THE EFFECTS OF PALBOCICLIB ON 2D AND 3D CELL CULTURES AND DOXORUBICIN RESISTANCE

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ABSTRACT

THE EFFECTS OF PALBOCICLIB ON 2D AND 3D CELL CULTURES AND DOXORUBICIN RESISTANCE

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Cell culture facilitates studying the mechanism of diseases and finding new treatment strategies. Traditionally used two-dimensional cell culture model is not quite sufficient in some studies due to the factors such as lack of cell to cell and cell-extracellular matrix interactions and changing cell morphology. Because of the limitations in 2D cell culture, nowadays three-dimensional cell cultures gain importance. The present study aims to identify the effect of Palbociclib on different cells when they cultured 2D and 3D.

Palbociclib is a dual cyclin-dependent kinase 4 and 6 inhibitor. Palbociclib has been shown to be effective against tumor cells administered alone or in combination, and it has widely been studied in breast cancer cell lines. However, its role in 3D culture of breast cancer cells which is the closest culture model mimicking tumor tissue has not totally been examined by regarding diverse subtypes of breast cancer cell.

The results underlined that MCF-7 and SKBR-3 cells which was capable of forming 3D spheroid structure more resistant to Palbociclib according to cell viability analysis. Additionally, the effect of Palbociclib was different between these two approaches in terms of cell cycle distribution and gene expression analysis, which demonstrated the importance of 3D culturing that was performed by Hanging drop method during the molecular pharmaceutical studies.

Palbociclib was also evaluated in doxorubicin-resistant cells with diverse tumor origin. Resistance to anticancer agents is the main obstacle of the success of the chemotherapy. The ABC-transporter MDR1/P-glycoprotein (MDR1/P-gp; ABCB1), which was the first defined factor, lies behind the multidrug resistance. The using of multiple anti-cancer agents in combination, which is resensitized the cell to drug, has come into prominence to treat drug resistance in cancer.

The results revealed that when the Doxorubicin combined with Palbociclib, the IC_{50} values of Doxorubicin significantly decreased in MCF-7/DOX, HeLa/DOX, K562/DOX and SKBR-3 cells, which had *MDR-1* gene expression. Moreover, Palbociclib re-sensitized the resistant cells towards doxorubicin by down-regulating the P-gp, which was the principal mechanism supporting the doxorubicin resistance in these cell lines. The results clarified that Palbociclib could be a critical agent for combination therapy in the treatments of Doxorubicin-resistant cancer types. Further molecular and clinical studies are needed to totally explore the influence of Palbociclib in cancer and cancer drug resistance.

Keywords: Palbociclib, 3D-Cell Culture, Cancer Drug Resistance, Breast Cancer

ÖZ

PALBOSİKLİBİN 2 VE 3 BOYUTLU HÜCRE KÜLTÜRLERİ VE DOKSORUBİSİN DİRENÇLİLİĞİ ÜZERİNDEKİ ETKİLERİ

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Hücre kültürü çalışmaları, hastalıkların mekanizmalarını anlamak, yeni tedavi yöntemleri bulmak için olanak sağlar. Hücre-hücre ve hücre-hücre dışı matris ile etkileşiminin eksikliği ve değişken hücre morfolojisi nedeniyle iki boyutlu hücre kültür modelleri bazı çalışmalarda yetersiz kalmaktadır bu nedenle günümüzde üç boyutlu hücre kültürü çalışmaları önem kazanmıştır. Bu çalışma, Palbociclib'in 2 ve 3 boyutlu hücre kültürlerinde üretilmiş farklı hücreler üzerindeki etkisini tanımlamayı amaçlamaktadır

Palbosiklib, bir siklin-bağımlı kinaz 4 ve 6 inhibitörüdür. Palbosiklib'in tek başına veya kombine halde uygulanması tümör hücrelerinde etkili olduğu özellikle meme kanseri hücre hatlarında geniş ölçüde çalışılarak gösterilmiştir. Ancak Palbosiklib'in tümör dokusunu taklit eden en yakın hücre kültür modeli olan 3 boyutlu hücre kültüründe geliştirilmiş farklı alt tipdeki meme kanseri hücreleri üzerindeki etkisi tam olarak gösterilmemiştir.

Hücre canlılığı analizleri göstermiştir ki 3 boyutlu küresel yapıyı kurabilen MCF-7 ve SKBR-3 hücreleri Palbosiklib'e karşı daha dirençlidir. Buna ek olarak, hücre döngüsü ve gen ekspresyon analizlerine göre, sonuçlar Palbosiklib'in etkisinin geleneksel iki boyutlu ve Asılı-Damla yöntemi kullanılarak oluşturulan üç boyutlu

hücre kültürleri arasında farklı olduğunu göstermiştir. Bu sonuç moleküler farmasötik çalışmalar sırasında 3 boyutlu hücre kültürünün önemini göstermiştir.

Bu çalışmada ayrıca Palbosiklib'in farklı tümör kökenine sahip Doksorubisin'e dirençli hücreler üzerindeki etkisi değerlendirilmiştir. Anti-kanser ajanlara karşı direnç, kemoterapideki başarısının ana engelinden biridir. İlk tanımlanmış faktör olan ABC-taşıyıcı MDR1 / P-glikoprotein (MDR1 / P-gp; ABCB1), çoklu ilaç direncinin arkasında yatan en önemli nedendir. Hücreyi ilaca yeniden duyarlı hale getiren anti-kanser ajanların kombinasyon halinde kullanılması, kanserdeki ilaç direncini tedavi etmekte önem kazanmıştır.

Sonuçlar göstermiştir ki, Doksorubisin'in IC_{50} değeri, Doksorubisin Palbosiklib ile birlikte hücrelere verilidiğinde, aktif *MDR-1* genine sahip olan MCF-7/DOX, HeLa/DOX, K562/DOX ve SKBR-3 hücrelerinde önemli derecede azalmıştır. Buna ek olarak, Palbociclib'in, bu hücre hatlarında Doksorubisin direncini destekleyen temel mekanizma olan P-gp'nin ekspresyonunu azaltarak ilaç dirençli hücreleri Doksorubisine karşı yeniden duyarlı hale getirdiğini ortaya koymuştur. Bu sonuçlar, Palbosiklib'in Doksorubisin'e dirençli çeşitli kanser türlerinin tedavisinde kritik bir ajan olabileceğini ortaya koymuştur.Palbociclib'in kanser ve kanser ilacı direncindeki etkisini tamamen araştırmak için daha ileri moleküler ve klinik çalışmalara ihtiyaç vardır.

Anahtar Kelimeler: Palbosiklib, 3-Boyutlu-Hücre Kültürü, Kanserde İlaç Dirençliliği, Meme Kanseri

To my valuable parents Melahat Yalçın & Rıza Yalçın,

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ABBREVIATIONS

BCL-2 B-cell lymphoma 2

BAX Bcl-2 associated X protein

DOX Doxorubicin

DMSO Dimethyl sulfoxide

MAPK Mitogen activated protein kinase

PAL Palbociclib

PBS Phosphate buffer saline

PUMA p53 upregulated modifier of apoptosis

SDS Sodium dodecyl sulfate

TBS Tris buffer saline

MCS Multicellular Spheroid

ECM Extra Cellular Matrix

RGD Arginine, Glycine, and Aspartate

CCND1 Cyclin D1

CCNE1 Cyclin E1

mTOR Mammalian Target of Rapamycin

CHAPTER 1

INTRODUCTION

1.1 Cancer

Cancer is a disease comprising dynamic changes in the genome leading to uncontrolled cell division. According to statistical information of the World Health Organization, cancer is the second major reason for death worldwide, and it is responsible for an estimated 9.6 million deaths in 2018. Lung (2.09 million cases), breast (2.09 million cases), colorectal (1.80 million cases), prostate (1.28 million cases), skin cancer (non-melanoma; 1.04 million cases), stomach (1.03 million cases) are the frequently diagnosed cancer types worldwide ("WHO | Cancer," 2018). Hence, cancer is a critical disease that risks the health of all humanity. Nevertheless, it originates from different kinds of tissues and therefore leads to several difficulties in diagnosis and treatment (Health (US) & Study, 2007).

Cancer results from a series of consecutive mutations in genes and these mutations disrupt the cell functions. These mutations could be sporadic or familial. Chemical compounds that have carcinogenic properties, bacteria, viruses, radiation, hormones, aging, stress, and chronic inflammation lead to sporadic mutations. Familial mutations are inherited from parents (Hassanpour and Dehghani, 2017; Lodish et al., 2000).

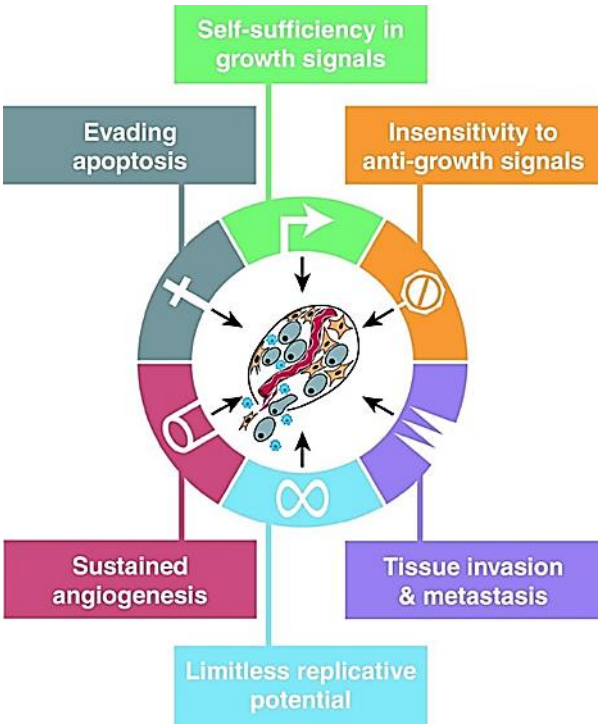

Figure 1.1 The hallmarks of the cancer (Hanahan and Weinberg, 2000).

According to Hanahan and Weinberg, there are acquired capabilities shared by cancer cells. Self-sufficiency in growth signals, blocking antiproliferative signals, overcoming apoptosis, unlimited multiplying potential, sustained angiogenesis, invasion and metastasis are six acquired capabilities that are titled as hallmarks of cancer. These capabilities cause genome instability, which creates genetic diversity (Figure 1.1; Hanahan and Weinberg, 2000).

1.2 Cell Cycle Regulation

The cell cycle is a highly regulated process and it must be maintained for cellular homeostasis. The cell cycle is checked by many events to make sure accurate cellular division (King and Cidlowski, 1998). The cell cycle process is regulated by two class of molecules; cyclins and cyclin-dependent kinases (Cdks; Morgan, 1997). In higher eukaryotic cells, about 20 Cdk-related proteins play a role in cell cycle events. In the G1, G1/S, S and G2/M phase-specific Cdks (Cdk1, Cdk2, Cdk3, Cdk5, Cdk4, Cdk6, Cdk7, Cdk8 and so on) and cyclins (A1, A2, B1, B2, B3, C, D1, D2, D3, E1, E2, F and so on) are combined to regulate complex system (King & Cidlowski, 1998). According to this system, Cylin-D activates the Cdk4 and Cdk6 and initiates phosphorylation of the retinoblastoma protein (Rb; Sherr, 1993). This causes the release of E2F transcription factors which are essential for cyclin E and A in the cell cycle process (Weinberg, 1996). Cylin E activates the Cdk2 in late G1 stage and this consummates the phosphorylation of Rb. This manner provides passing into the S stage. In the S stage, Cdk2 and Cylin A form a complex and Cylin A phosphorylate DNA replication (Petersen et al., 1999). In the G2/M stage, Cyclin A and Cdk 1 form a complex and this causes the starting of prophase (Furuno et al., 1999). At the end of the process, the Cdk1/cyclin B complex promotes the passage to the mitosis (Riabowol et al., 1989). Two families of Cdk inhibitors regulate Cylin-Cdk complexes; the INK family (p16INK4a, p15INK4b, p18INK4c, p19INK4d) that connect to Cdk4 and Cdk6 and inhibits Cyclin-D activity, and in the normal and extreme situations Cip/Kip family (p21Cip1/Waf1/Sdi1, p27Kip1, p57Kip2) that inhibits cyclin E, A, C B activity (Toyoshima and Hunter, 1994; Figure 1.2).

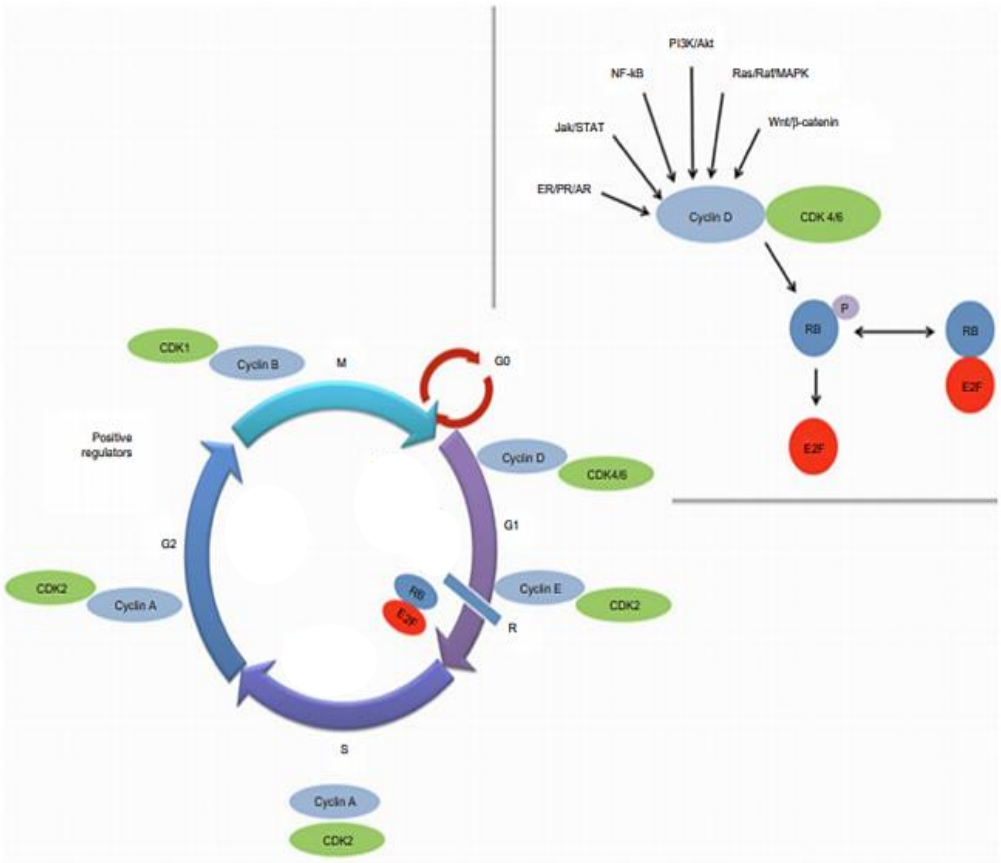

Figure 1.2 Cell cycle regulation (Cadoo et al., 2014).

1.3 Targeting D1-CDK4/6-Rb Signaling Cascade and Palbociclib

In cancer, deterioration of cyclin D1-CDK4/6-Rb signaling cascade induces unchecked cell proliferation (Finn et al., 2016; Malumbres & Barbacid, 2009). Studies imply that cyclin-dependent kinases 4/6 and cyclin D1 as potential therapeutic targets in breast cancer. Palbociclib is a small molecule that inhibits CDK4/6. Inıtial studies showed that Palbociclib had caused Rb dephosphorylation and G1 arrest in Rb-proficient breast cancer cell lines whereas there is no effect in Rb-deficient cell lines (Fry et al., 2004). Finn et al demonstrated the antiproliferative effect of Palbociclib on different types of breast cancer cells in order to reveal which subtype would be a suitable target (Finn et al., 2009). They found that reduced Rb phosphorylation and

G1 arrest were observed luminal A and B cell lines (ER+/HER2- and ER+/HER2+ cell lines, respectively) as opposed to treatment with Palbociclib. Only 15% of cases do not respond to Palbociclib *in vitro*, which were detected to be Rb-deficient cells and there was no connection with hormone receptor status (Dean et al., 2012). This finding indicates the Rb may be an important biomarker for CDK4/6 inhibitors.

According to *in vitro* breast cancer studies, overexpressing cyclin D1 caused aberrant cell cycle progression, and *in vivo*, transgenic mice with increased expression of cyclin D1 enhanced breast cancer (Musgrove et al., 1994; Wang et al., 1994). Loss of function mutation of cyclin D1 affected normal mammary growth and development (Landis et al., 2006). In addition, the ablation of cyclin D1 in HER2-mediated tumors prevented tumor proliferation and caused senescence (Choi et al., 2012).

The cyclin D1-CDK4/6-Rb signaling plays an active role in ER+ and HER2+ breast cancer types (Fantl et al., 1990). Cyclin D1 is amplified in 15% to 20% of all breast cancers and an increased level of cyclin D1 protein has been associated with Estrogen Receptor positivity (Zukerberg et al., 1995). However, basal tumors have increased expression of cyclin E compared with the overexpression of cyclin D1 in luminal subtypes (The Cancer Genome Atlas Network, 2012; Voduc et al., 2008). In this situation, cyclin E may have an alternative role driving Rb phosphorylation to induce S phase transition although cyclin D1-CDK4/6 complex is inhibited (Witkiewicz, et al., 2014).

Hormone signaling maintains most of the breast cancer and especially estrogen has an important role to regulate cell cycle as a mitogen (Garrido-Castro and Goel, 2017). Cyclin D1 is responsible for the regulation of mitogens and growth signals extrinsic to the cell (Sherr and Roberts, 1999). Evidence suggested that estrogen induces G1 to S transition in breast cancer cells and anti-estrogen therapies cause G1 arrest (Foster and Wimalasena, 1996; Patnaik et al., 2016).

Binding of estradiol to the nuclear Estrogen Receptor-alpha transcription factor causes transcription of CCND1 gene expression, subsequently inducing CDK4 activation and

Retinoblastoma phosphorylation. This mechanism represents estrogen-promoted cellular proliferation (Altucci et al., 1996; Said et al., 1997). Remarkably, cyclin D1 also directly binds ER-alpha and promotes cellular growth, even in the absence of estradiol (Neuman et al., 1997, 1997). This activation independent of CDK4/6 activity creates a different pathway for abnormal cell cycle regulation in hormone-driven breast cancer (Figure 1.3).

