

T.R.
GEBZE TECHNICAL UNIVERSITY
GRADUATE SCHOOL

THERAPEUTIC EFFECT OF POTENTIAL c-MET INHIBITORS
ON PANCREAS CANCER CELL LINES

ZEYNEP YALÇINKAYA

A THESIS OF MASTER OF SCIENCE
DEPARTMENT OF BIOTECHNOLOGY

ADVISOR: PROF. DR. ELİF DAMLA ARISAN

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GEBZE TEKNİK ÜNİVERSİTESİ
LİSANSÜSTÜ EĞİTİM ENSTİTÜSÜ

POTANSİYEL c-MET İNHİBİTÖRLERİNİN PANKREAS
KANSERİ HÜCRE HATLARI ÜZERİNDEKİ
TERAPÖTİK ETKİLERİ

ZEYNEP YALÇINKAYA

YÜKSEK LİSANS TEZİ
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*To my Family
and
Fiancé...*

ABSTRACT

Pancreatic ductal adenocarcinoma (PDAC) is among the most lethal cancers due to its aggressive course and frequent advanced stage at the time of diagnosis. Pancreatic cancer, which was the fourth leading cause of cancer-related deaths in 2018, is expected to become the second leading cause of cancer deaths by 2030. One of the important regulators of this type of cancer at the molecular level is the c-MET protein, a receptor tyrosine kinase activated by hepatocyte growth factor (HGF). Increased HGF/c-MET signaling is associated with cell proliferation, invasion, metastasis, and resistance to treatment. Pancreatic Stellate Cells (PSCs) are the main source of HGF, the ligand of the c-MET protein, in the PDAC microenvironment. Therefore, c-MET is known to be a potential target in the treatment of pancreatic cancer. In this thesis study, the therapeutic effects of novel triazolopyridazine derivative c-MET inhibitor candidates, BH836, BH733, BH851, BH867, BH892 and BH952, on human pancreatic cancer cell lines MIA PaCa-2 (KRAS^{G12C}) and PANC-1 (KRAS^{G12D}) were evaluated. In addition, clinically approved c-MET inhibitor cabozantinib was used as a reference molecule. MTT assay to determine cell viability, colony formation assay to measure long-term proliferative capacity, survival assay to assess cell survival under treatment conditions, fluorescent staining to analyze cellular stress responses (including ROS production, mitochondrial membrane potential, lipid accumulation, and detection of dead cells), and Western blot analyses to evaluate protein expression levels were performed. While BH836 and BH851 showed the strongest antiproliferative effect in MTT and colony formation tests; BH733 and BH952 exhibited moderate cytotoxicity. BH952 caused significant loss of viability in both cell lines starting from 10 μ M. Cabozantinib caused dose-dependent cell viability reduction in both cell lines; however, it could not completely suppress colony formation. Survival assay results showed that BH851 and BH836 reduced cell survival in MIA PaCa-2 cells, while BH733, BH952, and Cabozantinib were more effective on PANC-1 cells in a time- and dose-dependent manner. In fluorescent stainings, BH836, BH733 and BH851 increased ROS production and decreased mitochondrial membrane potential except for BH851 molecule applied to PANC-1. BH952 generated high levels of ROS at low doses and caused significant cell death at high doses. No significant decrease was observed in mitochondrial activity in cabozantinib despite the decrease in cell number, but nuclear changes were observed. BH836, BH733, and BH851 were observed to increase lipogenesis in both cell lines and BH867 MIA PaCa-2 cell line in a dose-dependent manner. However, BH952 showed suppressive effect on lipid accumulation. Western blot analyses showed that BH836 and BH733 decreased EGFR and AKT levels; BH851 increased c-PARP and enhanced apoptosis markers. BH952 suppressed MET pathway proteins specifically in PANC-1 cells. Cabozantinib targeted similar molecular pathways but showed more limited effect compared to some new inhibitors. The results show that BH836 and BH851 produced strong antitumor effects in PDAC cells; BH733 and BH952 triggered more limited but significant biological responses. These findings provide important preliminary data for the potential of new c-MET-targeted inhibitors in the treatment of pancreatic cancer.

Key Words: Pancreas Cancer, c-MET Inhibitors, HGF/c-MET Pathway, Cabozantinib, HGF

ÖZET

Pankreas duktal adenokarsinomu (PDAC), tanı anında sıklıkla ileri evrede olması ve agresif seyri nedeniyle en ölümcül kanserler arasında yer almaktadır. 2018'de kanserle ilişkili ölümlerin dördüncü önde gelen nedeni olan pankreas kanserinin, 2030 yılına kadar kanser ölümlerinin ikinci önde gelen nedeni olması beklenmektedir. Bu kanseri türünün moleküler düzeydeki önemli düzenleyicilerinden biri, hepatosit büyüme faktörü (HGF) tarafından aktive edilen bir reseptör tirozin kinaz olan c-MET proteinidir. HGF/c-MET sinyallesinin artışı, hücre proliferasyonu, invazyon, metastaz ve tedaviye dirençle ilişkilidir. Pankreatik stellat hücreler (PSC), PDAC mikroçevresinde c-MET proteininin ligandı olan HGF'nin ana kaynağıdır. Bu nedenle c-MET'in pankreas kanserinin tedavisinde potansiyel bir hedef olduğu bilinmektedir. Bu tez çalışmasında, triazolopiridazin türevi yeni c-MET inhibitörü adayından BH836, BH733, BH851, BH867, BH892 ve BH952 moleküllerinin, insan pankreas kanseri hücre hatları MIA PaCa-2 (KRAS^{G12C}) ve PANC-1 (KRAS^{G12D}) üzerindeki terapötik etkileri değerlendirilmiştir. Ayrıca klinikte onaylı c-MET inhibitörü Cabozantinib referans molekül olarak kullanılmıştır. Hücre canlılığını belirlemek için MTT testi, uzun vadeli çoğalma kapasitesini ölçmek için koloni oluşum testi, tedavi koşulları altında hücre sağ kalımını değerlendirmek için sağkalım testi, hücresel stres yanıtlarını analiz etmek amacıyla floresan boyama (reaktif oksijen türleri (ROS) üretimi, mitokondriyal membran potansiyeli, lipid birikimi ve ölü hücrelerin tespiti dahil) ve protein ekspresyon düzeylerini değerlendirmek için Western blot analizleri uygulanmıştır. BH836 ve BH851, MTT ve koloni oluşturma testlerinde en güçlü antiproliferatif etkiyi gösterirken; BH733 ve BH952 orta düzeyde sitotoksikite sergilemiştir. BH952, her iki hücre hattında 10 µM'den itibaren anlamlı canlılık kaybına neden olmuştur. Cabozantinib, her iki hücre hattında doz-bağımlı hücre canlılığı azalması sağlamış; ancak koloni oluşumunu tamamen baskılayamamıştır. Sağkalım testi sonuçları, BH851 ve BH836'nın MIA PaCa-2 hücrelerinde hücre yaşamını azalttığını, BH733, BH952 ve Cabozantinib'in ise PANC-1 hücrelerinde zaman ve doza bağlı olarak daha etkili olduğunu gösterdi. Floresan boyamalarda BH836, BH733 ve BH851, ROS üretimini artırmış ve mitokondriyal membran potansiyelini PANC-1'a uygulanan BH851 molekülü hariç düşürmektedir. BH952, düşük dozda yüksek düzeyde ROS oluşturmuş, yüksek dozda ise belirgin hücre ölümüne yol açmıştır. Cabozantinib'de mitokondriyal aktivitede hücre sayısı azalsada belirgin bir azalış gözlemlenmemiştir ancak nükleer değişiklikler izlenmiştir. BH836, BH733, ve BH851 her iki hücre hattında ve BH867 MIA PaCa-2 hücresinde doza bağlı olarak lipogenez artışına neden olduğu görülmüştür. Ancak, BH952, lipid birikimini baskılayıcı etki göstermiştir. Western blot analizleri, BH836 ve BH733'ün EGFR ve AKT düzeylerini azalttığını; BH851'in c-PARP artışı ile apoptoz belirteçlerini güçlendirdiğini göstermiştir. BH952, özellikle PANC-1 hücrelerinde MET yolak proteinlerini baskılamıştır. Cabozantinib benzer moleküler yolakları hedef almış, ancak bazı yeni inhibitörlere kıyasla daha sınırlı etki göstermiştir. Sonuçlar, BH836 ve BH851'in PDAC hücrelerinde güçlü antitümör etkiler oluşturduğunu; BH733 ve BH952'nin ise daha sınırlı fakat anlamlı biyolojik yanıtlar tetiklediğini göstermektedir. Bu bulgular, c-MET hedefli yeni inhibitörlerin pankreas kanseri tedavisindeki potansiyellerine dair önemli ön veriler sunmaktadır.

Anahtar Kelimeler: Pankreas Kanseri, c-MET Inhibitörleri, HGF/c-MET Yolağı, Cabozantinib, HGF

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LIST OF SYMBOLS AND ABBREVIATIONS

μ	: Micro
h	: Hour
μ L	: Microliter
ml	: Milliliter
mM	: Millimolar
xg	: Times gravity
%	: Percentage
$^{\circ}$ C	: Degree Celsius
<i>p</i>	: P value
BODIPY	: Fluorinated Boron-Dipyrromethene
CO ₂	: Carbon dioxide
DAPI	: 4',6-diamidino-2-phenylindole
DCFDA	: Dichlorodihydrofluorescein Diacetate
dH ₂ O	: Distilled water
DIOC6	: 3,3'-dihexyloxacarbocyanine iodide
DMEM	: Dulbecco's Modified Eagle Medium
DMSO	: Dimethyl sulfoxide
DNA	: Deoxyribonucleic Acid
MeOH	: Methanol
MET	: Mesenchymal Epithelial Transition Factor
EtOH	: Ethanol
HGF	: Hepatocyte Growth Factor
kDa	: Kilodaltons
Min	: Minute
NaCl	: Sodium Chloride
PBS	: Phosphate Buffered Saline
PI	: Propidium Iodide
ROS	: Reactive Oxygen Species
RPM	: Revolutions per minute
TBS	: Tris-Buffered Saline
TBS-T	: Tris-Buffered Saline-Tween 20

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1. INTRODUCTION

1.1 Pancreatic Cancer

Pancreatic cancer (pancreatic ductal adenocarcinoma (PDAC/PC)) is one of the deadliest cancer types that have the 5-year-survival-rate with less than 10% [Kim et al., 2024; Siegel, Miller, and Jemal, 2020]. By 2030, pancreatic cancer is expected to rank as the second most common cause of cancer-related fatalities, up from its fourth-place ranking in 2018 [Mortazavi et al., 2023]. The main reason for the poor prognosis is since the disease cannot be diagnosed at an advanced stage while it is metastatic. 80% of patients diagnosed have metastatic disease [Pothula et al., 2020]. For this reason, curative surgical application cannot be performed, or even if it is performed, there is a possibility of recurrence afterwards. The survival rate for tumors that cannot be removed is 3.5 months [Kim et al., 2024; Gradiz et al., 2016]. Since it is a type of cancer that has a high resistance to traditional cancer treatments, it shows resistance to various methods such as chemotherapy or immunotherapy [Kim et al., 2024]. The main characteristic of PC is the desmoplastic/stromal reaction, which is made up of extracellular matrix proteins and includes immune cells, endothelial cells, neuronal cells, and pancreatic stellate cells (PSCs) [Kim et al., 2024; Che et al., 2020]. All of these plays a vital role in cancer progression. Primary tumour growth is promoted by PSCs, which is the principal source of stromal collagen and chemo- and radiotherapy resistance is induced by these cells and facilitate metastasis [Che et al., 2020]. The characterized tumor microenvironment that occurs is one of the barriers to effective drug delivery, and the effectiveness of immune-based therapies is decreasing for this reason [Kim et al., 2024]. Several signaling molecules such as FGF-2, TGF- β , VEGF, PDGF and HGF play a role in the interaction of cancer cells and PSCs with each other. HGF-mediated signaling pathways are involved in the interaction of endothelial cells with PSCs. HGF is a ligand of c-MET and is upregulated in pancreatic cancer and plays a role in poor prognosis. By connecting HGF to its receptor, c-MET, various cell signaling pathways are activated, affecting many cell functions such as cell proliferation, migration, invasion and mobility [Che et al., 2020].

1.1.1 Epidemiology of Pancreatic Cancer

Pancreatic cancer is one of the few deadly cancers where the mortality rate is almost equal to the incidence [Che et al., 2020]. It has one of the lowest survival rates of any cancer, at 13% [Siegel, Miller, and Jemal, 2020]. By 2030, it is predicted to rank as the second most common cause of cancer-related deaths, with an annual incidence rate of 0.5% to 1.0% [Park, Chawla, and O'Reilly, 2021]. In the 28 countries of the European Union, an estimated 1,115,000 people will die from pancreatic cancer by 2025 [Hu et al., 2021]. The incidence of pancreatic cancer is higher in men (262,865 cases, 5.7 per 100,000) than in women (232,908 cases, 4.1 per 100,000) [Ushio et al., 2021]. Approximately 275,000 hospitalizations occur each year in the United States due to pancreatitis [Chadalavada, Simons-Linares, and Chahal, 2020]. According to statistics in 2018, the incidence of the disease is 458918, the mortality rate is 432242, and deaths constitute 94.2% of new cases [Hu et al., 2021]. Although the epidemiology of the disease is known, there is no definitive treatment yet because the underlying cellular mechanisms are not fully understood [Kolodecik et al., 2014]. Since the symptoms of the disease are not clear, it is difficult to use clinical symptoms for early diagnosis. In this case, only about 2% of PCs are detected in stages 0 and 1 [Ushio et al., 2021].

1.1.2 Risk Factors of Pancreatic Cancer

The risk factors that cause pancreatic cancer are divided into two groups: unchangeable and changeable [Hu et al., 2021]. There are various unchangeable factors such as age, ethnicity, gender, blood type, diabetes mellitus, family history of the disease, microbiota, gallstones, hyperlipidemia, allergies and genetic susceptibility, while there are also factors such as smoking, diet, obesity, socioeconomic status, toxins, traumas and infections that can be changed [Hu et al., 2021; Klein, 2021; Kolodecik et al., 2014]. Differences occur in the physiology of the pancreas because of these factors. These are conditions such as decreased enzyme secretions in the pancreas, harmful activation of digestive cells in the acinar cells, increased infection responses and cell death. People at risk of pancreatic cancer should be monitored with blood tests, various screening and imaging methods [Kolodecik et al., 2014].

1.1.3 Therapies for Pancreatic Cancer

There are clinical studies using different new agents and treatment methods against pancreatic cancer. However, it is a type of cancer that is resistant to traditional treatments such as chemotherapy and immunotherapy [Chen, Tsai, & Hung, 2015; Kim et al., 2024]. There are different treatment methods for pancreatic cancer. These are surgical operations, radiation therapy, chemotherapy, chemoradiation and targeted/personalized therapy methods. The most effective treatment is to remove cancer from the body after an early diagnosis. Otherwise, this may not be possible in cases of late diagnosis and aggressive metastasis resistant to chemotherapy [Kolodecik et al., 2014; Sharma and Malviya, 2023]. Only 10% of the tumors are resectable, while 60% of the remaining 90% have metastasis, while 30% have local tumors [Mekapogu et al., 2023]. There are three different methods for performing the surgery: Whipple procedure if the tumor is limited to the head of the pancreas, distal pancreatectomy if it is towards the tail end of the pancreas, and total pancreatectomy [Sharma & Malviya, 2023]. PC is more resistant to chemotherapy compared to other types of cancer, so several chemotherapy drugs such as Gemcitabine and FOLFIRINOX (leucovorin, fluorouracil, irinotecan, and oxaliplatin) have been approved and have shown good performance in killing cancer cells when combined with albumin-bound paclitaxel (nab-paclitaxel) nanoparticles. Approved drugs for pancreatic cancer include capecitabine, 5-fluorouracil (FU), erlotinib, leucovorin, gemcitabine nanoliposomal irinotecan, and oxaliplatin [Buckley and O'Reilly, 2024; Mekapogu et al., 2023; Mortazavi et al., 2023; Sharma & Malviya, 2023]. Another treatment method used to kill cancer cells is radiation therapy, which uses high-energy X-rays or other particles, such as those used in SBRT or cyberknife, proton beam therapy. The process in which radiation is used together with chemotherapy is called radiosensitization. Systemic treatments are administered in two different ways: oral chemotherapy and intravenous (IV) [Kim et al., 2024]. In recent years, targeted therapies have focused on the main effect that causes cancer by targeting specific genetic mutations and molecular pathways. Some of these are PARP inhibitors targeting pancreatic cancer caused by BRCA1 and BRCA2 mutations. Inhibitors are also used for various genes such as KRAS, NTRK and RET [Buckley & O'Reilly, 2024]. For example, erlotinib targets the EGFR, olaparib targets the BRCA mutation and larotrectinib targets the NTRK fusion

[Mortazavi et al., 2023; Kolbeinsson et al., 2023; Sharma and Malviya, 2023]. Patients with germline mutations in homologous recombination repair (HRR) genes are recommended by NCCN guidelines to receive first-line platinum-containing chemotherapy including FOLFIRINOX, mFOLFIRINOX or gemcitabine and cisplatin. For another common mutation in pancreatic cancer, KRAS G12C, Sotorasib and adagrasib have been approved by the FDA in NCCN guidelines **[Buckley and O'Reilly, 2024]**.

Immunotherapy has also been used in recent years to target cancer cells using the body's immune system. It is thought that the lack of effective response to PDAC immunotherapy is due to the immunosuppressive TME. Mismatch repair deficiency and/or microsatellite instability is seen in approximately 2% of patients **[Buckley & O'Reilly, 2024; Kolbeinsson et al., 2023]**.

Personalized medicine applications targeted according to the genetic profile of tumors are promising applications in the treatment of cancer. It increases the survival probability of patients and their quality of life because they target the protein, molecular or ecological origin to eliminate cancer **[Kolbeinsson et al., 2023; Sharma & Malviya, 2023]**. c-MET receptor tyrosine kinase, which is also overexpressed in PDAC and other certain types of cancer, is a protein that plays an important role in the growth, survival and metastasis of cancer cells. It can be said that HGF, the ligand of c-MET protein in the PDAC microenvironment, is mainly secreted from PSCs and therefore c-MET is one of the molecular targets in the discovery of anticancer treatments **[Firuzi et al., 2019; Sharma and Malviya, 2023]**.

1.2 Mechanisms of c-MET Activation

The mesenchymal–epithelial transition factor (c-MET) also known as MET, RCCP2, DFNB97, AUTS9, and hepatocyte growth factor receptor (HGFR) **[Park and Richardson, 2020]** is a receptor tyrosine kinase **[Qian et al., 2009]** which expressed mostly in endothelial, melanocytes and surface of epithelial cells of many organs like liver, prostate, pancreas, bone marrow and kidney **[Mohan et al., 2024; Raj et al., 2022]** stimulated by HGF also known as scatter factor (SF) **[Sharma and Malviya, 2023]**. The HGF gene is found in chromosome 7q21.11 **[Mohan et al., 2024]** and has a heterodimer consists of two distinct chains which are 69 kDa α - and 34 kDa β -chain **[Sharma & Malviya, 2023]**.

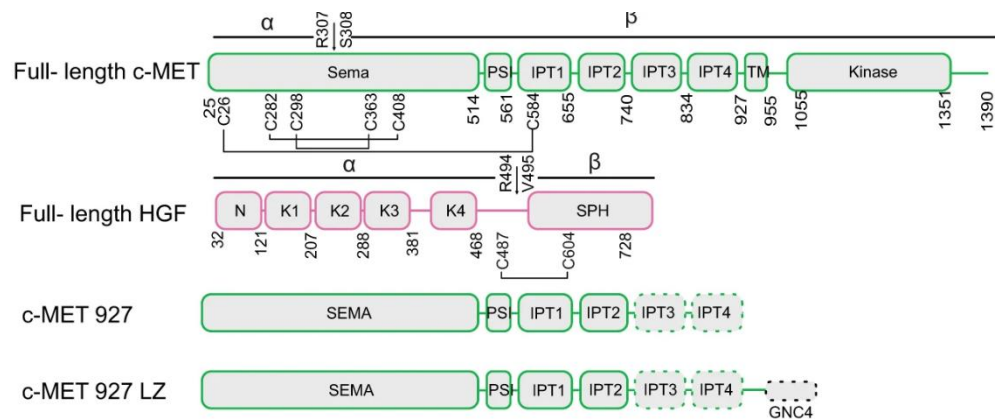


Figure 1.1: Illustration of the c-MET receptor and HGF, its ligand. The transmembrane β -chain and the extracellular α -chain make up the receptor tyrosine kinase known as the c-MET receptor [Uchikawa et al., 2021].

The primary ligand for initiating the c-MET receptor's biological activity is HGF. The precursor becomes a functional two-chain heterodimer upon cleavage by extracellular proteases [Raj et al., 2022]. Its twenty exons encode a 92 kDa glycoprotein called pro-HGF, an inactive single-chain precursor produced by mesenchymal cells. Pro-HGF is cleaved by extracellular proteases to produce its bioactive form [Tolbert et al., 2010]. A disulfide link holds the α and β chains together in the 103 kDa soluble heterodimer that is the mature form of HGF. The α chain contains an N-terminal hairpin loop, four kringle domains, and an 80 amino acid double-loop structure composed of three internal disulfide connections. It also features a high-affinity binding site for the c-MET receptor [Faiella et al., 2022]. Two c-MET heterodimers dimerize when Human Growth Factor (HGF) is identified by the c-MET immunoglobulin-like domains and binds to the extracellular region of the MET β domain [Tolbert et al., 2010]. This causes three tyrosine residues (Tyr1234, Tyr1230, and Tyr1235) inside the kinase catalytic domain to become autophosphorylated. Following this initial activation step, autophosphorylation also occurs at two additional tyrosine residues, Tyr1349 and Tyr1356, located in the carboxy-terminal region and serving as multifunctional docking sites for downstream signaling effects [Gherardi et al., 2012; Moosavi et al., 2019].

c-MET receptor consists of an internal KD, a single transmembrane helix (TM), four sequential immunoglobulin-plexin-transcription factors (IPT1-4) domains in the extracellular region, a semaphorin (SEMA) domain, and a plexin-semophorin-integrin (PSI) domain (Fig 1.1) [Uchikawa et al., 2021].

The catalytic domain and a C-terminal tail with two tyrosine essential to c-MET receptor function and carcinogenic potential make up the intracellular component of the receptor [Moosavi et al., 2019]. C-MET is initially synthesized as a 170 kDa single-chain precursor which is cleaved by furin protease into a 50 kD extracellular α -subunit and a 145 kDa transmembrane β -subunit [Faiella et al., 2022]. The single polypeptide chain that makes up the c-MET receptor splits into the β and α chains at the cell surface. The c-MET receptor heterodimer is composed of an extracellular α chain and a β chain with an extracellular domain, a single transmembrane domain, and a cytoplasmic protein kinase domain [Hellmich and Evers, 2006]. C-MET is essential for normal cell function, embryonic development, organ development, regeneration and wound healing [Mohan et al., 2024; Park & Richardson, 2020]. Paracrine or autocrine mechanism release HGF and HGF interact with c-MET and leads to dimerization and autophosphorylation of tyrosine in the cytoplasmic domain subsequently [Mohan et al., 2024; Park and Richardson, 2020].

The protein is activated through structural alterations caused by the enzymatic breaking of certain bonds within the protein. HGF functions as a particular ligand for c-MET once it is activated. HGF binding to the c-MET receptor initiates a series of events in addition to being a static interaction [Kim et al., 2024]. Following HGF binding to c-MET, signaling effectors such as GRB2 and GAB1 are recruited. It then stimulates different downstream signaling pathways [Kim et al., 2024; Xia, Huang, and Wang, 2025] such as ERK1/2, PI3K/AKT/mTOR, STAT3, and Rac1, Ras/Raf/MEK/MAPK, Ras/PAK, SCR, FAK [Kim et al., 2024], and Wnt/ β -catenin [Puccini, Marín-Ramos, and Battaglin, 2019] pathways involved in angiogenesis, cell proliferation, tumor formation, survival and motility [Cecchi, Rabe, and Bottaro, 2022; Dienstmann, Rodon, and Tabernero, 2013; Ma et al., 2008; Puccini, Marín-Ramos, and Battaglin, 2019]. Aberrant activation of the c-MET receptor can occur through several mechanisms, including autocrine or paracrine ligand stimulation, alternative splicing (especially skipping of exon 14 of the MET gene), c-MET overexpression, and genomic rearrangements (amplifications, translocations, and mutations) [Ben Dhiab et al., 2025; Puccini, Marín-Ramos, and Battaglin, 2019]. Furthermore, the HGF/c-MET signaling pathway promotes carcinogenesis and targeted therapy resistance by strong crosstalk with other RTK-mediated signaling pathways, including the EGFR pathway and RON (recepteur d'origine nantaïs) [Puccini, Marín-Ramos, and Battaglin, 2019]. Furthermore, increased c-MET

expression has been linked to a worse prognosis in additional tumor types, such as gastric, liver, breast, and brain cancer [Liu et al., 2013]. Anormal activation of HGF-c-MET axis caused by c-MET gene mutations, overexpression or amplification leads to cancer progression by activating several signaling pathways like Erk, Akt and STAT3 [Dienstmann, Rodon, and Tabernero, 2013]. According to research, cell sensitivity to chemotherapy is reduced by HGF [Wood et al., 2021]. The investigation of the possible consequences of its dysregulation in pathological states including cancer, fibrosis, and other disorders is crucial since the HGF/c-MET pathway has a wide range of effects. Extensive research on the processes and possible therapeutic targets of this pathway is necessary to comprehend its complicated structure, particularly in the context of illnesses like pancreatic cancer where it plays a significant role [Kim et al., 2024].

