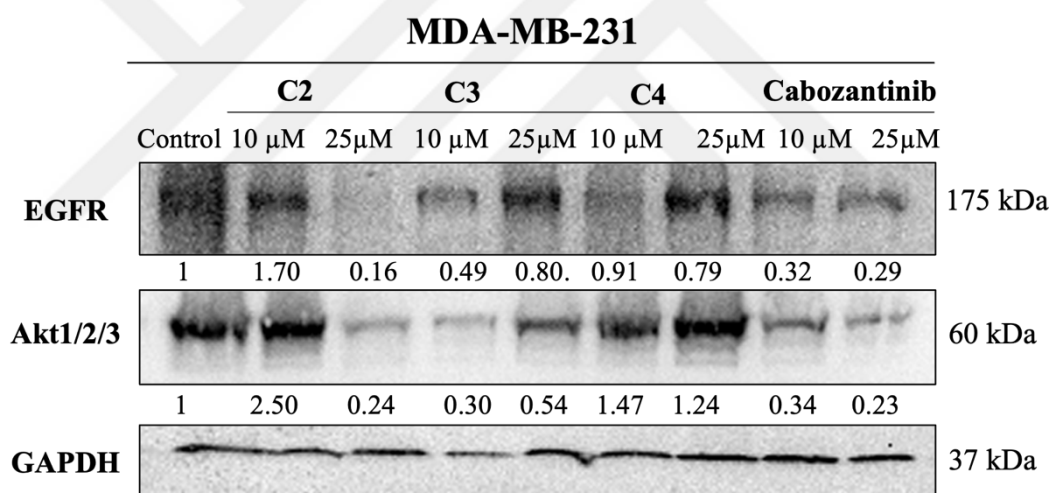


Figure 4.15: Western blot analysis of EGFR (175 kDa) and Akt1/2/3 (60 kDa) protein expression levels in MDA-MB-231 BC cells following 24 h treatment with 10 μ M and 25 μ M concentrations of C2, C3, C4, and Cabozantinib. GAPDH (37 kDa) was used as a loading control.

5. DISCUSSION AND CONCLUSION

BC is the most frequently diagnosed cancer among women worldwide and represents a major public health concern in terms of both incidence and mortality. According to 2025 projections from the International Agency for Research on Cancer (IARC) and the World Health Organization (WHO), there were an estimated 2.3 million new cases and 670,000 deaths from BC globally in 2022. If current patterns persist, it is anticipated that by 2050, there will be 3.2 million new cases and 1.1 million deaths from BC each year (**J. Kim et al., 2025**). TNBC is defined by the lack of ER, PR, and HER2 expression. Representing approximately 15–20% of all BC cases, TNBC is linked to a more aggressive disease progression, an increased likelihood of metastasis, and a less favorable prognosis compared to other breast cancer subtypes. Due to the absence of HR and HER2 amplification, endocrine therapies or HER2-targeted agents are not effective in TNBC patients, and treatment options are mainly limited to chemotherapy (**Yin et al., 2020**). However, TNBC can also rapidly develop resistance to chemotherapy, leading to disease progression with relapse. MDA-MB-231 cells are TNBCs that do not express hormone receptors. In contrast, HR+ BC (tumors expressing ER and PR) account for the vast majority of BC. For instance, MCF-7 cells express estrogen hormone. These tumors generally have a better prognosis and respond well to endocrine therapies such as tamoxifen or aromatase inhibitors. However, resistance to endocrine therapy is also a significant clinical problem in this patient group, resulting in disease recurrence and metastasis (**Pegram et al., 2023**). The complexity of BC biology includes tumor heterogeneity, genetic and epigenetic alterations, and interactions with the tumor microenvironment, which contribute to treatment resistance and disease progression. This highlights the urgent need for novel therapeutic strategies that target the molecular drivers of BC, overcome resistance mechanisms, and improve patient outcomes (**Baliu-Piqué et al., 2020**). Therefore, the c-Met signaling pathway has assumed a critical role in BC pathogenesis. c-Met, a receptor tyrosine kinase activated by HGF, regulates fundamental processes such as cellular proliferation, survival, motility, invasion, and angiogenesis (**Gherardi et al., 2012**). Overactivation of the c-Met signaling pathway can occur through overexpression, mutation, or autocrine/paracrine stimulation and has been associated with tumor growth, metastasis, and resistance to conventional therapies in BC.