When the estrogen receptor activity is lacking, different alternative pathways regulate mitogenic signaling to cyclin D1-CDK4/6 that thought to induce tumor growth in spite of endocrine therapy. For instance, as shown in Figure 1.3, HER2 signaling plays a role via multiple pathways in cyclin D1-CDK4/6 checkpoint. In HER2-amplified breast cancer cell lines, inhibition of the ErbB2 receptor led to decreased MAPK- and PI3K-dependent transcription of cyclin D1, decreased cyclin D1-CDK4 complexes in the cytosol, and induced reversible G1 arrest. (Lenferink et al., 2001). Besides, suppressing MAPK and PI3K pathways led to decreased cyclin D1 expression and cell cycle arrest (Kilker & Planas-Silva, 2006). Considering all these studies, targeting D1-CDK4/6 signaling pathway and its central control mechanism have importance in breast cancer pathogenesis, especially in ER+ and HER2-driven tumors.

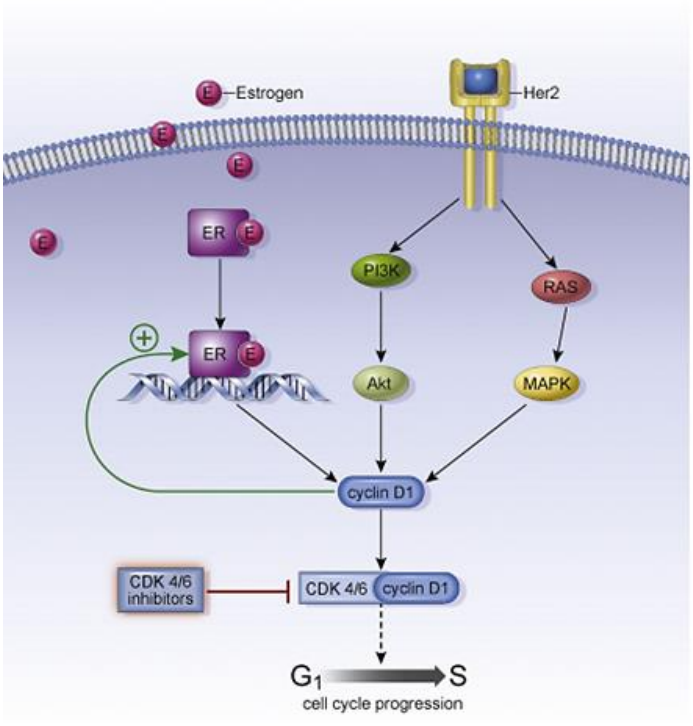

Figure 1.3 Hormonal control of the cyclinD1-CDK4/6 pathway (Scott et al., 2017)

1.4 The Importance of Using Three-Dimensional Cell Culture

In vitro cellular studies provide numerous improvements in cell biology like comprehending the constitution and function of tissues, organs, as well as diseases. Although the human system has demonstrably a complex three-dimensional (3-D) structure, most of these studies were procured using a traditional two-dimensional (2-D) cell culture method. In addition, 2-D cell cultures cannot represent complete *in vivo* characteristic features (Abbott, 2003).

In nature, cells interact with neighboring cells and extracellular matrix (ECM) to constitute a 3-D formation. The cell-to-cell and cell-to-ECM interactions create a complicated communication network of biochemical and mechanical signals that are substantial for normal cellular physiology and function. Also, the plastic petri dish

has an unnatural rigid substrate compared to the smoother environment that cells grow *in vivo* and this alters the cell function. Consequently, lacking *in vivo* features is prevalent for cells grown in 2-D monolayer cell cultures (Griffith and Swartz, 2006).

In recent years, many developments have been recorded to enhance 3D cell culture techniques to bridge the gap between cell studies and animal studies in for decreasing experimental uncertainties originating from 2D monolayer cell culture (Lin and Chang, 2008).

When the cells are lacking an attachment scaffold or surface, they will aggregate and show the self-assembly property and can form 3D multicellular spheroid (MCS) structure. In natural processes, self-assembly property seen in embryogenesis, morphogenesis, and organogenesis (Achilli et al., 2012).

There are studies that spheroids mimic tissue-specific functional characteristics; for instance, spheroids were formed from cardiomyocyte cells beat with heart-like rhythm and spheroids consisted of hepatocyte cells have liver-like properties (Desroches et al., 2012; Fukuda and Nakazawa, 2005).

Spheroids display an *in vivo*-like microenvironment through creating more complicated cell-to-cell and cell-to-ECM interactions. These interactions cause biochemical signals that can affect cell shape, proliferation, motility, and gene expression (Achilli et al., 2012). Therefore, using three-dimensional cell culture in the studies has gained great importance.

1.5 Molecular Mechanism of Spheroids Formation

There are several studies to reveal the mechanism of multicellular spheroid formation. One study was on hepatoma spheroids formation. Hepatoma MCS formation is a process that consists of three steps (Ruei-Zeng Lin et al., 2006). ECM fibers and cadherins are necessary components for the spheroid formation in hepatoma and also the other cell types (Robinson et al., 2004; Shimazui et al., 2004; Figure 1.4). ECM

fibers which have multiple Arginine, Glycine, and Aspartate (RGD) motifs induce aggregation of the cells. This aggregation increases cadherin expression. Lastly, cadherin-to-cadherin binding between two cells causes powerful adhesion. It is thought that β-catenin complex might initiate the signal transduction, and this causes different characteristics in spheroids. Additionally, *ERBIN* , *KLF5* and *TCFL5* genes, which attend spheroid formation, was overexpressed in colon cancer carcinoma spheroids (Dardousis et al., 2007). Another study demonstrated that hormones are essential to maintain cell structures such as that MCF-7 breast cancer cells form spheroids in the presence of 17P-estradiol (Villalobos et al., 1995)

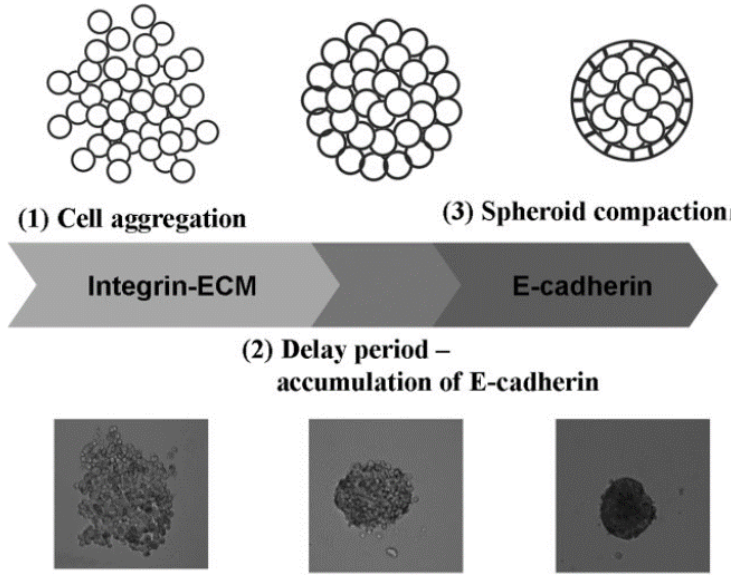

Figure 1.4 Spheroid Formation Process (Lin and Chang, 2008).

1.6 Microenvironment of Spheroids

In order to mimic in MCS, many mathematical models have been suggested to understand oxygen and nutrition consumption, waste transportation, and also therapeutic's diffusion (Curcio et al., 2007; Jiang et al., 2005). The results demonstrated that the MCS represents avascular tissue or a tumor mass. Also, it has a similar diffusion restriction of about 150–200 μm to several molecules, especially O_2

(Curcio et al., 2007). Tumor spheroids display spherical geometry with a concentric structure consisting of three cell types; proliferating, quiescent and dead cells. This formation leads to the formation of a layer-like structure. The inner layer is composed of necrotic cells; inner layer surrounded by quiescent viable cell zone; and finally, the outer layer creates the proliferating zone. Whereas the amount of carbon dioxide and waste are increased from the outer layer to the inner layer of the spheroid, the amounts of oxygen and nutrient growth factor is decreased (Lin and Chang, 2008; Figure 1.5).

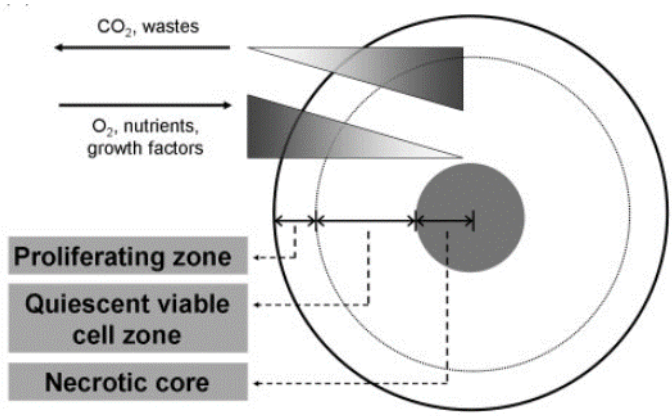

Figure 1.5 Microenvironment of Spheroid (Lin and Chang, 2008)

1.7 The Methods to Form Multicellular Spheroid

Up to now, several methods have been improved for the generation of multicellular spheroids. There are some necessities in order to form spheroids such as avoiding cells from attaching the culture surface, proliferation productivity, dimension uniformness, and feasibility applications. The different types of methods are demonstrated in Figure 1.6 (Lin and Chang, 2008).

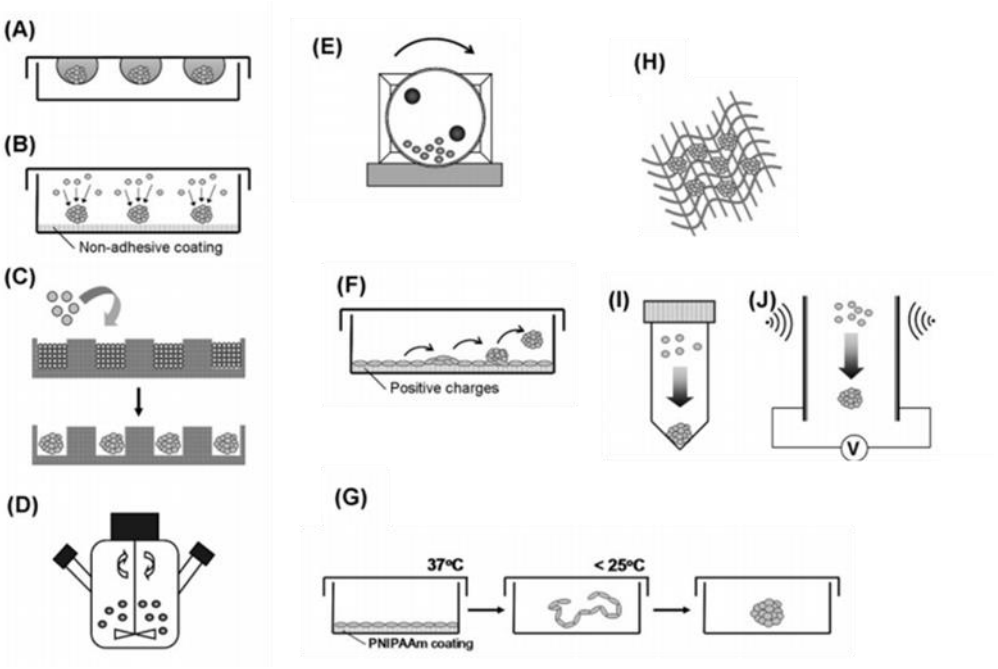

Figure 1.6 Techniques for MCS generation. (A) Hanging-drop culture. (B) Single cell culture on non-adhesive surface. (C) Microfabricated microstructures. (D) Spinner flask system. (E) Rotary system. (F)Primaria dishes. (G)Using of PNIPAAm in MCS formation. (H) Porous scaffold. (I) Centrifugation pellet culture. (J) Magnetic, Electric or acoustic force cell aggregation (Lin and Chang, 2008).

1.7.1 Hanging-Drop Cultures

The hanging-drop method was first used for cultivating embryonic stem cells. In order to make a hanging drop culture, firstly, cells are suspended and then 30 μL cell suspension drops are put onto inside of the cell culture petri dish lid. Each drop can involve a different range of cells (300 to 3000) depending on the cell type. When the petri dish lid is inverted, by the help of surface tension, drops keep their stability. This manner creates a microgravity environment for cells (Figure 1.6 (A)). This method is cheaper and particularly useful for forming spheroid cell culture. In addition, it facilitates working on the cellular or molecular process in cells (Lin and Chang, 2008).

1.7.2 Spheroid Formation Using Non-Adhesive Surface

In order to generate MCS with this method, non-adhesive Petri dishes and 96 well plates that are for non-adhesive cells can be used. These plastics also can be covered with agarose film or polymer with hydrophobic properties such as poly-HEMA (Figure 1.6(B)). Different size distribution is observed by this method (Landry et al., 1985)

1.7.3 Microfabricated Microstructures

Microstructure that helps the formation of multicellular spheroids, is a controllable powerful method to form 3D well-shaped cell geometry (Wu et al., 2008) This microstructure can be composed of non-adhesive materials such as, agarose or PEG. Suspended cell guides to form spheroid through non-adhesive microstructure (Figure 1.6 (C)). This method has been used for 3D liver and stem cell spheroid formation, in high-throughput drug screening (Powers et al., 2002).

1.7.4 Large-Scale Spheroid Production in Rotary Bioreactors

In order to form multicellular spheroid on a large scale, spinner flasks with surface treatment can be used (Nyberg et al., 2005; Song et al., 2004; Figure 1.6 (D)). Spheroid size can be controlled by initial cell seeding amount, medium content spinning and time. This technique has been used for large scale formation of porcine hepatocyte spheroid. However, this culture system may influence cellular physiology because of having powerful shear force. In order to overcome this challenge, the National Aeronautics and Space Administration (NASA) of the United States created a rotary cell culture system to form a microgravity environment with low shear force (Ingram et al., 1997; Unsworth and Lelkes, 1998; Figure 1.6 (E)). This system has the capability of forming multi cell-type spheroids.

1.7.5 Using Surface-modified Substrates or Scaffolds

Different type of modified surfaces has been used in culture for MCS production. One of them is charge-modified surfaces. Primaria plates (BD, Biosciences) have positively charged surfaces, and it has been used for primary hepatocytes spheroid formation (Figure 1.6 (F)). It can be easily observed to the transition of cells 2D into 3D spheroids via positively charged surfaces, after 48 hours of culture. The other one is galactosylated poly (vinylidene difluoride) or polyethylene terephthalate-treated surfaces (Du et al., 2007; Lu et al., 2003). These substances are suitable for forming hepatocyte spheroids although their effects on other cell types are not well known. Temperature-modulated systems are also used for the generation of spheroids. Thermo-responsive poly(N-isopropyl acrylamide; PNIPAAm)-grafted surface in the plates provides a floating cell layer (Figure 1.6 (G)). The floating cell layer gradually condenses and forms spheroid which can be easily obtained by centrifugation after lowering the incubation temperature to dissolve the hydrogel (Koide et al., 1990; Takezawa et al., 1990). However, using of PNIPAAm in MCS formation is not appropriate for routine studies. Using porous 3D scaffolds also enables the cell self-assembly. It is mostly used for hepatocyte cells (Figure 1.6 (H)). Cells are seeded onto scaffolds that can be consisted of biomaterials like alginate, gelatin or hyaluronan (Glicklis et al., 2000; K. H. Lin et al., 1995). Also, synthetic scaffolds can be used such as polyurethane foam, fructose/galactose-modified polyamidoamine dendrimer, galactosylated nanofiber meshes and poly-L-lactic acid matrices (Higashiyama et al., 2003; Török et al., 2006). Depending on the material properties such as availability, biodegradability, and biocompatibility, these scaffolds can be selected.

1.7.6 Formation of MCS Using External Forces

When the suspended cells are concentrated to a high amount, it can be enabled to generate cell aggregation and spheroid formation (Figure 1.6 (I)). Using some methods can improve the aggregation such as low-speed centrifugation, dielectrophoresis,

ultrasound wave traps and magnetic fields (Liu et al., 2007; Figure 1.6 (J)). However, spheroids generated by these techniques display physiological effects and necessity of special instrument limit the routine use of these methods in spheroid formation.

In the present study also aims to find out role of Palbociclib as a co-therapeutic agent on Doxorubicin-resistant cells. Therefore, multiple drug resistance in cancer and Palbociclib-drug resistance relationship are discussed in the next titles.

1.8 Multiple Drug Resistance (MDR) in Cancer

Cancer leads to the uncontrolled growth of cells by alterations in their genome (MacConaill and Garraway, 2010). Cancer progress diminishes the normal biological process of healthy cells (Goldenberg, 1999). There are many cancer treatment methods such as surgery, radiotherapy, chemotherapy and combination therapy. Although there are many advances in cancer treatment, especially in chemotherapy, drug resistance is the major factor limiting the efficacy of the treatment (Longley and Johnston, 2005). Therefore, drug resistance seems to be a critical problem in the field of cancer.

A cancer cell can develop resistance to chemotherapeutic agents through several mechanisms (Figure 1.7). These mechanisms include reduced intracellular drug levels that could be due to elevated drug efflux, suppressed apoptosis, reduced transformation of drug to an active form, changed amount of target enzyme or receptor and diminished affinity of target enzyme or receptor for drug, activation of DNA repair mechanisms, and altered expression of genes for cell survival (Young et al., 2003). In addition, many studies stated that overexpression of ABC transporters (P-glycoprotein/MDR1, and BCRP) confers resistance to various cytotoxic agents in various cancer cells (Işeri et al., 2010; Meltem Demirel Kars et al., 2007).

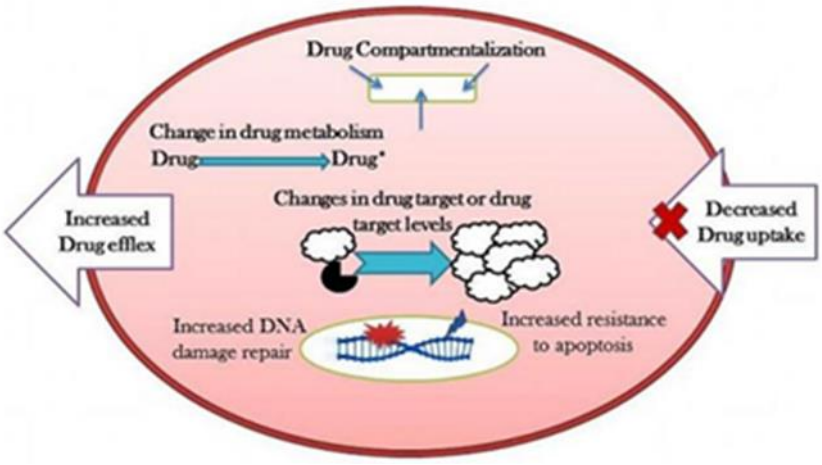

Figure 1.7 The mechanisms of drug resistance in cancer cells (Mansoori et al., 2017).