1.2.1. HGF/ c-MET Pathway in the Pancreatic Cancer

The HGF/c-MET signaling pathway is a key player in the development of several cancer types. High levels of HGF and/or c-MET expression have been linked to a poor prognosis in several malignancies, including those of the head & neck, lung, kidney, uterus, cervix, stomach, and blood [Pothula et al., 2020]. It has been discovered that HGF produced by fibroblasts stimulates the development of colon cancer cells via activating signaling dependent on c-MET. Researchers studying pancreatic cancer found that PSCs were the main source of HGF release and that the c-MET receptor was expressed by both cancer cells and endothelial cells, which is consistent with these findings [Sharma and Malviya, 2023]. For HGF in the inactive proform structure to fulfill its function, it must be broken down by proteases, and the urokinase plasminogen activator (uPA) that plays a role in this is involved in the regulation of the HGF/c-MET pathway. With the binding of HGF to cancer cells, uPA production is induced, and thus pro-HGF is converted into active HGF, creating a positive feed-forward loop (Fig. 1.2) [Pothula et al., 2020].

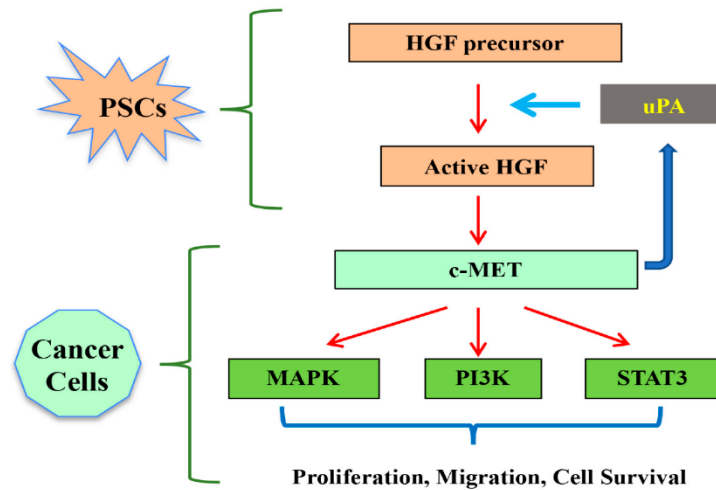


Figure 1.2: Diagrammatic representation of the HGF/c-MET pathway's possible function in pancreatic cancer. Proteases like urokinase plasminogen activator (uPA) facilitate the proteolytic cleavage of HGF, which is released by PSCs in a precursor form. The activation of downstream intracellular signaling cascades that control cancer cell functions that impact tumor progression occurs when HGF binds to its receptor, c-MET. A feed-forward loop is created when this binding causes cancer cells to produce more uPA, which in turn activates HGF from PSCs [Pothula et al., 2020].

In the study conducted by Buckley et al., it was seen that the uPA inhibitor which is called 6-substituted hexamethylene amiloride derivative can significantly reduce the incidence of metastasis in a pancreatic cancer model [Buckley et al., 2018; Pothula et al., 2020]. Plasma HGF levels were higher in pancreatic cancer patients than in healthy controls, as well as in patients with benign pancreatic tumors and chronic pancreatitis [Sharma and Malviya, 2023]. Studies have shown that c-MET-targeted drugs have antiproliferative and apoptosis-inducing properties in PDAC cells and significantly inhibit cancer growth in PDAC animal models. Furthermore, a number of lines of evidence indicate that PDAC patients who have activation of the HGF/c-MET pathway have a poorer prognosis. This has led to various clinical investigations evaluating the abnormal effectiveness of HGF/c-MET signaling pathway inhibitors in treating PDAC [Mortazavi et al., 2023]. MET dysregulation in malignancies has a bad prognosis due to its many functions in tumor pathobiology. Activated MET boosts cancer cell proliferation and survival while increasing motility and invasiveness, hence aiding tumor spread. Furthermore, HGF activates MET in endothelial cells, which promotes tumor angiogenesis. MET signaling dysregulation enhances tumorigenicity and metastatic potential in modified cells and transgenic animals. In contrast,

inhibiting MET signaling with ribozymes, antisense RNA, anti-HGF antibodies, or an inhibitory portion of HGF decreases tumor xenograft growth in mice [**Mortazavi et al., 2023; Pothula et al., 2020**].

Thus, c-MET is a prospective therapeutic target for a variety of tumors due to its significant influence on cancer growth, carcinogenesis, and the development of resistance to treatments [**Park and Richardson, 2020**].

1.3 Therapeutic Targeting of c-MET

Because it can trigger several mitogenic signaling cascades, including the PI3K/Akt/mTOR axis, MAPK pathway, STAT3 pathway, NF- κ B pathway, and many more, the c-MET pathway holds an important role in oncology. Each of these signaling axis' carcinogenic roles has been extensively investigated, and c-MET targeting when combined with other receptor tyrosine kinases may offer useful treatment approaches [**Mohan et al., 2024; Raj et al., 2022**]. Inhibiting a single kinase is advantageous to targeting each downstream pathway separately since c-MET acts as an activator of the several oncogenic proteins. Many c-MET inhibitors are undergoing preclinical research or clinical trials [**Raj et al., 2022**]. In many tumors, the c-MET protein acts as a proto-oncogene, promoting the spread, invasion, migration, and survival of cancer cells as well as their proliferation, angiogenesis, metastasis, and resistance to tyrosine kinase inhibitors [**Park & Richardson, 2020; Qian et al., 2009**]. Targeting many oncogenic proteins is possible, but c-MET is unique because of its combined mitogenic, morphogenic, and motogenic functions [**Mohan et al., 2024**]. Ras/Raf/MAPKK/ERK, Cdc42/Rac1, FAK, Src/FAK, PI3K/FAK, AKT/mTOR, PAK/N-WASP, AKT/NF κ B, STAT3, and Arp2/3, CD44, plexins, death receptors, tetraspanin, integrins, mucins, and RTKs like EGFR, HER2, and HER3 are among the proteins with which c-MET interacts [**Park and Richardson, 2020; Raj et al., 2022**]. When these various molecules interact, c-MET is either activated or inhibited, which has an impact on drug resistance, oncogenesis, cell migration and invasion, and cell survival. When considered as a whole, most of these interactions have a synergistic influence on the development of treatment resistance and the advancement of cancer. Because of its strong effects on carcinogenesis, treatment resistance, and cancer progression, c-MET is a prospective therapeutic target for a variety of malignancies [**Park and Richardson, 2020**].

According to studies, c-MET is a stem cell marker in pancreatic cancer, and progenitor cells express MET, which exhibits pro-survival (anti-apoptotic) signals. A resistant population of cancer cells with stem cell-like characteristics is left behind after gemcitabine treatment has its lethal effect on vulnerable cancer cells. They have demonstrated that both EMT and stem cell markers are highly expressed in gemcitabine-resistant pancreatic cancer cells. Notably, preclinical models of orthotopic pancreatic cancer and genetically modified mice have demonstrated improved chemotherapeutic response and improved drug delivery when HGF/c-MET inhibition is present. Using spheroid models, Firuzi and associates have more recently demonstrated that blocking the HGF-c-MET pathway aids in the resolution of drug resistance [Pothula et al., 2020].

There have been numerous investigations to find this pathway as a target since HGF is the sole known c-MET ligand and because aggressive disease is associated with both HGF and c-MET [Pothula et al., 2020]. Targeting the HGF/c-MET pathway, which enhances communication between cancer cells and pancreatic stellate cells (primary collagen-producing stromal cells) and between PSCs and endothelial cells, may be a promising target for the development of innovative drugs for the treatment of pancreatic cancer [Gherardi et al., 2012; Pothula et al., 2020]. The discovery of the HGF/c-MET pathway dates to the mid-1980s, during which time several c-MET inhibitors such as Tepotinib, Altiratinib, Capmatinib, Savolitinib and the ALK/c-Met inhibitor Crizotinib have been effective in demonstrating anticancer properties [Zhou, Zhang, and Wang, 2022]. HGF ligand is secreted by PSCs, and the receptor c-MET is found on pancreatic cancer cells. Interfering with this pathway reduces tumor growth and eliminates metastasis in immunodeficient xenograft models. Evaluation in the presence of an active host immune system will provide a clearer conclusion [Gallo, Folco, and Crepaldi, 2024]. As a result, extensive studies are underway to develop inhibitors for the pharmacological suppression of c-MET activity as an emerging approach in cancer treatment [Gallo, Folco, and Crepaldi, 2024].

1.3.1 Modes of Action of c-MET Inhibitors

With the understanding of MET signaling, various therapeutic intervention strategies targeting MET in oncology are emerging [Mekapogu et al., 2023]. Inhibition of the HGF/c-MET axis is achieved in two main ways: First, anti c-MET monoclonal antibodies that block the interaction of HGF with c-MET or c-MET dimerization; second, small molecule c-MET tyrosine kinase inhibitors (TKIs) that target the cytoplasmic activation sites of the receptor and stop phosphorylation and signal transduction are widely used in cancer treatment [Gallo, Folco, and Crepaldi, 2024; Jin et al., 2024; Puccini, Marín-Ramos, and Battaglin, 2019]. Thus, a variety of therapies are available that aim to prevent downstream signaling activation through targeted inhibition of c-MET or HGF [Gallo, Folco, and Crepaldi, 2024; Faiella et al., 2022; Puccini, Marín-Ramos, & Battaglin, 2019].

The first of these are selective Type I c-MET inhibitors are divided into two subgroups: Ia (e.g. crizotinib) and Ib (e.g. capmatinib, MK-2461). These inhibitors bind to the Y1230 site and the active structure of DFG-in. Subgroup Ib inhibitors have higher specificity for c-MET because they do not interact with G1163 [Jabbarzadeh Kaboli et al., 2024]. These inhibitors bind to the phosphorylated, or active, site of the receptor [Faiella et al., 2022; Kim et al., 2024]. Type I c-MET kinase inhibitors work by a competitive mechanism with ATP [Fu et al., 2021; Gallo, Folco, and Crepaldi, 2024]. Nevertheless, the inhibitors' affinity for c-MET is reduced by specific pocket point mutations in the ATP binding pocket of c-MET. As a result of these alterations, tumors treated with Type I c-MET kinase inhibitors eventually have a tendency to become resistant [Mekapogu et al., 2023].

The other therapy is nonselective MET kinase inhibitors such as Type II c-MET inhibitors (e.g. cabozantinib, foretinib, merestinib, BMS-777607, and glesatinib) bind to the ATP binding site in the DFG-out conformation (H1088, G1087, and K1110) and Type III c-MET inhibitors (e.g. tivantinib) have a mechanism that does not compete with ATP and does not fit the DFG model [Mekapogu et al., 2023; Jabbarzadeh Kaboli et al., 2024]. While selective c-MET inhibitors have the benefit of exhibiting strong antitumor efficacy in MET-driven cancers without off-target effects, nonselective inhibitors have broad-spectrum anticancer action due to their ability to target many tyrosine kinases at once [Dienstmann, Rodon, and Tabernero, 2013; Jabbarzadeh Kaboli et al., 2024]. Glesatinib can overcome resistance to Type-I c-

MET inhibitors by using a unique target blocking method [Fu et al., 2021]. These drugs downregulate c-MET protein by a variety of methods, such as (1) a reduction in MET mRNA levels, (2) lysosomal degradation of c-MET, and (3) metalloprotease activity stimulation that results in c-MET destruction [Park and Richardson, 2020]. The other c-MET inhibitors type are anti-MET monoclonal antibodies (mAbs) (e.g. emibetuzumab, onartuzumab, ficlatuzumab, rilotumumab) that aim to destroy HGF/c-MET interaction and /or c-MET dimerization [Dienstmann, Rodon, and Tabernero, 2013; Fogli et al., 2022; Moosavi et al., 2019]. Depending on whether they bind to the receptor c-MET or the ligand HGF, anti-MET mAbs are further categorized by their target.

Rilotumumab is a human HGF-neutralizing monoclonal antibody designed to prevent HGF from interacting with the c-MET receptor and negatively affecting cellular processes [Puccini, Marín-Ramos, and Battaglin, 2019]. Although it showed promise in phase I and II trials in patients with advanced gastric and gastroesophageal cancer, its studies were discontinued in phase III studies because it was observed to increase disease-related mortality [Jabbarzadeh Kaboli et al., 2024]. Another HGF-neutralizing monoclonal antibody is Ficlatuzumab (AV-299) [Mekapogu et al., 2023]. It is being investigated in pancreatic ductal adenocarcinoma, head and neck squamous cell carcinoma, and acute myeloid leukemia [Bauman et al., 2023; Perez et al., 2023; Ruff, Brown, and Pawlik, 2023]. Onartuzumab is also designed to prevent the interaction with HGF by targeting the extracellular domain of the c-MET receptor and works with knob-into-hole technology, thus facilitating precise one-to-one interaction with the receptor [Puccini, Marín-Ramos, & Battaglin, 2019]. However, it did not show the desired effect as rilotumumab in clinical studies [Wang et al., 2021]. Newly developed anti-c-MET antibodies hold promise in cancer treatment. Single-arm anti-c-MET antibody 2E6 significantly reduced tumor growth and cell migration by blocking HGF-c-MET interaction in hepatocellular carcinoma (HCC) cells. It also suppressed tumor growth in mouse models [Puccini, Marín-Ramos, and Battaglin, 2019]. On the other hand, rilotumumab has shown efficacy in gastric and gastroesophageal cancers [Fogli et al., 2022], and ficlatuzumab in combination with EGFR inhibitors in various advanced solid tumors [Puccini, Marín-Ramos, and Battaglin, 2019]. Emibetuzumab is a humanized MET-specific IgG4 monoclonal antibody targeting the c-MET protein [Kim, Barzi, and Rajdev, 2018; To, Leung, and Cho, 2023]. It primarily blocks the binding of HGF to the MET

receptor and blocks downstream HGF-mediated signaling pathways. Unlike onartuzumab, it also enables the functionalization and degradation of the Met receptor protein. Thus, it has anticancer effects in both HGF-dependent and HGF-independent cancers, including MET-amplified [To, Leung, and Cho, 2023]. ARGX-111 is an afucosylated IgG1 monoclonal antibody targeting the c-MET receptor. This antibody blocks the binding of HGF to MET, promoting its internalization and degradation. It has shown cytotoxic effects in both c-MET positive human cancer cells and patient-acquired leukemia cells [Kim and Kim, 2017]. ARGX-111 has been shown to suppress metastasis and reduce circulating tumor cells in an orthotopic mouse model and has shown favorable safety profiles in a phase I clinical trial in patients with advanced MET overexpression hematological and solid tumors (NCT02055066) [Kim & Kim, 2017; To, Leung, & Cho, 2023].

Therapeutic monoclonal antibodies (mAbs) directed against c-MET generally have higher target specificity, more predictable pharmacokinetic properties, less dose-limiting toxicity, and long-term sustained treatment efficacy compared to small molecules. All these developments indicate that new antibodies targeting the HGF/c-MET pathway will play an important role in cancer treatment [Hojjat-Farsangi, 2014; Mer et al., 2024].

In c-MET targeted therapies, in addition to small molecule inhibitors, antibody drug conjugates (ADCs) also known as smart chemotherapy targeting the HGF/MET axis in the tumor microenvironment are gaining importance. ADCs combine the specificity of mAbs with the strong effect of chemotherapeutic agents, delivering toxic substances so called payloads directly to MET-positive tumor cells and reducing side effects [Kim and Kim, 2017; Mekapogu et al., 2023]. Telisotuzumab vedotin formerly known as ABBV-399, is promising, especially in NSCLC patients showing MET overexpression. Several ADCs, such as SHR-A1403, TR1801-ADC, RC108, and BYON3521, are undergoing clinical trials [Kim and Kim, 2017].

In the tumor microenvironment, HGF secreted from stromal cells activates the MET receptor and contributes to tumor growth and drug resistance. For this reason, antibodies that neutralize HGF are included in treatment strategies [Raj et al., 2022; To, Leung, and Cho, 2023]. However, some clinical studies have not achieved the expected success, and limited benefit has been observed in some patient groups [Mekapogu et al., 2023]. In the future, it is aimed to develop more effective treatments with minimized side effects by combining small molecules, ADCs and antibodies

targeting the microenvironment in the design of c-MET inhibitors [Gallo, Folco, and Crepaldi, 2024; Mekapogu et al., 2023]. Additionally, a better understanding of the tumor microenvironment and resistance mechanisms will play a critical role in patient selection and increasing treatment success [Mekapogu et al., 2023].

New small molecule-based degrader approaches are being developed for MET-targeted protein degradation. This method allows the target protein to be selectively destroyed within the cell rather than simply blocking protein activity, thus offering the potential to reduce side effects and overcome drug resistance. The most widely used technology is PROTAC molecules, which activate the target protein binding unit and E3 ligase to achieve proteasomal degradation. Recent studies have shown that MET-targeting PROTACs reduce tumor growth, suggesting a new treatment option, especially in cancers with abnormal MET signaling [Kim, Barzi, & Rajdev, 2018; Mekapogu et al., 2023].

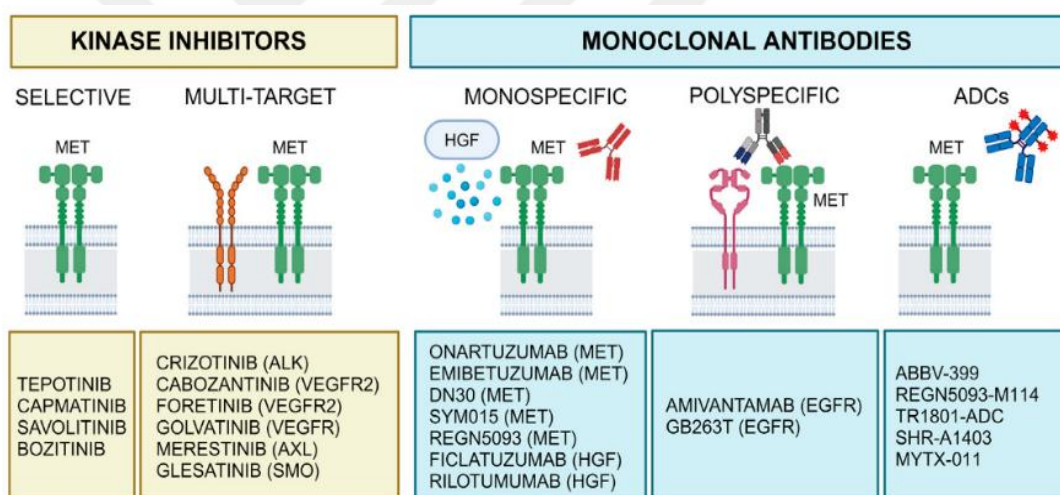


Figure 1.3: MET targeting strategies. Numerous strategies have been developed to target MET. MET kinase inhibitors selectively target MET or multiple RTKs, monoclonal antibodies specifically target MET or HGF, polyspecific antibodies target multiple RTKs, and MET-targeted antibody-drug conjugates (ADCs) combine the specificity of MET monoclonal antibodies with the cytotoxic potency of chemotherapeutic agents [An et al., 2023].

In addition, other protein degradation strategies, such as hydrophobic tag (HyT) technology, are being developed. HyT are molecules containing hydrophobic groups that bind to the protein surface, causing misfolding and degradation. Although

challenges such as delivery optimization and reduction of side effects remain, clinical application of this innovative strategy is ongoing [Mekapogu et al., 2023].

The development and approval of various inhibitors mentioned above demonstrates the importance of molecular-focused approaches in oncology. For example, one of the approved inhibitors, Cabozantinib which is a multi-kinase inhibitor targets MET together with VEGFR2 and is used in the treatment of various metastatic cancers [Fu et al., 2021; Mekapogu et al., 2023]. In preclinical pancreatic and neuroblastoma cancers, tumor severity can be reduced by targeting MET together with VEGF inhibition. Cabozantinib is also used in patients with thyroid and kidney cancer. Recently, structural optimizations have been made to improve the drug-like properties of anti-MET TKIs. In addition, multi-target TKIs (e.g. foretinib, golvatinib, merestinib, glesatinib) that target other receptor tyrosine kinases along with MET have been developed and clinical studies are ongoing. These multi-target inhibitors hold promise for increasing treatment efficacy and overcoming resistance problems seen in single-target drugs [Gallo, Folco, and Crepaldi, 2024; Mekapogu et al., 2023].

1.4 Rationale and Emerging Need for Novel c-MET Inhibitors

1.4.1 Cell Dependent Differentiated Responses Against c-MET Inhibition

PDAC tumors show a high level of intratumoral heterogeneity, that is, the tumor mass exhibits different characteristics at the phenotypic, epigenetic, genetic and functional levels [Evan et al., 2022; Fu et al., 2025]. This difference causes complexity between cell subpopulations in terms of response to treatment, making treatments difficult. These subpopulations are phenotypically divided into two main groups as “classical” and “basal-like/squamous cell-like”. Classical subtypes show low KRAS activity, high GATA6 expression and lipogenetic metabolism and have a good prognosis in the clinic, while subtypes with squamous features show features such as high KRAS activity, mutation in epigenetic regulators and glycolytic metabolism (**Figure 1.4**) [Evan et al., 2022].

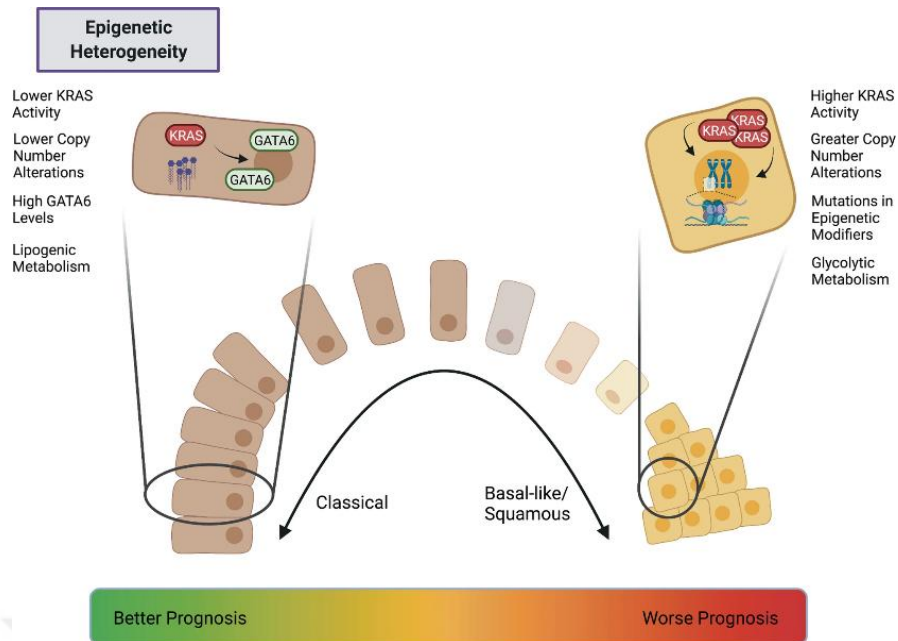


Figure 1.4: Pancreatic tumors may simultaneously contain both classical and squamous cell-like PDAC subtypes defined by different transcriptional and epigenetic programs [Evan et al., 2022].

These may facilitate the development of resistance to targeted therapies such as c-MET inhibitors in squamous-like subtypes, which causes single-agent treatment approaches to be inadequate. Although targeting epigenetic changes in PDAC has not yet been applied in the clinic, several studies suggest that it is a way to bypass the aggressive disease feature [Manoukian et al., 2025]. C-MET inhibitors that have shown preclinical efficacy have failed to demonstrate success in Phase I/II clinical trials due to inappropriate selection of certain patient populations and the emergence of resistance to MET-targeting drugs [Pothula et al., 2020].

Considering the prognosis difference between basal-like and classical structures, pharmacologically pushing PDAC cells towards a more classical subtype seems promising. Increasing GATA6 expression via MET inhibitors can transition basal cells to a classical phenotype. Although targeting the receptor tyrosine kinase MET is not common for PDAC, extensive clinical studies are needed in this area. Understanding the different sensitivities of subtypes and treatment-related phenotypes is critical for developing effective combination therapies [Manoukian et al., 2025].