Targeting the c-Met pathway stands out as a promising therapeutic approach, particularly in aggressive subtypes such as TNBC, due to the limited number of available treatment options. Various c-Met inhibitors, including small molecules and monoclonal antibodies, are being investigated in preclinical and clinical studies. These agents aim to prevent tumor progression and overcome drug resistance by disrupting c-Met-mediated signaling cascades (Tomuleasa et al., 2024). In conclusion, BC remains a complex and challenging disease with significant unmet clinical needs. Understanding the distinct biological behaviors of subtypes, such as TNBC and HR+ BC, and targeting critical pathways, such as c-Met, are key steps toward developing more effective and personalized therapies (Mahmoud et al., 2022). In this study, we evaluated the anti-cancer potential and mechanistic effects of novel C1, C2, C3, C4, C5 compounds and cabozantinib in MDA-MB-231 (TNBC) and MCF-7 (ER+) BC cell lines. The study combined cell viability, colony formation, ROS generation, mitochondrial membrane potential, lipid metabolism, and key signaling pathway analyses to provide a comprehensive assessment of the biological activities of these compounds. For this purpose, firstly, the effects of novel potent C1, C2, C3, C4, C5 c-met inhibitors, and Cabozantinib on cell viability when applied to MDA-MB-231 and MCF-7 cells for 24 hours were investigated. According to **Figure 4.1**, C1 showed a minimal cytotoxic effect in both cell lines after 24 hours of treatment. Even when applied at high doses, cell viability did not decrease below 50%. C2, C3, C4, C5 compounds caused a significant decrease in cell viability, with values below 25% in both cell lines at increasing doses. Cabozantinib was used as a positive control and demonstrated a strong cytotoxic effect in both cell lines, resulting in a dramatic decrease in cell viability. The results obtained in this study show that newly synthesized potent c-Met compounds exhibit significant cytotoxic effects in BC cells. Especially compounds C2, C3, C4, C5 suggest that they may be potential candidates for the treatment of MCF-7 HR+ BC. Similarly, newly synthesized Pyrazolo(1,2,4) triazolopyrimidine Derivatives have been investigated for their anti-cancer effects on BC and cervical cancer; they were found to exhibit anti-cancer, anti-proliferative effects in MCF-7 BC and HeLa cervical cancer cells (Szliszka et al., 2009). The fact that Cabozantinib exhibits high cytotoxic effects in both cell lines confirms its effectiveness in TNBC in clinical studies, and it is known to be effective in aggressive BC types by targeting VEGFR and Met pathways (Tolaney et al., 2017b). When **Figure 4.2** and **Figure 4.3** are examined, it is seen that C1, C2, C3, C4, C5 compounds

inhibit the long-term proliferation and colonization capacity of MDA-MB-231 cells. While similar colonization and proliferation formation were observed in the control group and the dose applied at 1 μ M, a significant decrease in colonization capacity was observed at doses of 10 μ M and above. According to the results obtained, it was concluded that these compounds not only have a short-term cytotoxic effect but also seriously reduce tumor growth potential in the long term. In a study investigating the effects of pyrazole-acridine derivatives on SKBR-3 BC cells, it was found that they exhibited dose and time-dependent cytotoxic and anti-proliferative effects (Küçükbağrıçık et al., 2024). When the colony formation results of MCF-7 cells given in **Figure 4.4** are examined, it was concluded that C1, C2, and C3 compounds inhibited the colonization capacity. A dramatic decrease in the number of colonies was observed with increasing doses of the toxin. The fact that colony formation was inhibited starting from 10 μ M concluded that these compounds suppressed the proliferation capacity in the long term in MCF-7 cells. For MCF-7 cells, C4 and C5 treatment resulted in a marked, concentration-dependent inhibition of colony formation. While the control and low concentrations (1 μ M, 5 μ M) exhibited numerous colonies, a significant reduction was observed at 10 μ M, and colony formation was almost completely abolished at 25 μ M and 50 μ M. This suggests that C4 possesses strong antiproliferative activity against MCF-7 cells at higher concentrations in **Figure 4.5**. Cabozantinib exhibited potent inhibitory effects on colony formation in both MDA-MB-231 and MCF-7 cell lines, but the degree of inhibition and concentration sensitivity differed between the two. In MDA-MB-231 cells, Cabozantinib led to a dramatic reduction in colony numbers beginning at 5 μ M, with nearly complete inhibition at 7.5 μ M and above. This indicates a high sensitivity of MDA-MB-231 cells to Cabozantinib. In MCF-7 cells, although Cabozantinib also reduced colony formation in a dose-dependent manner, the effect was less pronounced, and colonies persisted even at the highest concentrations tested. These results suggest that Cabozantinib is more effective in inhibiting the clonogenic capacity of the MDA-MB-231 cell line compared to the MCF-7 cells in **Figure 4.6**. In a study, the anti-cancer effect of 4-(3,4,5-trimethoxyphenoxy) benzoic acid derivatives in BC species such as MCF-7 and MDA-MB-468 was investigated, and it was concluded that they caused a significant decrease in colony formation (Kuan-Han Lee, 2014). **Figure 4.7** presents the effects of C1, C2, C3, C4, C5, and Cabozantinib on the survival and proliferation of MDA-MB-231 cells over 72 hours. The results provide a comprehensive view of