MDR1 or P-gp is one of the well-characterized ABC transporters. It is comprised of two transmembrane domains that form a passage for substrates and two nucleotide-binding domains that bind and hydrolyze ATP. The binding and the subsequent hydrolysis of ATP is led to a conformational alteration in the transporter, causing the pumping out of the substrates. MDR1 has multiple drug binding sites such as doxorubicin, paclitaxel and vinblastine that can pump a wide variety of substrates from the cell (Wilkens, 2015).

Doxorubicin (DOX), also known as Adriamycin, is an anthracycline drug frequently used in cancer chemotherapy. Doxorubicin binds directly to DNA via intercalation between base pairs on the DNA helix. It also blocks DNA repair by inhibiting topoisomerase II. These actions cause the prevention of DNA and RNA synthesis(Robert and Larsen, 1998). Unfortunately, its therapeutic potential has been restricted by its dose limited cardiotoxicity and the resistance developed by the tumour cells to the molecule after some time of treatment (Hanušová et al., 2011).

1.9 Palbociclib-Drug Resistance Relationship

Although the effectiveness and functionality of Palbociclib have been extensively studied on the different cancer cell, there are very few studies which evaluate the effect of Palbociclib on cancer cells with drug resistance. Up to now, the effect of Palbociclib in multidrug resistance has not been examined in detail. Nie et al., (2019) showed that when the Palbociclib was treated with Afatinib, a tyrosine kinase inhibitor, it overcame acquired Afatinib resistance on non-small cell lung cancer and murine xenograft. Furthermore, they indicated that the combination therapy significantly attenuated the proliferation of malignancy in the patient-derived xenograft model compared to monotherapy. De Gooijer et al., (2015) demonstrated that Palbociclib was a substrate of both P-gp and breast cancer resistance protein (BCRP) trough *in vitro* transwell assay on parental and human (ABCG2) and murine BCRP (Abcg2) overexpressing canine kidney epithelial cells (MDCK) and porcine kidney epithelial cells (LLC). Additionally, *in vivo* experiments on wild type and various ABC transporter-knockout mice showed that P-gp and BCRP substantially prevented Palbociclib brain penetration (de Gooijer et al., 2015). However, there are not extensive studies with Palbociclib on this subject.

1.10 Apoptosis

Apoptosis is the widely known form of programmed cell death via two main pathways, intrinsic and extrinsic (Figure 1.8; de Vries et al., 2006). Apoptosis is triggered when cell-surface death receptors such as Fas are bound by their ligands and the extrinsic pathway becomes active. It conveys death signals in 3 ways: to lysosome membrane permeabilization (LMP), to caspase-8-dependent activation of effector caspases, or to BH3- only-dependent mitochondrial outer-membrane permeabilization (MOMP). Various signal transducers and toxic agents trigger the intrinsic pathway through the activation of pro-apoptotic BH3-only proteins from the Bcl-2 family. Mitochondrial permeabilization leads to the release of caspase activators. Apoptosis starts at the

mitochondrial level through the Bcl-2 family of proteins which is categorized into 3 groups: 1- anti-apoptotic multi-domain members (Bcl-2, Bcl-X L and Mcl-1), that have four type of Bcl-2 homology domains (BH1, BH2, BH3 and BH4), 2- pro-apoptotic multi-domain members (Bax and Bak), which lack the BH4 domain and 3-proapoptotic BH3-only proteins (Bid, Bim and Bad; Martinou & Green, 2001; Vicencio et al., 2008).

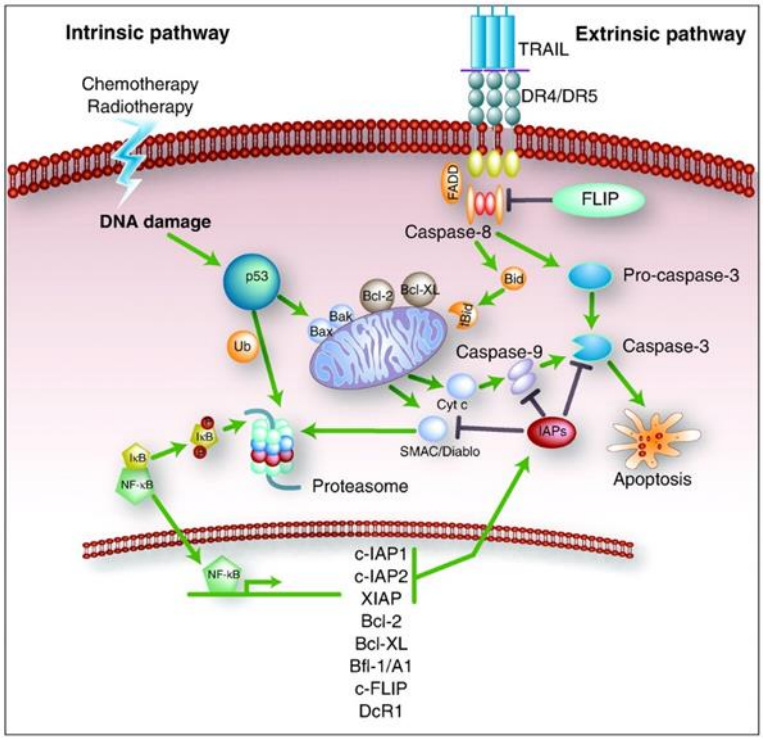

Figure 1.8 Extrinsic and intrinsic pathways of apoptosis (de Vries et al., 2006).

1.11 Aim of the Study

This study has two major aims and these two objectives are discussed under two titles. The first one is the effect of Palbociclib in different subtypes of breast cancer cells under monolayer, (2-dimension), and spheroid, (3-dimension) conditions. The second one is to find out the role of Palbociclib as a co-therapeutic agent on Doxorubicin-resistant cancer cells.

The objectives of this study are listed below.

- Comparing the effect of Palbociclib in different subtypes (MCF -7 (ER+, PR+, HER2-), MDA-MB-231 (ER-, PR-, HER2-) and SKBR-3 (ER-, PR-, HER2+)) of the breast cancer cell.
- Comparing the effect of Palbociclib in MCF-7, MDA-MB-231 and SKBR-3 cells grown in monolayer (2D), and spheroid (3D) cell cultures.
- Examination of the changes in expression levels of cell cycle and apoptosis-related genes in Palbociclib-treated 2D and 3D MCF-7, MDA-MB-231 and SKBR-3 cells.
- Finding out the effect of Palbociclib on different types of Doxorubicin-resistant cancer cell lines coming from different origins (Doxorubicin-resistant breast cancer cell line, MCF-7; Doxorubicin-resistant cervical cancer cell line, HeLa; and Doxorubicin-resistant chronic myeloid leukemia cell line, K562).
- Investigating the role of Palbociclib as a co-therapeutic agent on drug-resistant and drug-sensitive cancer cells.
- Examination of the changes in expression levels of cell cycle and apoptosis-related genes in Palbociclib-treated resistant cells (MCF-7/DOX, HeLa/DOX, K562/DOX and SKBR-3).

- Determination of the changes in expression levels of multiple drug-resistant-related genes in Palbociclib-treated P-gp-expressing resistant cells (MCF-7/DOX, HeLa/DOX, K562/DOX and SKBR-3).

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

Palbociclib was purchased from Sigma Aldrich, USA. Doxorubicin was obtained from Saba, Turkey. MTT and Propidium Iodide was purchased from Sigma-Aldrich (USA). Trypan blue was obtained from Thermo Scientific, USA. High Pure RNA isolation kit and The LightCycler® 480 SYBR Green I Master were purchased from Roche, Germany.

2.2 Cell Lines and Cell Culturing

MCF-7, MDA-MB-231 and SKBR-3 breast cancer cell lines were obtained from ATCC. Doxorubicin-resistant MCF-7, HeLa and K562 cell lines were developed previously in our laboratory by stepwise selection from their parental MCF-7, HeLa and K562 in incremental drug concentrations (Baran et al., 2007; Darcansoy İşeri, 2009; Erdem, 2014; Kars et al., 2006). All cell lines were maintained in RPMI 1640 medium (Biological Industries, Israel) supplemented with 10% (v/v) fetal bovine serum (Biological Industries, Israel) and 0.2% (v/v) gentamycin (Biological Industries, Israel). Cells were incubated at 37˚C in a humidified atmosphere with 5% (v/v) CO_2 in an incubator.

2.3 Sub-culturing and Cell Counting

When the cell population on the flask surface reached 80% confluency, the cells were sub-cultured under laminar flow (BioAir, Italy), at sterile conditions. The old medium was removed from the culture flask and cells were washed with PBS. In order to dissociate the cell-to-cell and cell-to-flask surface interactions, Trypsin-EDTA (Biological Industries, Israel) was added onto cells. The flask was incubated at 37ºC for 5 minutes. Fresh medium was added to stop the activity of Trypsin-EDTA and cells were centrifuged at 1500 rpm for 5 minutes. After centrifugation, detached cells were homogenized with fresh medium and passed into a new flask.

Trypan blue dye (Thermo Scientific, USA) was used for viable and dead cell counting. Trypan blue (4%) was mixed with cell suspension at the ratio of 1:10. Cell counting was achieved using the Neubauer hematocytometer (Thermo Fischer, USA). In Trypan blue assay, unstained brownish cells represent viable ones while the blue colored represent death cells (Altman et al., 1993). The cell number was calculated using equation 1.

Equation 1. Cell number/ml = Average cell count per square x Dilution factor x 4 x 10^6

2.4 Hanging Drop Method for 3D Cell Culture

Hanging drop cell culture protocol was followed as described by Foty for the formation of 3D cell cultures (Foty, 2011). When the cells reached 80% confluency, the medium was removed from the breast cancer cells MCF-7, SKBR-3, and MDA-MB-231 and after washing with PBS, 0.05% trypsin was applied and the cells were transferred to 15 ml falcon tubes, and centrifuged at 1500 rpm for 5 minutes. After the cells were counted by haemocytometer, the concentration was adjusted to $1x10^4$ cells/drop.

As shown in Figure 2.1, the lid of the 100 mm petri dish was inverted and 30 µl of each drop was put onto the bottom of the lid. Enough space was left between the drops. Then, 5 ml PBS added in the bottom of the petri dish in order to prevent evaporation and the lid was inverted onto the PBS-filled bottom chamber and incubated 4 days at 37˚C in the incubator.

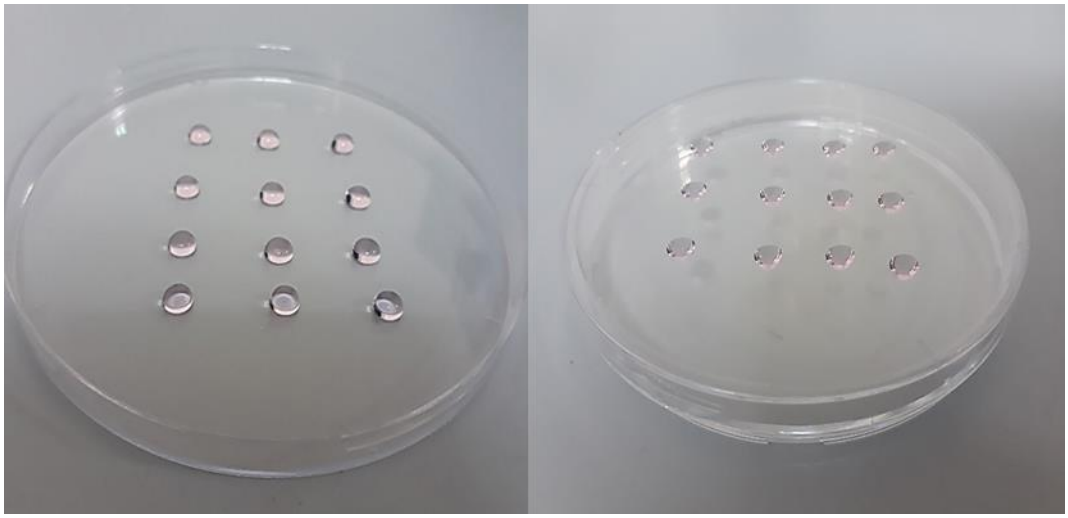

Figure 2.1 Hanging-Drop method using petri dish.

2.5 Cell Viability Analysis

2.5.1 MTT Analysis

3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) assay which is a colorimetric assay for determining cell viability (van Meerloo et al., 2011) was used to show the cytotoxic effect of Palbociclib (dissolved in sodium lactate; pH 4) and Doxorubicin (dissolved in water). All cell lines were seeded into 96-well plates as $15x10^3$ cells per well and incubated for 24 hours at 37°C in a 5% CO_2 incubator to adhere the cell to the plate. Then, different concentrations of Palbociclib, Doxorubicin and Palbociclib plus Doxorubicin were treated to the cells for 72 h. At the end of this time period, 10 µl of MTT solution (5 mg/ml) was applied onto cells and incubated for 4 h. Cells were disrupted with SDS-HCl solution (1 g SDS in 0.01 M HCI in 10 ml final volume) overnight and the microplates were read using a microplate spectrophotometer (Multiskan GO; Thermo Fisher Scientific, USA) at 570 nm. The viability of the untreated control group was accepted as 100% and the relative cell viability of treated cells was determined accordingly.

2.5.2 Cell Counting by Trypan Blue Cell Exclusion Method for 2D and 3D Cell Viability

The viabilities of 3D cells were determined by cell counting by Trypan-blue (Thermo Scientific, USA). Following to 3D sphere formation occurred after 96 hours, different concentrations of Palbociclib were applied to cells and the cells were incubated for 72 hours. As mentioned above, drug-treated and non-treated 10 spheroids were collected and trypsinized 5 minutes to digest the cell-to-cell interaction. Fresh medium was added to stop the activity of Trypsin-EDTA and cells were mixed with Trypan blue at the ratio of 1:10. Cell counting was achieved using the Neubauer haematocytometer. MTT cell viability analysis was also confirmed by the cell counting by Trypan-blue cell exclusion method for 2D cells. All 2D cell lines

were seeded into 48-well plates as $30x10^3$ cells per well and incubated for 24 hours at 37°C in a 5% CO_2 incubator to adhere the cell to the plate. Then, increasing concentrations of Palbociclib were treated to the cells for 72 h. Drug-treated and non-treated cells were collected and trypsinized 5 minutes. Fresh medium was added, and cells were mixed with Trypan blue at the ratio of 1:10. Cell counting was performed. Cell viability analysis was performed according to the ratio of drug-treated cells to untreated control cells whose viability was assumed as 100%

2.6 Cell Cycle Analysis

Cell cycle analysis was performed by flow cytometry (Riccardi & Nicoletti, 2006). After 72-hour exposure of Palbociclib at different concentrations, cells from both 2D and 3D cultures were trypsinized, washed with PBS, fixed in 70% ethanol and kept at -20°C overnight. Fixed cells were collected, washed and resuspended in Dulbecco's Phosphate Buffered Saline (Biological Industries, Israel). After incubation with RNAase, PI and Triton-X solution for 30 min, the DNA content of the cells was analysed using flow cytometer (BD-Accuri-C6, BD Biosciences, USA). Data were analysed by BD Accuri C6 Software.

2.7 Gene Expression Analysis

2.7.1 Total RNA isolation

Total RNA was isolated by the High Pure RNA isolation kit (Roche, Switzerland) according to the manufacturer's instructions. Briefly, the cells were harvested as described above and centrifuged for 5 min at 1000 rpm. Then, the supernatant was removed, the cell pellet was homogenized with 200 µl PBS and protocol was performed. All RNA samples were checked by running the samples on 2% agarose

gel (w/v;). Concentrations of the isolated RNA samples were determined using BioDrop spectrophotometer (BioDrop, UK).

2.7.2 cDNA synthesis

After total RNA isolations, RNAs were further treated by DNase to completely remove the possible DNA contamination. DNase treatment was performed by DNase I (Thermo Fisher Scientific, USA) for 1,5 µg of RNAs at 37ºC for 1 h and the reaction was terminated by adding 1 µl of 50 mM EDTA solution (Thermo Scientific, USA) at 65ºC for 10 min. DNase-treated samples were further treated with 1 µl of 0,2 mg/ml Random Hexamer (Thermo Scientific, USA) at 65ºC for 5 min. cDNA synthesis was carried out by DNase-treated RNA samples and 40 units of M-MuLV Reverse Transcriptase (Thermo Fisher Scientific, USA) according to Table 2.1. The final solutions were used to synthesize cDNAs by incubating at 25ºC for 10 min, 42ºC for 60 min and 70ºC for 10.

Table 2.1 The chemicals and their volumes used to synthesize cDNAs.

Chemicals/Sample	Volume (µl)
DNase-treated RNA samples + Random Hexamer (0.2 µg/µl)	12,5
Buffer Reverse Transcriptase (5X)	4
Dntp (10 mM)	2
Reverse Transcriptase (200 U/µl)	1
RiboLock (40 U/µl)	1
Total	20

2.7.3 Quantitative Reverse Transcriptase PCR (qRT-PCR)

cDNAs were diluted as 1:10 for qRT-PCR studies. Expression levels of related genes (*BCL-2*, *BAX*, *Survivin*, *PUMA*, *CCND1*, *CCNE1*, and *mTOR*) were determined using The LightCycler® 480 SYBR Green I Master (Roche, Germany) according to Table 2.2 and Bio-Rad Connect Real-Time System (Bio-Rad Laboratories Inc., USA). *ACTB* gene was used as an internal control. Primers used for the qRT-PCR studies were given in Table 2.3. To be able to quantify the gene amplification, four standards with serial dilutions (1:2, 1:10, 1:50 and 1:250) of the one of the synthesized cDNA were run for all primer pairs. Each run was performed in triplicates and three independent experiments. The cycles of the qRT-PCR were carried out as 95ºC 3 min and 95ºC 30 sec-60ºC 30 sec-72ºC 30 sec (40 cycles) in the presence of temperature cycle from 50-99ºC for 5 sec/1ºC to obtain melting curve. Melting curves for each primer pairs were checked properly before analysing the data. The fold changes analysed by $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001).