1.4.2 Economic and Market Perspective of c-MET Inhibitors

Advances in molecular biology and personalized medicine are increasing the demand for targeted therapies such as c-MET inhibitors. The c-MET inhibitor market is also growing with the increase in various cancer types. More than 50 companies are working on c-MET inhibitors with more than 500 clinical trials. Globally, the United States, France, Italy, Germany, Japan, Spain, and the United Kingdom are conducting studies [DelveInsight, 2023]. As of 2024, the MET-targeted therapies market was valued at ~4.35 billion USD and is expected to reach a market volume of ~30 billion USD by 2034 [Business Research Insights, 2024; DelveInsight, 2024]. There are leading companies and start-ups such as Novartis, Merck, AstraZeneca, GSK, Pfizer, Exelixis, Ipsen in the global market, which shows that R&D studies and competition in this area are high [Business Research Insights, 2024]. For example, the c-MET inhibitor market for Non-Small Cell Lung Cancer (NSCLC) is reported to be approximately 590 million USD in 2023 [Pharma Intelligence, 2024]. The marketplace is increasing with the trials of selective and new generation c-MET inhibitors such as capmatinib, tepotinib and savolitinib, telisotuzumab vedoitin [DelveInsight, 2023]. The high research and development cost required for the development of a product in the market is one of the obstacles to the commercialization of the product. The confusion in the HGF/c-MET pathway is among the factors that make targeting difficult. Especially in PDAC, c-MET inhibitors are not widely used now due to the complex stroma and lack of effective biomarkers. However, with the increase in approved drugs in the market over time, the groundwork is being laid for PDAC treatment with new generation agents that apply stroma target and combined therapy approaches [DelveInsight, 2024; Li et al., 2024]. This growth in the market is supported by the trend towards personalized treatment, biomarker-based clinical studies and various pharmaceutical initiatives [DelveInsight, 2023].

1.5 Design and Future Directions of c-MET inhibitors

Chemotherapeutic and cytotoxic drugs are widely used in cancer treatment, but despite their potential to improve patient quality of life, they have several disadvantages that limit their effectiveness. To minimize these side effects, new strategies that specifically target tumor cells are gaining importance. A small number of clinical trials

with MET inhibitors have shown effective results in certain diseases. Therefore, it is anticipated that HGF/c-MET targeted inhibitors will achieve more effective results when combined with other agents **[Hojjat-Farsangi, 2014]**.

Along with targeted therapies and personalized medicine, approaches are emerging in which small molecule inhibitors (SMIs) play an important role. The interest in receptor tyrosine kinase inhibitors (RTK-TKIs) in oncology is increasing over time. Most of the current RTK-TKIs are multitargeted and associated with significant side effects, whereas selective RTK-TKIs have been developed more recently and tend to cause fewer side effects. Therefore, selective TKIs emerge as safer, more manageable cancer treatments **[Mer et al., 2024]**.

The HGF/MET signaling pathway, which plays a critical role in cancer, is considered an important therapeutic target in various malignancies. Intensive efforts have been made to develop selective c-MET inhibitors such as small molecules and monoclonal antibodies, and these agents have shown promising clinical efficacy and manageable toxicity profiles. However, most phase III clinical trials have not achieved the expected success. Approved c-MET inhibitors such as crizotinib and cabozantinib have expanded the treatment options in certain cancer types and generally show mild to moderate and drug-specific side effects. Therefore, ongoing safety monitoring studies and combination strategies with other treatments are of great importance to better understand drug tolerance and reduce toxicity **[Fogli et al., 2022; Mer et al., 2024]**.

Detailed investigation of the activation of the c-MET pathway and the mechanisms of resistance to this pathway will allow for targeted drug development and patient selection to optimize treatment outcomes. Although most clinical studies focus on tumors overexpressing c-MET, reliable biomarkers and diagnostic methods that predict patient response to treatment are still limited. At this point, next-generation sequencing (NGS) technologies and innovative testing methods are widely used to improve patient selection and support targeted therapy development processes **[Jabbarzadeh Kaboli et al., 2024; Mer et al., 2024]**.

To prevent drug-drug interactions (DDIs) and to optimize programs for prolonged in vivo reduction of c-MET phosphorylation, it is critical to comprehend the clinical pharmacokinetics of c-MET inhibitors **[Jabbarzadeh Kaboli et al., 2024]**.

2. PURPOSE OF THE STUDY

The thesis aims to investigate the therapeutic effects of potential c-MET inhibitors derived from triazolopyridazine/pyrimidine structures on pancreatic cancer cell lines. To assess their anti-cancer effects of different chemical molecules, with different chemical structures, including approved c-MET inhibitor cabozantinib, were applied. MTT assay, colony formation assay, survival assay, fluorescence imaging, and Western blot analysis were conducted to evaluate cellular and molecular responses. After MTT and colony formation assay, two different dosages, 10 μ M and 25 μ M, were chosen to see the effect of the inhibitor on the molecular changes, proliferation and viability of pancreatic cancer cells.

3. MATERIALS AND METHODS

3.1. Materials

3.1.1 Cell Lines and Reagent

Cell lines and reagents that were used in the thesis study listed in APPENDIX A.

3.1.2 Laboratory Materials and Equipment

Laboratory equipment and devices that were used in the thesis listed in APPENDIX B.

3.1.3 Chemicals and Antibodies

All the chemicals, antibodies, molecular biology kits and solutions that were used in this thesis were listed in APPENDIX C.

3.1.4 Buffers and Solutions

Buffers and solutions that were used in this study are shown in APPENDIX D.

3.2. Methods

3.2.1. Cell Culture and Treatments

PDAC-derived cell lines MIA PaCa-2(CRL-1420) and PANC-1(CRL-1469) were maintained in DMEM supplemented with 10% FBS, 1% penicillin/streptomycin. The cultures were kept in a humidified environment with 5% CO₂ at 37°C. The cell medium was changed at intervals of 2–3 days by passage of cells depending on their proliferation rate.

Potential c-MET inhibitors were dissolved with DMSO and stored at an initial stock concentration of 50 µM as aliquots at -20 °C.

3.2.2. Cell Freezing

The old medium was first removed from the cells, washed with 1 mL of PBS and enzymatically removed from the surface to which the cells adhered with the help of 500 μ L of trypsin-EDTA. After five minutes, 1 mL of complete medium was added to inhibit the activity of trypsin-EDTA, and the cells were collected in sterile falcon tubes and centrifuged at 5000 rpm for 5 minutes to sediment. The supernatant was discarded, and the pellet was dissolved with freezing medium containing FBS-DMSO prepared in a 9:1 ratio and added to cryotubes. It was stored first at -20 °C and then at -80°C.

3.2.3. Cell Thawing

Cell lines stored at -80°C frozen with freezing medium (90% FBS- 10% DMSO) were quickly thawed and the cells were transferred to centrifuge tubes by adding medium in a ratio of 1:5. The cells were centrifuged at 5000 rpm for 5 minutes. Following centrifugation, the pellet was dissolved in growth media and the supernatant was discarded. 3 ml of full medium was added to T25 flask, and all the cells were seeded. Cultures were maintained at 37°C in a humidified 5% CO₂ atmosphere. The medium was changed until the cells reached a sufficient density.

3.2.4. MTT Cell Viability Assay

The effects of the BH836, BH733, BH851, BH867 and BH892 c-MET inhibitors on pancreatic cancer cell lines' viability were determined by MTT assay. In the MTT assay, a colorimetric analysis, metabolically active cells reduce yellow tetrazolium salt (MTT) to form purple formazan crystals by cleaving the tetrazolium ring of the MTT with the increase of mitochondrial dehydrogenase enzyme activity. The intensity of the color of the resulting solution is directly proportional to the amount of living and metabolically active cells. PANC-1 and MIA PaCa-2 cells were seeded at 5×10^3 cells per well in 96-well plates and treated with five different concentrations of c-Met inhibitors which are BH733 and BH836 (1, 10, 25, 50 and 100 μ M) for 24 h. After the treatment, 10 μ L MTT reagent (5 mg/ml) was added to each well and incubated at 37°C for 4 h. After 4 hours, the culture medium is removed and 100 μ L DMSO was added to each well. The 96-well plate is wrapped with aluminum foil and incubated

for 15 minutes on a shaker at RT and 45 rpm. Lastly, the absorbance was measured with microplate reader at 570 nm wavelength.

$$\text{Percent (\%) viability} = \text{OD of drug-treated sample} / \text{control} \times 100 \quad (3.1)$$

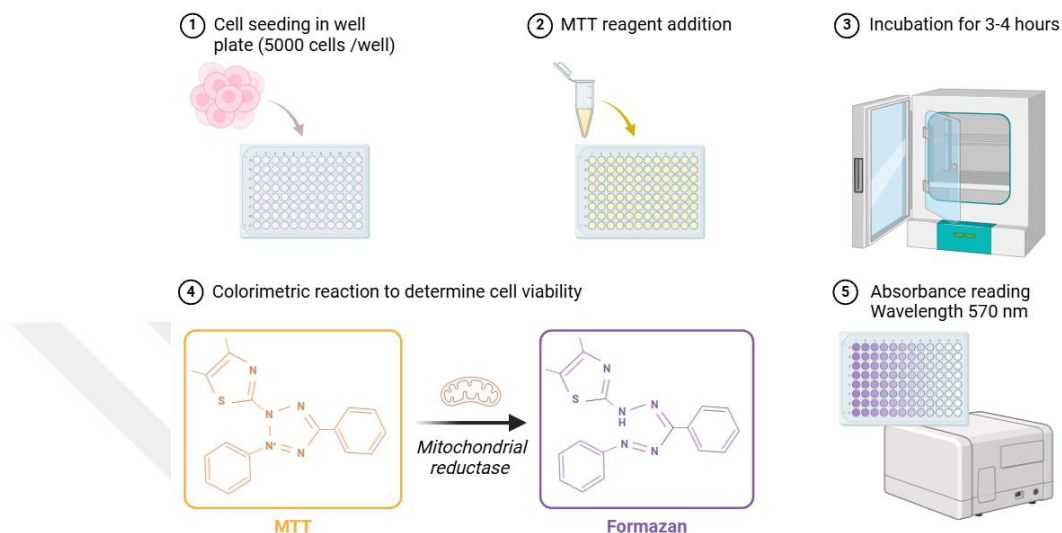


Figure 3.1: Overview of the MTT assay steps used to assess cell viability. MIA PaCa-2 and PANC-1 cells were treated with BH836, BH733, BH851, BH867, BH892, and BH952 for 24 hours prior to the assay.

3.2.5. Colony Formation Assay

The cells were seeded at 2.5×10^3 density per well in 6-well plates and allowed to adhere to for 24h. After attachment, the cells were treated with five different concentrations of c-MET inhibitors which are BH733, BH851, BH836 (1, 10, 25, 50 and 100 μM) and BH867 (1,5,10, 25 and 50 μM) for 14 days and incubated at 37°C in an environment with 5% CO_2 . After 24 h, the culture media was removed, and new media was added. After colonies were formed, culture media was removed and washed with 1000 μL PBS. 1000 μL of the acetic acid/methanol (1:3) solution was added and fixed for 15 minutes. After 15 min fixation solution was removed, and the cells were stained with 1000 μL of 0.5% crystal violet was added for 15 minutes. Later, the dye was removed, and excess dye was washed with 2 mL distilled water. The images were captured, and colonies were counted with Image J.

3.2.6. Survival Assay

Pancreatic cancer cells were seeded at 1×10^4 density per well in 24-well and allowed to adhere for 24 hours. The cells were treated with selected dosages for 24h and 48h. After 24 and 48 hours of treatment, cells in the wells were counted in two replicates, the average cell counts were noted and converted into graphs.

3.2.7. Measurement of Cellular ROS Generation and Mitochondrial Membrane Potential

Pancreatic cancer cells were seeded at 3×10^4 density per well in 24-well plates and allowed to adhere for 24 hours in a 37°C in an environment with 5% CO_2 . Cells were treated with selected two different dosages for 24h. After 24 hours of treatment, the cells were stained with 2',7'-dichlorodihydrofluorescein ($\text{H}_2\text{-DCFHDA}$). 1 μL of DCFDA dye and MitoSpy were dissolved with serum-free DMEM / RPMI and added 150 μL into each well. Absorbance was measured by Varioskan LUX Multimode Microplate Reader.

3.2.8. Fluorescent Imaging

Pancreatic cancer cells were seeded at 3×10^4 density per well in 24-well plates and allowed to adhere for 24 hours at 37°C in an environment with 5% CO_2 . Cells were treated with selected two different dosages for 24 h. After 24 hours of treatment, the cells were stained with a different dye mixture. One of them consists of MitoSpyTM Red CMXRos, DAPI, and DCFH-DA. The other one is a compromise of ,PI, and BODIPY. MitoSpyTM Red CMXRos is a cell-permeable red fluorescent dye that selectively stains mitochondria in living cells, DCFH-DA, a cell-permeable green, fluorescent dye, deacetylated by cellular esterase into DCFH, which is a non-fluorescent compound. In the presence of ROS, DCFH is oxidized to high fluorescent DCF, and DAPI stains DNA both in live and fixed cells by binding to adenine-thymine rich regions in DNA. PI is a red-fluorescent dye that only stains the chromosomes of dead cells because of not permeant to live cells. BODIPY, a green fluorescent dye, stains lipid droplets DAPI and PI dyes are diluted as 1:1000 in serum-free DMEM / RPMI, 1 μL DCFDA is diluted as 0.5:1000 in serum-free DMEM / RPMI and 1 μL BODIPY is diluted as 4.5:1000 in serum-free DMEM / RPMI. 150 μL of dye mix

added into each well. The plate is incubated in a dark area for 15 minutes. The plates visualized by using fluorescent microscope (SOPTOP ICX4I).

3.2.9. Total Protein Isolation

MIA PaCa-2 and PANC-1 pancreatic cancer cells were seeded in 6-well plates and maintained at 37°C in a humidified 5% CO₂ atmosphere until the cells reached a sufficient confluency. Cells were treated with 10 µM and 25 µM dose of BH836, BH733, BH851 and BH867 c-MET molecules for 24 h. After 24 h, media was discarded and well washed with 1X PBS. Then, 200 µL Trypsin was added for 5 minutes. Cells were collected with 1000 µL complete DMEM into the sterile falcon tubes and centrifuged at 5000 rpm for 5 minutes. Following those steps, supernatant was discarded and pellet washed with 300 µL PBS and centrifuged at 15000 rpm for 1 minute. Total protein is extracted from cultured MIA PaCa-2 and PANC-1 cells using RIPA buffer that contain cOmplete™ ULTRA Tablets for protease and phosphatase Inhibition. Centrifuge is performed for 25 minutes at 13200 rpm in a centrifuge machine previously cooled to +4°C. The supernatant is collected for immunoblotting.

3.2.10. Bradford Assay

Protein concentrations were calculated for Western Blot using Bradford assay. 2 µL of isolated protein samples were taken and added to the 96-well plates and 100 µL of Bradford reagent was added into well. The absorbance measured at 570 nm wavelength.

3.2.11. Western Blotting

3.2.11.1. Sample Preparation

The protein samples are mixed with 4X Laemmli Sample Buffer and separated by %12 SDS- PAGE Gel. PageRuler Prestained Protein ladder is used as ladder.

3.2.11.2 Gel Electrophoresis

The protein samples are incubated for 5 minutes on the heat block at 95°C. Run the gel at 80V at first, after samples leave from well setting are changed as 130 V and run for 1.5-2 h.

3.2.11.3. Protein Transfer into PVDF Membrane

Prepare a 1X transfer buffer solution by mixing 20 mL 10x Transfer buffer, 140 mL distilled water and 40 mL methanol. PVDF membrane (GVS, North America Sanford) is activated in methanol for 1 min and then put into distilled water for 5 min. After a quick water rinse, equilibrate the gel in 1X transfer buffer. A sandwich transfer model was made by filter paper with 1X transfer buffer vertically between sponge pads that had been previously wet. The transfer cassette was filled with 1X transfer buffer, and the transfer module was inserted into a mini gel tank. After soaking the transfer module in the 1X transfer buffer, proteins were transferred for 90 minutes at a constant 20V voltage.

3.2.11.4 Membrane Blocking and Antibody Incubation

After the membrane transfer, the membrane is blocked with 5% Skim milk in TBS-T on shaker for 1h at RT. Then, the membrane is placed onto the prepared antibody (1:1000) and incubate on roller overnight at +4°C. After primary antibody incubation, the membrane was washed with 1X TBS-T 5-15-5-5 minutes at RT on shaker. The membrane was incubated with HRP-linked secondary antibody (1:1000 in 5% skim milk/1X TBS-T) compatible with primary antibody for 2-3 h at RT. After 2-3h, the membrane was washed the same as after the primary antibody. Used monoclonal antibodies: EGF Receptor, AKT, PARP and PTEN antibodies.

3.2.11.5 Protein Detection and Visualization

The membrane was incubated in the prepared Enhanced chemiluminescence (ECL) solution to detect and quantify the proteins on membrane for 1 min in dark. ChemiDoc XRS+ Imaging System was used to visualize.

3.2.12. Statistical Analysis

Microsoft® Excel® was used to calculate the cell viability. A two-way analysis of variance (ANOVA) was performed on all collected data with a 95% confidence level. ImageJ and GraphPad software were used for all analysis.



4. RESULTS

4.1. Assessment of Cytotoxicity and Cell Viability in Pancreatic Cancer Cell Lines Treated with potential c-MET Inhibitors and clinically approved c-MET inhibitor Cabozantinib

The effects of the potential c-MET inhibitors cell viability of MIA PaCa-2 (KRAS^{G12C}) and PANC-1 (KRAS^{G12D}) pancreatic cancer cell lines were determined by MTT assay. Cytotoxic responses were first evaluated against five different concentrations (1, 10, 25, 50 and 100 μ M) for 24 hours as shown in **Figure 4.1**. for molecules BH836, BH733, and BH851 c-MET inhibitors. Other doses were determined for molecules BH867, BH892, and BH952 c-MET inhibitors as shown in **Figure 4.1**. BH836 molecule decreased cell viability to approximately 50% after 10 μ M in both cell lines. BH733 molecule viability started to show a significant decrease after 10 μ M for MIA PaCa-2 (* p =0.0186), while a significant decrease was observed after 25 μ M for PANC-1. BH851 decreased viability to approximately 50% at 25 μ M in both MIA PaCa-2 and PANC-2 cell lines. It was concluded that all three molecules decreased viability by less than 10% at 100 μ M. Therefore, the 100 μ M dose was removed due to its toxicity and an intermediate dose of 5 μ M was added (**Figure 4.1**). The BH867 molecule did not reduce the average viability to below 50% at any of the applied doses. The first significant decrease in viability was observed at 25 μ M for MIA PaCa-2 (** p =0.0041) and at 50 μ M for PANC-1 cell line (** p =0.0042). When the BH892 molecule was examined, the viability for PANC-1 cell line decreased to ~70% at 25 μ M and to ~50% at 50 μ M, while it remained above 50% for MIA PaCa-2 cell line even at 25 μ M. When the BH952 molecule was examined, the viability for both cell lines showed a significant decrease after 10 μ M (*** p <0.0001). (**Figure 4.1**).

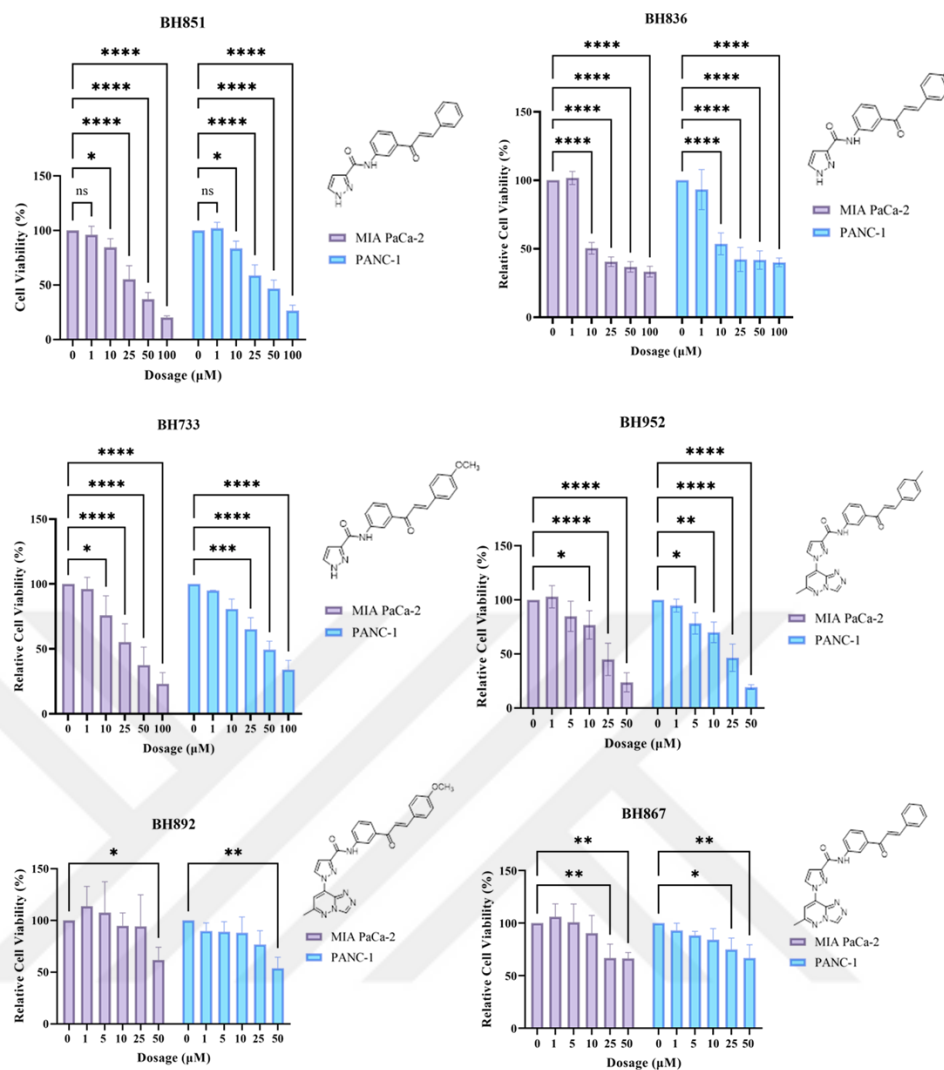


Figure 4.1: MTT cell viability assay of MIA PaCa-2 and PANC-1 pancreas cancer cells. The cells (5000 cells / well) were exposed to 5 different concentrations (1, 10, 25, 50 and 100 μM) of BH836, BH733, and BH851 and 5 different concentrations (1, 5, 10, 25 and 50 μM) of BH867, BH892, and BH952 c-MET inhibitors for 24 h. The mean ± standard deviation of three independent experiment with four repetitions is shown in the columns. Dunnett's multiple comparisons test was used after two-way ANOVA to determine P-values. **** $p < 0.0001$.

In summary, when these six molecules were evaluated, the sharpest decrease was observed in BH836 and BH851 molecules. For both molecules, the first significant decrease in cell viability started at 10 μM, and at 25 μM, viability decreased to ~60%, and at 50 μM, viability decreased to 25-30%. BH733 and BH952 molecules showed a significant decrease at 25 μM and at 50 μM, viability decreased to 25-30%. It can be said that these two molecules have moderate activity. Although the BH892 molecule caused more loss of viability compared to MIA PaCa-2 for PANC-1, it can be said that

its activity was limited because of the need for a dose application of more than 25 μM for both. One of the weakest candidates is the BH867 molecule, as it requires a dose of more than 50 μM to show a significant effect. In the light of these data, it was observed that the BH851 and BH836 molecules have the highest lethality, followed by the BH733 and BH952 molecules, which also show potential, but the BH892 and BH867 molecules are not very effective.

The effects of the potential c-MET inhibitors cell viability of pancreatic cancer cell lines were determined by MTT assay. Cytotoxic responses were evaluated against five different concentrations (1, 5, 7.5, 10 and 25 μM) of approved c-MET inhibitor Cabozantinib for 24 hours which was shown on **Figure 4.2**.

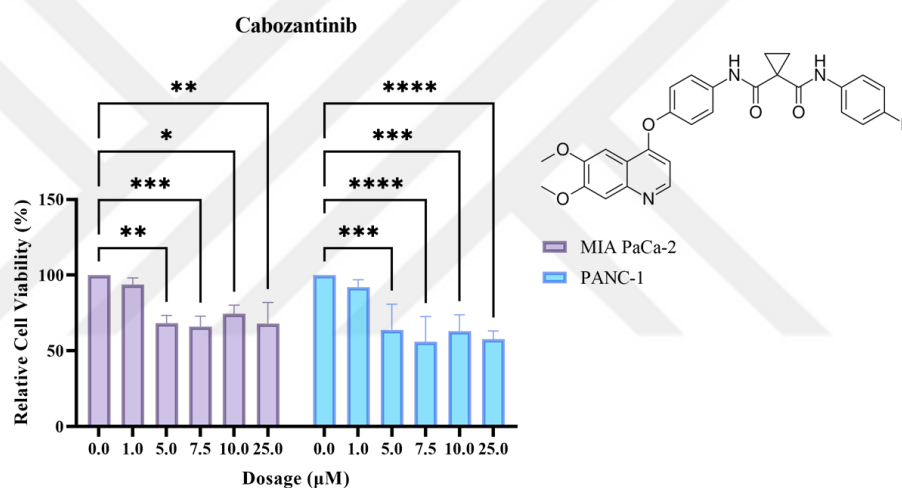


Figure 4.2 MIA PaCa-2 and PANC-1 pancreatic cancer cells (5000 cells/well) were subjected to five different concentrations (1, 5, 7.5, 10, and 25 μM) of Cabozantinib c-MET inhibitors for a duration of 24 hours using the MTT cell viability assay. The mean $\pm$ standard deviation of three separate trials with four repetitions is shown in the columns. Dunnett's multiple comparisons test was used after two-way ANOVA to determine P-values. **** $p < 0.0001$.