both dose- and time-dependent cytotoxicity, highlighting significant differences in the potency and kinetics of these agents. **Figure 4.8** presents the C1, C2, C3, C4, C5, and cabozantinib on the proliferation and survival of MCF-7 BC cells over 72 hours at concentrations of 10 μ M and 25 μ M. The results reveal significant differences in cytotoxicity among the compounds, with precise dose- and time-dependent trends. C1 displayed minimal cytotoxic activity. Both 10 μ M and 25 μ M concentrations resulted in only slight reductions in cell number compared to the control, and the overall cell population continued to increase over time. This suggests that C1 does not significantly inhibit proliferation or induce cell death in MCF-7 cells under these conditions. C2, C3, C4, and C5 demonstrate the dose- and time-dependent cytotoxicity. At 10 μ M, all four significantly reduced cell numbers by 48 and 72 hours. At 25 μ M, the effect was even more dramatic, with cell numbers dropping to or below baseline, indicating not only growth inhibition but also substantial cell death. C3 and C5, in particular, caused a rapid and sustained decrease in cell count, suggesting potent cytotoxic mechanisms. Cabozantinib is the approved type of multikinase inhibitor, serving as a positive control, and the results showed robust cytotoxicity in MCF-7 cells. In a study, the effects of DOX (doxorubicin) were investigated, and the results showed a dose-dependent reduction in cell viability in MCF-7 cells (**Ramazi et al., 2023**). The potent and sustained cytotoxicity of C2, C3, C4, and C5 comparable to cabozantinib, underscores their promise as lead compounds for further preclinical evaluation. Their ability to induce cell death in MCF-7 cells suggests potential for application in this clinically significant cancer subtype. The lack of effect of C1 also highlights the importance of chemical structure and target specificity in determining cytotoxic potential. This fluorescent staining analysis of MDA-MB-231 cells reveals the differential effects of six compounds on mitochondrial function, oxidative stress, and cellular morphology. The triple staining approach, using MitoSpy, H2DCFDA, and DAPI, provides a comprehensive insight into cellular responses to treatment. In **Figure 4.9**, C1 shows minimal impact on MDA-MB-231 cells across all parameters. The MitoSpy staining remains intense and well-distributed at both concentrations, indicating preserved mitochondrial integrity. H2DCFDA fluorescence shows only slight increases, suggesting minimal ROS generation. Nuclear morphology appears essentially unchanged, with maintained cell density. This pattern is consistent with the compound's low cytotoxic activity observed in previous viability assays. C2 demonstrates moderate but dose-dependent effects. At 10 μ M, there is a slight

reduction in MitoSpy intensity with some mitochondrial fragmentation visible. H2DCFDA staining increases moderately, indicating elevated ROS production. At 25 μ M, these effects become more pronounced with further mitochondrial dysfunction and increased oxidative stress. Cell density appears reduced, and some nuclear condensation is evident in the DAPI staining, suggesting early apoptotic changes. C3 exhibits potent effects on cellular metabolism and viability. At 10 μ M, significant mitochondrial fragmentation is visible with reduced MitoSpy intensity in a punctate pattern. H2DCFDA staining is markedly increased, indicating substantial ROS production. At 25 μ M, the effects are dramatic, with severely compromised mitochondrial networks, intense ROS generation, and significant cell loss. The remaining cells show nuclear fragmentation and condensation characteristic of apoptosis. C4 shows similar patterns to C3 but with slightly different kinetics. Mitochondrial dysfunction progresses dose-dependently, with apparent fragmentation at 10 μ M and severe disruption at 25 μ M. ROS levels increase substantially, particularly at higher concentrations. Cell density is markedly reduced at 25 μ M, with surviving cells displaying apoptotic nuclear morphology. C5 demonstrates the most pronounced effects among the test compounds. Even at 10 μ M, there is significant mitochondrial fragmentation and elevated ROS production. At 25 μ M, the cellular response is severe, with extensive cell death, minimal remaining mitochondrial signal, and intense ROS generation in the few surviving cells. This corresponds with the compound's high cytotoxic potency in previous assays. Cabozantinib, serving as the positive control, exhibits the expected dose-dependent cytotoxic effects. At 10 μ M, moderate mitochondrial dysfunction and ROS elevation are evident. At 25 μ M, substantial cell death is observed, accompanied by fragmented mitochondria and elevated ROS levels in the remaining cells, consistent with its established mechanism of action. These findings align with several studies examining mitochondrial dysfunction and ROS generation in MDA-MB-231 cells. Research has shown that MDA-MB-231 cells can acquire mitochondria from platelets. MitoSpy Red staining has been used to monitor mitochondrial dynamics in these cells (**Cereceda et al., 2023**). The dependency of MitoSpy dyes on mitochondrial membrane potential makes them excellent indicators of mitochondrial health, as compromised mitochondria lose their ability to retain these dyes. The H2DCFDA staining results are consistent with the literature, showing that effective anti-cancer agents often induce oxidative stress as part of their mechanism of action. The correlation between mitochondrial