Table 2.2 qRT-PCR mixes.

Solutions	Volume (µl)
The LightCycler® 480 SYBR Green I Master	5
Forward primer (5 µM)	1
Forward primer (5 µM)	1
cDNA (1:10 diluted)	3

Table 2.3 Primer pairs used in the qRT-PCR studies.

Primer Name	Sequences (5'-3')	Product Length	Tm°C (Melting Temperature)
mTOR FP	CTCTGGCATGAGATGTGGCA	284 bp	60
mTOR RP	GGCAGCTGCTTTGAGATTCG		
CCND1 FP	TCTGCGAGGAACAGAAGTGC	281 bp	60
CCND1 RP	GAAATCGTGCGGGGTCATTG		
CCNE1 FP	TGAGCAACACCCTCTTCTGC	134 bp	60
CCNE1 RP	TCGCCATATACCGGTCAAAGA		
ACTB FP	CCAACCGCGAGAAGATGA	97 bp	59
ACTB RP	CCAGAGGCGTACAGGGATAG		
BCL-2 FP	CCCGCGACTCCTGATTCATT	166 bp	60
BCL-2 RP	AGTCTACTTCCTCTGTGATGTTGT		
Survivin FP	AGCCAGATGACGACCCCATAGAGG	60 bp	65
Survivin RP	AAAGGAAAGCGCAACCGGACGA		
BAX FP	TCTGACGGCAACTTCAACTG	188 bp	58
BAX RP	TTGAGGAGTCTCACCCAACC		
MDR-1 FP	ACAGAAAGCGAAGCAGTGGT	62 bp	60
MDR-1 RP	ATGGTGGTCCGACCTTTTC		

2.8 Quantification Spheroid Dimensions by Microscopy

Following 3D sphere formation occurred after 96 hours, increasing (15-100 μM) concentrations of Palbociclib were applied to cells and the cells were incubated for 72 hours. Different 5 spheroid images were taken by Leica DMLB Fluorescence microscopy (Leica, Germany) for each cell line and conditions and vertical and horizontal dimensions were analysed.

CHAPTER 3

RESULTS AND DISCUSSION

The present study aims to decipher the effect of Palbociclib in different subtypes of breast cancer cells under monolayer (2D), and spheroid (3D) cultures, and also to find out the role of Palbociclib as a co-therapeutic agent on drug-resistant cancer cells. Thus, the study was decided to be presented under two subtitles.

3.1 The Effects of Palbociclib on 2D and 3D MCF-7, MDA-MB-231 and SKBR-3 Breast Cancer Cells

3.1.1 Anti-proliferative Effect of Palbociclib on 2D and 3D Cells

Cyclin-dependent kinase 4/6 (CDK4/6) and cyclin D1 are the potential therapeutic targets in breast cancer and Palbociclib is a small molecule agent that inhibits CDK4/6 and causes cell cycle arrest; hence, it is an anti-cancer agent used as single or in combination with other chemotherapeutic agents (Fry et al., 2004). Moreover, Palbociclib has frequently been studied in breast cancer tumour tissues and cell lines (Bollard et al., 2017). On the basis of the receptor status, Palbociclib has been proposed to be critical agent to prevent cell proliferation, especially for receptor-positive breast cancer cells since they promoted the production of cyclin D1 and cell cycle progression through CDK4/6-cyclin D1 interaction via the function of the receptors, estrogen receptor and/or epidermal growth factor receptor (Scott et al., 2017). To further study the functionality of Palbociclib in the breast cancer cell lines with differentiated receptor status under diverse conditions, the anti-proliferative effect of Palbociclib was evaluated in those cell lines. The anti-proliferative effect of the drug was examined on monolayer (2D) and spheroid (3D) cell culture model. 3D

cell culture is a condition where the cellular organizations mimic the tumour tissue by which the results mostly correlates with that from the human specimens; thus, the method supplies a systemic approach *in vitro* (Lv et al., 2017). Therefore, the 3D effect of Palbociclib, for the first time, was followed and compared to that of 2D counterpart in the present study.

In order to determine cell viability in response to different concentrations of Palbociclib on 2D MCF-7, MDA-MB-231 and SKBR-3 cell cultures, Trypan-blue cell viability analysis was performed. The results demonstrated that Palbociclib critically decreased the cell viabilities in all cell lines. The inhibitory concentration 50 (IC_{50}) values were 12 μM for 2D-MCF-7, 23 μM for 2D-MDA-MB-231 and 26 μM for 2D-SKBR-3 cells (Figure 3.1).

According to Finn et al, the subtypes that most sensitive to growth inhibition by Palbociclib were ER-positive cells such as the MCF-7 cell line (Finn et al., 2009). In parallel to previous studies, Palbociclib was most effective against hormone receptor-positive breast cancer cell cells (MCF-7) compared to negative ones (MDA-MB-231 and SKBR-3). 2D-SKBR-3 cell line was the most resistant in response to Palbociclib, pointing possible molecular mechanisms responsible for cell cycle regulation which will be evaluated in the next sections.

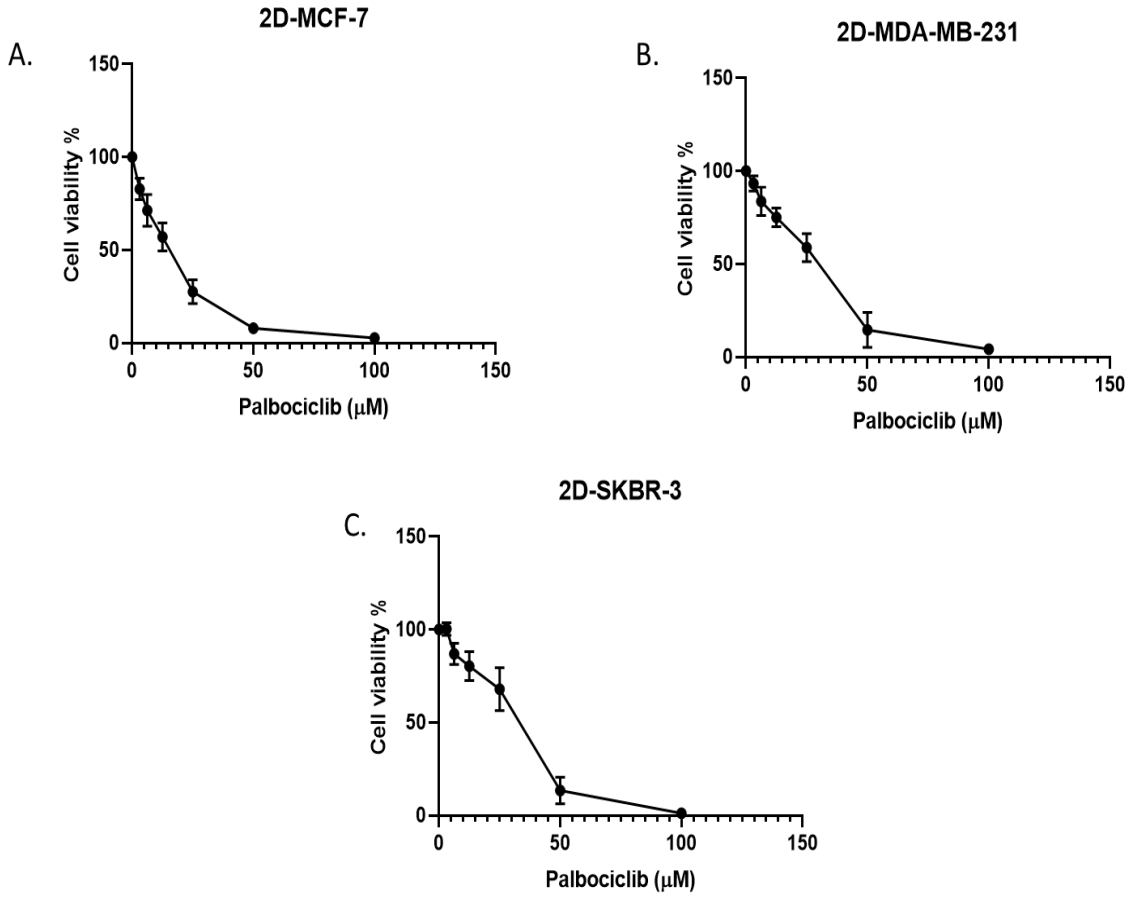

Figure 3.1 The anti-proliferative effect of Palbociclib on A. 2D-MCF-7, B.2D-MDA-MB-231 and C.2D-SKBR-3 cell line. Cells were treated with increasing concentrations of Palbociclib when they were 80% confluent for 72 h. Trypan-blue cell viability analysis was performed. IC_{50} value of Palbociclib was analyzed and compared by non-linear regression.

The critical issue in studying cell proliferation would be the cycle status of the cells when a cell cycle arresting molecule is focused (Gérard and Goldbeter, 2014). In

order to check whether the effect of the Palbociclib on cell viability would increase, cells were treated with Palbociclib after the cell cycle synchronization. The cell cycle synchronization was procured by serum starvation, which was a common method to synchronize different types of cells in G1/G0 (Krek and DeCaprio, 1995).

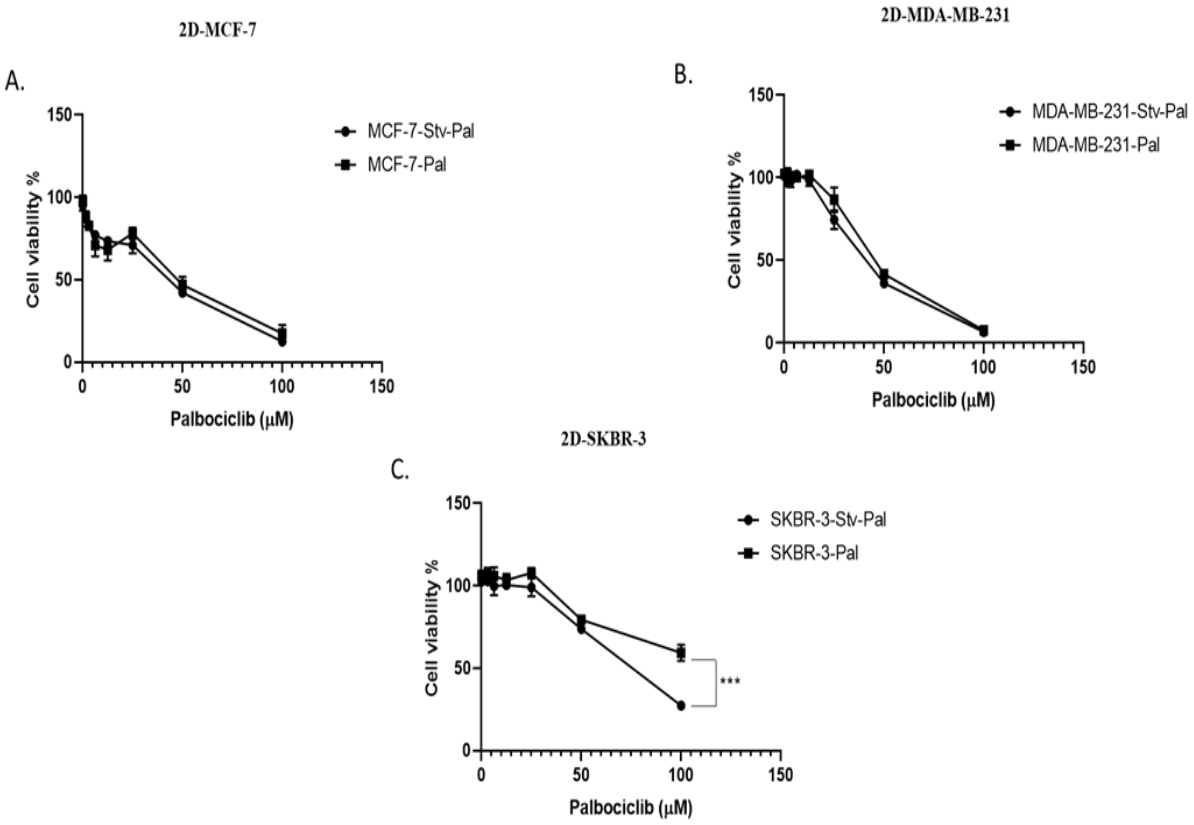

Figure 3.2 The comparison of anti-proliferative effect of Palbociclib after 24 h serum starved and non-starvation conditions on A.2D-MCF-7, B.2D-MDA-MB-231 and C. 2D- SKBR-3 cells. Cells, which were serum starved and non-starved for 24 h, treated with increasing concentrations of Palbociclib for 72 hours. Cell viability was measured using MTT (Sigma Aldrich, USA) assay. IC_{50} value of Palbociclib was analyzed and compared by non-linear regression. ***$p<0.001$

According to the result (Figure 3.2), applying Palbociclib after the serum starvation did not have a different effect on the viabilities of MCF-7 and MDA-MB-231 cell lines. The cell viability of SKBR-3 cells, however, was significantly decreased in the high concentrations compare to non-starvation conditions. The results illustrated that SKBR-3 cells were needed to strictly be at G1 phase to trigger Palbociclib-dependent cell death.

In order to compare the cytotoxic effect of Palbociclib on cells grown in 2D and 3D cell culture, 3D spheroids were formed using the hanging drop method. The cell viability of spheroids was measured by Trypan blue assay which is based on counting cells under the light microscopy. The cytotoxic effect of Palbociclib 3D MCF-7, MDA-MB-231, and SKBR-3 cell cultures was shown in Figure 3.3.

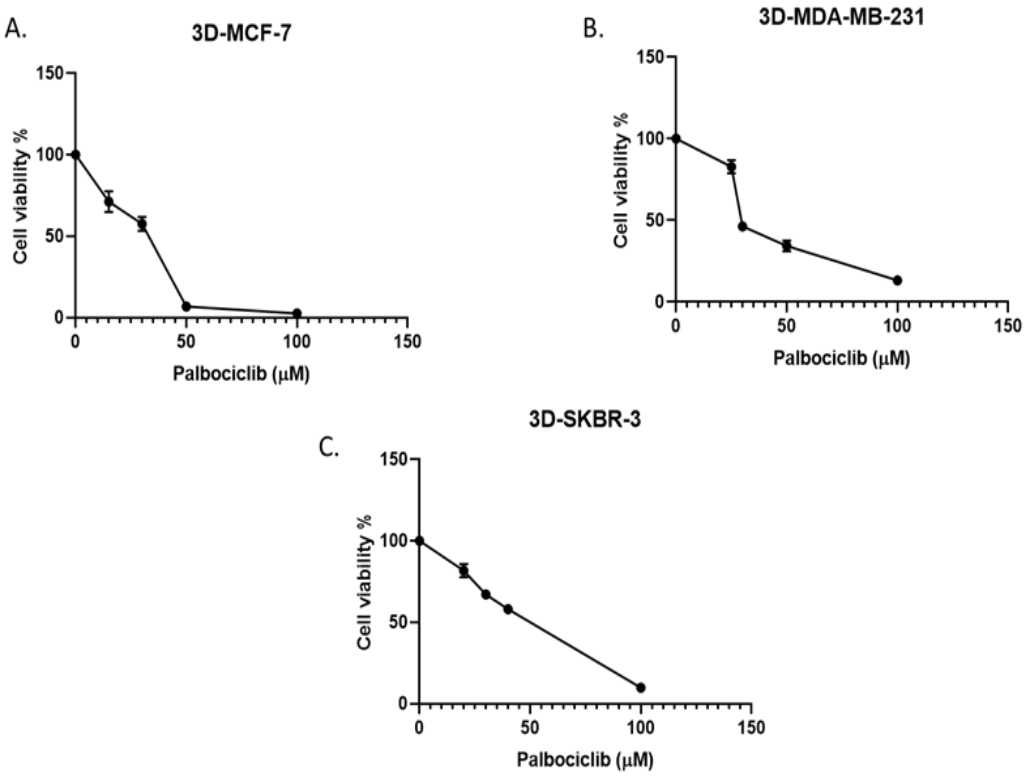

Figure 3.3 The anti-proliferative effect of Palbociclib on A. 3D-MCF-7, B.3D-MDA-MB-231 and C.3D- SKBR-3 cell line. Cells were treated with increasing concentrations of Palbociclib for 72 h when formed spheroid after 96 hours. Trypan-blue cell viability analysis was performed IC_{50} value of Palbociclib was analyzed and compared by non-linear regression.

According to cell viability results (Figure 3.3), the IC_{50} values were increased for MCF-7 and SKBR-3 cell line while there were not any alterations for MDA-MB-231 cells. The IC_{50} values were 32 μM for 3D-MCF-7, 24 μM for 3D-MDA-MB-231 and 43 μM for 3D-SKBR-3 cells. The previous studies showed that MCF-7 and SKBR-3 cell lines can generate 3D spheroid while MDA-MB-231 cells can form only lose aggregate after the four days with the hanging drop method (Ivascu and Kubbies, 2006). Our results were also similar to the literature as shown in Figure 3.4.

Since the MDA-MB-231 cell line cannot form proper and intact spheroid shape, the Palbociclib may exhibit the same effect on cell viability of 3D-MDA-MB-231 spheroid cells.

The averaged IC_{50} values of Palbociclib in 2D and 3D MCF-7, MDA-MB-231 and SKBR-3 cell lines were shown comparatively in Table 3.1. The IC_{50} value of Palbociclib on 3D-MCF7 cells was increased 2.6-fold. Also, the inhibitory concentration 50 of Palbociclib on 3D-SKBR-3 was enhanced 1.6-fold. However, 3D-MDA-MB-231 did not show significant alteration.

Table 3.1 The IC_{50} values of Palbociclib in 2D and 3D MCF-7, MDA-MB-231 and SKBR-3 cell lines.

	IC_{50} Values of Palbociclib	
Cell-Lines	**2D**	**3D**
MCF-7	12±1.28 μM	32±5.6 μM
MDA-MB-231	23±3.29 μM	24±1.8 μM
SKBR-3	26±4.94 μM	43±10.5 μM

In order to examine the effect of the drug on spheroids for all cell lines, spheroid images were taken, and horizontal and vertical dimensions were also measured. Spheroids were treated with Palbociclib in different concentrations (15-100 μM) and the differences according to control was visualized using fluorescence microscopy under the white light. According to the results, 3D MCF-7, MD-MB-231 and SKBR-3 cell lines gave different responses to the different concentrations of Palbociclib.