It was concluded that the dose-response curves were parallel in both cell lines and the mean viability values were $78.40 \pm 3.13\%$ in MIA PaCa-2 and $72.11 \pm 3.13\%$ in PANC-1, with a difference of +6.30%. While the viability in the range of 1-7.5 μM showed a significant decrease at the level of $p < 0.01$ (**), it showed stronger effects at the level of $p < 0.001$ – 0.0001 (***-****) (**Figure 4.2**).

4.2. Colony formation potential of MIA PaCa-1 and PANC-1 cells treated potential c-MET inhibitors

The effects of potential c-MET inhibitors BH836, BH733, BH851, BH867, BH952 and approved c-MET inhibitor Cabozantinib on the long-term proliferation and survival capacity of MIA PaCa-2 and PANC-1 pancreatic cancer cell lines were evaluated by colony formation assay. Cells were treated with 1, 10, 25, 50 and 100 μ M doses of BH836, BH733, BH851 inhibitors, 1, 5, 10, 25, 50 doses of BH952 and BH867 molecules and 1, 5, 7.5, 10 and 25 doses of Cabozantinib for 24 hours (**Figure 4.3 and Figure 4.4**). BH836 molecule completely abolished colony formation after 10 μ M in both cell lines. The BH733 molecule reached complete inhibition at 25 μ M for PANC-1 cell line, while it reached complete inhibition after 10 μ M for MIA PaCa-2 cell line. For BH851 molecule, very few colonies were observed at 10 μ M for MIA PaCa-2 cell line, while complete inhibition was observed at 25 μ M. In PANC-1 cell line, more colonies were observed at 10 μ M compared to MIA PaCa-2, but zero colony formation was observed in this cell line after 25 μ M (**Figure 4.3**).

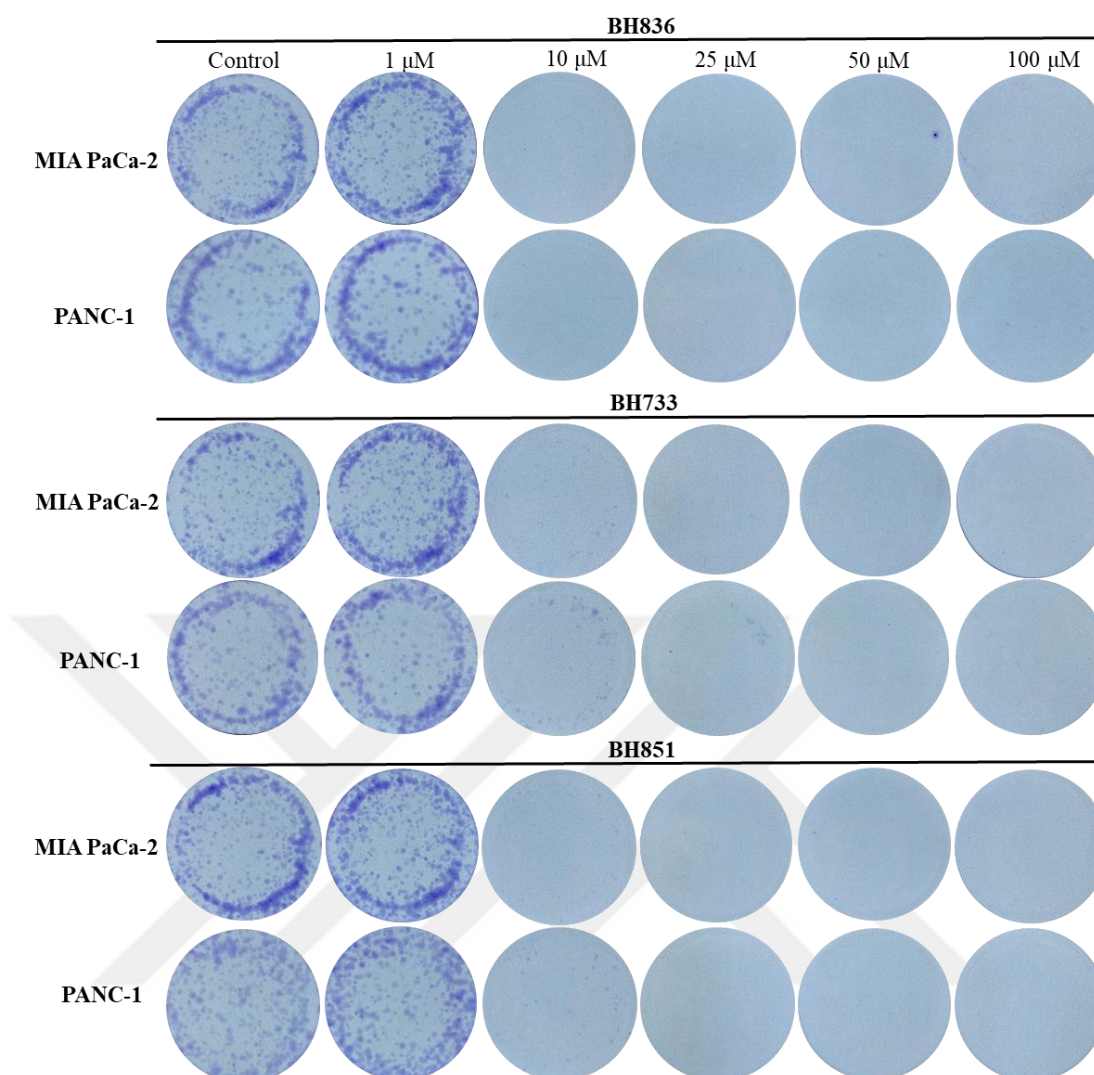


Figure 4.3: Colony formation assay of MIA PaCa-2 and PANC-1 cells treated with potential c-MET inhibitors. PANC-1 and MIA PaCa-2 pancreas cancer cells (2500 cells / well) were treated with 5 different concentrations (1, 10, 25, 50 and 100 μ M) of BH836, BH733 and BH851 c-MET inhibitors for 24 h. After treatment, the media was replaced, and the colonies were allowed to form for 10-14 days. Colonies were fixed and stained with Crystal Violet.

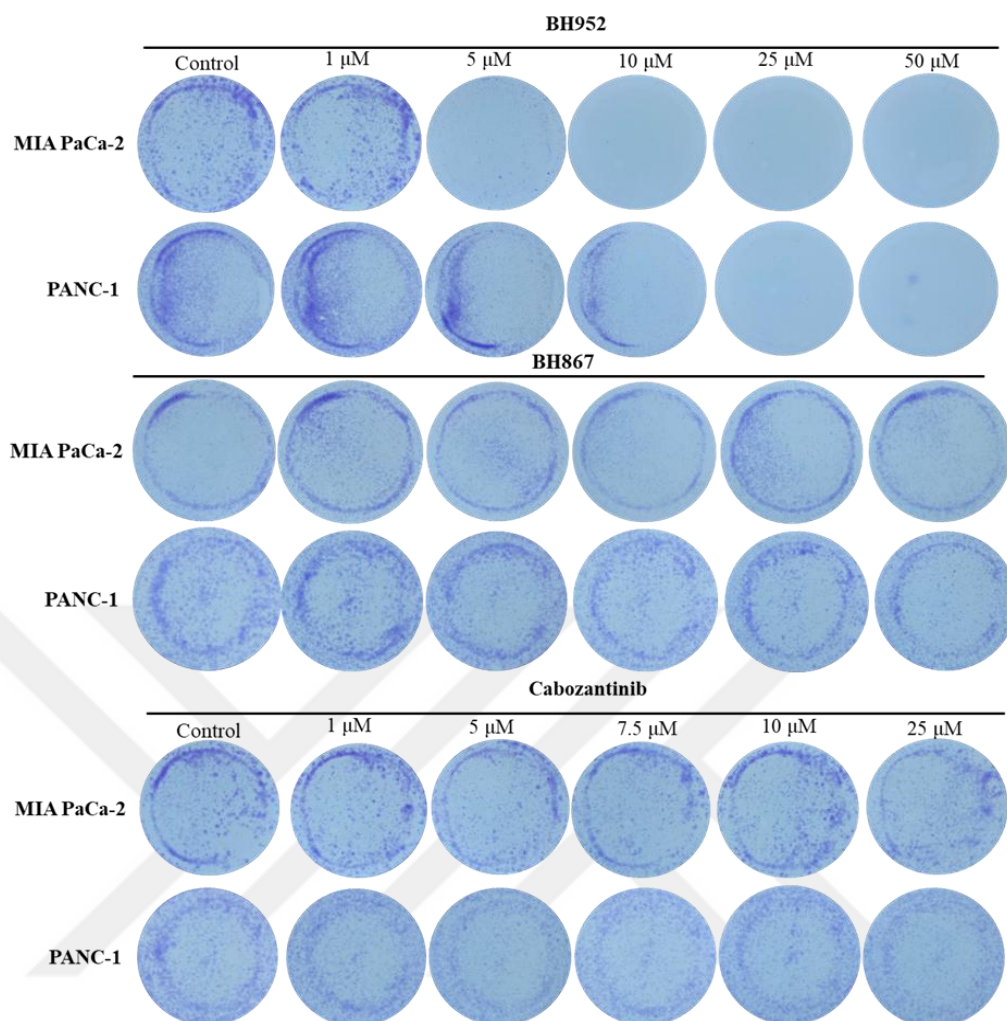


Figure 4.4.: Colony formation assay of MIA PaCa-2 and PANC-1 cells treated with potential c-MET inhibitors. PANC-1 and MIA PaCa-2 pancreas cancer cells (2500 cells / well) were treated with 5 different concentrations of BH952/BH867 (1, 10, 25, 50 and 100 μ M) and Cabozantinib (1, 5, 7.5, 10 and 25 μ M) for 24 h. After treatment, the media was replaced, and the colonies were allowed to form for 8-10 days. Colonies were fixed and stained with Crystal Violet.

For BH952 molecule, very few colonies were observed at 5 μ M for MIA PaCa-2 cell line, while no colony formation was observed after 10 μ M. In PANC-1 cell line, no colony formation was observed after 10 μ M compared to MIA PaCa-2. In the colony results obtained from Cabozantinib, an approved c-MET inhibitor, colony formation was observed for each dose. The BH867 molecule, which exhibits a similar profile to the reference drug cabozantinib, failed to show a statistically significant suppressive effect on the long-term proliferation and colony-forming capacity of both cell lines (Figure 4.4).

Colonies in each treatment group consist of two replicates. The graphs in **Figure 4.5** were created by counting the colonies in two replicates using ImageJ Software (**Figure 4.5**). Repetitions of colony formation assay for each molecule are included in Appendix E.

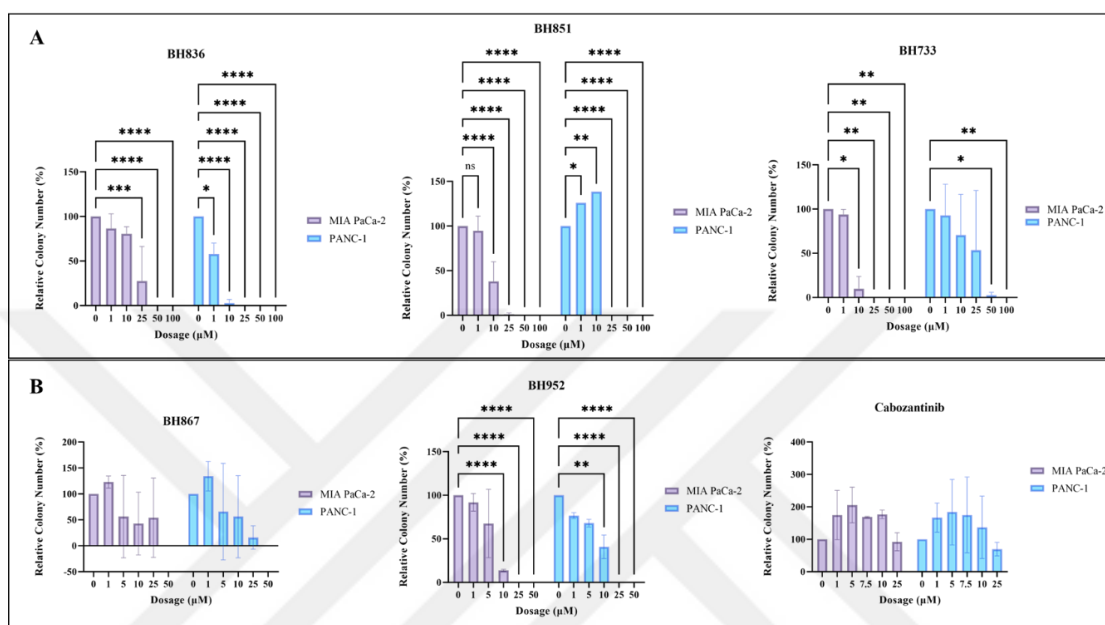


Figure 4.5: Quantification of colony formation in MIA PaCa-2 and PANC-1 cell lines after treatment with c-MET inhibitors. Cells were treated with BH836, BH733, BH851, BH952 and Cabozantinib c-MET inhibitors were exposed to 5 different concentrations (1, 10, 25, 50 and 100 μM) of BH836, BH733, and BH851 c-MET inhibitors for 24 h. (B) The cells were exposed to 5 different concentrations (1, 5, 10, 25 and 50 μM) of BH867, BH892, and BH952 c-MET inhibitors for 24 h, followed by an 8–10-day colony growth period in drug-free medium. Colonies were stained with Crystal Violet and counted using ImageJ software. The mean $\pm$ standard deviation (SD) of two independent experiments is shown in the columns. Dunnett's multiple comparisons test was applied after two-way ANOVA to determine P-values. **** $p < 0.0001$.

4.3. Assessment of Cell Survival in Pancreatic Cancer Cells Treated with c-MET Inhibitors

Survival assay results showed significant changes with the BH851 molecule applied to the MIA PaCa-2 cell line, while no significant changes were observed at the doses applied for PANC-1. However, it still killed cells compared to the control group. With the BH836 molecule, a decrease in cell count was observed on MIA PaCa-2 at 48 hours compared to the control group (**Figure 4.6**). While the BH733 molecule did not reduce cell count in the MIA PaCa-2 cell line, it reduced cell count in the PANC-1 cell line at 25 μ M dose at both 24 and 48 hours. The BH952 molecule reduced cell count in the PANC-1 cell line in a time- and dose-dependent manner (**Figure 4.7**). Cabozantinib was not effective on MIA PaCa-2 cell count, but reduced cell number in the PANC-1 cell line in a dose- and time-dependent manner (**Figure 4.8**).

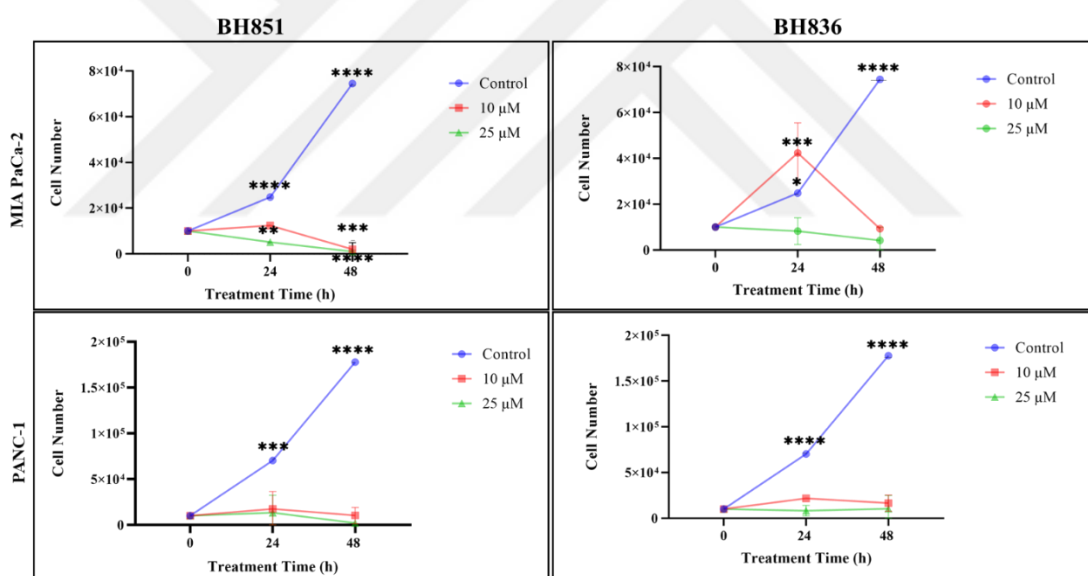


Figure 4.6: Line graph representing the survival rates of MIA PaCa-2 and PANC-1 pancreatic cancer cells (10,000 cells/well) treated with BH851 and BH836 novel c-MET inhibitors at 10 μ M and 25 μ M for 24 and 48 hours. Cell survival was assessed using the survival assay. The mean $\pm$ standard deviation of three separate experiments with four technical replicates is used to express the data. Dunnett's multiple comparisons test and two-way ANOVA were used for statistical analysis.. *** $p < 0.0001$.

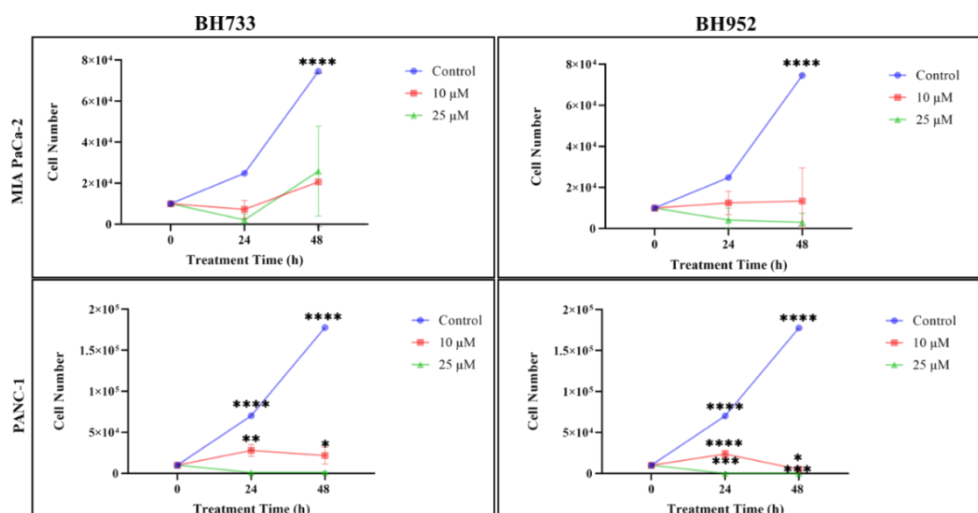


Figure 4.7: Line graph representing the survival rates of MIA PaCa-2 and PANC-1 pancreatic cancer cells (10,000 cells/well) treated with BH733 and BH952 novel c-MET inhibitors at 10 μ M and 25 μ M for 24 and 48 hours. Cell survival was assessed using the survival assay. The mean $\pm$ standard deviation of three separate experiments with four technical replicates is used to express the data. Dunnett's multiple comparisons test and two-way ANOVA were used for statistical analysis. *** $p < 0.0001$.

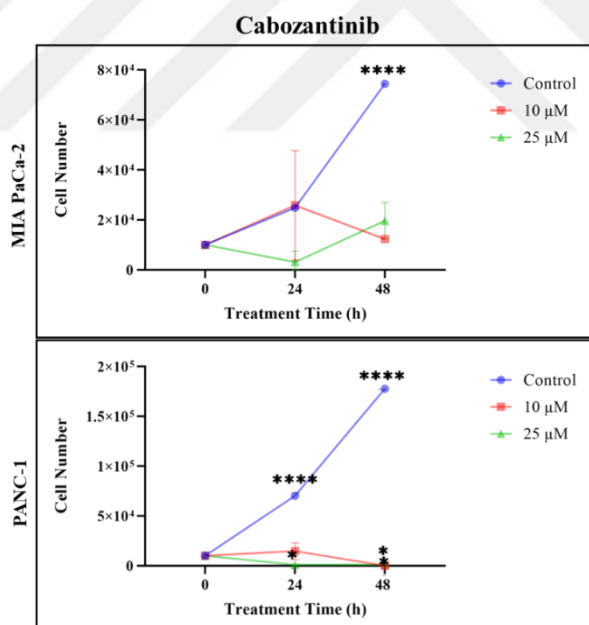


Figure 4.8: Line graph representing the survival rates of MIA PaCa-2 and PANC-1 pancreatic cancer cells (10,000 cells/well) treated with Cabozantinib at 10 μ M and 25 μ M for 24 and 48 hours. Cell survival was assessed using the survival assay. The mean $\pm$ standard deviation of three separate experiments with four technical replicates is used to express the data. Dunnett's multiple comparisons test and two-way ANOVA were used for statistical analysis. *** $p < 0.0001$.

4.4. Assessment of Mitochondrial Activity, Nuclear Morphology, and Reactive Oxygen Species Induction in Pancreatic Cancer Cells Treated with c-MET Inhibitors

The responses of MIA PaCa-2 and PANC-1 pancreatic cancer cells to 10 μ M and 25 μ M concentrations of BH836, BH733, BH851, BH867, BH952 and the approved inhibitor Cabozantinib were evaluated by fluorescent staining. BODIPY was used to stain lipids, membranes and other lipophilic compounds, H₂DCFDA was used as an indicator for reactive oxygen species (ROS) in cells, MitoSpy was used to show changes in mitochondrial membrane potential, Propidium Iodide (PI) was used to detect dead cells and DAPI was used to reference nuclear morphology.

The effect of BH836 molecule on cells has been examined, a decrease in cell number and a change in cell morphology are observed for both cell lines MIA PaCa-2 and PANC-1 at 10 and 25 μ M doses compared to the control group. When the obtained MitoSpy images are examined, a slight decrease is seen at 10 μ M compared to the control group, but the decrease in signal is greater at 25 μ M. According to the DCFDA result, it can be said that ROS formation increases significantly at 10 μ M for MIA PaCa-2. At 25 μ M, it is observed that the number of cells decreases and the green signal increases in a few of the remaining cells. According to the DAPI result, bright spots are observed in the MIA PaCa-2 cell line at 10 μ M and 25 μ M, and it can be said that these are the result of nuclear fragmentation. For PANC-1, the ROS signal is seen to be less than MIA PaCa-2. Similarly, DAPI signal decreases with increasing dose. For PANC-1, at 10 μ M, the mitochondrial membrane potential and cell morphology began to change in the MitoSpy image. At 25 μ M, the signal and cell number decreased significantly (**Figure 4.9**).

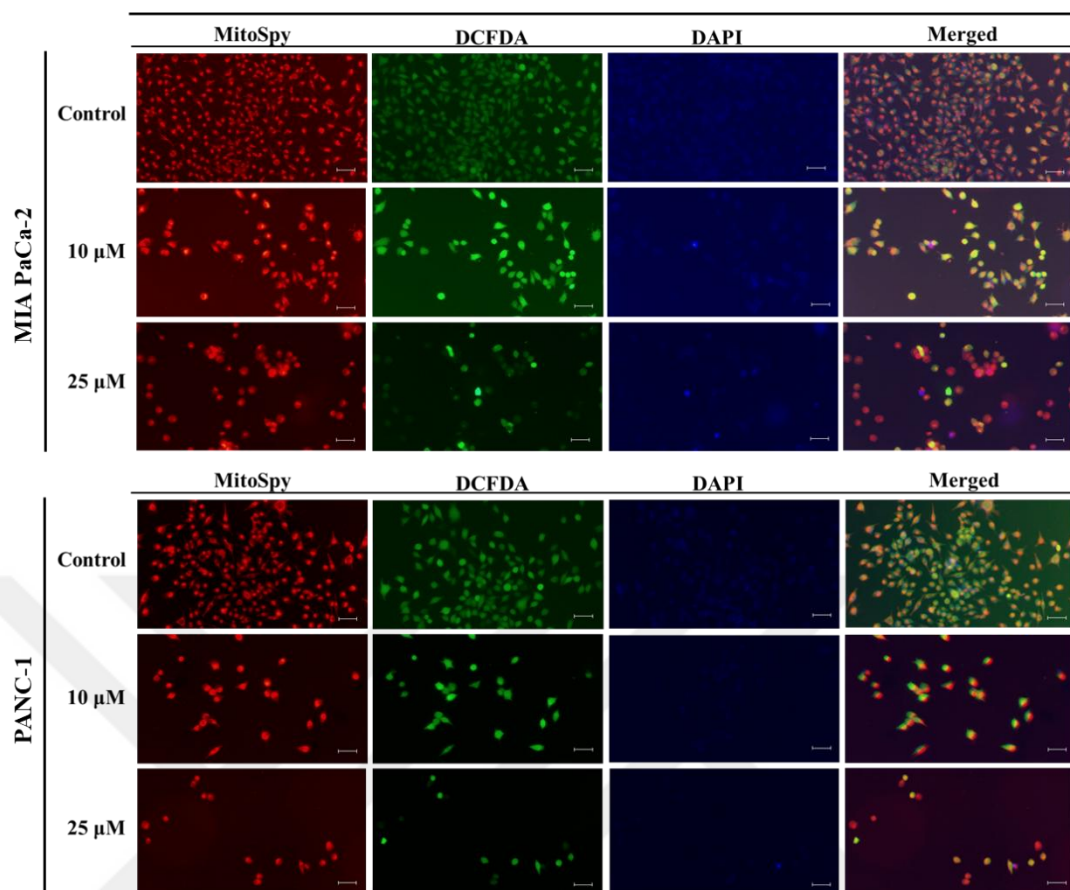


Figure 4.9: Fluorescent staining of wt MIA PaCa-2 and PANC-1 cell lines with MitoSpy, H₂DCFDA and DAPI after treatment with BH836 c-MET inhibitor. Cell viability was observed by using MitoSpy, cell nucleus examined with DAPI while ROS formations examined with H₂DCFDA. The scale bar is 100 μ m for MitoSpy, H₂DCFDA and DAPI.

