DETERMINATION OF THERAPEUTIC EFFECTS OF MULTIFUNCTIONAL ANTIBODY AND PEPTIDE MICELLE-BASED NANOCARRIERS ON BREAST CANCER CELLS

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ABSTRACT

DETERMINATION OF THERAPEUTIC EFFECTS OF MULTIFUNCTIONAL ANTIBODY AND PEPTIDE MICELLE-BASED NANOCARRIERS ON BREAST CANCER CELLS

Breast cancer is the most prevalent type of cancer and a major cause of death among women globally. Currently, many treatments are developed to reduce breast cancer death risks. Targeting therapy represents an advanced and successful approach. It provides targeting specific tumor sites by using specific ligands and modifying physicochemical characterization of nanocarriers to increase drug efficiency.

In this study, we aim to determine and compare the therapeutic effects of doxorubicin (DOX)- loaded nanocarrier that was synthesized by using two properties a core cross-linked and pH sensitivity to increase drug stability and DOX releasing at the tumor site. The effects of DOX-loaded micelles (DM), HER2 targeting peptide (LTVSPWY)-conjugated-DOX-loaded micelles (DMP), and antibody (Herceptin) conjugated-DOX-loaded-micelles (DMA) on HER2 positive SKBR-3 cell line and HER2 negative MCF-10A normal epithelial breast cell line were determined by using cytotoxic, apoptotic, cytostatic, and genotoxic assays.

According to the cytotoxic assay, the IC_{50} value of DM, DMA, and DMP were 0.71-, 0.49-, 0.34-μM, respectively. Additionally, the fluorescence image showed higher DOX uptake by SKBR-3 cells treated with DMP. According to the apoptotic assays, the mitochondrial membrane potential on SKBR-3 cells with treated DMP decreased as well as higher apoptosis and necrosis rate that was regulated by Bcl-2, Pro-Caspase-3, PARP1, Bax, Bak, and Bcl-xL. Besides, the application of DMP caused cell cycle arrest at the G2/M phase. Lastly, DNA damage was observed in response to DMP determined by comet assay. This study provides a novel and effective therapeutic option for breast cancer through using this nanocarrier system with targeting properties.

ÖZET

ÇOK FONKSİYONLU ANTİKOR VE PEPTİT MİSEL NANOTAŞIYICILARININ MEME KANSERİ HÜCRELERİ ÜZERİNDEKİ TERAPÖTİK ETKİLERİNİN BELİRLENMESİ

Meme kanseri tüm dünyada kadınlarda görülen en yaygın kanser türüdür ve Kadınlarda en çok ölüme neden olan kanser türüdür. Son yıllarda meme kanseri ölüm risklerini azaltmak için birçok tedavi yöntemi geliştirilmektedir. Kansere yönelik hedef tedavi, geliştirilen ve başarılı bir yaklaşımdır. Hedef tedavi, fizikokimyasal karakterizasyon modifiye edilen Nanotaşıyıcılar ile ilaç etkinliği artırılması ve spesifik ligand kullanarak spesifik tümör bölgesinin hedeflenmesi sağlamaktadır

Bu çalışmada, çapraz bağlı ve pH duyarlılığı olmak üzere iki özellik kullanılarak ilaç stabilitesi ve tümor bölgesinde DOX salınımı artırmak için sentezlenen doksorubisin (DOX) yüklü farklı nanotaşıyıcıların terapötik etkilerini belirlemeyi ve karşılaştırmayı amaçladık . DOX-yüklü miseller (DM), HER2 hedefleme peptidi (LTVSPWY)-konjuge-DOX-yüklü miseller (DMP), antikor (Herceptin) konjuge-DOX yüklü miseller (DMA) HER2 pozitif meme kanseri SKBR-3 hücreleri ve HER2 negatif MCF-10A normal epitelyal meme hücresi üzerindeki etkileri sitotoksik, apoptotik, sitostatik ve genotoksik testler kullanılarak belirlendi ve karşılaştırıldı.