The molecular mechanisms through diverse gene regulation patterns via cell-to-cell interactions have been shown to be differentiated in 3D spheroids compared to 2D cells (Lv et al., 2017). The spheroids have a three-layer structure: the inner layer is composed of necrotic cells; the inner layer surrounded by quiescent viable cell zone; and finally, the outer layer creates the proliferating zone (Lin and Chang, 2008). When MCF-7 and SKBR-3 spheroids were treated with Palbociclib, the outer layer, which was composed of the proliferating cell, was assumed to be more exposed to the drug at the lower concentration of Palbociclib 15μM and 20 μM, respectively (Figure 3.4 A. and C.). In addition, the lower concentrations of Palbociclib were chosen according to 2D IC_{50} values. As shown in Table 3.2, vertical and horizontal dimensions of 15 μM and 20 μM Palbociclib-treated 3D-MCF-7 and 3D-SKBR-3 cells were decreased compared to control. Although the size of the spheroids was reduced, they still had a compact spheroid shape, hence, the drug may not reach to the quiescent viable zone. Therefore, the viability of spheroids might be high in the lower concentration of Palbociclib (Figure 3.3 A. and C.). However, when the highest concentration of Palbociclib, 100 μM was administered to all cell lines, it was showed a high toxic effect and spheroids formation was dispersed (Figure 3.3 and 3.4). Increasing Palbociclib concentrations (30, 40 and 50 μM) led to the increasing size of spheroids in all cell lines (Table 3.1). This effect was thought to be due to increased diffusion of the drug into the spheroid. The results showed that the cells generating proper spheroid form were less sensitive to Palbociclib than 2D cell cultures, suggested that the traditional cell culture method was insufficient to show the exact effect of anticancer drugs *in vitro* as proposed previously (Melissaridou et al., 2019). This chemoresistance in tumor spheroids could be based upon several factors including the slower proliferation rate of spheroids, the limitation in the diffusion of anti-cancer drugs and increased level of hypoxia in the core zone of the spheroids (Zhao et al., 2019). All in all, these results demonstrated the efficacy of Palbociclib in 3D spheroids of different subtype breast cancer cells compared to 2D cells, for the first time.

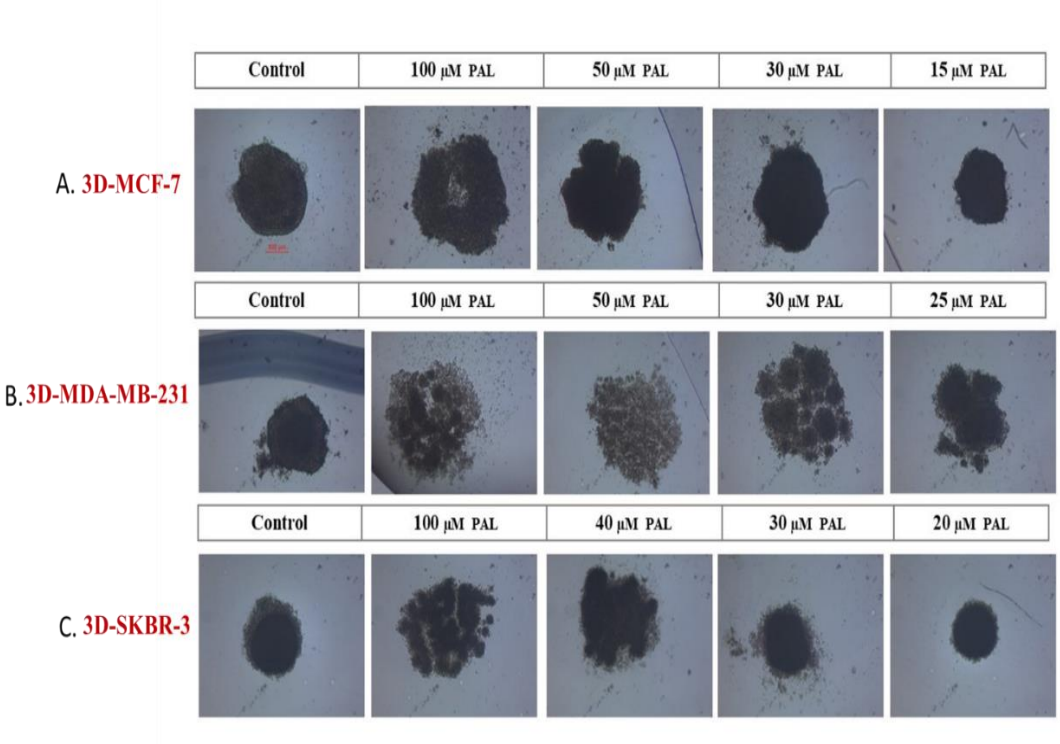

Figure 3.4 The microscopy images of spheroids A.3D-MCF-7, B.3D-MDA-MB-231 and C.3D- SKBR-3 in response to different concentrations of Palbociclib. 3D spheroids were formed by hanging drop method for 4 days and the spheroids were treated with increasing concentrations of Palbociclib 72 hours. Images were taken by Leica DMLB Fluorescence microscopy (Leica, Germany).

Table 3.2 The vertical and horizontal dimensions for increasing concentrations of Palbociclib-treated MCF-7, MDA-MB-231 and SKBR-3 spheroids (n=5).

3D-MCF-7	Cont	100µM Pal	50µM Pal	30µM Pal	15µM Pal
Horizontal	1526µm±85µm	1560µm±99µm	1832µm±129µm	1640µm±78µm	1300µm±129µm
Vertical	1495µm±43µm	1630µm±155µm	1613µm±55µm	1463µm±106µm	1337µm±66µm

3D-MDA-MB-231	Cont	100µM Pal	50µM Pal	30µM Pal	25µM Pal
Horizontal	2240µm±98µm	2030µm±70µm	1802µm±227µm	1766µm±70µm	1795µm±388µm
Vertical	2270µm±42µm	2036µm±40µm	1902µm±340µm	1963µm±30µm	1725µm±106µm

3D-SKBR-3	Cont	100µM Pal	40µM Pal	30µM Pal	20µM Pal
Horizontal	1170µm±104µm	1765µm±180µm	1425µm±205µm	1206µm±49µm	995µm±77µm
Vertical	1190µm±70µm	1672µm±222µm	1400µm±209µm	1256µm±60µm	1120µm±14µm

3.1.2 The Effect of Palbociclib on Cell Cycle in 2D and 3D Cultures of MCF-7, MDA-MB-231, SKBR-3 Cells

Palbociclib inhibits the G1-S phase transition in the cell cycle by inhibiting cyclin-dependent kinases 4 and 6 and prevents the proliferation of cancer cells (Finn et al., 2009). The role of Palbociclib in the blocking of G1-to-S progression has widely been studied in the literature (Pernas et al., 2018). By this function, Palbociclib displays anti-proliferative function as illustrated above. The cell cycle was shown to be diversely regulated in 3D spheroids compared to 2D cells (Laurent et al., 2013). Thus, even the mechanism of the drug would be expected, the cell cycle distribution of the cells treated with Palbociclib was followed to see whether there could be differences between 2D and 3D cells.

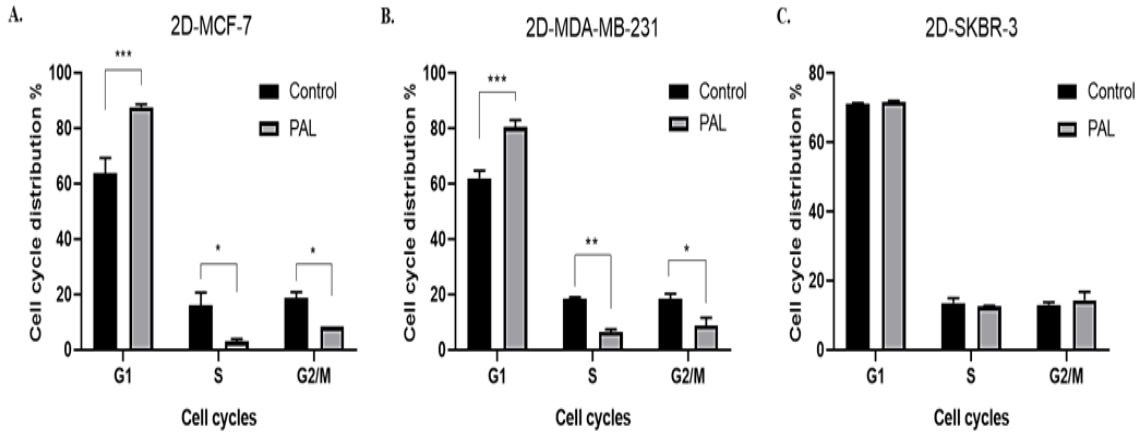

Figure 3.5 According to flow cytometry analysis, cell cycle distribution of A. 2D MCF-7 B. 2D- MDA-MB-231 and C. 2D-SKBR-3 cells treated with 1.5, 2.5 and 2 μM Palbociclib, respectively. Multiple comparison analysis was performed by student t-test. n=3; * = $p<0.05$; ** = $p<0.01$ and *** = $p<0.001$.

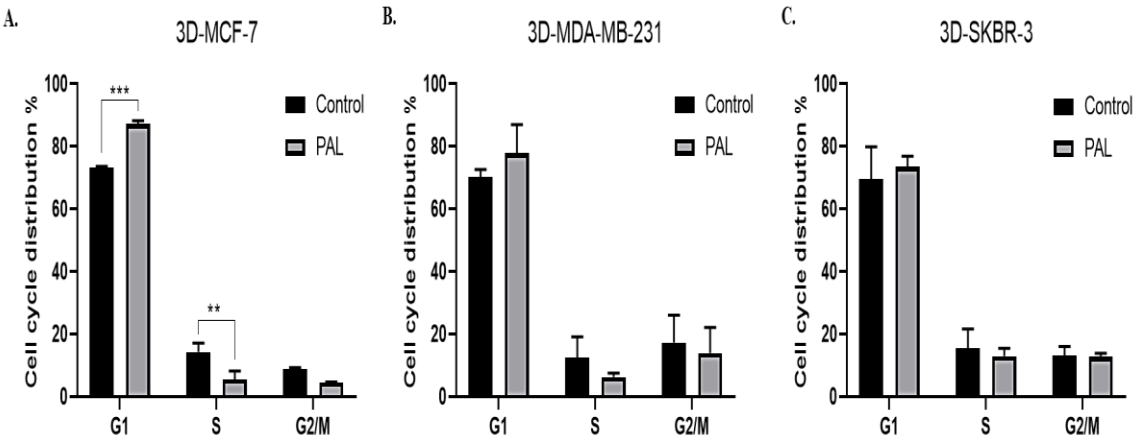

Figure 3.6 According to flow cytometry analysis, cell cycle distribution of A. 3D MCF-7, B. 3D- MDA-MB-231 and C. 3D-SKBR-3 cells treated with 1.5, 2.5 and 2 µM Palbociclib, respectively. Multiple comparison analysis was performed by student t-test. n=3; * = $p<0.05$; ** = $p<0.01$ and *** = $p<0.001$.

According to the cell cycle analysis of 2D cell cultures, G1 arrest was observed in 2D-MCF-7, MDA-MB-231 whereas 2D-SKBR-3 cells were unaffected after 72 hours of exposure to minimum lethal concentration of Palbociclib (1.5µM for MCF-7, 2.5 µM for MDA-MB-231 and 2 µM for SKBR-3; Figure 3.5). This can be due to the high-level expression of *CCNE1* in SKBR-3 (Figure 3.8 G.). It has been demonstrated that over-expression of cyclin E can overcome the effect of Palbociclib (Witkiewicz et al., 2014). Minimum lethal concentration of Palbociclib was used for all cell lines in order to detect G1 arrest. It is known that treatment with Palbociclib at concentrations of 1 µM or less caused reversible G1 arrest (Vijayaraghavan et al., 2017). In order to find out the effect of Palbociclib on 3D cell lines, the same concentrations were applied to the spheroids and cell cycle distributions were analyzed. G1 arrest only was observed in 3D-MCF-7 cells (Figure 3.6). 3D-MDA-MB-231 and 3D-SKBR-3 cells were unaffected statistically. These data showed that 2D and 3D MCF-7, MDA-MB-231 and SKBR-3 cells were showed the same cell cycle distribution tendency.

3.1.3 The *CCND1*, *CCNE1* and *mTOR* Expression Analysis in Palbociclib treated 2D and 3D Cultures of MCF-7, MA-MB-231 and SKBR-3 cell lines

When the mitogenic signals trigger cell proliferation, the cell cycle mechanism begins to work. Firstly, cyclin-D and CDK4/6 activation that is essential for the entrance into the G1 phase occurred. Then, retinoblastoma (Rb) and related proteins are phosphorylated, and this causes the release of E2F transcription factors which is essential for transcription of target genes such as cyclin E, cyclin A, CDK2 and CDK1. Subsequently, cyclin E activates the Cdk2 and Rb hyper-phosphorylation is completed. Induction into the S-phase entry and starting of DNA replication are performed (Weinberg, 1996).

The cells need enough nutrients and hormones in order to proliferate and grow. The connection between cell growth and proliferation is necessary for cell cycle progression and cell survival since incoherent signals can promote cell death or senescence. Senescence is an active arrest that occurred advanced points of G1, G1/S, and even G2 (Blagosklonny, 2011). The mTOR has a crucial role in cell metabolism and is affected by intracellular and extracellular signals; hence, it is thought that mTOR is the main player in the regulation of cell growth and division. Cell cycle arrest at the G1 phase happens when the mTOR activity was inhibited by rapamycin or nutrient starvation (Cuyàs et al., 2014). There are many pathways in the cell that provide mitogenic signaling such as PI3K/AKT/mTOR pathway, MAPK and ERs. These pathways can connect to cyclin D and CDK4/6 interaction (Niu et al., 2019) . Therefore, studying the expressions of *CCND1*, *CCNE1* and *mTOR* genes were important to understand the Palbociclib mechanism of action.

When the 2D-MCF-7 cells treated with Palbociclib, the expression of *CCND1* and *mTOR* genes were upregulated compared to the non-treated control. Approximately, a 2.1-fold increase in expression of the *CCND1* gene, 1.6-fold increase in expression of the *mTOR* gene were found. The expression of *CCNE1* did not show any

significant changes after Palbociclib treatment (Figure 3.7 A., B. and C.) Elevated expression of *CCND1* was a common feature of Palbociclib (Maria Teresa Herrera-Abreu et al., 2016). Palbociclib treatment in 2D-MDA-MB-231 cells did not alter *CCND1, CCNE1* and *mTOR* genes expression compared to the untreated control, statistically (Figure 3.7 D., E. and F.). The *CCND1* and *CCNE1* genes expression were upregulated more than 2-fold, the *mTOR* gene was upregulated 1.5-fold in Palbociclib treated 2D-SKBR-3 cells (Figure 3.7 G., H. and I.).

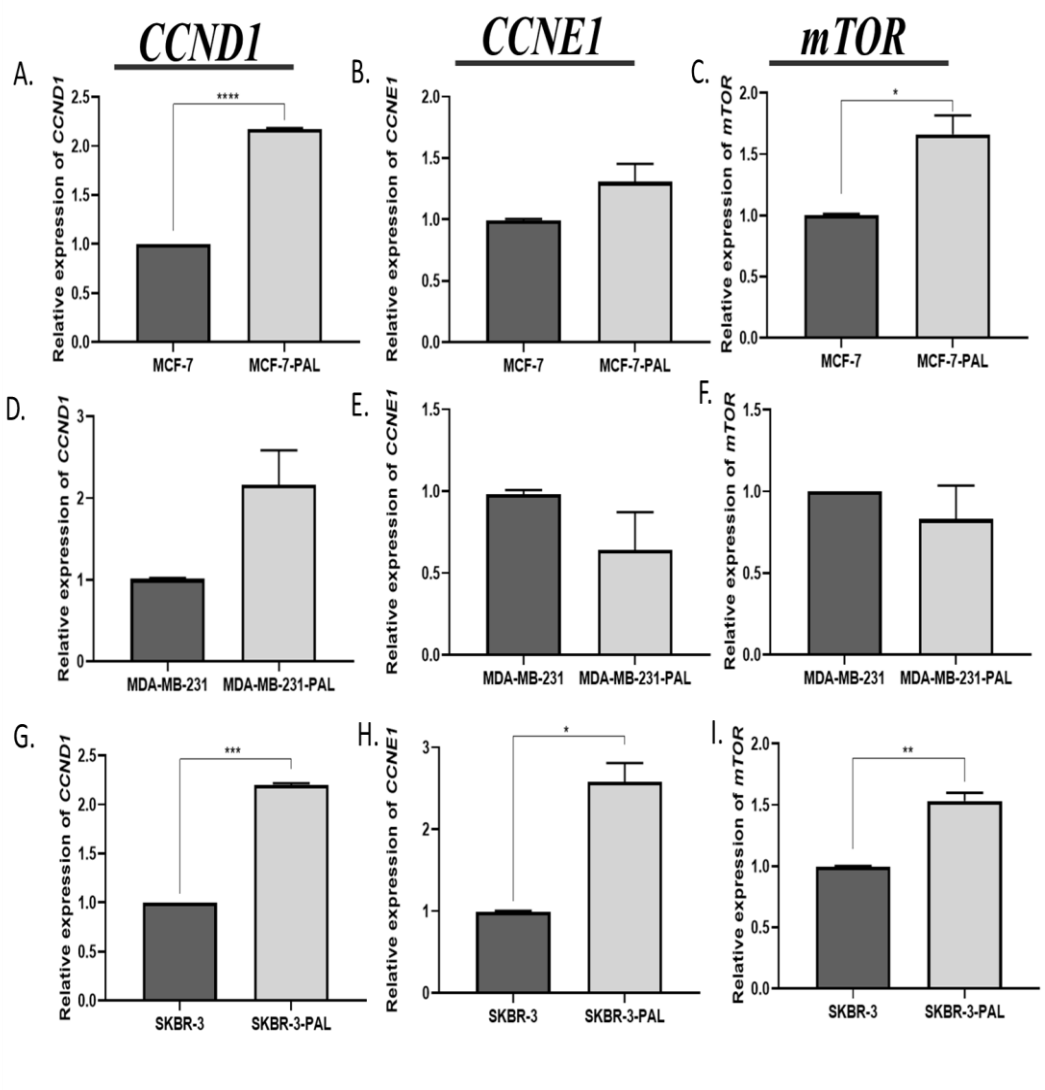

Figure 3.7 Expression of *CCND1*, *CCNE1* and *mTOR* genes in 2D-MCF-7, MDA-MB-231 and SKBR-3 cells. Expressions of cell cycle related genes were determined using The LightCycler® 480 SYBR Green I Master. *ACTB* was used as internal control. Fold changes were calculated by $2^{-\Delta\Delta Ct}$ method (n=3; * = $p<0.05$, ** = $p<0.01$; *** = $p<0.001$).