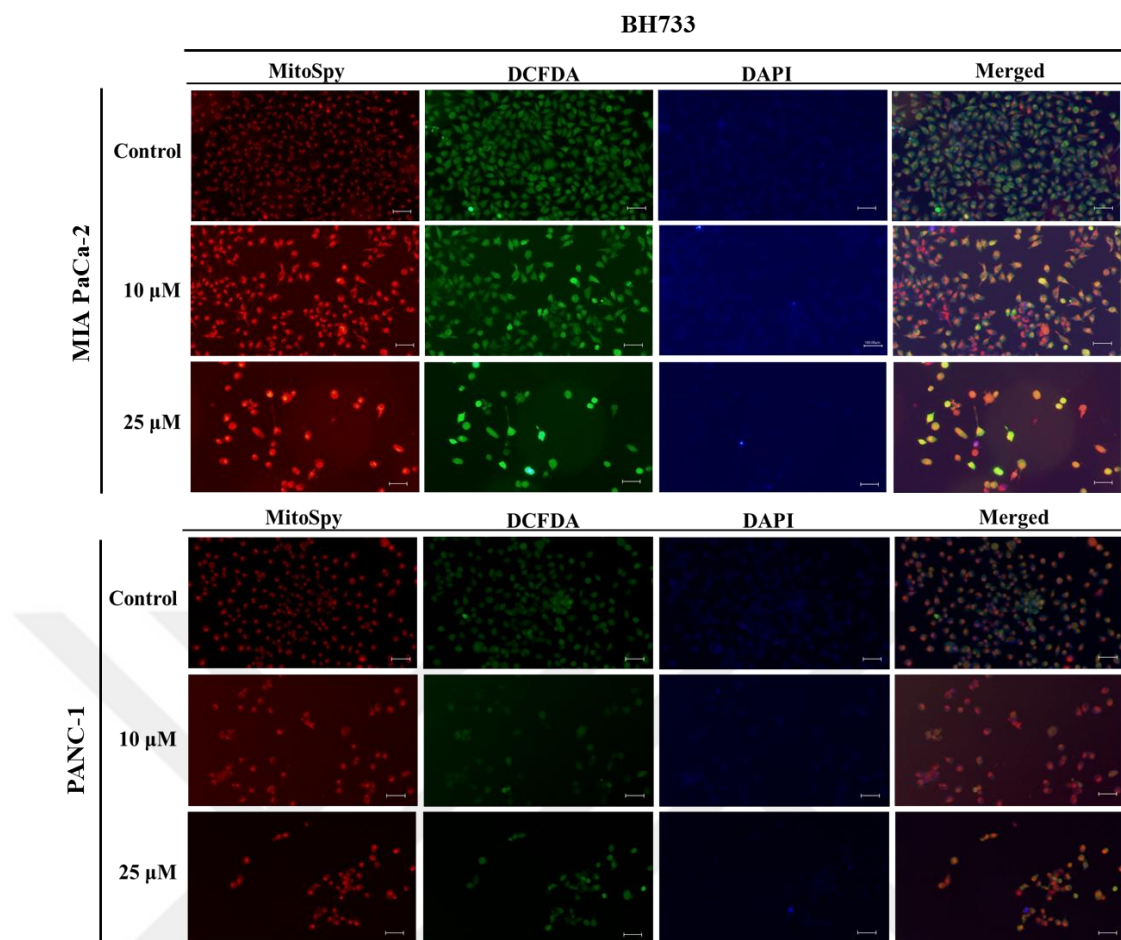


Figure 4.10: Fluorescent staining of wt MIA PaCa-2 and PANC-1 cell lines with MitoSpy, H₂DCFDA and DAPI after treatment with BH733 c-MET inhibitor. Cell viability was observed by using MitoSpy, cell nucleus examined with DAPI while ROS formations examined with H₂DCFDA. The scale bar is 100 μ m for MitoSpy, H₂DCFDA and DAPI.

BH733 molecule decreased the cell number with increasing dose compared to the control group in both MIA PaCa-2 and PANC-1 cell lines. For MIA PaCa-2 cell line mitochondrial activity rate increased with increasing dose. ROS signal also increased in cells with very low cell numbers. Bright and condensed nuclei in DAPI are seen to be more intense in MIA PaCa-2 compared to PANC-1. For PANC-1 cell line, ROS and MitoSpy signal decreased compared to the control group at 10 μ M and 25 μ M doses (Figure 4.10).

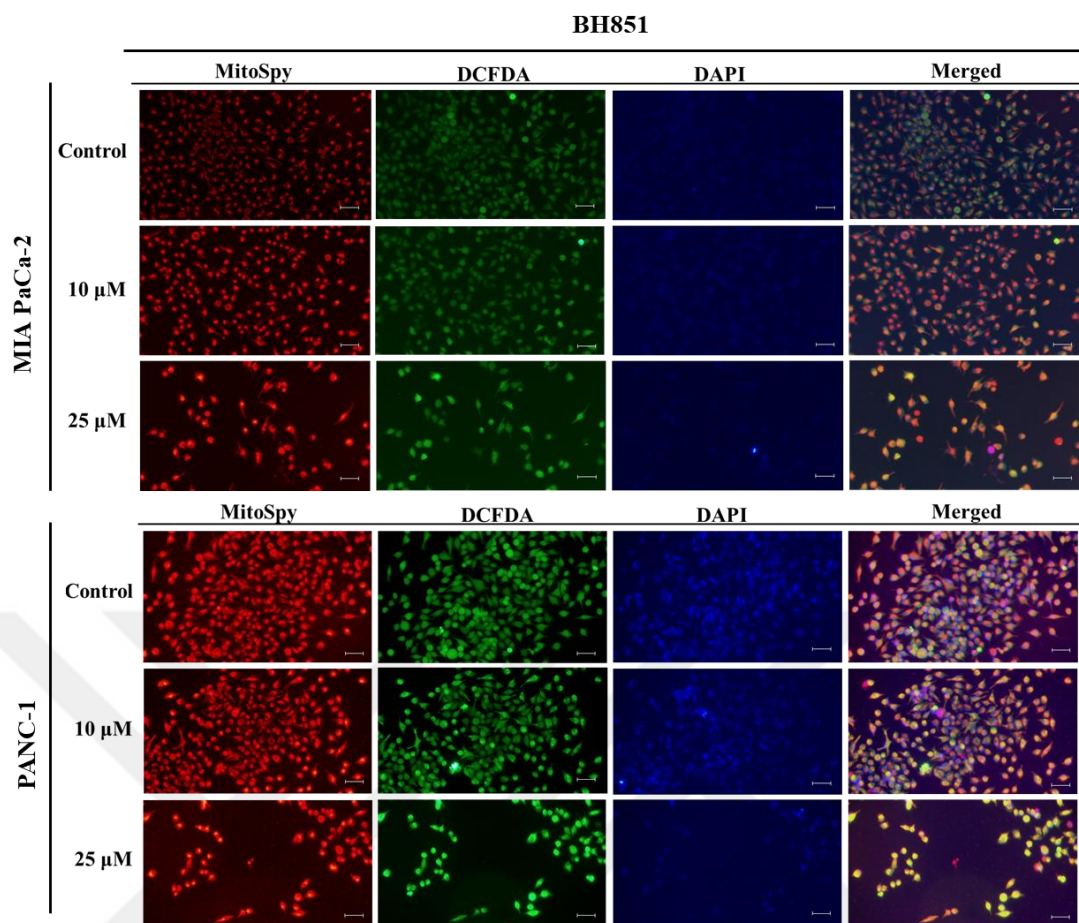


Figure 4.11: Fluorescent staining of wt MIA PaCa-2 and PANC-1 cell lines with MitoSpy, H₂DCFDA and DAPI after treatment with BH851 c-MET inhibitor. Cell viability was observed by using MitoSpy, cell nucleus examined with DAPI while ROS formations examined with H₂DCFDA. The scale bar is 100 μ m for MitoSpy, H₂DCFDA and DAPI.

BH851 molecule decreased the number of cells with increasing doses compared to the control group in both cell lines. An increase in MitoSpy signal was observed for the MIA PaCa-2 cell line compared to the control group. The ROS signal increased in a few cells. The DAPI signal decreased with increasing doses, and a single glow signal was observed at 25 μ M. No obvious decrease was observed in the MitoSpy signal for PANC-1. Although the number of cells decreased at 25 μ M, the signal intensity increased. It was observed that the ROS signal increased with increasing doses, the cells became rounded at 10 μ M and the signal increased in a few cells and the ROS signal increased more at 25 μ M compared to the control group. It was observed that the DAPI signal increased with increasing doses compared to the control group (Figure 4.11).

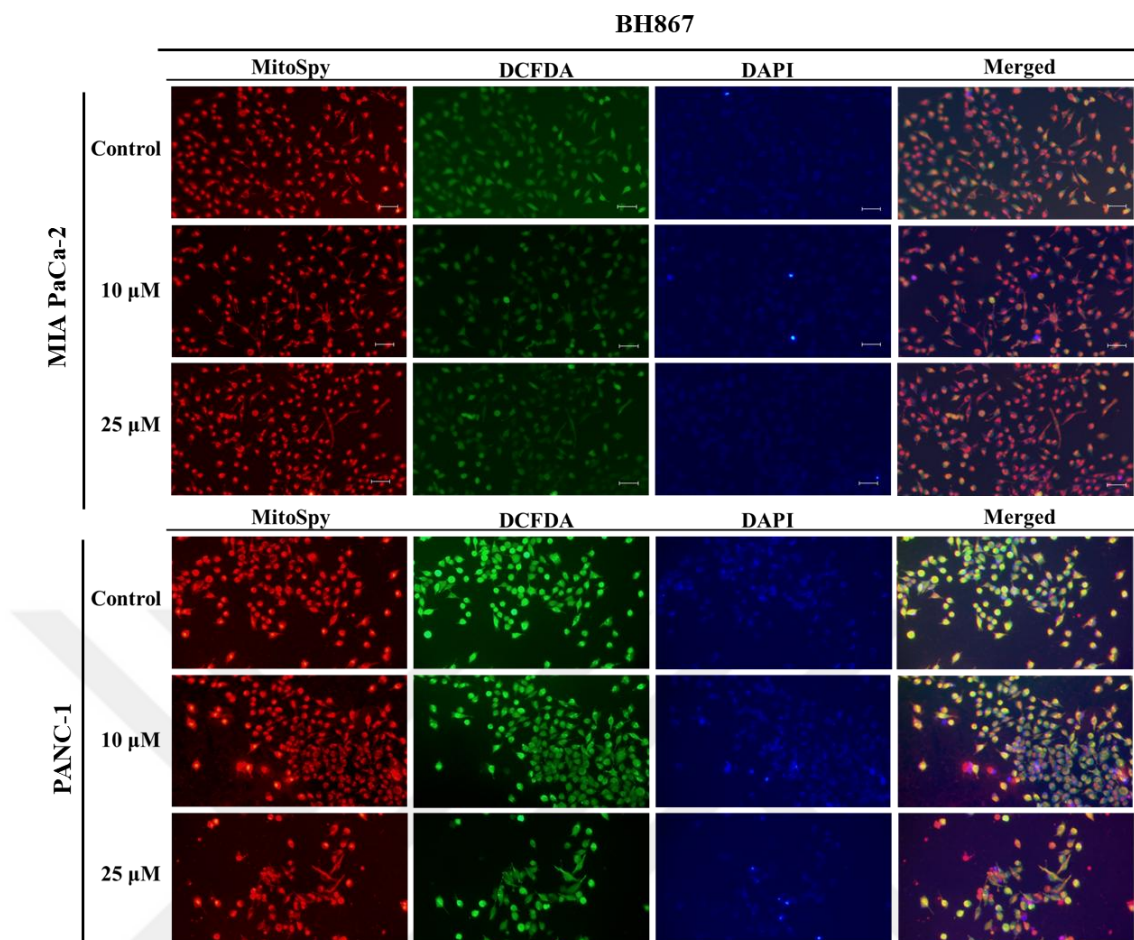


Figure 4.12: Fluorescent staining of wt MIA PaCa-2 and PANC-1 cell lines with MitoSpy, H₂DCFDA and DAPI after treatment with BH867 c-MET inhibitor. Cell viability was observed by using MitoSpy, cell nucleus examined with DAPI while ROS formations examined with H₂DCFDA. The scale bar is 100 μ m for MitoSpy, H₂DCFDA and DAPI.

It is observed that BH867 molecules do not cause a decrease in cell number in MIA PaCa-2 cell line, but decreases with increasing dose compared to control group in PANC-1 cell line. ROS signal did not create significant brightness for MIA PaCa-2, for PANC-1 it was observed that cells in control group showed bright signal, decreased at 10 μ M and decreased with cell number at 25 μ M. DAPI signal showed similar signal to control group in dosed cells in both cell lines. Nuclear fragmentation traces are observed for MIA PaCa-2 at 10 μ M. DAPI signal is observed more in PANC-1 compared to MIA PaCa-2. Nuclear fragmentation is observed at 10 and 25 μ M (**Figure 4.12**).

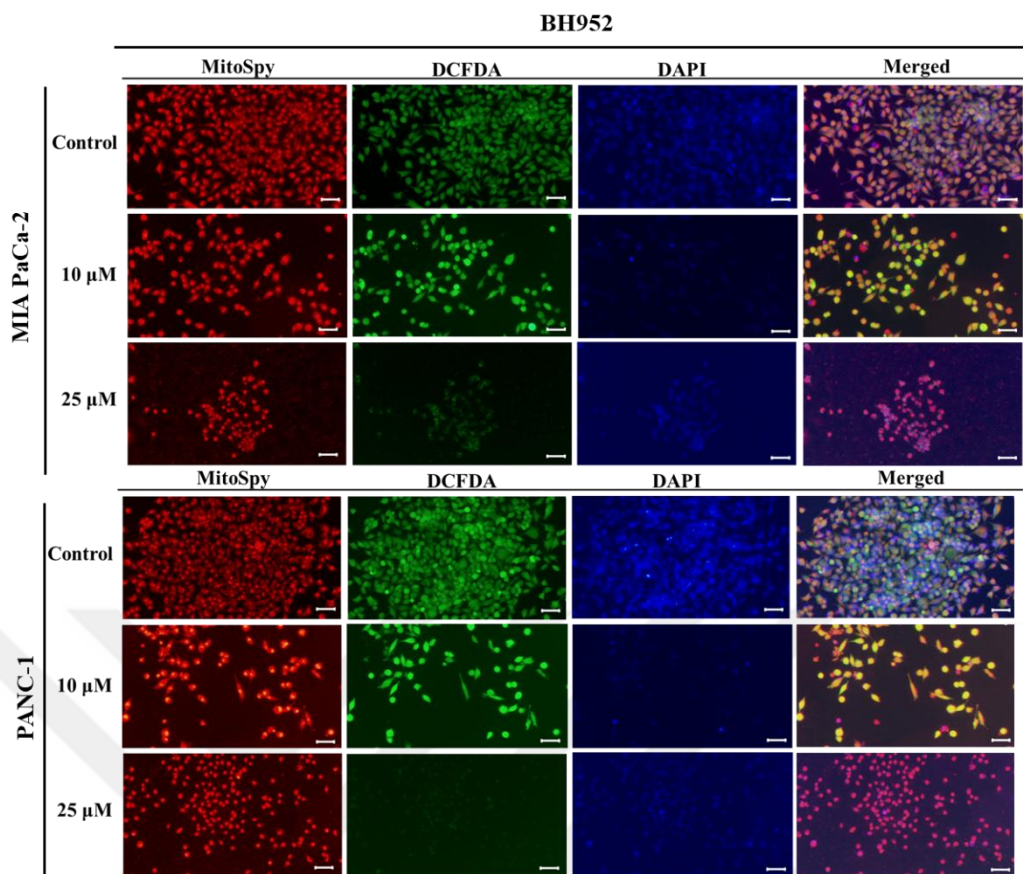


Figure 4.13: Fluorescent staining of wt MIA PaCa-2 and PANC-1 cell lines with MitoSpy, H₂DCFDA and DAPI after treatment with BH952 c-MET inhibitor. Cell viability was observed by using MitoSpy, cell nucleus examined with DAPI while ROS formations examined with H₂DCFDA. The scale bar is 100 μ m for MitoSpy, H₂DCFDA and DAPI.

The effect of BH952 molecule is seen to be extremely strong in both cell lines. In the control group of MIA PaCa-2, mitochondrial activity is intense and the cells appear healthy. At 10 μ M, a decrease in cell numbers is observed compared to the control group and a significant increase in ROS is observed. At 25 μ M, the morphology of the cells has changed, and they have died, but the remaining cells still show mitochondrial activity. The ROS signal has decreased. While the DAPI signal was high in the control group, it decreased at 10 μ M and a glow was observed in a few cells. At 25 μ M, most of the cells have died. Similarly, for PANC-1, it is seen that mitochondrial activity continues at 10 μ M but a decrease in cell numbers is observed compared to the control. The ROS signal also increased at 10 μ M and disappeared at 25 μ M. Although the DAPI signal is quite intense in the control group, nuclear fragmentation is observed in a few cells. At 10 μ M and 25 μ M, the DAPI signal is significantly reduced (**Figure 4.13**).

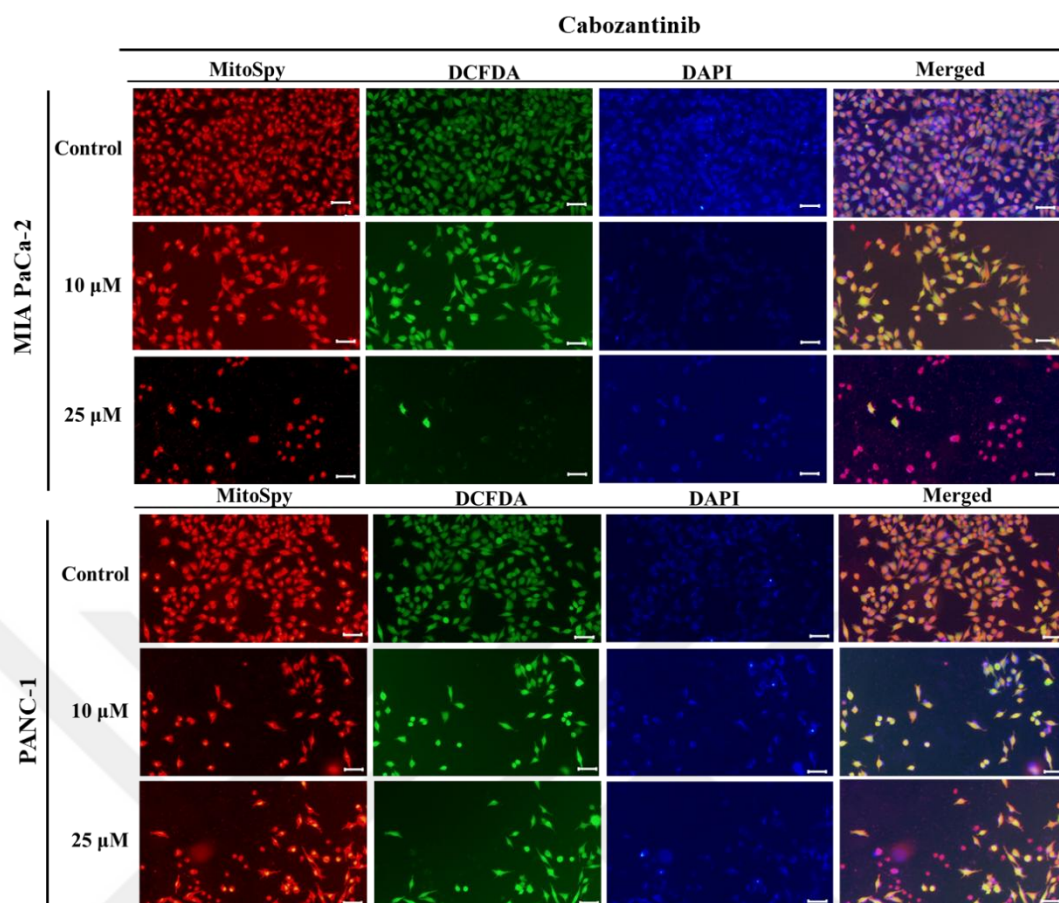


Figure 4.14: Fluorescent staining of wt MIA PaCa-2 and PANC-1 cell lines with MitoSpy, H₂DCFDA and DAPI after treatment with Cabozantinib approved c-MET inhibitor. Cell viability was observed by using MitoSpy, cell nucleus examined with DAPI while ROS formations examined with H₂DCFDA. The scale bar is 100 μ m for MitoSpy, H₂DCFDA and DAPI.

Cabozantinib showed significant dose-dependent effects. In the control groups, homogeneous MitoSpy signal, low basal H₂DCFDA intensity and smooth DAPI stained nuclei were observed. At 10 μ M, there was a decrease in mitochondrial membrane potential and an increase in cytoplasmic ROS, especially in MIA PaCa-2 cells. Although MitoSpy fluorescence was still seen at a dose of 25 μ M, most cells died. DAPI also decreased with increasing doses. PANC-1 cells showed a decrease in mitochondrial signal at 10 μ M, while an increase in signal was observed at 25 μ M. ROS signal also increased with increasing doses. It was observed that the DAPI signal decreased at 10 μ M compared to the control group due to the cell starting to die (Figure 4.14).

4.5. Evaluation of Lipid Droplet Accumulation and Cell Death in Pancreatic Cancer Cells Treated with c-MET Inhibitors

The role of potential c-MET inhibitors on lipid metabolism and cell viability was investigated. For this purpose, MIA PaCa-2 and PANC-1 cell lines were treated with 10 μ M and 25 μ M doses of BH836, BH733, BH851, BH867, BH952 and Cabozantinib and then lipid droplets were labeled with a neutral dye, BODIPY 493/503 (green fluorescence) and dead cells were labeled with Propidium Iodide (PI) (red fluorescence), which stains the nuclei of cells with impaired cell membrane permeability, and examined under a fluorescence microscope.

While no lipid droplets were observed in MIA PaCa-2 control cells treated with BH836, a minimal accumulation was triggered at 10 μ M and a significant lipogenesis (lipid formation) was triggered at 25 μ M. Similarly, lipid accumulation was not detected in the control group in PANC-1 cells, and the accumulation that started in rare cells at 10 μ M became more pronounced at 25 μ M (**Figure 4.15**).

BH733 exhibited a different effect profile in MIA PaCa-2 cells. While basal levels and a small number of lipid droplets were observed in the control group, it was determined that lipid accumulation was inhibited, and droplets were not observed at 10 μ M and 25 μ M doses. In PANC-1 cells, the control group had a significant basal lipid accumulation, but no significant increase was detected at 10 μ M. At the 25 μ M dose, there was a serious decrease in the number of cells due to the cytotoxic effect of the drug, and this prevented the observation of lipid droplets (**Figure 4.16**).