Sitotoksik teste göre, DM, DMA ve DMP' nin IC_{50} değerleri sırasıyla 0.71-, 0.49-, 0.34-uM'dır. Buna ek olarak floresan görüntüsü, DMP uygulanan SKBR-3 hücreleri tarafından alınan DOX miktarı diğer misellere göre daha yüksek olduğu gösterilmiştir. DMP uygulanan SKBR-3 hücrelerindeki mitokondriyal membran potansiyeli düşmekte ve yüksek apoptoz ve nekroz oranı görülmektedir. Bu durum Bcl-2, Pro-Caspase-3, PARP1, Bax, Bak ve Bcl-xl tarafından tarafından düzenlenmektedir. Buna ek olarak, DMP uygulandığında SKBR-3 hücrelerinde G2/M fazında hücre döngüsünün durmasına neden olmaktadır. Son olarak, comet testi ile DMP′ in DNA hasarına sebep olduğu gözlemlendi. Bu çalışma, hedefleme özelliklerine sahip bu nanotaşıyıcı sistemi kullanarak meme kanseri için yeni ve etkili bir tedavi seçeneği sunmaktadır.

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CHAPTER 1

INTRODUCTION

Cancer disease is a worldwide health problem and the second cause of death after heart disease (Siegel, Miller, and Jemal 2019), following lung cancer, the second most common type of cancer is breast cancer, cancer incidence in men account mostly for lung cancer, prostate cancer, colorectal cancer respectively, while in the female, most incidence and death occur due to breast cancer (Bray et al. 2018). Based on the world health organization estimated data by 2040, the newly diagnosed case of cancer is expected to reach 29.5 million people, and cancer death cases might reach 16.5 million annually. Cancer cases are found higher in low and middle-income countries compared to high-income countries, due to a lack of developed early detection diagnosis tools besides, lifestyle, obesity, and weak health care (Shah et al. 2019).

At the tissue level, cancer is known for its variety which creates a big challenge for its diagnosis and treatment (Hassanpour and Dehghani 2017). At the cellular level, cancer is a genetic disease, the development of the cancer mechanism firstly starts when mutation accumulates, thus lead to altering DNA sequence, genomic instability, causing abnormal gene expression, variation in the gene copy, number, and loss of heterozygosity (LOH). These mutations induce cellular abnormalities that induce uncontrolled cell division, continuous cell growth, and invasion to different organs of the body (Harrington 2016; L.-H. Wang et al. 2018). The second mechanism is epigenetic alternation, DNA modification such as methylation, chromatin composition alternation, and histone post-translational modification. Many genes are responsible to regulate cellular processes. However, the mutations on genes execute overexpressed or repressed genes, mRNA, protein, etc. that also induce dysregulation on the cellular processes of normal cells. Two classes of genes that are also called oncogenes can derive cancer cells. these oncogenes are classified into two types. One is called proto-oncogene, in cancer cell oncogene found to be dominant in which a single mutated copy is enough to enhance uncontrolled gene expression (Harrington 2016). The other class is called tumor suppressor gene (TSG), this

type of gene is responsible to inhibit cell growth, control DNA damage repair, suppress cell invade, metastasis, and regulating cell apoptosis (L.-H. Wang et al. 2018).

Many therapies have been developed to treat cancer disease. Such as local treatment; surgery, radiation therapy, and systemic therapy; hormonal therapy, chemotherapy, targeted therapy, and immunotherapy. Surgery and radiation treatment are used for removing the solid tumor and shrinking the tumor to reduce its spread and invasiveness.

Chemotherapy is a chemical drug therapy that is used to inhibit cancer cell growth and metastasis. The chemotherapeutic agents could be a synthetic compound or a natural product, also, microbiologically produced drug (Miller et al. 2019; Alam et al. 2018). Determining therapy options depends on patient recurrence risk, age, and comorbidity. Hormonal therapy is applied to breast cancer and prostate cancer, due to hormone overexpression to prevent cancer progression by blocking a hormonal-dependent pathway that stimulates neoplastic. However, the treatment should be monitor properly to avoid subsequent side effects (Drãgãnescu and Carmocan 2017; Namiki, Ueno, and Kitagawa 2012).

To overcome traditional treatment problems, targeted therapy has been developed, in which a specific molecule can target specific overexpressed protein receptors, or target some proteins involved in cell tumorigenesis, invasion, migration, and reduce toxicity on healthy cells (Gerber 2008). Drug delivery and tumor targeting achieved by nanoparticle system, including monoclonal antibody (mAb), peptide and siRNA, miRNA depend on specific selecting tumor properties against solid and hematological cancer. The human mAb was generated by phage display technique and transgenic mice (Scott, Allison, and Wolchok 2012). Trastuzumab (mAb) can target epidermal growth factor receptor (EGFR) overexpressed to target the progression of cancer including, head and neck cancer, lung ovary, colon, and malignant glioma (Z. Zhang et al. 2010). In addition to that, human epidermal growth factor receptor 2 (HER2) amplified in 30% of breast cancer patients (Revillion, Bonneterre, and Peyrat 1998), pancreatic cancer, and other types of cancer (Stoecklein et al. 2004). Trastuzumab successfully targets HER2 (Cuello et al. 2001) and combining chemotherapy with targeted therapy, improved drug efficiency such as doxorubicin and trastuzumab together were more effective in HER2 positive breast cancer (Tokuda et al. 2009; Rimawi, Schiff, and Osborne 2015). Some short peptide sequences with targeting prosperities increase drug efficiency through easily penetrating inside the tumor cell, they can be easily synthesized and combined with chemotherapy