dysfunction and increased ROS production observed with C3, C4, and C5 suggests these compounds may target mitochondrial function, leading to oxidative stress-induced apoptosis. Fluorescence-based discrimination studies have shown that cancer cells, including MDA-MB-231, can be effectively distinguished from normal cells using fluorescent markers, with drug treatments significantly altering fluorescence patterns (Morita et al., 2019). The dramatic changes in cellular fluorescence patterns observed with the most potent compounds, C3, C4, and C5 support their potential as effective anti-cancer agents. The fluorescent staining analysis reveals that C3, C4, and C5 induce significant mitochondrial dysfunction and oxidative stress in MDA-MB-231 cells, correlating with their high cytotoxic potency. These effects parallel those of cabozantinib and are consistent with literature reports of effective anti-cancer agents that target mitochondrial function and induce ROS-mediated apoptosis (N. Kumar et al., 2024). The minimal impact of C1 further validates the structure-activity relationships observed in previous cytotoxicity assays. The effects of C1, C2, C3, C4, C5, and Cabozantinib visualized by triple fluorescent staining on MCF-7 cells are given in **Figure 4.11**. Each compound was tested at 10 μ M and 25 μ M concentrations. The expected results included increasing ROS levels, indicating oxidative stress and activation of apoptosis pathways; a reduction in red fluorescence, reflecting a loss of mitochondrial membrane potential and dysfunction; and the presence of apoptosis-induced compounds, which should cause nuclear condensation and fragmentation, visible as changes in blue fluorescent intensity and nuclear morphology. Minimal changes are observed in mitochondrial membrane potential, ROS levels, and nuclear morphology at both concentrations. This suggests that C1 does not induce significant mitochondrial dysfunction, oxidative stress, or apoptosis in MCF-7 cells. At a higher concentration of C2, there is a moderate reduction in mitochondrial membrane potential and a slight increase in ROS, with some evidence of nuclear condensation. This indicates moderate cytotoxic and pro-apoptotic activity. A pronounced reduction in mitochondrial membrane potential and a marked increase in ROS are observed, particularly at 25 μ M of C3. DAPI staining reveals evident nuclear fragmentation and condensation, indicating strong activation of the intrinsic apoptotic pathway. Similar to C3, there is a dramatic loss of mitochondrial membrane potential, substantial ROS elevation, and significant nuclear fragmentation at higher concentrations, confirming potent cytotoxic and pro-apoptotic effects for C4. C5 exhibits the most severe effects observed here, characterized by extensive loss of mitochondrial membrane potential,

high ROS production, and widespread nuclear fragmentation, even at 10 μ M, indicating rapid and overwhelming cell death. As a positive control, cabozantinib exhibits the expected dose-dependent mitochondrial dysfunction, ROS increase, and apoptotic nuclear changes, thereby validating the experimental system. The observed results are consistent with numerous studies that demonstrate the effectiveness of anti-cancer agents in inducing apoptosis in BC cells by disrupting mitochondrial membrane potential, increasing reactive oxygen species (ROS), and causing nuclear fragmentation. For example, deltonin treatment in MDA-MB-231 cells results in increased ROS, collapse of mitochondrial membrane potential, and caspase-dependent apoptosis (S. Zhang et al., 2013). Similarly, combination therapies such as gemcitabine and arsenic trioxide in both MCF-7 and MDA-MB-231 cells trigger mitochondrial dysfunction and ROS elevation, leading to apoptosis (Maleki et al., 2025). In MCF-7 cells, mitochondrial depolarization and ROS production are well-established mechanisms for apoptosis induction by various chemotherapeutic agents. Interestingly, C1 did not increase ROS or induce mitochondrial dysfunction, highlighting that not all agents with anti-cancer potential act through oxidative stress or mitochondrial pathways. This is supported by studies showing that certain drugs can induce apoptosis via ROS-independent mechanisms or that cancer cells with robust antioxidant defenses may buffer ROS increases, masking oxidative stress at the cellular level. In summary, C3, C4, and C5 as well as cabozantinib, induce potent cytotoxic effects in MCF-7 cells, characterized by loss of mitochondrial membrane potential, increased ROS production, and nuclear fragmentation, hallmarks of intrinsic apoptosis. C2 shows moderate effects, while C1 is essentially inactive. These findings align with the existing literature on mitochondrial dysfunction and ROS-mediated apoptosis in BC cells, supporting the further development of these compounds as potential anti-cancer agents. Notably, the lack of ROS increase in some treatments underscores the importance of considering alternative cell death pathways and the role of cellular antioxidant capacity in drug response. BodiPy and PI staining results in MDA-MB-231 cells are presented in **Figure 4.10**, illustrating the dose-dependent responses to the six different compounds tested. BodiPy staining specifically targets lipid droplets, while PI staining identifies cells with disrupted membrane integrity, indicating cell death. Treatment with C1 did not cause notable changes in either BODIPY or PI staining compared to the control. Lipid droplet content and distribution remained similar, and PI positivity was absent, indicating that C1 does not significantly