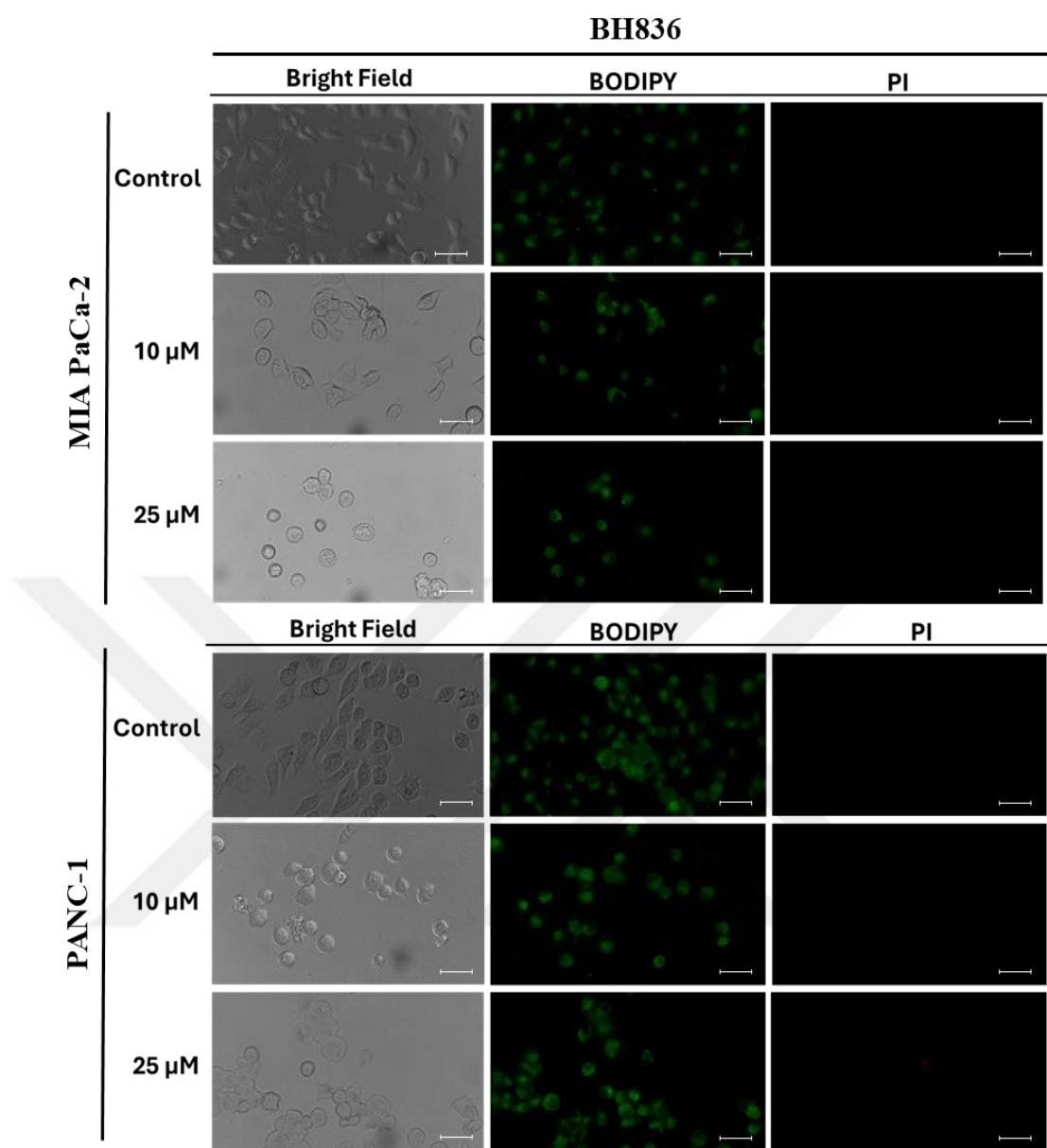


Figure 4.15: Fluorescent staining of wt MIA PaCa-2 and PANC-1 cell lines with BODIPY and PI after treatment with BH836 c-MET inhibitor. Lipid droplet was observed by using BODIPY and death cells examined with PI. The scale bar is 100 μ m for BODIPY and PI.

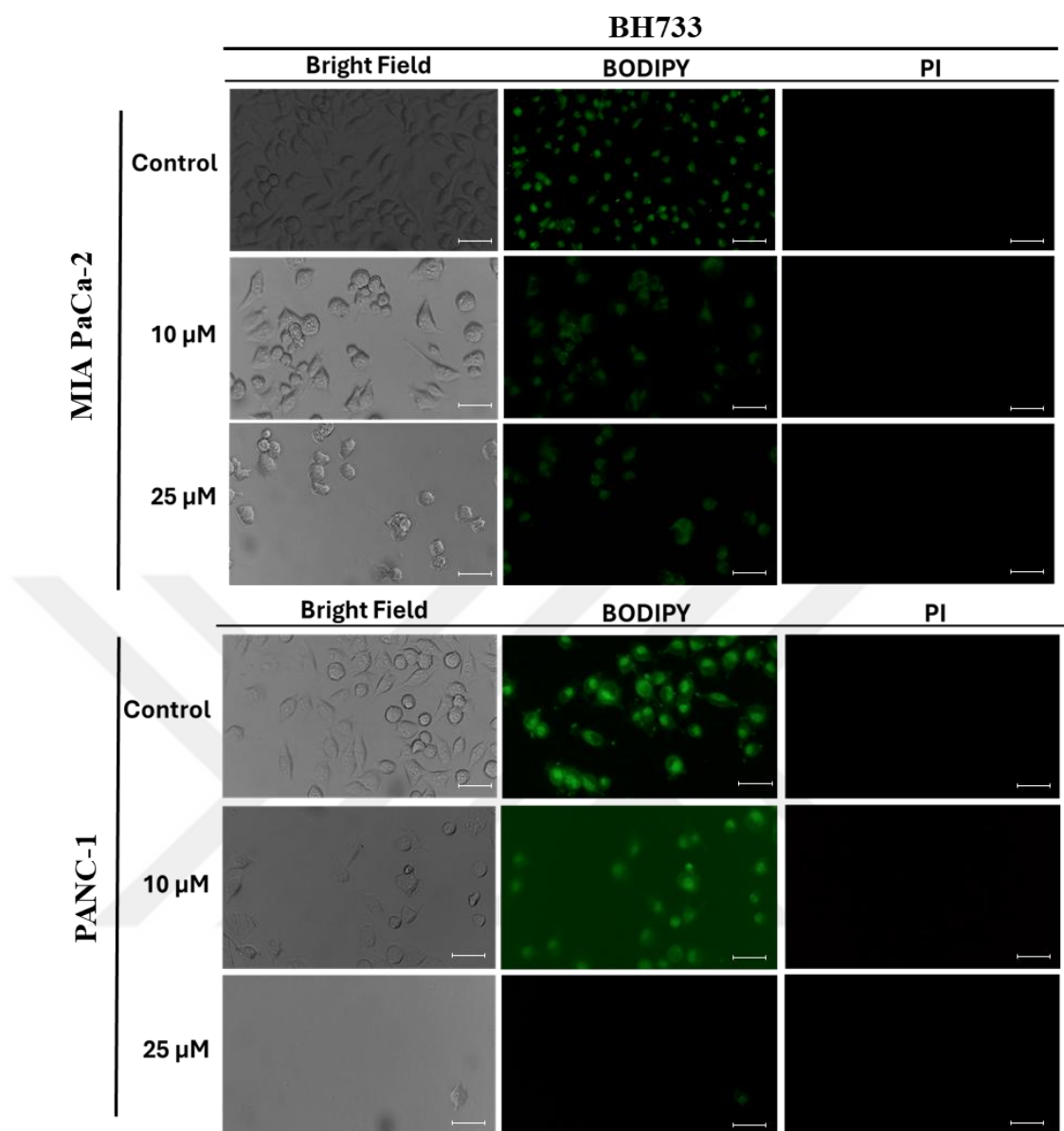


Figure 4.16: Fluorescent staining of wt MIA PaCa-2 and PANC-1 cell lines with BODIPY and PI after treatment with BH733 c-MET inhibitor. Lipid droplet was observed by using BODIPY and death cells examined with PI. The scale bar is 100 μ m for BODIPY and PI.

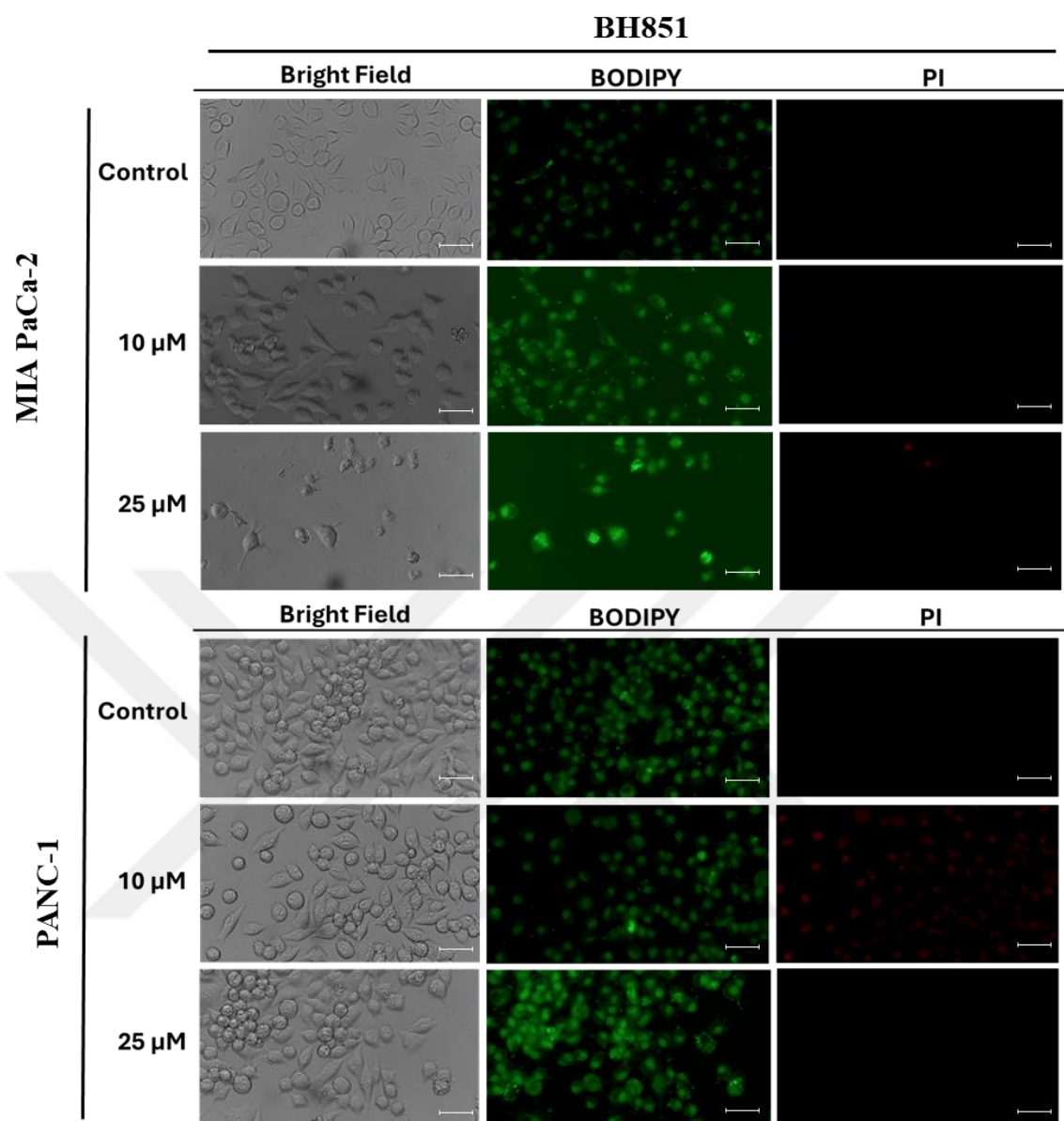


Figure 4.17: Fluorescent staining of wt MIA PaCa-2 and PANC-1 cell lines with BODIPY and PI after treatment with BH851 c-MET inhibitor. Lipid droplet was observed by using BODIPY and death cells examined with PI. The scale bar is 100 μ m for BODIPY and PI.

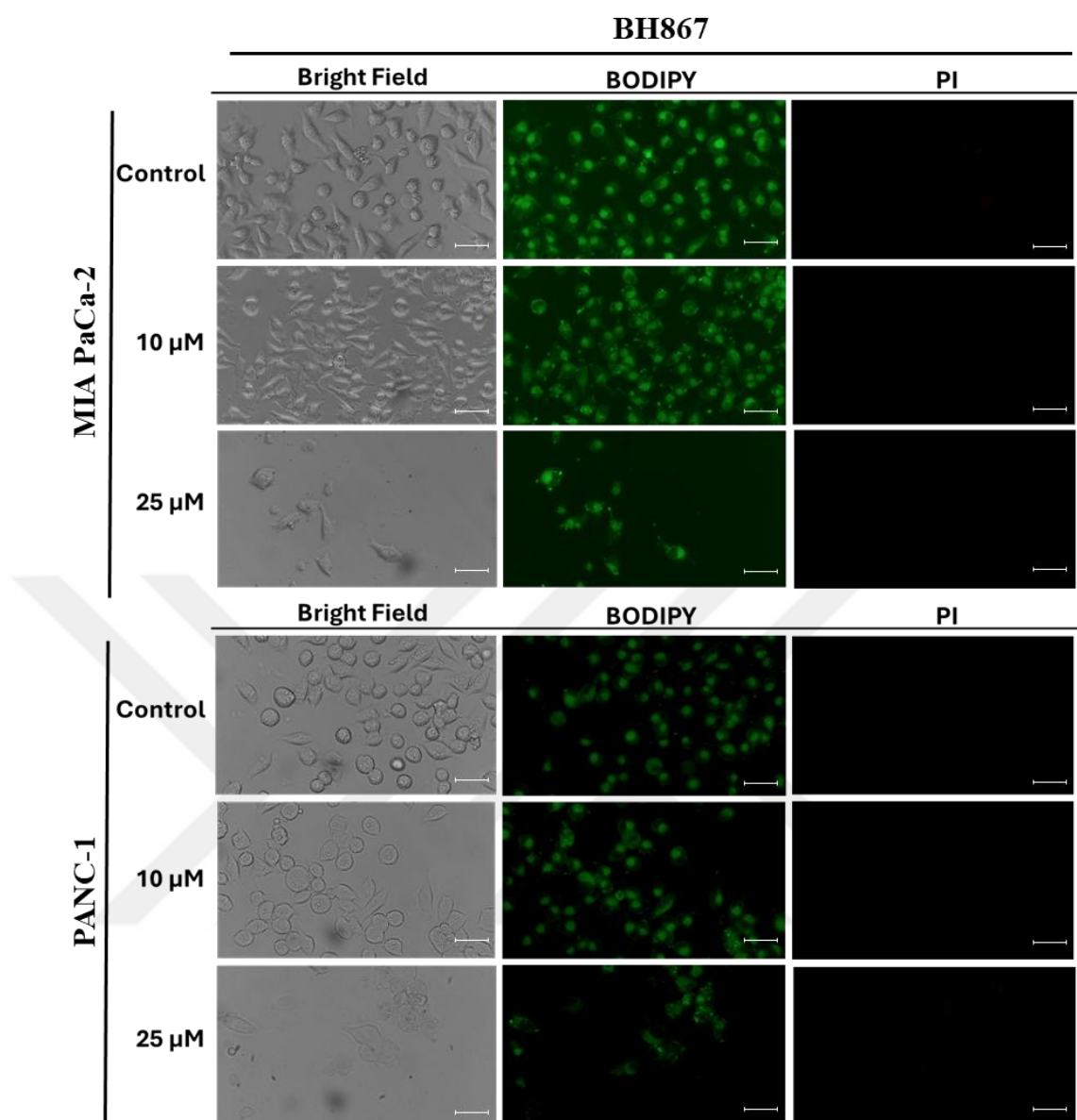


Figure 4.18 Fluorescent staining of wt MIA PaCa-2 and PANC-1 cell lines with BODIPY and PI after treatment with BH867 c-MET inhibitor. Lipid droplet was observed by using BODIPY and death cells examined with PI. The scale bar is 100 μ m for BODIPY and PI.

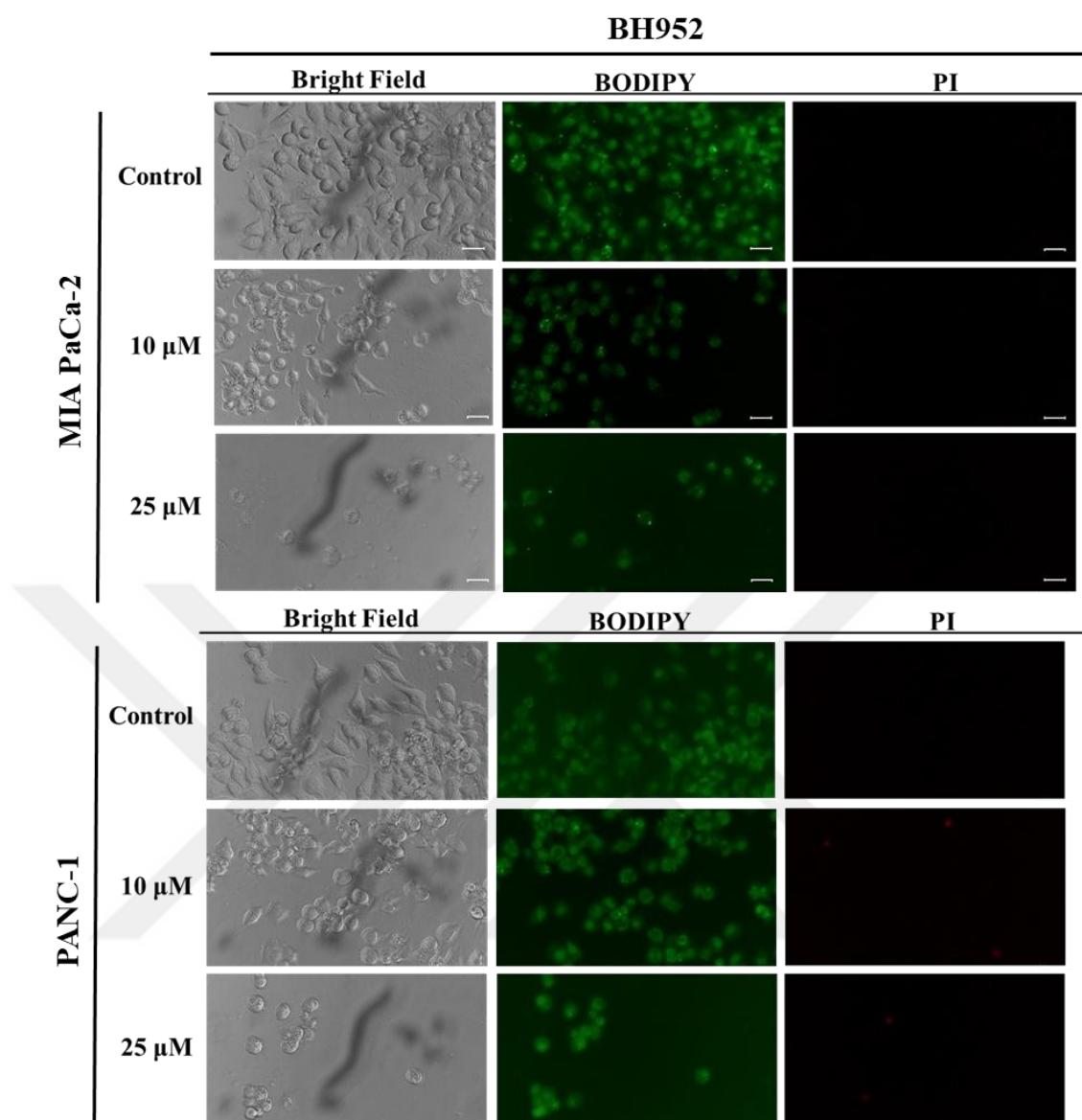


Figure 4.19: Fluorescent staining of wt MIA PaCa-2 and PANC-1 cell lines with BODIPY and PI after treatment with BH952 c-MET inhibitor. Lipid droplet was observed by using BODIPY and death cells examined with PI. The scale bar is 100 μ m for BODIPY and PI.

While there was minimal lipid accumulation in BH851, MIA PaCa-2 control cells, lipogenesis was significantly stimulated at the 10 μ M dose. Despite the decrease in cell number at the 25 μ M dose, the intense lipid signal continued in the remaining cells and the presence of dead cells labeled with PI was detected. In PANC-1 cells, while no lipid accumulation was observed in the control group, a serious increase in the number of PI-positive dead cells was noted with a minimal signal at the 10 μ M dose. At the 25 μ M dose, the formation of lipid droplets was clearly observed (**Figure 4.17**).

In MIA PaCa-2 cells treated with BH867, the basal accumulation observed in a few cells in the control group increased to cover a large portion of the cells at the 10 μ M dose. Although the cell number decreased at 25 μ M, lipid droplets continued to be observed in the remaining cells. Lipid accumulation started at the 10 μ M dose in PANC-1 cells, and despite the serious decrease in cell number at the 25 μ M dose, the presence of lipid droplets was preserved in the remaining cells (**Figure 4.18**).

The lipid signal, which was already present in the BH952, MIA PaCa-2 control group, showed a decreasing trend at the 10 μ M dose and was almost completely extinguished at 25 μ M except for a few droplets. In the PANC-1 control cells, although there was a scattered green signal, no distinct droplets were detected, and a small number of lipid droplet formations were observed at 10 μ M. The cell number almost completely decreased at 25 μ M, and a small number of dead cells labeled with PI were detected at both doses (**Figure 4.19**).

When the effects of the approved c-MET inhibitor Cabozantinib were examined, there was a basal lipid accumulation in the control groups in both cell lines. After treatment, a decrease in the overall green signal and cell number was observed with increasing dose. However, even at a high dose of 25 μ M, lipid droplets and dead cells were found in the remaining few cells (**Figure 4.20**).

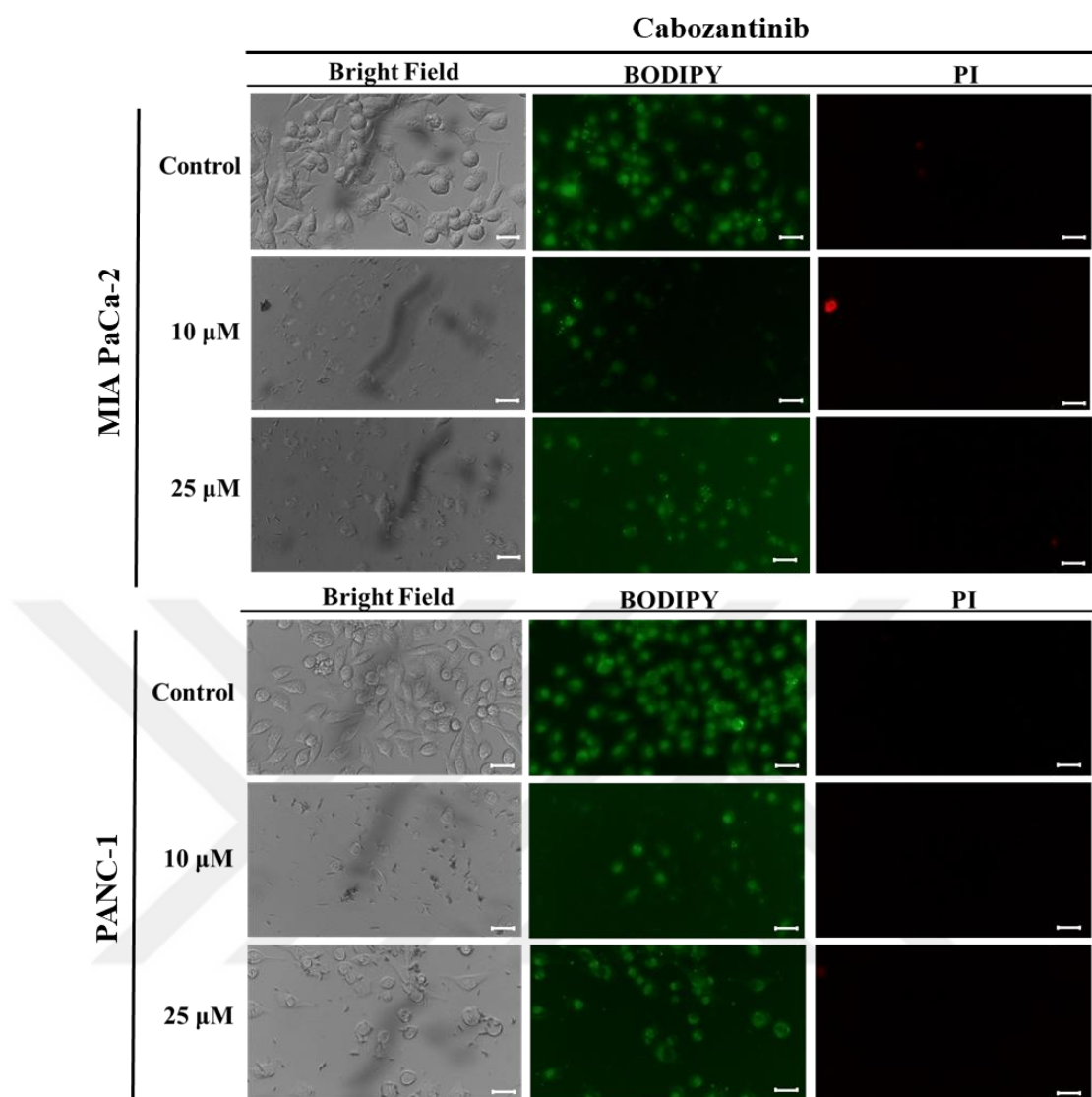


Figure 4.20: Fluorescent staining of wt MIA PaCa-2 and PANC-1 cell lines with BODIPY and PI after treatment with Cabozantinib c-MET inhibitor. Lipid droplet was observed by using BODIPY and death cells examined with PI. The scale bar is 100 μ m for BODIPY and PI.