such as LTVSPWY peptide which targets HER2 receptor (Shadidi and Sioud 2003). Moreover, understating the physicochemical characteristic of used nanoparticles in the development of targeted therapy is important to increase drug stability, efficiency and reduce toxicity on healthy cells.

1.1. Breast Cancer

Breast cancer (BC) is the most common cancer type among women globally, it is considered a major health problem. Among women, the occurrence risk on BC is 100 times higher than its occurrence among men, due to BC aggressiveness, advanced treatment is still needed, in the biochemical research field, the priority was given to BC research. The incidence of breast cancer reached around 1,700,000 annually. However, the mortality rate was improved in early diagnosed cases, while in metastatic cases the survival rate is declined to around (24 months). For the next 5-10 years, the mortality rate of BC is expected to increase (Anastasiadi et al. 2017; Greaney et al. 2015). The cumulative risk percentage from the date of birth to the 74-year age is 5.03% (Bray et al. 2018). The dangerous characteristic of BC is the lack of symptoms which leads to late detection. However, frequent screening can overcome this problem. Such as examination and BC education, early-stage detection can increase patient survival, and reduce BC mortality rate (Sun et al. 2017), this has been achieved by organized screening programs, by educating healthy women to undergo an examination, to detect any change in breast tissue, this effectively reduces mortality percentage among screened women from 38-48% (Broeders et al. 2012).

The point at which breast tumors started is from the ductal hyperproliferation, the consistent stimulation from carcinogenic factor lead to the development of a benign or malignant tumor or even metastatic carcinoma. The tumor microenvironment is affected by many factors that influence the initiation of BC such as stromal and macrophage (Sun et al. 2017). They can defend against tumors or develop tumor growth and metastasis (Noy and Pollard 2014), or even contribute to chemotherapy resistance. Developing BC could be related to injured deoxyribonucleic acid (DNA), or hereditary problems, by inheriting fault genes such as TP53, BRCA1, BRCA2, and PTEN (Filippini and Vega 2013). The classification of BC is based on their ability to spread or not, the noninvasive called ductal in situ (DCIS), the abnormal cell in DCIS is found in breast duct lining

without spreading outside the breast tissue. The lobular carcinoma in situ (LCIS), the abnormal cells are in the breast lobular, which increases the breast cancer risk and rarely invades out of the breast, while invasive BC including both ductal and lobular can be very dangerous and metastasize to other body organs, invasive ductal carcinoma (IDC) known as the most common subtype of BC (Makki 2015; Zangouri et al. 2018).

Breast cancer can be diagnosed with many different methods, starting with a physical examination in a clinic, mammography screening is a valuable diagnostic method (Smetherman 2013), in addition to, tumor marker, ultrasound breast imaging, magnetic resource imaging (MRI) (Banin Hirata et al. 2014; Gartlehner et al. 2013; Sardanelli et al. 2004). Patient education and awareness are important to lower the risk of late detection, breast-self examination (BSE) is a regular breast examination, in which, abnormal shape or size in the breast can be detected by the women themself (Gursoy et al. 2009). The treatment strategies are determined based on BC stage, location, and tumor size, in an early stage, surgery is general standard care, followed by radiotherapy for the whole breast (Whelan et al. 2010). For more aggressive types and early stages of BC, chemotherapeutic agents are favorable, Their treatment approaches based on factors like age and comorbidities, such as doxorubicin and cyclophosphamide are often used for more than 1cm tumor, those agents show effective response on a patient with hormone receptor-negative (Berry et al. 2006). However, the patient treated with doxorubicin show a cardiotoxicity effect, doxorubicin can bind to DNA and inhibit topoisomerase-II leading to cell death (Zhao and Zhang 2017).

Hormonal therapy such as estrogen synthetic inhibitors, aromatase inhibitors are used for women with BC during their postmenopausal, also, combining chemotherapy and hormone can be applied to some patients. Hormonal therapy causes many side effects such as hot flashes, vomiting, nausea (Coombes et al. 2004; Pan et al. 2018).