affect lipid metabolism or induce cell death in MDA-MB-231 cells at the tested concentrations. C2 led to a modest reduction in BODIPY fluorescence at higher concentrations, suggesting a slight decrease in lipid droplet content or altered lipid metabolism. However, PI staining remained minimal, indicating that this compound does not induce substantial cell death under these conditions. C3 treatment resulted in a clear reduction of BODIPY-positive lipid droplets, especially at 25 μ M, alongside the appearance of PI-positive cells. This suggests that C3 disrupts lipid droplet homeostasis and induces cell death, possibly by interfering with lipid metabolism and a vulnerability in aggressive BC cells (**Antalis et al., 2011**). C4 showed a pattern similar to C3, with a decrease in lipid droplet staining and increased PI positivity at higher doses. This suggests that C4 also impairs lipid storage and synthesis, compromising cell viability, and supports its potential as a cytotoxic agent targeting cancer cell metabolism. C5 caused a pronounced reduction in BODIPY staining and a marked increase in PI-positive cells at both concentrations, especially at 25 μ M. This dramatic effect suggests a substantial disruption of lipid metabolic processes and induction of cell death, consistent with the literature, which shows that targeting lipid droplets can sensitize TNBC cells to therapy (**Hershey et al., 2020**). Cabozantinib also led to a reduction in lipid droplet content and increased PI staining at higher concentrations. This supports previous findings that some targeted therapies can affect both metabolic and viability parameters in aggressive BC cells. Lipid droplets are increasingly recognized as important regulators of cancer cell metabolism, survival, and therapy resistance, especially in TNBC (**Antalis et al., 2011**). MDA-MB-231 cells are known for their high lipid droplet content, which supports their aggressive phenotype and migration capacity. Studies have shown that disrupting lipid droplet formation or function can impair cell proliferation, migration, and survival (**Y. Wang et al., 2021**). Recent research has also revealed that lipid accumulation in tumor and immune cells can promote immune suppression and treatment resistance in TNBC and that targeting lipid droplets can help reverse these effects (**Rifaat et al., 2025**). The observed dose-dependent reduction in lipid droplets and increased cell death with C3, C4, C5, and cabozantinib aligns with these findings, highlighting the therapeutic potential of metabolic targeting in TNBC. The BODIPY and PI staining results of MDA-MB-231 TNBC cells treated with C1, C2, C3, C4, C5, and cabozantinib at 10 μ M and 25 μ M concentrations. BODIPY staining visualizes lipid droplets, while PI staining indicates loss of membrane integrity and cell death (**Qiu & Simon, 2016**). C1

treatment did not cause notable changes in BODIPY or PI staining compared to the control group. Lipid droplet content and distribution remained similar, and PI staining was absent, indicating that C1 does not significantly affect lipid metabolism or induce cell death in MDA-MB-231 cells at the tested concentrations. This aligns with the compound's previously observed minimal cytotoxicity, suggesting a lack of metabolic or membrane-disruptive effects. C1 is metabolically and cytotoxically inert in MDA-MB-231 cells under these conditions. C2 led to a slight reduction in BODIPY fluorescence at higher concentrations, suggesting a modest decrease in lipid droplet content or altered lipid metabolism. However, PI staining remained minimal, indicating that this compound does not induce substantial cell death. This may reflect early or mild metabolic stress without acute cytotoxicity. C2 may modestly affect lipid metabolism but does not compromise cell viability in MDA-MB-231 cells. C3 treatment resulted in a visible reduction in BODIPY-positive lipid droplets, particularly at 25 μ M, but PI positivity was still absent. This suggests that while C3 disrupts lipid droplet homeostasis, it does not induce cell death within the experimental timeframe. Literature indicates that lipid droplet depletion can sensitize cells to stress or promote cell death under additional insults, but here, the effect is limited to metabolic modulation (**Jarc Jovičić et al., 2021**). C3 disrupts lipid droplet metabolism without causing acute cell death in MDA-MB-231 cells. C4 showed a similar pattern to C3, with a decrease in lipid droplet staining at higher doses and no PI positivity. This indicates impaired lipid storage or synthesis but preserved membrane integrity and cell viability. Such metabolic effects may precede or sensitize cells to later cytotoxicity, as suggested by studies showing the role of lipid droplets in supporting cancer cell survival and migration (**Olzmann & Carvalho, 2019**). C4 impairs lipid homeostasis but does not induce immediate cell death in MDA-MB-231 cells. C5 caused a marked reduction in BODIPY staining at both concentrations, especially at 25 μ M, but PI staining remained negative. This dramatic effect on lipid droplets, without accompanying cell death, suggests a substantial metabolic disruption. Literature supports that targeting lipid droplet metabolism can weaken cancer cells' resistance to stress and therapy, even if it does not cause acute cytotoxicity (**Antalis et al., 2011**). C5 robustly disrupts lipid droplet content but does not induce cell death in the short term. Cabozantinib also led to a reduction in lipid droplet content at higher concentrations, with no PI positivity. This is consistent with reports that some targeted therapies can affect both metabolic and viability parameters in aggressive BC