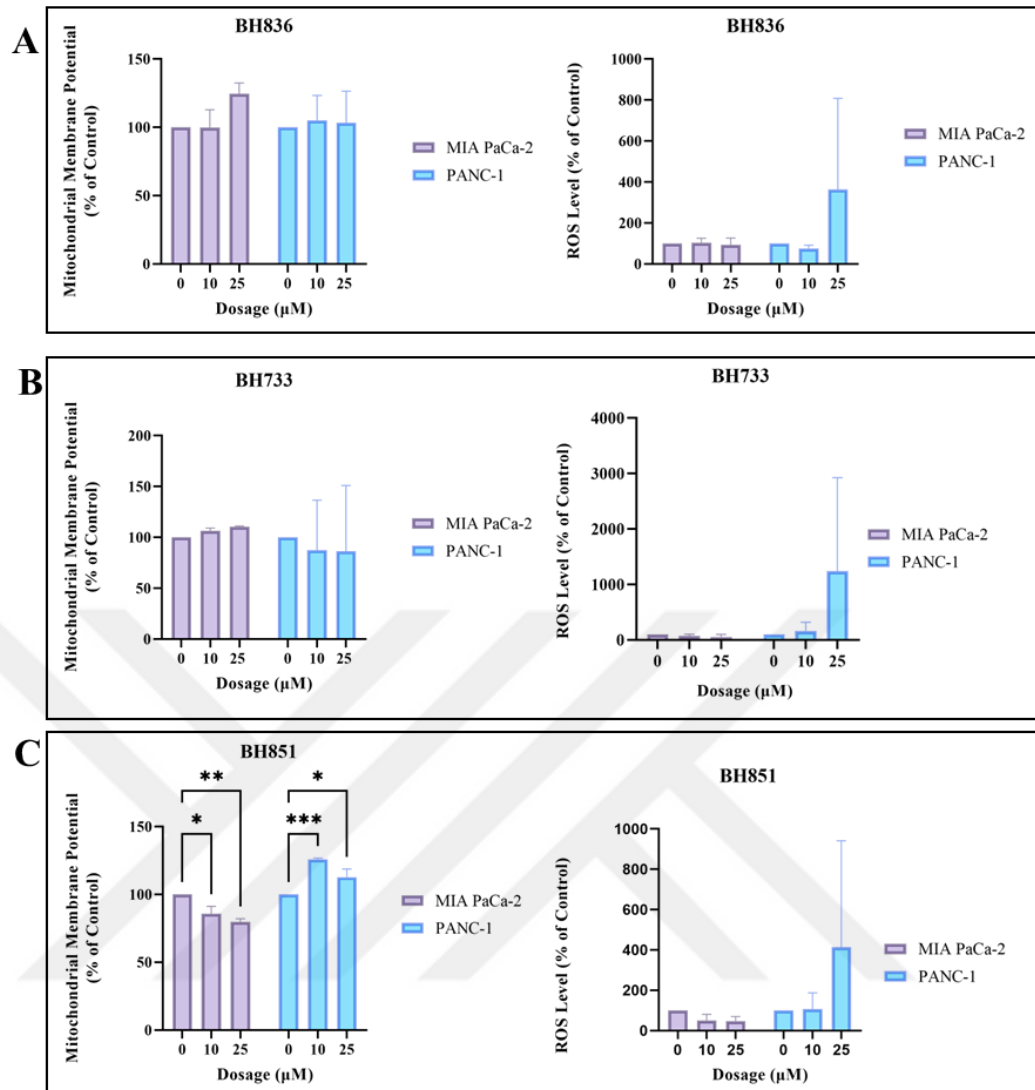


Figure 4.21: Relative fluorescent intensity of MIA PaCa-2 and PANC-1 cell lines after 24h treatment of potential c-MET inhibitors and Cabozantinib. Cells were treated for 24 h with BH836, BH733, and BH851 at 10 μM and 25 μM . Reactive oxygen species (ROS) were quantified using H_2DCFDA (Ex/Em = 485/535 nm), and mitochondrial membrane potential was measured with MitoSpy Deep Red (Ex/Em = 488/520 nm). Fluorescence values are expressed as Relative Fluorescence Intensity (% of Control). Columns represent the mean $\pm$ standard deviation of two independent experiments with 2 repeats. *P*-values were calculated using two-way ANOVA, followed by Dunnett's multiple comparisons test. *****p*<0.0001.

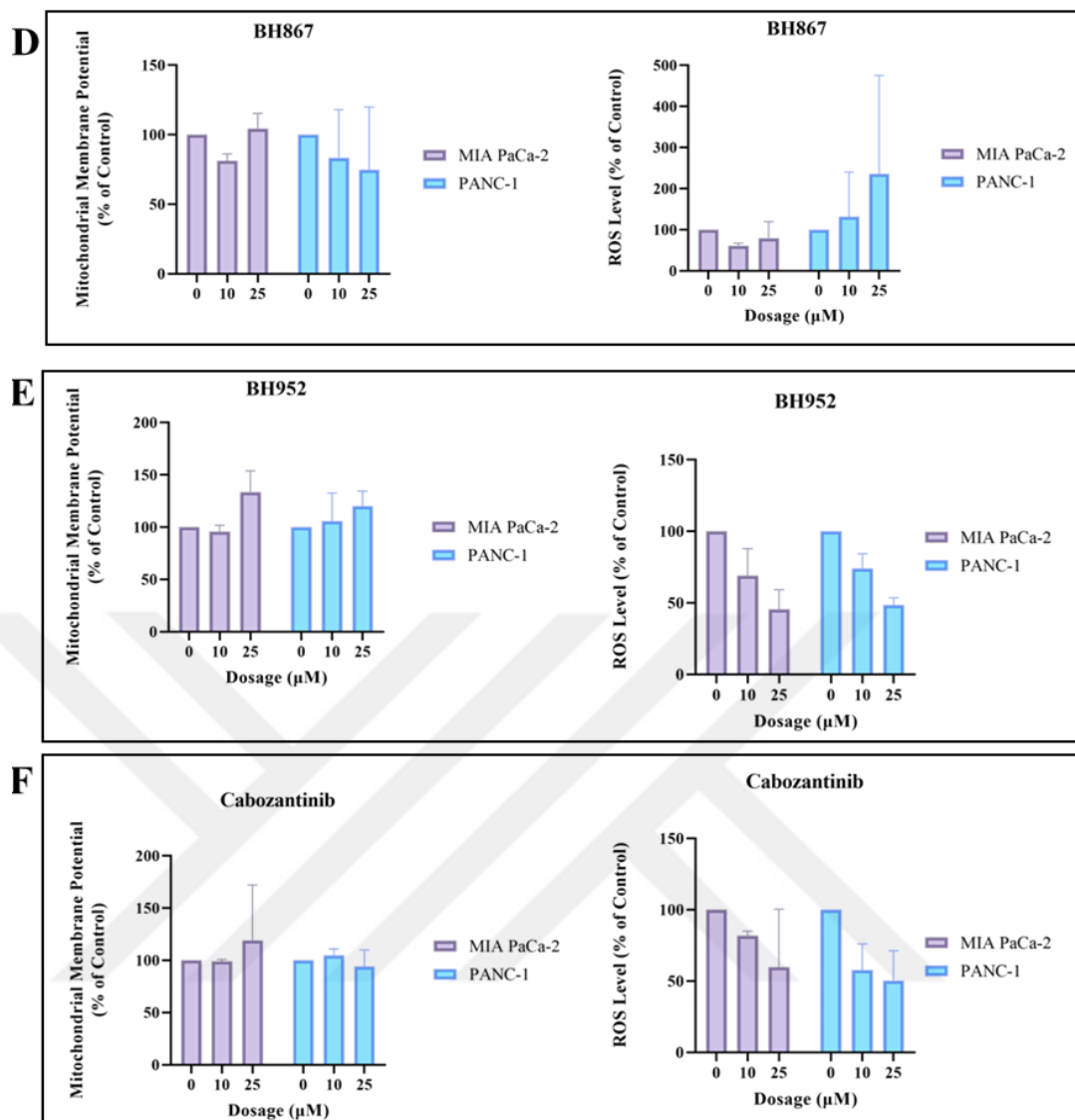


Figure 4.22: Relative fluorescent intensity of MIA PaCa-2 and PANC-1 cell lines after 24h treatment of potential c-MET inhibitors and Cabozantinib. Cells were treated for 24 h with BH867, BH952 and Cabozantinib at 10 μ M and 25 μ M. Reactive oxygen species (ROS) were quantified using H₂DCFDA (Ex/Em = 485/535 nm), and mitochondrial membrane potential was measured with MitoSpy Deep Red (Ex/Em = 488/520 nm). Fluorescence values are expressed as Relative Fluorescence Intensity (% of Control). Columns represent the mean $\pm$ standard deviation of two independent experiments with 2 repeats. *P*-values were calculated using two-way ANOVA, followed by Dunnett's multiple comparisons test. *****p*<0.0001.

MitoSpy and DCFDA signals of BH836, BH733, BH851, BH867, BH952 and cabozantinib molecules applied to MIA PaCa-2 and PANC-1 cell lines for 24 hours were evaluated (**Figure 4.21-4.22**). The purpose of using these dyes is to follow the changes in mitochondrial membrane potential ($\Delta\Psi_m$) for MitoSpy™ and total intracellular reactive oxygen species (ROS) for H₂DCFDA. According to the results, a significant difference was only observed for the BH851 molecule. It is seen that there is a significant decrease in mitochondrial membrane potential at 25 μ M for MIA PaCa-2 cell ($p=0.0120$). An increase ($p=0.0006$) was observed in MitoSpy signal in PANC-1 cell from control group to 10 μ M. There is still an increase at 25 μ M, but not as much as at 10 μ M ($p=0.0200$). The obtained fluorescence intensity graphs present different expressions compared to the results in the fluorescence images. For other molecules (BH836, BH733, BH867, BH952 and Cabozantinib), no statistically significant change was detected in mitochondrial membrane potential ($\Delta\Psi_m$) or ROS measurements compared to the control.

4.6. Protein Expression Profiles of MIA PaCa-2 and PANC-1 treated with potential c-MET inhibitors and Cabozantinib

Western blot analysis was performed to check whether short-term c-MET blockade modulates multiple receptor-tyrosine-kinase (RTK) effectors in a cell line-dependent manner and its effects on apoptosis and other pathways (**Figure 4.6.1**). MIA PaCa-2 (KRAS^{G12C}) and PANC-1 (KRAS^{G12D}) cell lines treated with c-MET inhibitors BH836, H733, BH851, BH952 and the clinically approved c-MET inhibitor Cabozantinib for 24 h showed a noticeable change in epidermal growth factor receptor (EGFR) band intensity compared to control, indicating partial suppression of the MET-EGFR crosstalk axis. In MIA PaCa-2 cells, a decrease in EGFR band intensity was observed with 10 μ M BH836, 10 μ M BH733, 25 μ M BH851, and 10 and 25 μ M Cabozantinib treatments. In the same cell line, AKT signal intensity was most significantly reduced with 25 μ M BH836 and 10 μ M BH733 and further decreased at both cabozantinib concentrations, indicating that MET inhibition compromises downstream PI3K/AKT signaling (**Figure 4.6.1. A**).

In PANC-1 cells (KRAS^{G12D}), a decrease in EGFR and MET pathway protein expression levels was observed after inhibitor applications compared to the untreated control group. According to densitometric analysis, MET pathway proteins were decreased with 10 μ M BH836, 10 μ M BH733, 25 μ M BH851 and 25 μ M BH952 applications. These results reveal that RTK signaling is suppressed in a dose-sensitive manner. Although vimentin has been included as an epithelial-to-mesenchymal transition (EMT) marker, its bands were faint in all lanes and fell below the threshold for reliable quantification, precluding definitive conclusions regarding EMT modulation under these conditions (**Figure 4.6.1.B**). According to the cPARP result, an increase in band thickness was observed in PANC-1 cell line with BH836 10 μ M dose application. This may be a sign of apoptosis. When looking at PTEN expressions, their expressions increased as a result of treatment with 10 μ M BH836 and BH733 molecules. This means that an additional lock was put on the PI3K/AKT pathway (**Figure 4.6.1.C**).

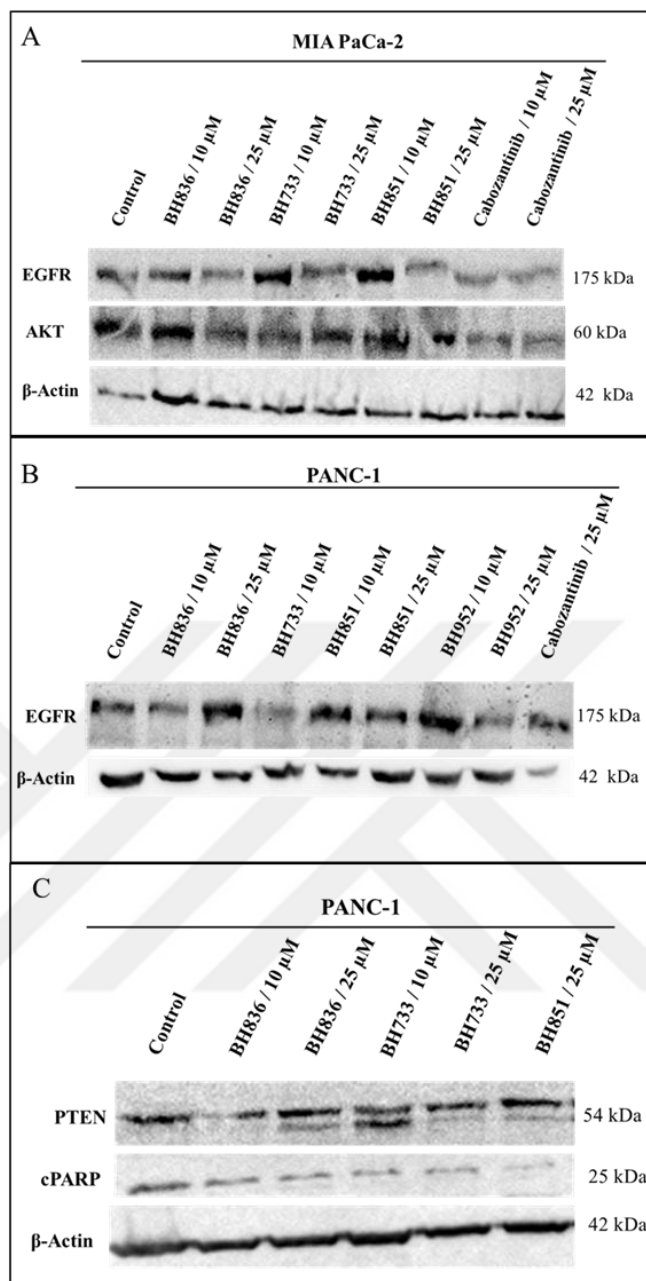


Figure 4.23: Western Blot analysis of apoptosis-related proteins and c-MET signaling in MIA PaCa-2 and PANC-1 cells treated with approved c-MET inhibitors, such as Cabozantinib, and potential c-MET inhibitors. For 24 hours, MIA PaCa-2 and PANC-1 cells were exposed to 10 μ M and 25 μ M concentrations of the clinically approved c-MET inhibitor Cabozantinib as well as the c-MET inhibitors BH836, BH733, BH851, and BH952. The protein expression levels of cPARP, EGFR, AKT, and PTEN were assessed by Western blotting. In order to control loading, β -actin was employed. The findings show that c-MET signaling is inhibited. ImageJ software was used to normalize each sample against β -actin and determine its relative band intensities.

5. DISCUSSION

Pancreatic ductal adenocarcinoma (PDAC) is one of the most lethal malignancies among cancer types with aggressive spread, latent onset, and associated with a very poor prognosis. Its incidence has been increasing worldwide over the years. There are fundamental challenges in the treatment of pancreatic cancer due to the resistance mechanism it develops against traditional chemotherapy and the tumor microenvironment that supports tumor growth and metastasis. One of the reasons for this complexity is the signaling pathway in which c-MET receptor tyrosine kinase and its ligand HGF play a role [Kim et al., 2024; Xia et al., 2025]. Another abbreviation for c-MET is Hepatocyte Growth Factor Receptor (HGFR), a tyrosine kinase receptor encoded by the MET proto-oncogene that is generated as a single-chain precursor that undergoes post-translational modification to create a structure connected by disulfide bonds [Xia et al., 2025; Zhang et al., 2024]. The primary source of HGF in the TME is activated PSCs, and communication between them and cancer cells is a major reason why the c-MET pathway plays a role in pancreatic cancer. PSCs secrete HGF, which in turn activates the c-MET receptor on cancer cells, triggering downstream signaling cascades that promote invasion, metastasis, and tumor progression. Increased c-MET expression in pancreatic cancer and many other cancers is associated with poor prognosis, making this pathway a therapeutic target [Mekapogu et al., 2023, Xia et al., 2025]. For this purpose, many clinical studies targeting this pathway have been conducted and are ongoing. Cabozantinib (XL 184), which was used in this study, is a multikinase inhibitor that targets tumor growth and cancer stem cell (CSC) signaling pathways by inhibiting multikinases such as VEGFR1, AXL, RET, along with c-MET [Denize et al., 2022; Hage et al., 2013]. In 2016, it was approved by the FDA for hepatocellular carcinoma (HCC) [Storandt et al., 2022], and medullary thyroid cancer (FDA approval in 2012) [Tappenden et al., 2019]. Its effectiveness has been investigated for various types of cancer, including pancreatic cancer, in clinical studies. However, although targeted studies are intended to be continued, resistance mechanisms or various side effects emerge in this process. For example, tyrosine kinase inhibitors such as Cabozantinib have been associated with a series of dermatological side effects called “class effects” [Tutunaru et al., 2025]. Decreased

c-MET expression, decreased proliferation indicator Ki-67, and increased anti-apoptotic proteins such as Bcl-2, Survivin, and XIAP are indicators of this resistance [Hage et al., 2013; Shichi et al., 2019].

The cytotoxic and antiproliferative activities of BH892 and BH952 triazolpyridazine/pyrimidine derived molecules on MIA PaCa-2 and PANC-1 pancreatic cancer cell lines were evaluated by various cell assays in vitro. It was also aimed to compare these molecules with Cabozantinib, an FDA approved multikinase inhibitor [Tutunaru et al., 2025]. According to FDA databases, more than 60% of the current drugs used in cancer treatment have heterocyclic rings containing at least one nitrogen atom in their structure, demonstrating the structural importance of N-based heterocycles in drug design and drug discovery. Basically, the anti-cancer activity of N-based heterocyclic agents is largely due to their ability to interact with DNA through hydrogen bonding [Heravi & Zadsirjan, 2020]. Especially in the field of oncology, 70% of small molecule drugs approved by the FDA in 2021 contain pyrimidine rings, proving the central role of such scaffolds in drug design [Mullard, 2022]. Since pyrimidine and its fused ring systems are considered biologically like the purine skeleton that forms ATP, they have the potential to inhibit protein kinases by locating them in the ATP binding pocket. Based on this basic principle, this thesis aims to design and evaluate new potential c-MET inhibitors by combining the pyrazole and triazolopyrimidine core structures, which are known to have anticancer potential in the literature, with the chalcone skeleton, which also has a wide therapeutic activity [McCluskey & Russel, 2021; He et al., 2021].

Research on c-MET inhibitors in pancreatic cancer remains relatively limited, which increases the scientific value of each new study in the field [Ahmet et al., 2019; Huang et al., 2024; Jiang et al., 2016]. This study aims to discuss the response of two different KRAS mutant pancreatic cancer cell lines, MIA PaCa-2 and PANC-1, to c-MET inhibitors within the framework of the unique genetic mutations of the cells and to interpret the experimental data obtained in this context. Both cell lines have an activating KRAS mutation (G12D in PANC-1, G12C in MIA PaCa-2), which is seen in more than 90% of pancreatic cancers and is the main driver of the disease. The KRAS oncogene is located downstream of receptor tyrosine kinases such as c-MET and therefore means that KRAS can remain permanently “on”, sending a multiplex signal to the cell and “bypassing” the inhibition of an upstream target such as c-MET.

This is the primary mechanism of resistance to targeted therapies and may underlie the fundamental biological reason why even the most potent molecules in the study have activity at the micromolar (μM) level. The MIA PaCa-2 cell line (nTPM:6.1) has a lower c-MET expression level compared to the PANC-1 (nTPM:97.8) cell line. Based on this data, MIA PaCa-2 with low MET expression is expected to be inherently resistant, whereas PANC-1 with moderate MET expression is expected to be a more sensitive model [**The Human Protein Atlas, n.d.**].

When the chemical structures of the molecules tested in this thesis were evaluated: BH733 molecule contains a pyrazole, that is, a small ring structure that allows binding to c-MET in the cell. This hetero ring interacts with the hinge region in the ATP pocket of kinases independently of hydrogen, and thus it is expected to replace ATP and prevent phosphorylation [**Liu et al., 2022; Pan et al., 2022**]. The establishment of the hydrogen bond and the flexibility provided by the amide bridge allow the molecules to come into a suitable conformation for the target region. In this way, it is expected to trigger apoptosis [**Xiong et al., 2020; Wang et al., 2028**]. Thanks to the α,β -conjugated system, it easily passes through the cell membrane, enters the hydrophobic pockets and greatly strengthens the π - π stacking bonds. It is known that stilbene-like drugs inhibit telomerase and proliferation pathways to easily enter the cell [**Lee et al., 2019**]. The methoxy ($-\text{OCH}_3$) group at the end of the phenyl ring makes the molecule lipophilic, and thus it is expected that the molecule will pass through the cell membrane more easily and adhere better to the kinase pockets [**He et al., 2021; Wang et al., 2021**]. The methoxy group is slightly larger than the methyl group and can act as a hydrogen bond acceptor due to the oxygen atom [**Chiodi & Ishihara, 2024**]. If the binding pocket of c-MET is suitable for the size and electronic properties of this group, this molecule may show stronger activity. However, if the pocket is too narrow, this large group may not fit and may decrease activity [**Huang et al., 2021; Martorana et al., 2020**]. The BH851 molecule consists of a pyrazole ring, amide group, and aromatic phenyl group, which can suppress phosphorylation in the ATP binding pocket of MET kinase. There is no additional group on the terminal phenyl ring [**Okun et al., 2025**]. In addition to BH851, the BH836 molecule has a methyl group ($-\text{CH}_3$) group, which is a hydrophobic structure. Thus, it is expected to increase the hydrophobic binding and bind more tightly to MET [**Pinheiro et al., 2023**]. The BH952 molecule aims to bind to the ATP binding site with high affinity with its

pyridazine triazol structure and to facilitate its passage through the cell membrane with a stilbene-like aromatic system [Li et al., 2021; Ai et al., 2012]. The BH892 molecule consists of a structure containing a pyridazine triazol core, an amide bridge and a methoxy-conjugated structure [Huang et al., 2021; Martorana et al., 2020].

Cabozantinib, a quinoline derivative, contains chemical blocks that provide specific binding to the ATP pockets of kinases on the one hand, and hydrophobic tails and stable cofactors that support this effect on the other hand, so it can bind to various kinases. It plays an active role in cancer and angiogenesis processes due to its ability to target both c-MET and VEGFR-2 pathways simultaneously. The lipophilicity of the molecule increases with the dimethyl group it contains, but this can cause side effects such as hepatotoxicity [Wang et al., 2024].

In the literature, it has been stated that triazolopyridazine and triazolopyrimidine rings have potential as c-MET inhibitors, and for example, **Zhang et al. (2020)** obtained promising results in their studies with a molecule with this skeleton. One of the main rationales for selecting the triazolopyrimidine nucleus (BH867, BH952, BH892) was these studies. However, contrary to expectations, MTT experiment results revealed that the weakest activity was seen in BH867 and BH892 molecules carrying this structure. In contrast, the highest cytotoxic activity was determined in BH836 and BH851 molecules carrying the simpler pyrazole nucleus, which is known for a wide range of biological activities in literature (**Jaballah, Serya, & Abouzid, 2017**). According to pharmacological data, it was found that the effective doses of the tested compounds showed activity at micromolar levels, indicating a potential comparable to the effective concentration range of BH836 and BH851. This situation indicates that a core structure alone is not sufficient for activity; The overall 3-D structure of the molecule, its orientation with the chalcone bridge, and steric factors play a more critical role in binding to the target. The triazolopyrimidine core in chalcone hybrids may have caused a steric mismatch in the ATP pocket of c-MET. When the effects of the groups at the ends of the molecules, which are expected to be in the selectivity pocket, on the activity are examined, the most active pyrazole series, the most potent derivatives without a substitute (BH851) and carrying a small hydrophobic methyl group (BH836) support that the target pocket has a small and hydrophobic character. This finding emphasizes the importance of small structural modifications for full compliance with the target protein in drug discovery [Zhang et al., 2023].