BC targeted therapy was developed such as a monoclonal antibody (mAb), pertuzumab, and trastuzumab. They are used against HER2, a specific receptor. mAb also were combined and show a better pathological response (Gianni et al. 2016). For a metastasized patient with HER2 negative, olaparib is an option for those patient, it acts by polymerase inhibition (Robson et al. 2017), despite many approved drugs for BC, the development of targeted therapy combined with chemotherapy is nowadays more favorable for nanobiotechnology research, due to the ability of targeted therapy to reduce drug dose. This can lower the drug side-effect, besides, targeting specific tumor which characterized in each patient group can improve better response to treatment.

1.2. Breast Cancer Risk Factors

Breast cancer is affected by many risk factors including sex, aging, family history, lifestyle, hormones, and reproductive factors. Breast cancer incidence is highly to occur in old age patients, based on 2016 statistical analysis, the breast cancer reported death in America was approximately 99.3% for those over 40 years old, and 71.2% for those over 60 years, for this reason, early detection on 40 years old is necessary among women (Siegel, Miller, and Jemal 2016; Sun et al. 2017).

The relation between age and BC incidence was explained by the change in the normal mammary gland and the decline in estrogen which reflects its effect on the breast. In an aged patient, the bone marrow which is known for its heterogeneity undergoes an overall change in its architecture, which contributes to BC metastasis and decreases aged patient survival(Place, Huh, and Polyak 2011; Greco 2019).

In BC patients, family history correlates significantly to BC risk factors. based on a study performed in women UK population, comparing patients with one first-degree relative to those with two or more first-degree relatives with BC. The risk of getting BC increase from 1.75 to 2.5 fold and almost a quarter of the patient was attributed to family history (Brewer et al. 2017). The reproduction factor also contributes to BC development such as late age at first pregnancy. Furthermore, delay in both menopause and menarche some studies revealed that first birth after 35 age increases the risk of BC compared to childless women. On the other hand, reducing estrogen receptor alpha plays an important role in reducing BC risk by dysregulation of a different mechanism (Horn and Vatten 2017; G. Dall, Risbridger, and Britt 2017).

Estrogen in its both endogeneous and exogeneous types is a major risk factor. high exposure to estrogen leads to an earlier age at first menarche. However, high estrogen during women pregnancy can decrease BC risk. Estrogen effects on the mammary gland or breast are age-dependent (G. V. Dall and Britt 2017). Lifestyle including alcohol consumption, saturated fat diet also is implemented in BC development and poor prognosis. Studies revealed that estrogen hormone level was elevated due to alcohol consumption which triggers the estrogen receptor pathway and develops BC (Jung et al. 2016; Makarem et al. 2013).

1.3. The Molecular Classification of Breast Cancer

The heterogeneity of BC makes it important to understand BC subtype and classification in each patient, analyzing gene expression and advanced technologies contributed widely to the establishment of BC subtype. There are six different subtypes were determined, luminal A, luminal B, HER-2 enriched, claudin-low, basal-like, and normal breast. They are different from each other in terms of their diagnosis, prognosis, treatment, risk factors, and incidence.

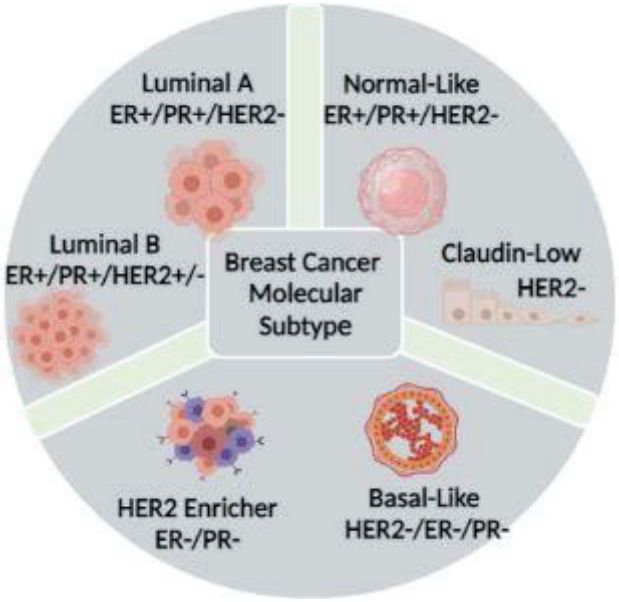

Figure 1.1. The molecular subtype of breast cancer.
(Figure drawn by BioRender).