cells but may require more prolonged exposure or combination with other agents to induce cell death (Antalis et al., 2011). Cabozantinib reduces lipid droplets but does not cause acute cytotoxicity in MDA-MB-231 cells under these conditions. Across all treatments, none of the compounds induced significant cell death in MDA-MB-231 cells, as evidenced by the lack of PI staining. However, C3, C4, C5, and cabozantinib notably reduced lipid droplet content, indicating that these agents disrupt lipid metabolism, a recognized vulnerability in aggressive BC (Moghtaderi et al., 2023). These metabolic effects may sensitize cells to further stress or serve as an early indicator of therapeutic efficacy, even in the absence of immediate cytotoxicity. **Figure 4.12.** presents BODIPY and PI staining results for MCF-7 cells treated with six different compounds at 10 μ M and 25 μ M concentrations. BODIPY (green) specifically stains lipid droplets, while PI (red) indicates cells with compromised membrane integrity and cell death. C1 treatment shows minimal impact on both lipid droplet content and cell viability. BODIPY staining remains intense and well-distributed across all concentrations, indicating preserved lipid droplet homeostasis in chemoresistant BC cells (Moghtaderi et al., 2023). PI staining is absent, confirming that C1 does not induce membrane permeabilization or cell death in MCF-7 cells. This suggests that C1 exhibits neither metabolic nor cytotoxic effects at the tested concentrations. C1 is metabolically inert and non-cytotoxic in MCF-7 cells under these experimental conditions. C2 demonstrates moderate effects on lipid droplet dynamics. There is a slight reduction in BODIPY fluorescence intensity at 25 μ M, suggesting decreased lipid droplet content or altered lipid metabolism (Silva et al., 2023). However, PI staining remains minimal, indicating that membrane integrity is largely preserved. This pattern suggests early metabolic stress without progressing to cell death. C2 modestly affects lipid droplet metabolism but does not compromise cell viability in MCF-7 cells. C3 shows a more pronounced effect on lipid droplet homeostasis, with a visible reduction in BODIPY staining at both concentrations, particularly at 25 μ M. This suggests significant disruption of lipid droplet formation or maintenance (Chen et al., 2023). Despite this metabolic disruption, PI staining remains negative, indicating that membrane integrity is maintained and cell death has not occurred within the experimental timeframe. C3 significantly disrupts lipid droplet metabolism without inducing acute cell death in MCF-7 cells. C4 exhibits a marked reduction in BODIPY fluorescence, especially at the higher concentration, indicating substantial depletion of lipid droplets. This aligns

with the literature, which shows that lipid droplet depletion can sensitize cancer cells to therapeutic interventions. The absence of PI staining suggests that while metabolic disruption is significant, it has not yet progressed to membrane failure or cell death. C4 causes substantial lipid droplet depletion but maintains cell membrane integrity in MCF-7 cells. C5 demonstrates the most dramatic effects among the test compounds. There is a severe reduction in BODIPY staining at both concentrations, indicating extensive lipid droplet depletion. Notably, some PI-positive cells are visible at 25 μ M, suggesting that the metabolic stress induced by C5 is beginning to compromise membrane integrity in some cells. This represents the strongest correlation between lipid droplet loss and cell death among all tested compounds. C5 causes severe lipid droplet depletion and initiates cell death in a subset of MCF-7 cells, indicating both metabolic and cytotoxic effects. Cabozantinib, as a clinically approved multikinase inhibitor, shows a significant reduction in BODIPY staining with minimal PI positivity. This pattern is consistent with its known mechanisms of targeting multiple cellular pathways that could affect lipid metabolism (**Moghtaderi et al., 2023**). The metabolic effects without extensive cell death suggest that cabozantinib's primary effects in this experimental setting are on cellular metabolism rather than direct cytotoxicity. Cabozantinib significantly affects lipid droplet metabolism with minimal acute cytotoxicity in MCF-7 cells under these conditions. The BODIPY and PI staining analysis reveals a spectrum of effects on MCF-7 cells, from metabolically inert (C1) to both metabolically and cytotoxically active (C5). Most compounds (C2, C3, C4, and Cabozantinib) primarily affect lipid droplet metabolism without inducing significant cell death, suggesting that lipid droplet disruption may be an early indicator of therapeutic efficacy that precedes cytotoxicity. Only C5 shows the progression from metabolic disruption to cell death, highlighting its potential as the most potent compound among those tested. These findings support the growing recognition of lipid droplets as important therapeutic targets in BC. **Figure 4.14** demonstrates the ROS fluorescence intensity measured after the application of C1, C2, C3, C4, C5, and cabozantinib compounds at 10 μ M and 25 μ M concentrations in MDA-MB-231 and MCF-7 cells. The findings show that, contrary to the expected increase in ROS levels, there is no significant increase in most compounds and, in some groups, even a decreasing trend. Effective anticancer compounds such as C2, C3, C4, C5, and cabozantinib do not increase ROS levels in MDA-MB-231 and MCF-7 cells, contrary to expectations and even reduce them in some cases. This shows that cell death can