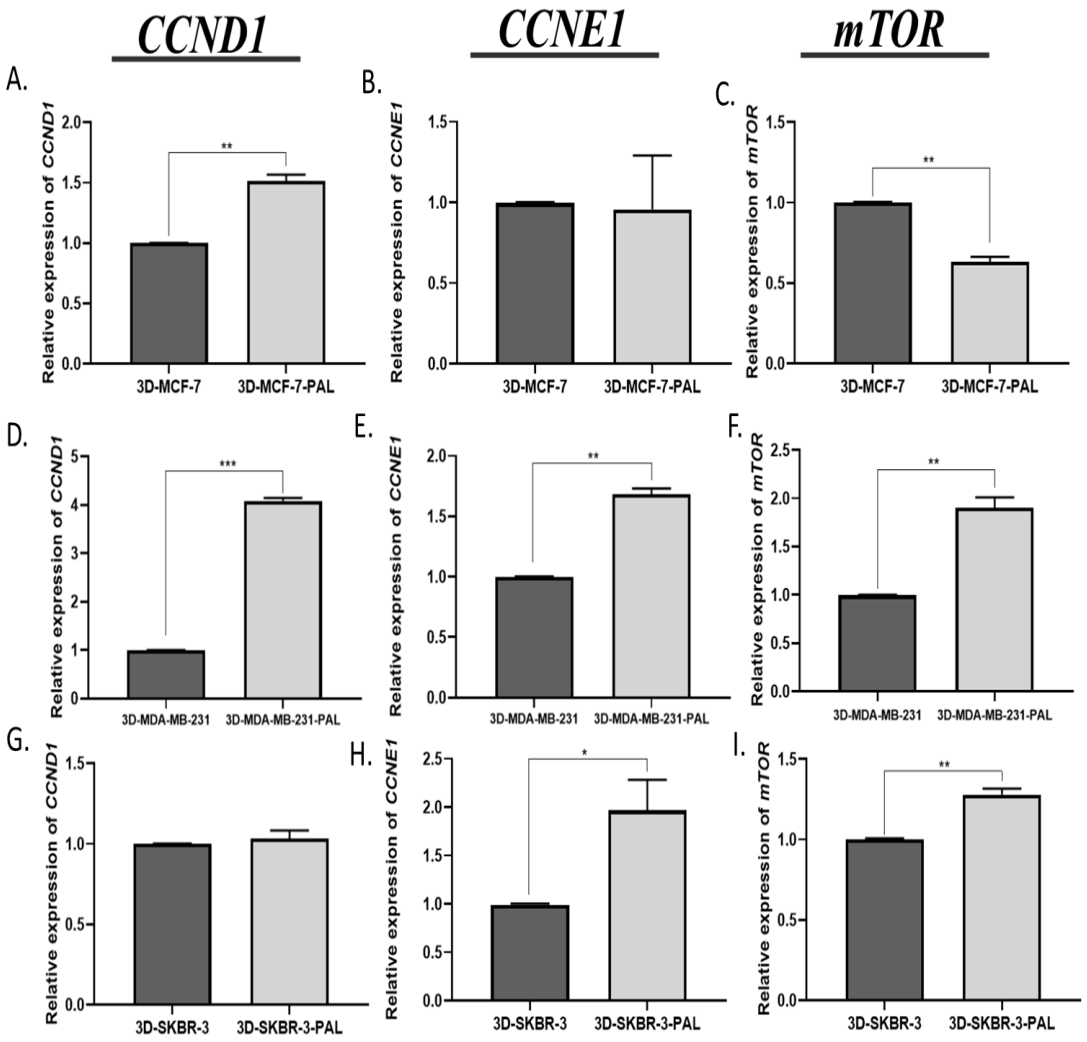

Figure 3.8 Expression of *CCND1*, *CCNE1* and *mTOR* genes in 3D-MCF-7, MDA-MB-231 and SKBR-3 cells. Expressions of cell cycle-related genes were determined using The LightCycler® 480 SYBR Green I Master. *ACTB* was used as an internal control. Fold changes were calculated by $2^{-\Delta\Delta Ct}$ method (n=9; *p<0.05, **p<0.01; ***p<0.001).

In order to compare the effect of Palbociclib in 2D and 3D cell lines, the expression of *CCND1*, *CCNE1* and *mTOR* genes were determined in 3D-MCF-7, MDA-MB-

231 and SKBR-3 cells. In the Palbociclib-treated 3D-MCF-7 cells, *CCND1* gene expression was upregulated whereas the *mTOR* gene was downregulated, *CCNE1* gene did not exhibit any significant change compared to control (Figure 3.8 A., B. and C.). In the Palbociclib-treated 3D-MDA-MB-231 cells, *CCND1* gene expression was increased. Additionally, *CCNE1* and *mTOR* genes were upregulated in Palbociclib-treated 3D-MDA-MB-231 cells compared to control (Figure 3.8 D., E. and F.). In Palbociclib-treated 3D-SKBR-3 cells, the *CCNE1* and *mTOR* genes expression were upregulated whereas *CCND1* gene expression did not show significant changes compared to the non-treated control. (Figure 3.8 G., H. and I.).

The expression of CCND1 has previously been correlated to the expression of mTOR for nasopharyngeal carcinoma (Huang et al., 2009). Thus, G1 arrest via Palbociclib may trigger the expression of mTOR in addition to CCND1 in the cells in which expressions of both CCND1 and mTOR increases. However, this could be cell-specific as seen for 3D-MCF-7 and SKBR-3 cells.

3.1.4 The Expression Analysis of Apoptosis-Related Genes, *BCL-2, BAX and SURVIVIN* Expression in Palbociclib-treated 2D and 3D MCF-7, MA-MB-231 and SKBR-3 cell lines

Apoptosis is a kind of cell death mechanism involved in a variety of biological events such as developmental process, homeostasis, and the eliminating of unwanted cells. Deterioration of the regulation of apoptosis might result in various diseases, including cancer. Bcl-2 was initially defined as an oncogene involved in human follicular lymphoma B cell origin. In1988, the anti-apoptotic activity of this protein was found. Then, Bcl-2 was demonstrated to have the ability to prevent apoptosis and enhancing proliferation through different stimuli, including serum deprivation, heat shock, and therapeutic agents (Tsujimoto, 1998).

The cancer cells need a high level of Bcl-2 activity to survive and inhibit apoptosis by blocking the activation of pro-apoptotic Bcl-2 family proteins such as Bax and

Bak (Brunelle and Letai, 2009). Upregulation of BCL-2 is correlated with decreased levels of caspase activation and cytochrome c release, enhanced carcinogenesis, inhibition autophagy and opposition to apoptosis (Czabotar et al, 2014)

The Bax is found in the cytosol. When the cells activate apoptosis, a conformational change in the Bax protein occurred, and the oligomeric form of Bax is integrated into the mitochondrial membrane. This causes loss of membrane potential and the releasing cytochrome c into the cytoplasm. Hence, elevated BAX expression is mainly associated with apoptosis (Finucane et al., 1999).

Survivin is necessary for the regulation of cell cycle and suppressing cell death. The major function of Survivin protein is to inhibit apoptosis (Humphry and Wheatley, 2018). In addition, overexpression of *SURVIVIN* causes resistance to chemotherapy (Zaffaroni and Daidone, 2002).

In order to find the role of Palbociclib on the expressions of pro- and/or anti-apoptotic genes, *BAX*, *BCL-2* and *SURVIVIN* were checked and compared between 2D and 3D cells. The results showed that in the Palbociclib-treated 2D-MCF-7 cells, the pro-apoptotic gene *BAX* expression was upregulated while anti-apoptotic *SURVIVIN* gene was 5-fold downregulated, and the other anti-apoptotic gene *BCL-2* did not show significant alteration (Figure 3.9 A., B. and C.).

After Palbociclib treatment, 2D-MDA-MB-231 cells displayed 1.6-fold upregulation in *BAX* expression and 1.5-fold downregulation in *BCL-2* expression. Also, the anti-apoptotic gene *SURVIVIN* significantly downregulated (34-fold) in Palbociclib treated 2D-MDA-MB-231 cells compared to control (Figure 3.9 D., E. and F.).

In 2D-SKBR-3 cells, Palbociclib treatment caused 3-fold upregulation of pro-apoptotic *BAX* gene, and 2-fold upregulation anti-apoptotic *SURVIVIN* gene compared to their controls. The *BCL-2* expression did not display significant differences after Palbociclib treatment compared to control (Figure 3.9 G., H. and I.).

This data showed that 2D- MCF-7, MDA-MB-231, and SKBR-3 cell lines display different attitudes after Palbociclib treatment compared to their control. The previous studies showed that the attenuation in the *BCL-2/BAX* ratio is a sign of induced apoptosis. The Palbociclib treatment in the all cell line led to decrease *BCL-2/BAX* ratio. In addition, the *SURVIVIN* gene whose the main function is to inhibit apoptosis was highly downregulated in 2D-MCF-7 and 2D-MDA-MB-231 cell lines. However, in the 2D-SKBR-3 cell, the anti-apoptotic *SURVIVIN* gene was upregulated. This may be due to the fact that the SKBR-3 was the most Palbociclib resistant cell line and also SKBR-3 cell had active *MDR-1* gene regulation.

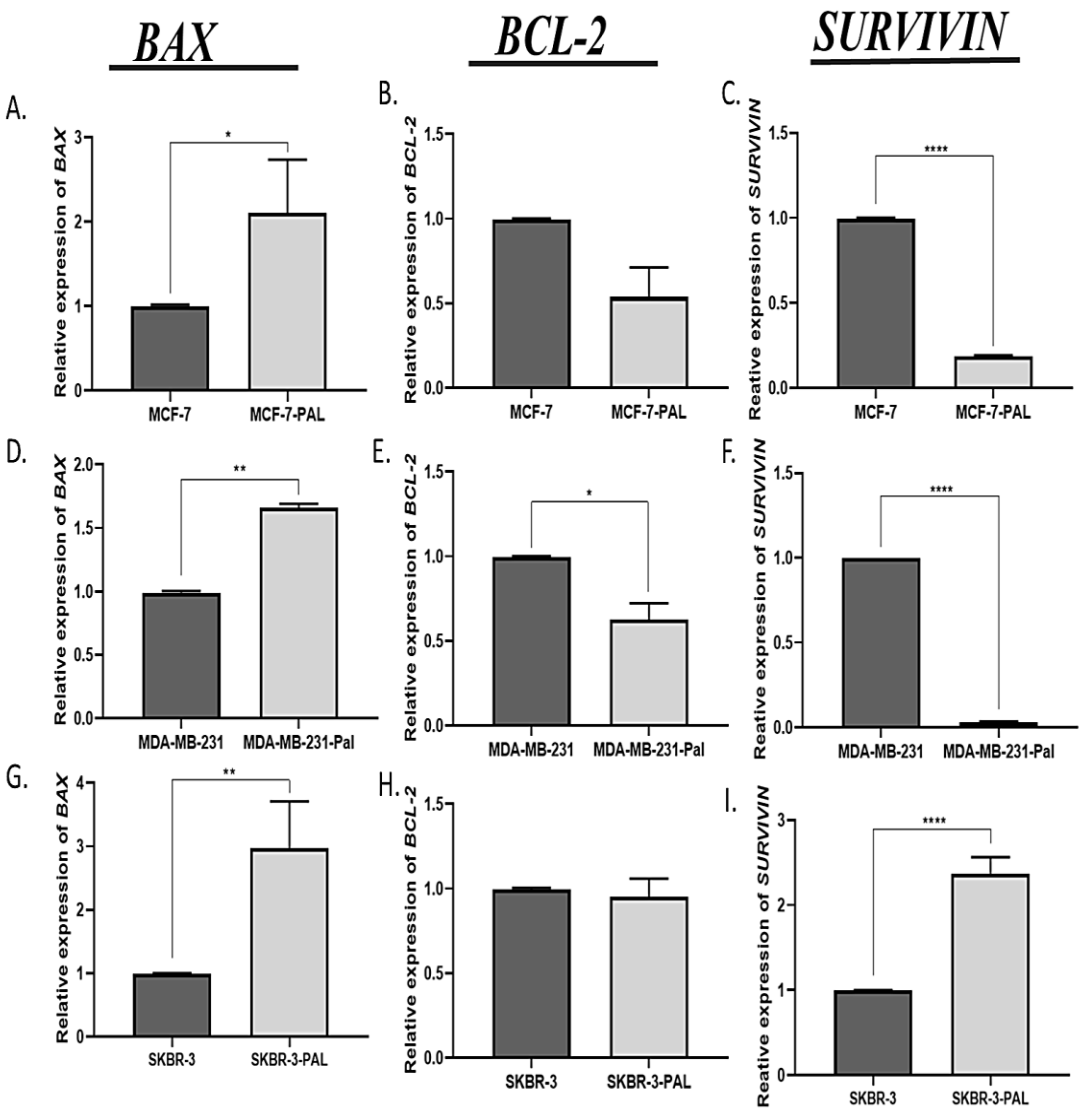

Figure 3.9 Expression of *BAX, BCL-2* and *SURVIVIN* genes in 2D-MCF-7, MDA-MB-231 and SKBR-3 cells. Expressions of apoptosis related genes were determined using The LightCycler® 480 SYBR Green I Master. *ACTB* was used as internal control. Fold changes were calculated by $2^{-\Delta\Delta Ct}$ method (n=9; * = $p<0.05$, ** = $p<0.01$; *** = $p<0.001$).

As seen in Figure 3.10, the expressions of apoptosis-related genes also were determined in 3D-MCF-7, MDA-MB-231 and SKBR-3 cells. After treatment with Palbociclib in 3D-MCF-7, the pro-apoptotic *BAX* expression was 2-fold upregulated and anti-apoptotic *BCL-2* expression was 2-fold down-regulated which was similar to that of 2D-MCF-7 cell line. However, *SURVIVIN* expression was 20-fold decreased compared to control (Figure 3.10 A., B. and C.). Interestingly, in 3D-MDA-MB-231 cells, *BAX* expression was 2-fold downregulated while *BCL-2* expression was 4-fold upregulated. However, *SURVIVIN* expression was significantly (40-fold) downregulated in 3D-MDA-MB-231 cells after Palbociclib treatment (Figure 3.10 D., E. and F.). In the 3D-SKBR-3 cell line, *BAX* and *BCL2* expressions were upregulated. Unlike 2D-SKBR-3 cells, the *SURVIVIN* expression was 2.5-fol downregulated after Palbociclib treatment (Figure 3.10 G., H. and I.).

The results demonstrated that 3D-MCF-7, MDA-MB-231 and SKBR-3 cells displayed different behavior in response to Palbociclib treatment compared to their controls. Although *BCL-2/BAX* ratio was increased 3D-MDA-MB-231 and 3D-SKBR-3 cells, the tendency to cell death was also observed in each 3D cell line due to the downregulation of *SURVIVIN* gene. All in all, results showed that diverse culture methods of the same cell lines caused different responses to the Palbociclib.

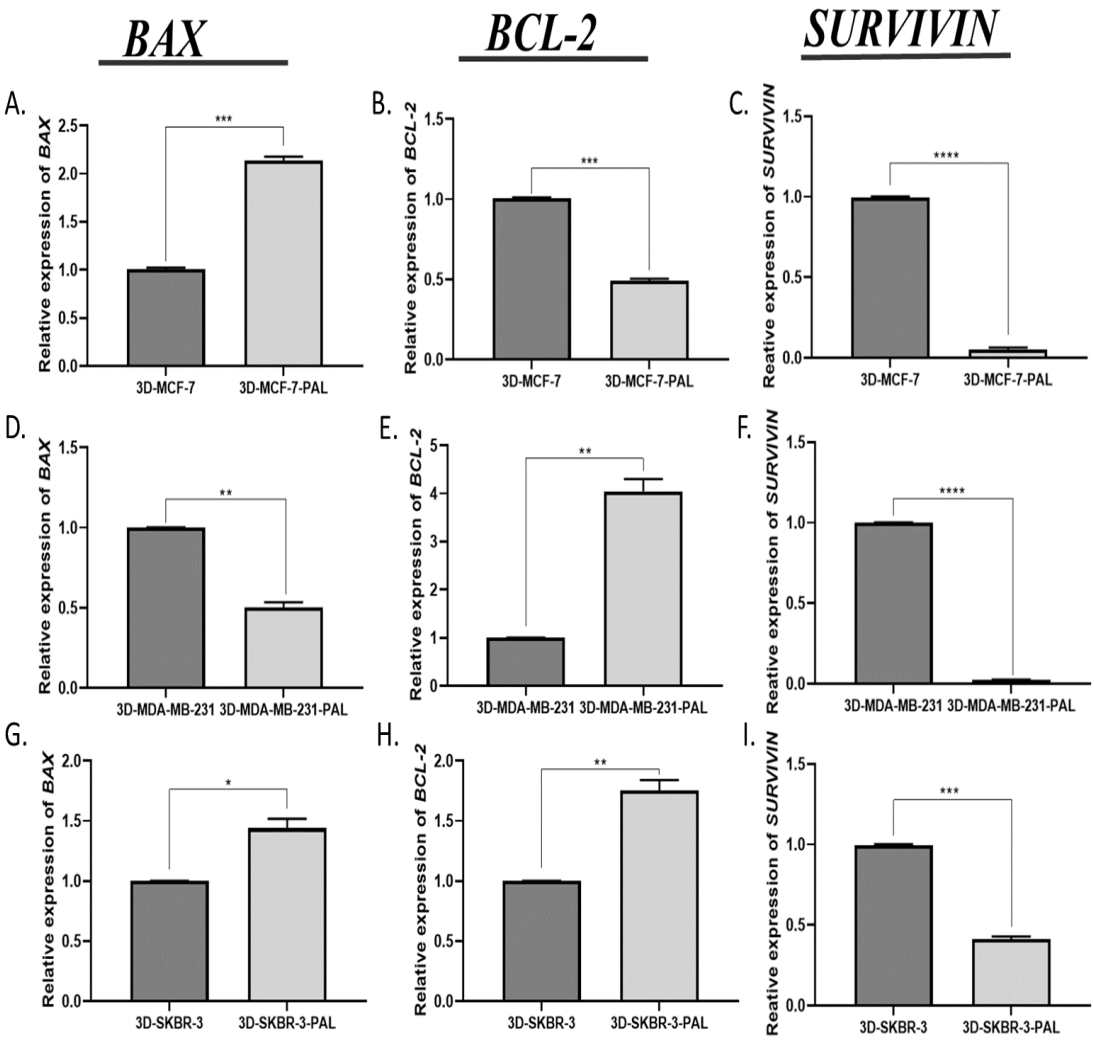

Figure 3.10 Expression of *BAX*, *BCL-2* and *SURVIVIN* genes in 3D-MCF-7, MDA-MB-231 and SKBR-3 cells. Expressions of apoptosis related genes were determined using The LightCycler® 480 SYBR Green I Master. *ACTB* was used as internal control. Fold changes were calculated by $2^{-\Delta\Delta Ct}$ method (n=9; * = $p<0.05$, ** = $p<0.01$; *** = $p<0.001$)

3.2 The Role of Palbociclib as a Co-Therapeutic Agent on Drug-Resistant Cells

3.2.1 The Anti-proliferative Effect of Palbociclib on Doxorubicin Resistance and Sensitive Cancer Cell Lines

As mentioned above, Palbociclib is a selective dual inhibitor of cyclin-dependent kinases (CDK) 4 and 6 and was improved by Pfizer for the treatment of cancer. CDKs are the crucial organizer of cell cycle entry and progression in response to mitogenic signals, so inhibition of these kinases with Palbociclib could promote the activity of other anticancer agents (Dhillon, 2015). Moreover, the combination of Palbociclib is common in the literature. Although the effectiveness and functionality of Palbociclib have been extensively studied on the different cancer cells, there are very few studies about the effects of Palbociclib on drug-resistant cancer cells. In this part, the impact of Palbociclib on drug-resistant cells was evaluated.