MTT experiments were performed to test the capacity of these molecules to inhibit the proliferation of cancer cells and reduce their viability. According to the MTT assay, the BH836 molecule was biologically active and showed a dose-dependent cytotoxic effect starting from 10 μ M, while BH733, BH851 and BH952 molecules started to have a cytotoxic effect after 25 μ M. However, the effectiveness of molecules such as BH867 and BH892, which carry the triazolopyrimidine core, was quite limited and required doses higher than 25-50 μ M for a significant effect [Adawy et al., 2024]. One of the major obstacles to the treatment of cancer is that the small number of cancer cells remaining after treatment proliferate again and cause cancerous tissue to regenerate. Therefore, how a drug affects the proliferation potential of cells in the long term is as important as its short-term effectiveness [Franken et al., 2006].

According to the results obtained with the colony formation assay, it is seen that BH851, BH733, BH836 and BH952 molecules have a destructive effect on clonogenic potential. However, colony formation was observed at every dose applied for BH867 and Cabozantinib molecules. According to the study of Escorcia et al. (2020), cabozantinib starts to reduce cell viability in the MIA PaCa-2 cell line starting from the dose of 14.3 μ M in 72-hour incubation; this finding shows that optimizing the incubation time in future studies may increase the efficacy of the drug. Cell-based screening methods play an indispensable role in drug discovery, but the correct interpretation of the results obtained depends on a good understanding of the biological "endpoint" and format measured by the selected assay. In this study, it was observed that Cabozantinib decreased viability to approximately 50% according to MTT results, but the same effect was not observed in the colony formation inhibition assay. The main reason for this is that the differences between short-term tests such as MTT that measure metabolic activity and long-term proliferative capacity such as colony formation are in distinguishing whether a drug is cytotoxic or only cytostatic [Forgie et al., 2024]. While a compound may appear active in the MTT assay by temporarily arresting the cell cycle, if the effect of the drug is reversible, the cells may re-proliferate and form colonies in the long term. Similarly, there are fundamental differences between high-content screening (HCS) methods such as fluorescence microscopy and plate reader-based high-throughput screening (HTS) methods. While microscopy allows the visualization of morphological changes in individual cells and heterogeneous responses within the population (e.g. only some cells undergoing

apoptosis), plate reader-based assays reduce the average response of thousands of cells to a single quantitative value. This may cause a strong biological response affecting only a small portion of the population to be missed in an average measurement. This explains why there was no significant relationship between ROS and DCFDA absorbance measurements of molecules seen to be effective in fluorescent staining images in this study. Therefore, current drug discovery approaches recommend the combined use of multiple experimental formats that measure different endpoints and provide both quantitative and qualitative data to holistically understand the true biological effect of a molecule [Anttila et al., 2019; Ghasemi et al., 2021; Wei, Wang, & Gou, 2021].

Western blot analyses were performed to determine whether the observed cytotoxic effect was due to a targeted mechanism. In this way, it was aimed to understand the role of c-MET in PDAC [Jin et al. 2021]. Inhibition of AKT levels, a key protein of the PI3K/AKT pathway, which is the downstream pathway of c-MET signaling, explains the cellular consequences of this targeted effect. AKT, also known as protein kinase B (PKB), plays a role in cell growth and survival and is increased in PDAC due to hyperphosphorylation and gene amplification. Suppression of this pathway is directly related to the inhibition of cell survival. When AKT levels in the MIA PaCa-2 cell line are examined, it is seen that the AKT band is intense in the control group and in 10 μ M BH836 application. However, it can be said that the band thickness decreases in 25 μ M BH826 and 10 μ M BH733 applications. Band thickness was significantly reduced, especially in cabozantinib treatment. (Figure 3.5.1) [Kim et al., 2024; Ouissam et al., 2024]. Epidermal Growth Factor Receptor (EGFR), which plays a key role in cell growth, proliferation and survival, stands out as an important escape mechanism used by cancer cells against targeted therapies [Zhang et al., 2022]. The new c-MET inhibitors evaluated within the scope of this study were shown to be biologically active in pancreatic cancer cells and target critical survival pathways (PI3K/AKT) [Wood et al., 2021]. However, according to the findings, it also revealed the complexity of targeted therapies [Fernandes et al., 2021]. As shown in Figure 3.5.1 A and Figure 3.5.1 B, EGFR levels for the MIA PaCa-2 cell line increased especially after the application of 25 μ M BH733 molecules and 10 μ M BH851 molecules. In the PANC-1 cell line, an increase in EGFR was observed with the application of 25 μ M BH836, 10 μ M BH851, and 10 μ M BH952 molecules. While the

inhibitors showed a targeted effect on the one hand, they also led to an adaptive resistance in which the cells increased EGFR receptor production as an escape mechanism. More importantly, it was found that they could increase the Vimentin protein, which is associated with the transition of cells to a more invasive phenotype under some conditions, after the application of 10 μ M BH951 and Cabozantinib for PANC-1 [Clement et al., 2020]. These results show that the use of these molecules alone carries potential risks and that rational combination strategies that also target resistance mechanisms are required to achieve success in pancreatic cancer treatment [Zhang et al., 2022].

In the analysis performed in PANC-1 cells, it was observed that c-MET inhibitors did not cause a significant change in the amount of tumor suppressor PTEN protein. PTEN acts as a lipid phosphatase that negatively regulates the PI3K/AKT pathway and converts PIP₃ to PIP₂, and thus plays a role in cell survival, proliferation and metabolism. The fact that the level of PTEN protein did not change indicates that the AKT pathway is not fully suppressed by c-MET inhibitors [Stanciu et al., 2022]. Analysis of cleaved PARP, an apoptosis marker, is interrupted by caspase-3 and reveals mixed results regarding the cessation of DNA repair. In particular, the BH836 molecule showed a promising effect by significantly triggering apoptosis at low doses, while other inhibitors and high doses of the same molecule decreased apoptosis markers below control levels instead of inducing cell death. This suggests that the effects of these molecules on cell death vary depending on the dose and the molecule itself, and that their primary effects may be cytostatic rather than cytotoxic (Figure 3.5.1.C). Therefore, it was observed that BH836, BH952, BH851 and BH733 molecules from the synthesized molecules affected c-MET and inhibited both the survival and proliferation mechanisms of cancer cells with different mechanisms [Tapera et al., 2025].

When the study is evaluated in terms of cell lines, it is expected that MIA PaCa, which normally has low c-MET expressions, will show low activity against inhibitors, but it showed generally similar responses to PANC-1 cell line [Harper et al., 2022]. In fact, when the colony formation results of BH952 molecule are examined (Figure 3.2.2), MIA PaCa-2 cell line did not show colony formation after 5 μ M, while PANC-1 started not to show colony formation after 10 μ M. The reason for this response may be that

the inhibitor used targets not only c-MET but also other kinases that play a vital role in cells [Liu et al., 2023].

Future in vivo studies are needed to evaluate the pharmacokinetics, toxicity, and antitumor efficacy of these molecules, which are currently limited to in vitro studies, as well as to understand the complex crosstalk in metastasis. These in vitro studies are limited by the lack of hypoxic niches that regulate MET signaling in 2D cultures [Ayoub, Ibrahim, & Alkhalifa, 2021; Miquel, Zhang, & Pilarsky, 2021; Wang et al., 2024]. To increase the efficacy and specificity of these molecules, their therapeutic potential can be further increased by combining them with chemotherapy or immunotherapy. Monotherapies are generally inadequate due to the activation of pathways such as PI3K/AKT/mTOR in addition to HGF-MET [Jabbarzadeh et al., 2024; Ouissam et al., 2024]. For example, it has been shown that the combination of gemcitabine or FOLFIRINOX with c-MET inhibitors can increase cytotoxic T-cell infiltration and suppress tumor growth and metastasis [Mekapogu et al., 2023]. For this reason, there is a need to develop multidrug and multitarget combination approaches for these pathways, which are of great importance in the development of PDAC, and to develop promising strategies to treat this aggressive disease. MAPK, Sonic Hedgehog pathways or pan-histone deacetylase inhibitors can be combined with these pathways [Ouissam et al., 2024; Pathala et al., 2020]. Combining PANC-1 with MEK inhibitors or PI3K inhibitors may have a synergistic effect by interrupting both downstream and parallel pathways. Applying MIA PaCa-2 directly with KRAS^{G12C} inhibitors or combining these inhibitors with other agents may be a logical strategy. Pyrazole derivatives target not only c-MET but also many different cancer-related kinases and proteins such as VEGFR, EGFR, CDKs, and PARP. There are several potential targets that can be focused on for future studies [Nehra et al., 2025; Zhang et al., 2023]. In the advanced stages, pharmacokinetic tests such as microsomal stability, oral bioavailability, and plasma protein binding should be performed. Recent studies indicate the need for increased clinical studies along with silico studies [Jin et al., 2024; Nehra et al., 2025]. Studies with orthotopic xenografts containing pancreatic stellate cells can be carried out to further evaluate stromal HGF buffering and pharmacodynamics [Ayoub, Ibrahim, & Alkhalifa, 2021]. In addition, biomarker studies can be performed by classifying data such as circulating HGF levels, p-MET IHC and cell-free DNA for MET exon14-skip mutations. In this way, clinical

response can be seen much better and understanding of resistance mechanisms becomes easier because the role of c-MET is not limited to intracellular signals only, it is important to create a comprehensive map. In addition, understanding the multifaceted effects of the cell on immunotherapy and moving towards targeted treatments is also important for the future of cancer therapy **[Jabbarzadeh et al., 2024]**.



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BIOGRAPHY

Zeynep YALÇINKAYA enrolled in the Molecular Biology and Genetics Department at Abdullah Gül University in 2018 and received her B.Sc. degree in 2023. During her undergraduate studies, she completed Erasmus+ internships at the Leibniz Institute for Neurobiology (LIN) in Magdeburg and the Helmholtz Institute for Pharmaceutical Research Saarland (HIPS) in Saarbrücken, Germany, where she gained experience in neurobiology, molecular biology, and natural product research. In 2023, she started her M.Sc. studies in Biotechnology at the Institute of Biotechnology, Gebze Technical University. She has been involved in the ADEB project (2023-A-113-05) since the same year.



PUBLICATIONS AND PRESENTATIONS FROM THE THESIS

Yalçinkaya Z., Hamur B., Kulu İ., Arısan E. D., (2025), “Therapeutic Effect of Potential c-MET Inhibitors on Pancreas Cancer Cell Lines”, 9th Graduate Research Symposium, 26, Kocaeli, Türkiye, 26-27 May.



APPENDICES

APPENDIX-A: CELL LINES AND REAGENTS

Table A.1: Cell lines, reagents and cell culture consumables.

Material Name	Cat no. / Product no.	Company
PANC-1 Cell Line	CRL-1469™	ATCC
MIA PaCa-2 Cell Line	CRL-1420™	ATCC
Dimethyl sulfoxide (DMSO)	67-68-5 5E3D4IA3	BioFroxx
Gibco™ DMEM	11965092	Life Technologies
Gibco™ Fetal Bovine Serum (FBS)	16000044	Life Technologies
Penicillin, Streptomycin (10000 U/ml) Penicillin, 10 mg/ml Streptomycin	15140122	Life Technologies
Trypsin 0.05%/EDTA 0.02% in DPBS	P10-0231SP	PAN Biotech

APPENDIX-B: LABORATORY MATERIALS AND EQUIPMENT WERE USED IN THIS STUDY

Table B.1: Equipments used in this study.

Product Name	Model/Cat no.	Company
+4°C refrigerator	454270	Vestel
10 µL pipette	7030301004	DLAB
10 µL tips	5130010C	CAPP
1000 µL pipette	7030301016	DLAB
1000 µL tips	5130130C	CAPP
15 ml centrifuge tubes	5100015C	CAPP
200 µL pipette	7030301009	DLAB
200 µL tips	5130070C	CAPP
-20°C freezer	FLV1003	Flavel
24-well plate	TCP 011024	BIOFIL
3D orbital shaker	WT 12	BIOMETRA
50 ml centrifuge tubes	5100050C	CAPP
6-well plate	92006	TPP
-80°C freezer	DF490	Nüve
96-well plate	92096	TPP
Cryotube	190321	Microcult
Heat Block	5032141212	DLAB
Hemocytometer	0640010	Marienfeld
Ice maker machine	GB 601	BREMA Ice Flaker
Inverted microscope	ICX41	SOIF
Microcentrifuge tubes	5100505C	CAPP
Mini Blot Transfer Module	B1000	Invitrogen
Safe 2020 Class II Biological Safety Cabinet	51026638	Thermo Fisher Scientific
Spin	HB20CAV0005823	DLAB
Tissue Culture Flasks	9002625	TPP
Tube roller	MX-T6-S/S+	DLAB
Vortex	8031102000	DLAB
Waterbath	M 24 K	Electromag

APPENDIX-C: CHEMICALS AND ANTIBODIES USED IN THIS STUDY

Table C.1: Chemicals and antibodies used in this study.

Product Name	Cas no. / Lot no.	Company
4X Bolt™ LDS Sample Buffer	B0007	Invitrogen
Acetic acid	27225	Sigma-Aldrich
Acrylamide Xtra	6538C9BD	neoFroxx
BODIPY 493/503	121207-31-6	Sigma-Aldrich
Bradford Reagent	BR05/BR0422	Clear Band
β-merkaptoetanol	M3148	Sigma Aldrich
CM-H2DCFDA (General Oxidative Stress Indicator)	C6827	Invitrogen
Coomassie brilliant blue G250	A3480	Applichem
Crystal violet	C0775	Sigma
DAPI	28718-90-3	BioShop
DiOC6	318426	Sigma Aldrich
DMEM	P04-03590	PAN BioTech
DMSO	67-68-5 5E3D4IA3	BioFroxx
2-Propanol	STBH9873	Sigma Aldrich
Luminol	A2185	PanReac AppliChem
MitoSpy™ Red CMXRos		BioLegends
Methanol	O0780	Honeywell
RIPA Lysis Buffer	RL1023	ECOTECH
PageRuler™ Plus Prestained Protein Ladder	26619	Thermo Scientific
Propidium iodide	P3566	Invitrogen
PVDF Membrane	7177798	GVS
Sodium dodecyl sulfate (SDS)	368131-1KG	AFG Bioscience
Skim Milk powder	5E81FA7E	BioFroxx

Continue Table C.1: Chemicals and antibodies used in this study.

N,N,N',N'- Tetra methylethylenediamine (TEMED)	7J51301	BioShop
Thiazolyl blue tetrazolium bromide (MTT)	1334GR001	BioFroxx
Tris-Base	77-86-1	BioShop
Tris-HCl	1185-53-1	BioShop
Tween-20	0B64696	BioShop
EGF Receptor (D38B1)	3019T	Cell Signalling Technology
Recombinant Human HGF (carrier-free)	584014	BioLegend
BH836	Novel c-MET inhibitors	
BH733		
BH851	Novel c-MET inhibitors	
BH867		
BH892		
BH952		
Cabozantinib	RD769	Deva Pharmacy

APPENDIX-D: BUFFERS AND SOLUTIONS USED IN THIS STUDY

Table D.1: 5% Skim milk preparation.

Skim Milk Powder	2.5 g
TBS-T	50 mL

Table D.2: Recipes for Resolving and Stacking Gels.

	12% Resolving gel (Bottom)	6% Stacking Gel (Upper)
dH₂O	1.7 mL	1.35 mL
Acrylamide (30%) (NeoFroxx)	2 mL	0.4 mL
1.5M Tris (pH 8.8)	1.25 mL	-
1M Tris (pH 6.8)	-	0.25 mL
10% SDS (Sodium Dodecyl Sulfate, AFG Bioscience)	0.1 mL	0.04 mL
10% APS (Ammonium Persulfate, BioShop)	0.1 mL	0.04 mL
TEMED	0.01 mL	0.004 mL

Table D.3: Chemiluminescence imaging buffer.

Ingredient	Solution A	Solution B
dH₂O	9 mL	9 mL
1M Tris-HCl (pH 8.5)	1 mL	1 mL
p-Coumaric Acid (SigmaAldrich)	45 µL	-
Luminol (Merck)	100 µL	-
H₂O₂ (30%, SigmaAldrich)	-	6 µL

Table D.4: 10X Running Buffer Recipe.

Ingredient	Amount for 1 L (pH 8.3)
Glycine (NeoFroxx)	144 g
Tris base (121.14 g/mol, NeoFroxx)	30.2 g
SDS (%10/10 g)	100 mL
dH₂O	to 1 L

Table D.5: The recipe of 10X transfer buffer.

Ingredient	Amount for 1 L (pH 8.3)
Glycine	144 g
Tris base	30.2 g
dH₂O	to 1 L

Table D.6: Preparation of 10X TBS.

Ingredient	Amount for 1 L (pH 7.6)
Tris base	24 g
NaCl (Kimyalab)	88 g
dH₂O	to 1 L

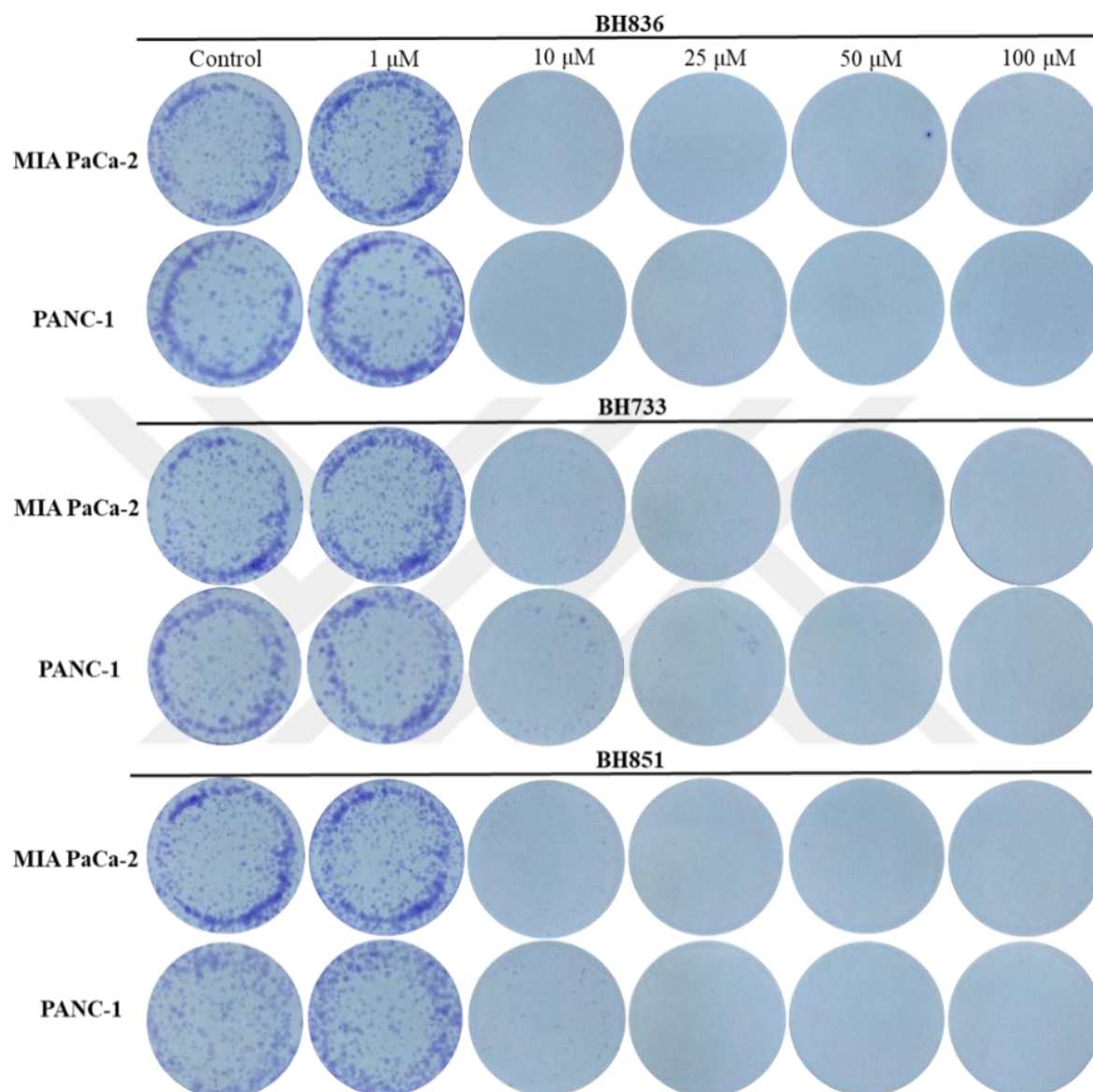
Table D.7: Preparation of Tris-HCl for ECL Solution

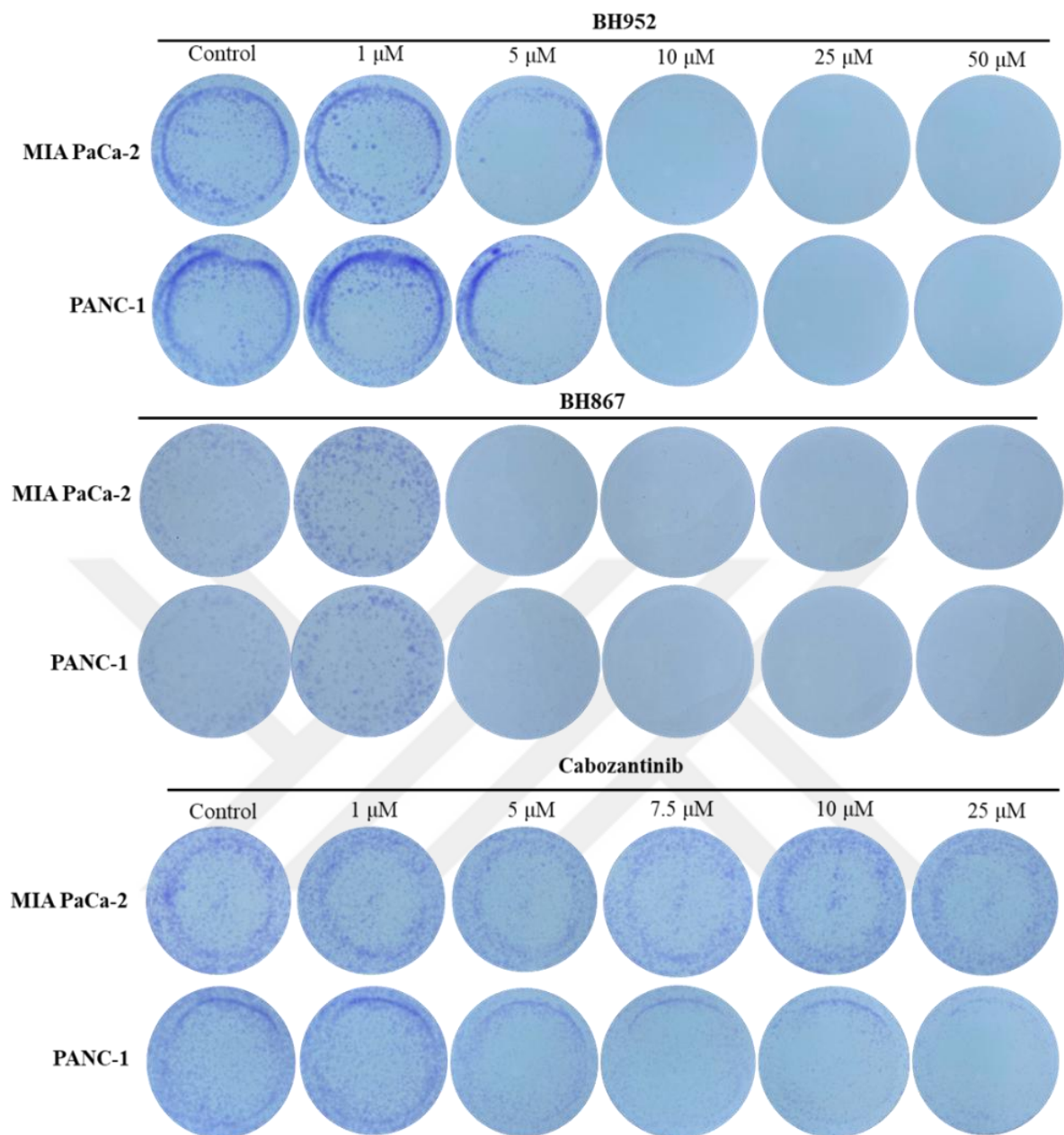
Ingredient	Amount for 100 mL (pH 8.5)
Tris base	8.72 g
Tris HCl (157.60 g/mol, BioShop)	4.42 g
dH₂O	to 100 mL

Table D.8: Preparation of 1X Transfer Buffer

Ingredient	Amount for 500 mL
10x Transfer Buffer	50 mL
Methanol	100 mL
dH₂O	250 mL

APPENDIX-E: COLONY FORMATION ASSAY REPLICATES OF MIA PACA-2 AND PANC-1 CELL LINES





APPENDIX-F: WESTERN BLOT RESULTS OF MIA PACA-2 AND PANC-1 CELL LINES