occur through ROS-independent mechanisms such as disruption of mitochondrial function and metabolic stress (Suski et al., 2012). The findings show that other cellular death pathways are also important and that the rise in ROS is not the only sign of the anticancer action. This method might aid in the creation of fresh therapeutic approaches, particularly for subtypes of BC that are resistant. **Figure 4.13** demonstrates quantitative fluorometric measurements of mitochondrial membrane potential in MDA-MB-231 and MCF-7 cells following treatment with C1, C2, C3, C4, C5, and cabozantinib at concentrations of 10 μ M and 25 μ M. The results provide crucial insights into the differential effects of these compounds on mitochondrial function across two distinct BC subtypes. Shows minimal impact on mitochondrial membrane potential in both cell lines across all tested concentrations. The stability of mitochondrial function correlates with its previously observed lack of cytotoxic effects, suggesting that C1 does not interfere with fundamental mitochondrial processes. Demonstrates the most significant effect, with a statistically significant increase in mitochondrial membrane potential in MCF-7 cells at 25 μ M. This paradoxical increase in membrane potential, rather than the expected decrease associated with cytotoxicity, suggests a complex mechanism of action. The compound may initially trigger compensatory mitochondrial responses before inducing dysfunction, or it could be affecting mitochondrial dynamics in a way that temporarily enhances polarization. These compounds maintain relatively stable mitochondrial membrane potential values in both cell lines despite their demonstrated cytotoxic effects in previous assays. This unexpected finding suggests that these compounds may induce cell death through mitochondrial-independent mechanisms or that the mitochondrial effects occur at different time points than measured here. Cabozantinib maintains stable mitochondrial membrane potential in both cell lines. This finding is consistent with its mechanism of action, which primarily targets receptor tyrosine kinases rather than directly affecting mitochondrial function. In **Figure 4.15**, the effects of C2, C3, C4, and cabozantinib at 10 μ M and 25 μ M concentrations on EGFR and Akt1/2/3 protein expression in MDA-MB-231 cells were shown. GAPDH is used as a loading control. In control group, both EGFR and Akt pathways are highly active in untreated MDA-MB-231 cells, which is consistent with the aggressive, proliferative phenotype of TNBC. C2 induces a dose-dependent decrease in both EGFR and Akt expression, though the effect is less dramatic compared to the other compounds. This suggests partial inhibition of the EGFR-Akt axis, which may lead to reduced

proliferation and survival signaling but may not be sufficient for strong cytotoxicity. C3 is the most potent among the tested molecules, causing dramatic, dose-dependent downregulation of both EGFR and Akt. This suggests robust inhibition of the EGFR/PI3K/Akt pathway, which is crucial for TNBC cell proliferation, migration, and survival. Such strong suppression is expected to result in significant anti-tumor effects. C4 also shows clear, dose-dependent inhibition of both EGFR and Akt, though not as pronounced as C3. This indicates effective targeting of the EGFR-Akt signaling pathway, which should impair cell growth and survival. Cabozantinib, a clinically used multi-kinase inhibitor, serves as a positive control and shows expected dose-dependent inhibition of both EGFR and Akt. This confirms the reliability of the assay and provides a benchmark for the efficacy of the new compounds. EGFR supports proliferation, survival, metastasis, and invasion in cancer cells (Uribe et al., 2021a). High EGFR expression in MDA-MB-231 TNBC cells is associated with the aggressiveness of the cell. For this reason, EGFR is an important therapeutic target in TNBC. There is a cross-talk mechanism between Met and EGFR, so when Met is inhibited, EGFR expression may also be affected. C2 compound inhibited EGFR expression dose-dependently, but Akt1/2/3 inhibition was less compared to EGFR. It was concluded that the C3 compound dramatically inhibited EGFR and Akt1/2/3 expression. C4 compound was observed to inhibit EGFR and Akt1/2/3 expression dose-dependently. GAPDH housekeeping serves as a control, supporting the reliability of the results across all lanes. Akt is a serine/threonine kinase that regulates processes such as cell survival, growth, proliferation, migration, and angiogenesis as the central node of the PI3K/Akt pathway and is frequently overactivated in many cancers (Mundi et al., 2016). Protein expression analysis was performed in MDA-MB-231 and MCF-7 cells using the Receptor Tyrosine Kinase Antibody Kit and the Met Signaling Antibody Kit. In our findings, while successful results were obtained with the EGFR antibody, no detectable signal was observed with the other antibodies from either kit. This is a problem frequently encountered in the literature, which can be attributed to various technical and biological factors. In this Western blot study, protein expression analysis was performed in MDA-MB-231 and MCF-7 cells using the Receptor Tyrosine Kinase Antibody and the Met Signaling Antibody. However, no results were obtained from antibodies other than EGFR. This condition is mostly caused by the combination of technical variables, variations in target protein expression levels, and the different optimization needs of the kit antibodies. The