In order to determine cell viability in response to increasing concentrations of Palbociclib on Doxorubicin-resistant MCF-7, HeLa and K562 cells and their drug-sensitive parents, MTT cell viability analysis was performed. As seen in Figure 3.11, increasing concentrations of Palbociclib critically decreased the cell viabilities in Doxorubicin-resistant MCF-7 breast cancer, HeLa cervical cancer, and K562 chronic myeloid leukemia cells. Furthermore, Doxorubicin resistant cells and their drug-sensitive parents were compared. When compared to their drug-sensitive parents, MCF-7/DOX and K562/DOX cells had higher IC_{50} values. As mentioned in Figure 3.1, the effect of increasing concentrations Palbociclib treatment on the three types of drug-sensitive breast cancer cell lines was shown.

According to viability results, the highest nonlethal Palbociclib concentration was selected for each cell line, and selected concentrations were combined with Doxorubicin. The selected Palbociclib concentrations for each cell lines were shown in Table 3.3.

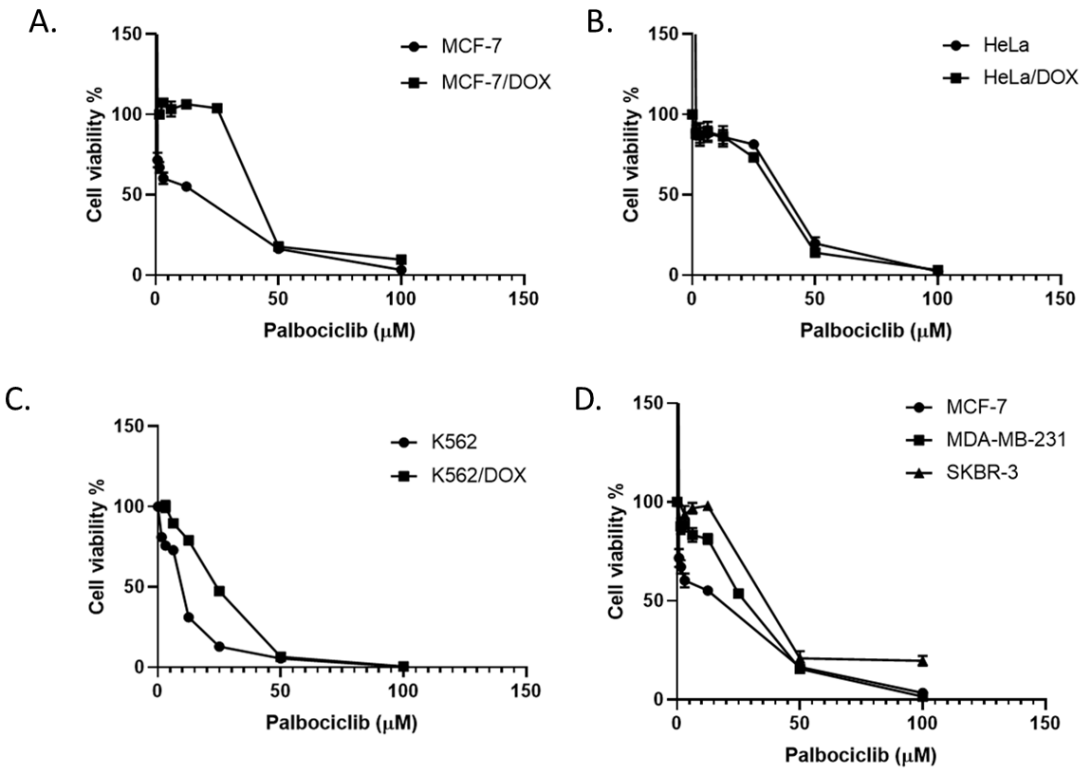

Figure 3.11 The anti-proliferative effect of Palbociclib on A. MCF-7 and Doxorubicin resistant MCF-7, B. HeLa and Doxorubicin resistant HeLa and C. K562 and Doxorubicin resistant K562 D.MCF-7, MDA-MB-231 and SKBR different breast cancer cell lines. Cells were treated with increasing concentrations of Palbociclib for 72 h. Cell viability was measured using MTT (Sigma Aldrich, USA) assay. IC_{50} value of Palbociclib was analyzed and compared by non-linear regression.

Table 3.3 The selected Palbociclib concentrations combined with Doxorubicin.

Cell-Lines	Concentration of Palbociclib
MCF-7/DOX	25 μM
HeLa/DOX	6.25 μM
K562/DOX	6.25 μM
SKBR-3	6 μM
MCF-7	3 μM
MDA-MB-231	3 μM
HeLa	6 μM
K562	2 μM

3.2.2 The Effect of Palbociclib as a Co-Therapeutic Agent on Doxorubicin Resistance and Sensitive Cell Viability

Although the treatment of certain malignancies with chemotherapeutic agents has been successful and promising, the efficacy has been limited by drug resistance. Furthermore, many tumor types are naturally resistant to several cytotoxic agents that are used in cancer treatment. Some tumors are initially sensitive to the drug but gain resistance to drugs over time. Due to the critical problem of clinical drug resistance, much effort has been expended to advance our understanding of the mechanisms of drug resistance in cancer cells (Tsuruo et al., 2003). Doxorubicin is

a cytotoxic anthracycline antibiotic and used for many types of cancer treatment. Doxorubicin binds DNA by intercalation between base pairs and blocks the activation of topoisomerase II (Weiss, 1992). In this study, we firstly showed the activity of Palbociclib combined Doxorubicin on Doxorubicin-resistant cell lines originated from different sources and drug-sensitive cell lines.

According to Figure 3.12, the result demonstrated that when the Dox-resistant cells treated with Palbociclib, the effect of Doxorubicin on cell viability significantly increased in Doxorubicin (DOX)-resistant MCF7, HeLa, K562 cell lines, compared to their parental cell. Interestingly, Doxorubicin combined with Palbociclib also more effective on cell viability for SKBR-3 cells compared to Doxorubicin monotherapy. On the other hand, the combination of Palbociclib with Doxorubicin did not show any difference in cell viability in drug-sensitive MCF-7, MDA-MB-231, HeLa and K562 cell lines compared to only Doxorubicin (Figure 3.13).

The averaged IC_{50} values of Doxorubicin and Palbociclib-combined Doxorubicin in Doxorubicin resistant and sensitive cell lines were also compared and summarized in Table 3.4. When the Doxorubicin applied with Palbociclib, the IC_{50} value of Doxorubicin was 28-fold decreased in MCF/DOX cells. Additionally, the IC_{50} value of Doxorubicin was 5-fold decreased in HeLa/DOX and SKBR-3 cells, when the cells treated with Palbociclib. Also, the inhibitory concentration 50 value of Doxorubicin was 7.5-fold diminished in K562/DOX cells, when the Doxorubicin applied with Palbociclib. On the other hand, the IC_{50} value of Doxorubicin in Doxorubicin sensitive cell lines, MCF-7, MDA-MB-231, HeLa and K562 did not show significant alteration in cell viability with Palbociclib.

These results indicated that Palbociclib re-sensitized different types of Doxorubicin-resistant cancer cells. Furthermore, this effect was also observed in SKBR-3 cells which had an active *MDR-1* gene (Faneyte et al., 2001). As a result, these data can be demonstrated that Palbociclib promoted the effect of Doxorubicin in P-gp-expressing cells.

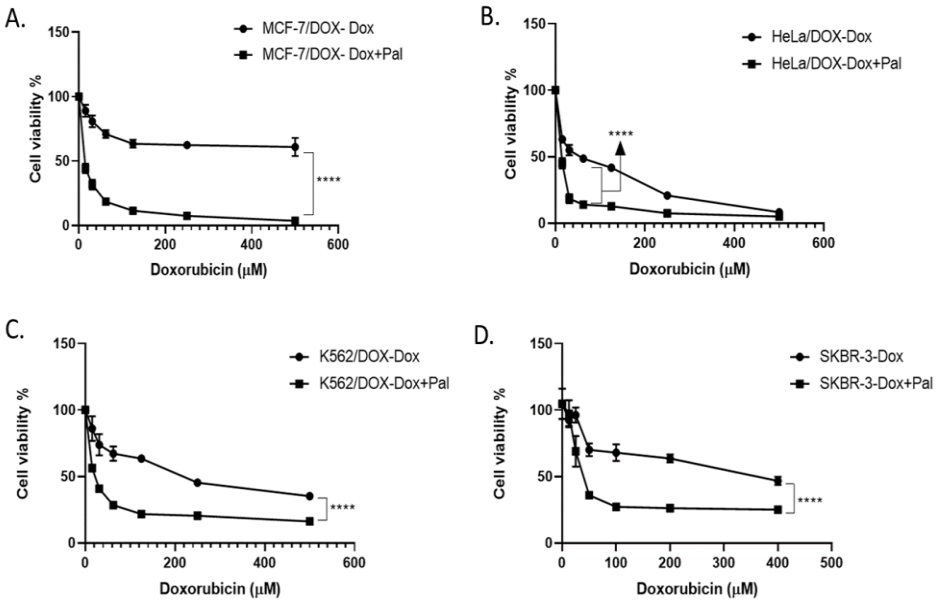

Figure 3.12 The comparison of only Doxorubicin and Palbociclib combined Doxorubicin effect on cell viability in A. Doxorubicin resistant MCF-7, B. Doxorubicin resistant HeLa C. Doxorubicin resistant K562 D. SKBR-3 cell lines. ****p<0.0001.

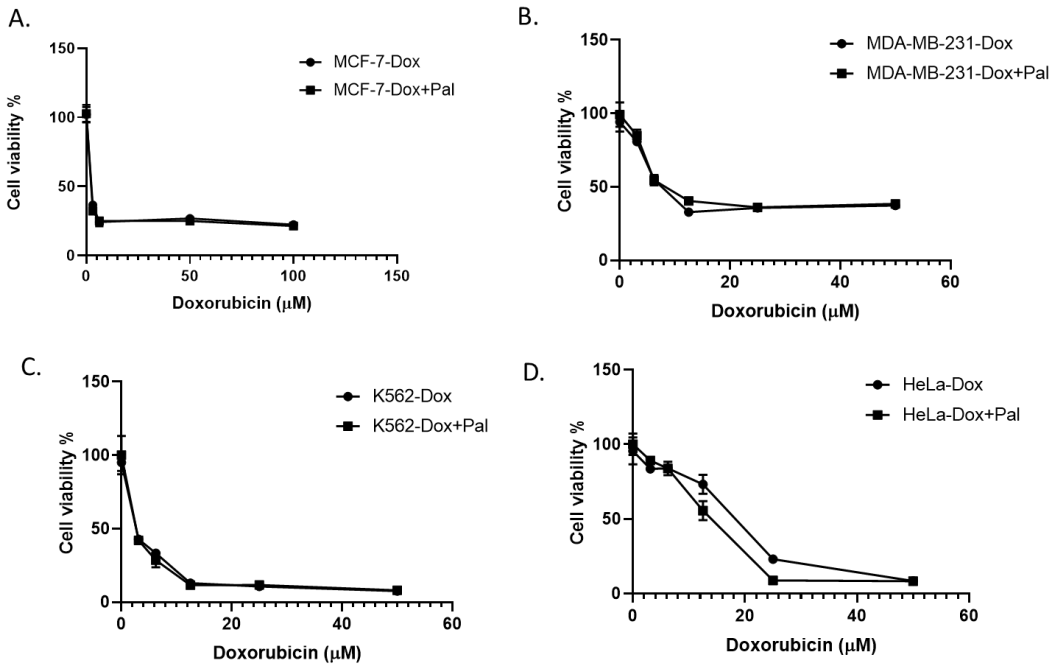

Figure 3.13 The comparison of only Doxorubicin and Palbociclib combined Doxorubicin effect on cell viability in drug-sensitive A. MCF-7, B.MDA-MB-231, C. K562 and D. HeLa cell lines. IC_{50} value of Doxorubicin was analyzed and compared by non-linear regression.

Table 3.4 The IC_{50} values of Doxorubicin and Palbociclib combined Doxorubicin in different cancer cell lines.(****p<0.0001; ns = no significance)

Cell-Lines	IC_{50} values of Doxorubicin	IC_{50} values of Doxorubicin+Palbociclib	Significance
MCF-7/DOX	373±55 µM	13±0.4 µM	****
HeLa/DOX	50±4.6 µM	10±0.7 µM	****
K562/DOX	183±17.1 µM	24±2.12 µM	****
SKBR-3	276±29 µM	51±6 µM	****
MCF-7	2±0.5 µM	1.8 ±0.9 µM	ns
MDA-MB-231	10±1.2 µM	12±1.4 µM	ns
HeLa	15±2.3 µM	12±1.9 µM	ns
K562	2±0.15µM	2±0.18 µM	ns

3.2.3 Expression of Cyclin-D, Cyclin-E and mTOR in Palbociclib-Treated Doxorubicin-Resistant Cells, and in SKBR-3

In order to explore the effect of Palbociclib treatment on molecular pathways of the P-gp-expressing cells, the gene expression of *CCND1*, *CCNE1* and *mTOR* were determined by qRT-PCR. Palbociclib has previously been shown to trigger the expression of Cyclin D and Cyclin E genes in different cell lines that were sensitive to Palbociclib (Herrera-Abreu et al., 2015; Min et al., 2018). Moreover, the relationship between Palbociclib and the mTOR-containing pathway has been well documented. Accordingly, mTOR inhibition was increased the synergistic effect of Palbociclib in different cancers (Chen et al., 2019; M.T. Herrera-Abreu et al., 2015; Yamamoto et al., 2019) and Palbociclib treatments have been demonstrated to affect the levels of mTOR and phosphorylated mTOR in different cell lines (Cretella et al., 2018) as mTOR-mediated pathways can interconnect with cell cycle progression (Haines et al., 2018).

In order to see the effect of selected Palbociclib concentrations, which was shown in Table 3.3, in mRNA level in P-gp-expressing cell lines, *Cyclin D*, *Cyclin E* and *mTOR* gene expression were analyzed. When the MCF-7/DOX cells were treated with Palbociclib, the expression of *CCND1* and *CCNE1* genes were unaffected compared to the non-treated control. On the other hand, approximately, a 2-fold increase was found in the expression of the *mTOR* gene (Figure 3.14. A., B. and C.). The *CCND1*, *CCNE1* and *mTOR* genes expressions were upregulated in Palbociclib-treated Doxorubicin resistant HeLa cells (Figure 3.14. D., E. and F.). This phenomenon was also observed in the P-gp-expressing SKBR-3 cell line (Figure 3.14. J., K. and L.). In Palbociclib-treated K562/DOX cells, the *CCND1* gene was 1.5-fold upregulated and the *CCNE1* gene was 2.8-fold upregulated. However, Palbociclib treatment in K562/DOX cells did not alter *mTOR* genes expression compared to the untreated control, statistically (Figure 3.14 G., H. and I.).