successful detection of EGFR can be attributed to its high expression level in both cell lines, which exhibits stable behavior during transfer and detection processes, given its molecular weight of 175 kDa. In contrast, phosphorylated proteins such as Phospho-Met (Tyr1234/1235), Phospho-Met (Tyr1003), and Phospho-Gab1 (Tyr307) in the Met Signaling Kit are found at very low levels under basal conditions without HGF stimulation and remain below the detection limit of Western blot. In addition, although c-Met protein is reported to be high in MDA-MB-231 cells in the literature, this information may not correlate with direct Western blot detectability, as there is no linear relationship between protein expression level and antibody affinity, extraction efficiency, and epitope accessibility. Kit antibodies are generally optimized for standardized conditions. However, they may require specific optimization for each cell line and protein, such as antibody concentration, incubation time, choice of blocking buffer, protein loading amount, fresh phosphatase inhibitors, more potent extraction buffers (such as RIPA), and growth factor stimulation, such as HGF, especially for phosphorylated proteins. The successful detection of EGFR and Akt thus indicates that the fundamental methodology is effective, while the failure of kit antibodies indicates the need for technological adjustment rather than the lack of protein present. In this study, the biological activities of pyrazole-chalcone (C1, C2) and pyridazine-triazole-chalcone (C3, C4, C5) derivatives in breast cancer cells (MDA-MB-231 and MCF-7) are directly related to their structural differences. Moreover, the comparative analysis with cabozantinib, a clinically used multi-tyrosine kinase inhibitor, has provided an important reference point for evaluating the potential of the newly synthesized compounds. The pyrazole scaffold has emerged as a promising pharmacophore in breast cancer therapy (**Ardevines et al., 2022**). However, the relatively low to moderate cytotoxic and antiproliferative effects observed for C1 and C2 in experimental results can be attributed to the limited number of aromatic rings and functional group diversity in their structures. According to the literature, the antiproliferative activity of pyrazole-chalcone hybrids is enhanced, particularly when electron-withdrawing groups (such as halogens or nitro groups) or additional aromatic rings are present on the phenyl ring (**Elkanzi et al., 2022**). While the methoxy group in C2 increases lipophilicity and cellular uptake, the core structure remains insufficiently complex, resulting in lower biological activity compared to C3, C4, and C5. The pyridazine-triazole core is known for its high target specificity and strong inhibitory potential in kinase inhibitors (**Zhang et al., 2020**). Compounds containing

this core, such as C3, C4, and C5, demonstrated dramatic reductions in both cell viability and colony formation, as well as marked mitochondrial dysfunction and significant disruption of lipid droplet metabolism in experimental studies. Notably, C5 stands out in the literature among similar derivatives for its ability to inhibit c-Met kinase at the nanomolar level, attributed to its aromatic ring richness and planar structure **(Zhang et al., 2020)**. The suppression of tyrosine kinases such as c-Met and EGFR which are critically important in breast cancer by these compounds is particularly significant for overcoming therapy resistance observed in TNBC **(Iweala et al., 2024)**. Cabozantinib is an agent that inhibits multiple tyrosine kinases, and its efficacy has been demonstrated in aggressive breast cancer subtypes in clinical settings. Experimentally, cabozantinib exhibited high cytotoxic and antiproliferative effects in both MDA-MB-231 and MCF-7 cells. According to the literature, cabozantinib has been shown to significantly reduce tumor growth and metastasis in TNBC models, and simultaneous targeting of c-Met and EGFR has been reported to enhance therapeutic efficacy. In conclusion, C3 and C5 showed the most promising effect as potent c-Met inhibitors for MDA-MB-231 BC cells. C3, C4, and C5 had the most promising effect as potent c-Met inhibitors on MCF-7 BC cells. C2 had a moderate impact on both these BC cells.

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BIOGRAPHY

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PUBLICATIONS AND PRESENTATIONS FROM THE THESIS

Aktürk B., Hamur B., Kulu İ., Arısan E.D.,” The Investigation of Potent c-Met Inhibitors Pyridazine Triazole Derivatives on Estrogen Receptor Positive and Negative Breast Cancer Cell Lines”, 9th GTU Graduate Research Symposium, Gebze, Türkiye, 26-27 May, 2025 (Poster Presentation).