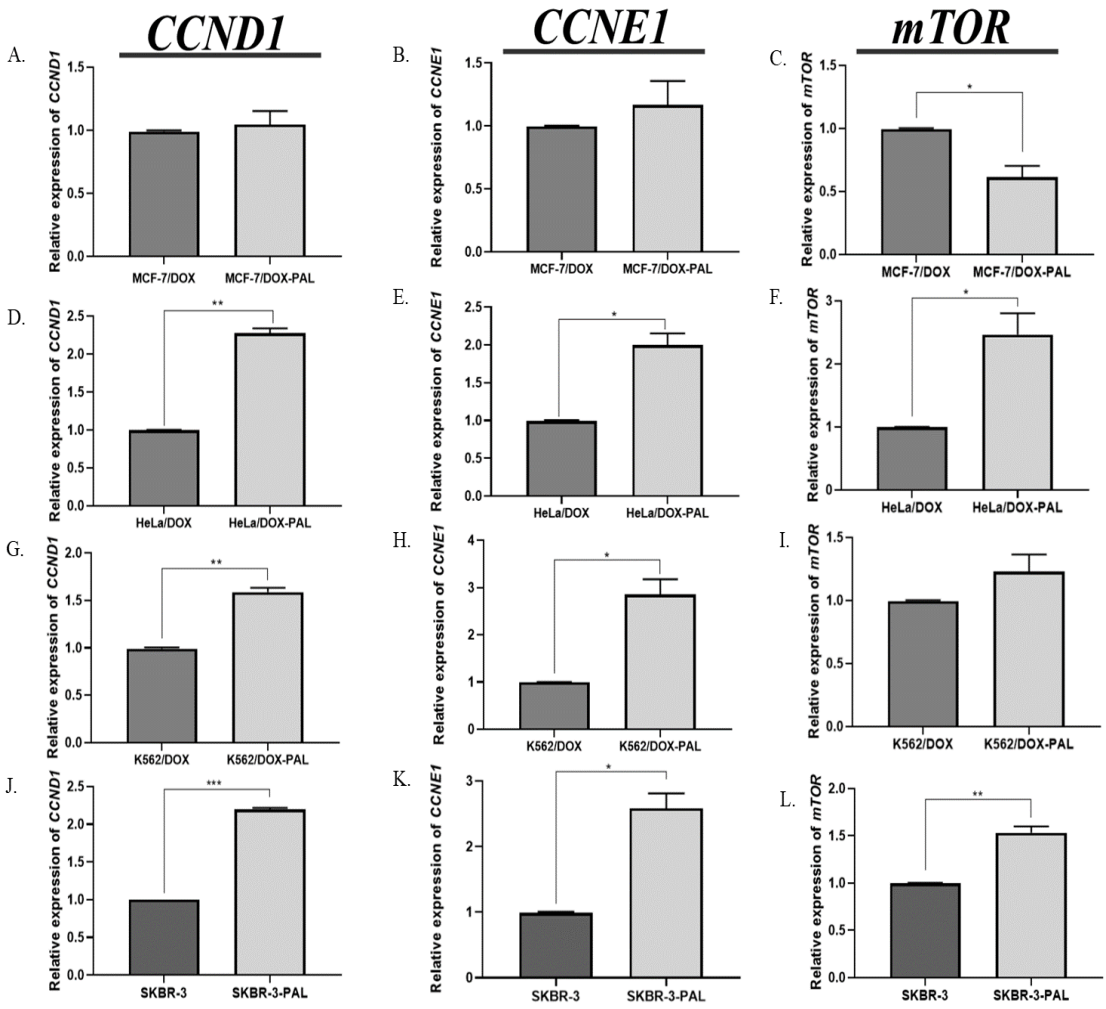

Figure 3.14 Expression of *CCND1*, *CCNE1* and *mTOR* genes in P-gp-expressing MCF-7/DOX, HeLa/DOX, K562/DOX and SKBR-3 cells. Expressions of cell cycle related genes were determined using The LightCycler® 480 SYBR Green I Master. *ACTB* was used as internal control. Fold changes were calculated by $2^{-\Delta\Delta Ct}$ method (n=9; * = $p<0.05$, ** = $p<0.01$; *** = $p<0.001$).

3.2.4 The Expression Analysis of Apoptosis-Related Genes in Palbociclib-Treated Doxorubicin-Resistant Cells and SKBR-3

In order to find out the effect of Palbociclib treatment on apoptosis-related molecular pathways of the P-gp-expressing cells, the expression profiles of *BAX, BCL-2, SURVIVIN,* and *PUMA* were determined by qRT-PCR. After applying the selected Palbociclib concentrations was shown in Table 3.3 on MCF-7/DOX, HeLa/DOX, K562/DOX and SKBR cell lines, all cell lines displayed different expression patterns.

According to Figure 3.15., when the anti-apoptotic and pro-apoptotic genes expressions were evaluated all cell lines, *BCL-2/BAX* ratio did not show any significant change compared to their control in MCF-7/DOX and HeLa/DOX cell lines. On the other hand, *BCL-2/BAX* ratio was decreased in K562/DOX and SKBR-3 cell lines after Palbociclib treatment. In addition, as mentioned above, in SKBR-3 cells *SURVIVIN* gene was significantly upregulated (Figure 3.15.L).

In the light of all results, all cell lines increasing the pro-apoptotic genes conversely promoted the anti-apoptotic genes. In addition, the up- and down-regulations in the expressions of the apoptosis-related genes seemed to trigger apoptosis in general. These results suggested that anti-cancer agent Palbociclib helped to increase the lethal effect of Doxorubicin.

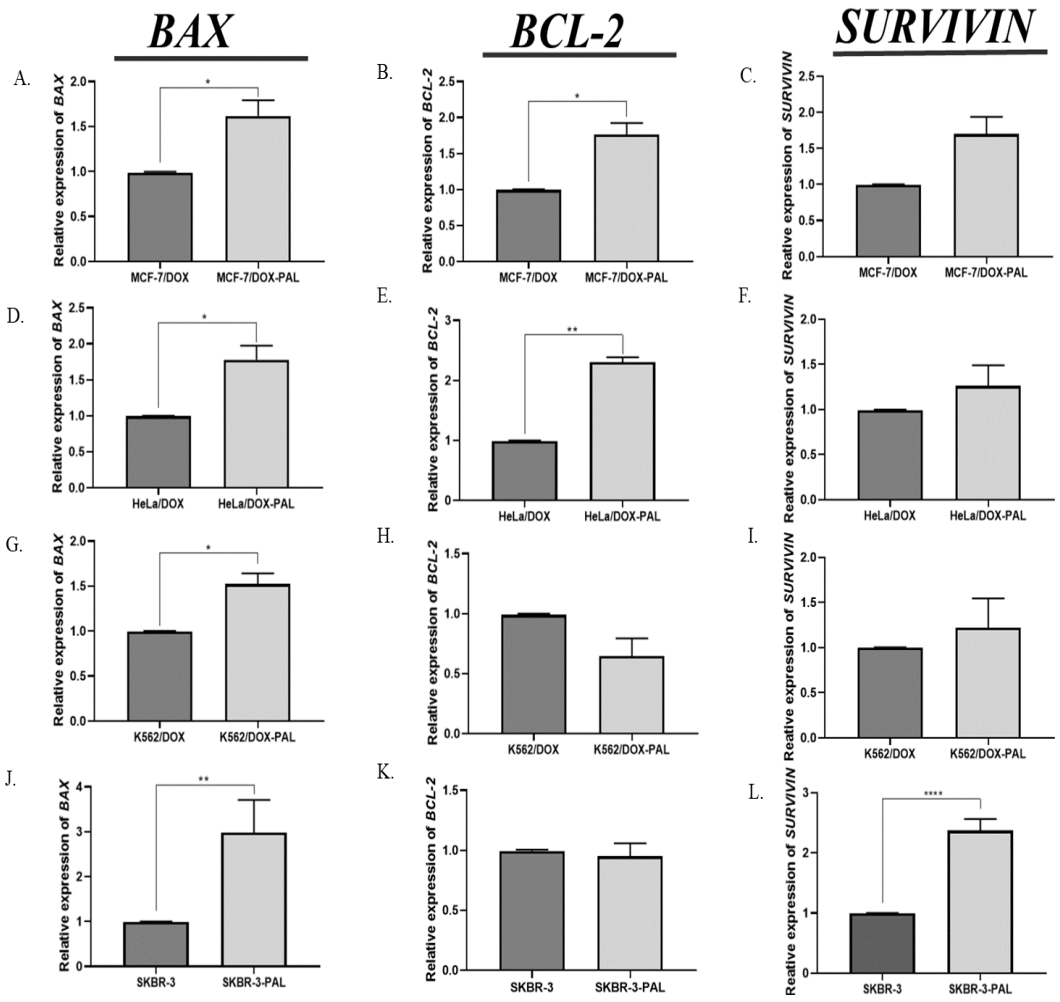

Figure 3.15 Expression of apoptosis related *BAX, BCL-2 and SURVIVIN* genes in P-gp-expressing MCF-7/DOX, HeLa/DOX, K562/DOX and SKBR-3 cells. Expressions of apoptosis related genes were determined using The LightCycler® 480 SYBR Green I Master. *ACTB* was used as internal control. Fold changes were calculated by $2^{-\Delta\Delta Ct}$ method (n=9; * = $p<0.05$, ** = $p<0.01$; *** = $p<0.001$).

3.2.5 The effect of Palbociclib on Expression of *MDR-1* and *BCRP* Genes in Doxorubicin-Resistant Cell Lines and SKBR-3 cells

In vitro experiments identified that cancer cells may become relatively resistant to anti-cancer agents by elevated drug efflux that results from the upregulation of ATP-binding cassette (ABC) transporters such as MDR protein 1 (P-gp), multidrug resistance-associated protein 1 and breast cancer resistance protein (BCRP). Also, studies demonstrated that the significance of finding out efficient drug resistance modulators for enhancing the effect of chemotherapy (Abaan et al., 2009; Meltem Demirel Kars et al., 2008).

Although Palbociclib is currently approved for breast cancer treatment and effectiveness, and functionality have been extensively studied, the potential use of Palbociclib in other indications is under investigation. Especially, there are very few studies focussing on the effects of Palbociclib on cancer cells with drug resistance. A study showed that when the Palbociclib was treated with Afatinib, it overcame acquired Afatinib resistance on non-small cell lung cancer and murine xenograft and significantly attenuated the proliferation of malignancy in the patient-derived xenograft model compared to monotherapy (Nie et al., 2019). The other study demonstrated that Palbociclib was a substrate of both P-gp and breast cancer resistance protein (BCRP) trough *in vitro* transwell assay. Additionally, *in vivo* experiments on wild type and various ABC transporter knockout mice showed that P-gp and BCRP substantially prevented Palbociclib brain penetration (de Gooijer et al., 2015). According to the US prescribing information of Palbociclib, their *in vitro* studies showed that at clinically relevant concentrations has low potential to inhibit the activities of the drug transporters P-glycoprotein (P-gp), breast cancer resistance protein (BCRP).

In the light of this information, in this study *MDR-1* and *BCRP* gene expression profiles were evaluated in the P-gp-expressing cells after Palbociclib treatment.

The result showed that Palbociclib treatment caused inhibition of the *MDR-1* gene in MCF-7/DOX, HeLa/DOX, K562/DOX and SKBR-3 cell lines (Figure 3.16. A., C., E. and G.). This result also explained that why the combination of Palbociclib and Doxorubicin re-sensitized P-gp-expressing cells to Doxorubicin. The possible mechanisms that can cause this situation are described below. E2F1 is a constantly overexpressed protein in human tumor cells that increases the activity of the MDR1 promoter, resulting in higher P-gp levels. Moreover, previous studies showed that downregulation of E2F-1 significantly increased intracellular accumulation of doxorubicin in doxorubicin-resistant human gastric carcinoma (Yan et al., 2014). Therefore, Palbociclib-dependent RB dephosphorylation consequently non-releasing E2F may cause decreasing *MDR-1* expression. The other possible mechanism is that Palbociclib may cause competitive inhibition of Pgp in resistant cancer cell lines. The previous studies demonstrated that Palbociclib was a substrate of both P-gp and BCRP, so it may compete with the Doxorubicin and lead to increase the concentration of Doxorubicin (de Gooijer et al., 2015).

On the other hand, the *BCRP* gene was downregulated only in HeLa/DOX cells after Palbociclib treatment (Figure 3.16. D.). Additionally, *BCRP* gene expression was upregulated in Palbociclib treated K562/DOX and SKBR-3 cells compared to control (Figure 3.16. F. and H.). The *BCRP* expression did not show any significant change compared to their control in MCF-7/DOX cell line (3.16. B.) This result showed that the inhibition of *BCRP* expression may not be seen in all cell lines.

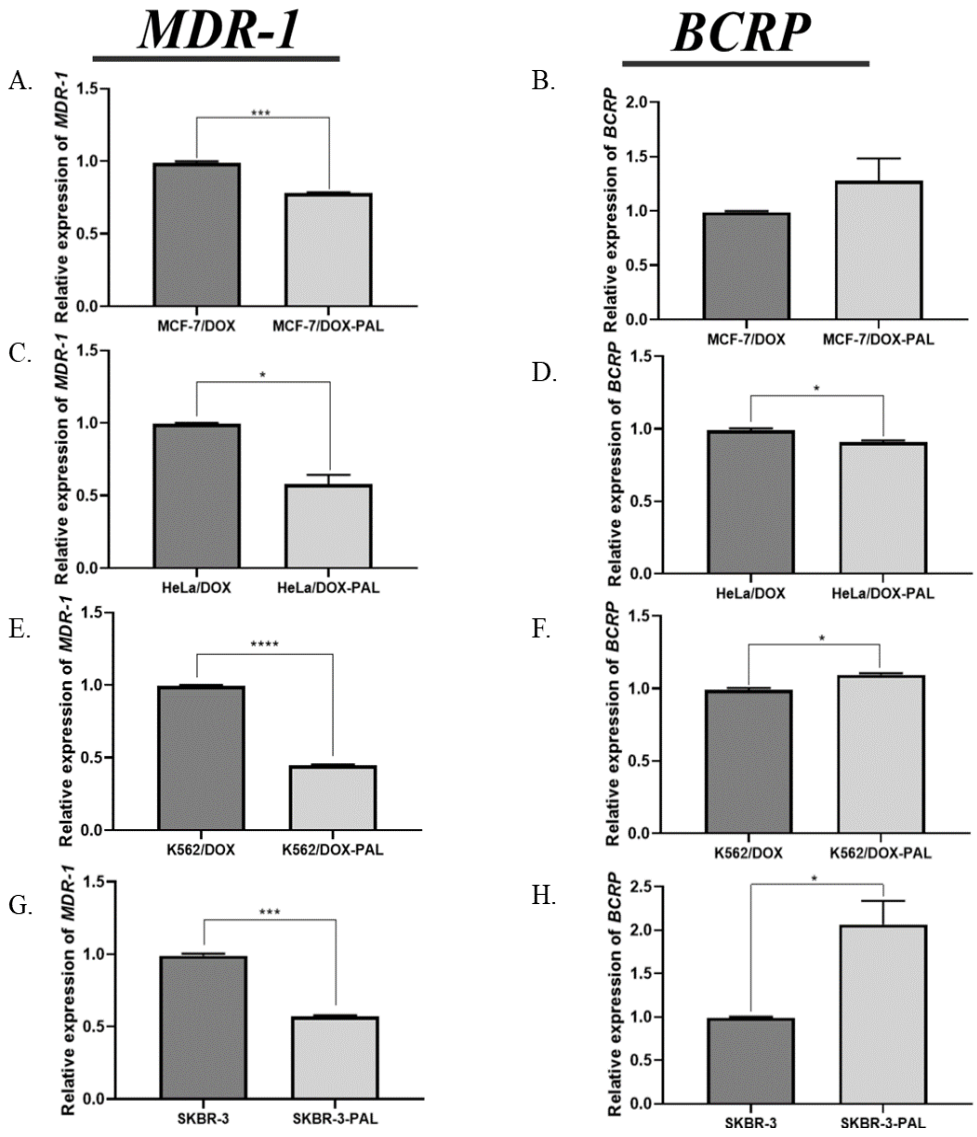

Figure 3.16 Expression of *MDR-1* and *BCRP* genes in P-gp-expressing MCF-7/DOX, HeLa/DOX, K562/DOX and SKBR-3 cells. Expressions of cell cycle related genes were determined using The LightCycler® 480 SYBR Green I Master. *ACTB* was used as internal control. Fold changes were calculated by $2^{-\Delta\Delta Ct}$ method (n=9; * = $p<0.05$, ** = $p<0.01$; *** = $p<0.001$).

CHAPTER 4

CONCLUSION

Uncontrolled cell cycle progression is a Hallmark of cancer. Studies showed that CDK4/ 6 and cyclin D1 as potential therapeutic targets in cancer. Palbociclib is a novel CDK4/6 inhibitor approved by the FDA. Palbociclib mechanism of action and the effect on different cancer type has been well documented. However, the effect of Palbociclib on the different subtypes of breast cancer (MCF -7 (ER+, PR+, HER2-), MDA-MB-231(ER-, PR-, HER2-) and SKBR-3 (ER-, PR-, HER2+)) grown in 2D and 3D cell culture conditions has not been studied yet.

This study revealed that the 2D and 3D MCF-7, MDA-MB-231 and SKBR-3 cells gave different responses to Palbociclib according to cell viability results. The MCF-7 and SKBR-3 cells could form spheroid (3D) were more resistant to Palbociclib compared to MCF-7 and SKBR-3 cells grown in traditional monolayer cell culture. On the other hand, MDA-MB-231 cells could not form spheroid properly, they could form loose aggregate. Therefore, 2D and 3D cell cultures did not show the same response to Palbociclib.

Palbociclib induces G1 arrest. When the cell cycle was analysed in both 2D and 3D cells, G1 arrest was observed in 2D-MCF-7 and 2D- MDA-MB-231 cell line but not in 2D-SKBR-3 cells owing to high-level expression of Cyclin E. Interestingly, G1 arrest was only seen in 3D-MCF-7 cells in the same concentrations of Palbociclib. 3D-MDA-MB-231 and 3D-SKBR-3 cells were unaffected.

Palbociclib has previously been shown to trigger the expression of *Cyclin D* and *Cyclin E* in different cell lines that were sensitive to Palbociclib. This study showed that after the Palbociclib treatment 2D and 3D MCF-7, MDA-MB-231 cells

displayed different expression patterns related to cell cycle genes, *Cyclin D*, *Cyclin E* and *mTOR*.

Although the expression patterns were different, 2D-MCF-7 and MDA-MB-231 exhibited significant upregulation in the expressions of pro-apoptotic gene BAX and downregulations in those of anti-apoptotic one's *SURVIVIN* and *BCL-2*. However, pro-apoptotic *BAX* and anti-apoptotic *SURVIVIN* genes were upregulated in 2D-SKBR-3 due to more resistance to Palbociclib. The previous studies showed that the decrease in the *BCL-2/BAX* ratio is an indication of induced apoptosis. The Palbociclib treatment in the all 2D cell line led to a decrease in *BCL-2/BAX* ratio.

After treatment of Palbociclib, the significant downregulation of the anti-apoptotic *SURVIVIN* gene was seen in 3D-MCF-7, MDA-MB-231 and SKBR-3 cells. Even though the *BCL-2/BAX* ratio was increased 3D-MDA-MB-231 and 3D-SKBR-3 cells, the tendency to cell death was also observed in each 3D cell line due to the downregulation of *SURVIVIN* gene. As a result, this study showed that diverse culture methods of the same cell lines caused different responses expression of genes.

This study also showed that Palbociclib resensitized Doxorubicin-resistant cells from different origins to Doxorubicin. Moreover, this effect was also observed in SKBR-3 cells which were P-gp-expressing cells. As a result, experiments demonstrated that Palbociclib promoted the effect of Doxorubicin by inhibiting the *MDR-1* gene in P-gp-expressing cells. However, *BCRP* gene inhibition only was observed in K562 /DOX cells.

This study also demonstrated that the only Palbociclib treatment in P-gp-expressing cells increased the pro-apoptotic genes conversely promoted the anti-apoptotic genes. Moreover, the up- and down-regulations in the expressions of the apoptosis-related genes exhibited to trigger apoptosis. This result also proposed that the Palbociclib helped to enhance the lethal effect of Doxorubicin.

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