The pathological marker includes estrogen receptors (ER), progesterone receptors (PR), and human epidermal growth factor receptor 2 (HER2) contribute to BC molecular subtyping. They are used to determine treatment approaches such as chemotherapy, hormonal therapy, or anti-HER2 therapy. BC subtype can be identified by immunohistochemistry and gene-based assay (Prat et al. 2015). Protein and RNA that control hormone regulation, cell proliferation, and cell cycle are highly distinguished between luminal A and luminal B. The latter express higher proteins or genes control cell cycle and proliferation (Goldhirsch et al. 2013) in terms of prognosis. The luminal A has a better outcome, comparing to another subtype across an early diagnosed patient. Also, patient survival and mortality are different in each subtype. The luminal A survival is the

highest. On the other hand, the most aggressive subtypes with the lowest survival are basal-like and HER2 subtypes (Milioli et al. 2017; Fallahpour et al. 2017). The mortality rate is affected by patient race, a study show, basal-like mortality rate of Africans with basal-like is higher than white with luminal subtype (Carey et al. 2006). Therefore, determine the patient BC subtype is essential for the patient treatment approach and determining survival rate.

1.3.1. Luminal A

The most common subtype of breast cancer is luminal A, found with ER+ and PR+. The cell of this type does not express HER2. The cell proliferation marker, Ki-67, is expressed in a low amount in this subtype (J. J. Gao and Swain 2018). Patients with luminal A had the best outcome and better prognosis (Hennigs et al. 2016). The luminal A is found with low mitotic activity, it affects approximately 50-60% of total BC. The tumor marker includes GATA binding protein3 (GATA3), the receptor tyrosine kinase erbB3, erbB3, BCL2, hepatocyte nuclear factor 3 alpha (HNF3), and luminal epithelial cytokeratins (CK8 and CK18). In addition to, another endoplasmic reticulum (ER) gene-related function such as g or X-box binding protein (XBP1) (Yersal and Barutca 2014; Carey 2010), since this subtype is hormone receptor the treatment approach for the patient diagnosed with luminal A. Tamoxifen and aromatase inhibitors are used for treatment to improve patient survival (Hwang et al. 2018; Ignatiadis and Sotiriou 2013).

1.3.2. Luminal B

Luminal B subtype comprises around 15-20% of BC, It is characterized by ER+ and PR+, while HER2 can be positive or negative, HER2 status makes luminal B subtype more aggressive than luminal A with worse patient outcome. Luminal B biomarkers also found in luminal A, which are, cyclin B1 gene, a baculoviral inhibitor of apoptosis repeat-containing (BIRC5), and surviving gene Ki-67 gene can be used to differentiate between lumina A and B. Since it is found to be highly expressed in luminal B, the molecular marker is important to develop targeting therapy. The luminal B target includes HER2, EGFR, PI3K, Akt, and mTOR, (Creighton 2012; Santamaria and Nebreda 2010).

Luminal B patients show bad outcomes despite using antiestrogen therapy (Loi et al. 2007) index such as ER and PR, HER2 and Ki-67 are used to distinguish patient prognosis good or bad in luminal BC (Cheang et al. 2009; Jackisch et al. 2015). Luminal B aggressiveness is similar to basal-like and HER2 enriched subtype in terms of the hazard ratio for patient relapse-free survival (Tran and Bedard 2011). There are around 30% of assigned HER2-positive tumors are luminal B (Sørlie et al. 2003). Due to many challenges, several studies show their insensitivity to both endocrine and chemotherapy, comparing to luminal A and HER2 enriched patients (Tran and Bedard 2011), to overcome this problem the anti-HER2 treatment such as trastuzumab and lapatinib are applied for those patients (Geyer et al. 2006). However, efforts are still needed to overcome many challenges and develop new strategies to treat the luminal B subtype.

1.3.3. Basal Like

Basal-like breast cancer (BLBC) is an aggressive, heterogeneous subtype. It accounts for approximately 12-17% of BC and affects younger women. In this subtype, hormone receptors are negative (ER, PR), and HER2 not amplified, for this reason, basal-like named by triple-negative breast cancer (TNBC). TNBC had a higher patient recurrence, around 25% of patients experience recurrence which can be distant in lung, liver, bone, and brain, and/or locoregional. Based on a cohort study recurrence can vary and it might occur in the first 3 years (Milioli et al. 2017; Steward et al. 2014), which increases the mortality rate. TNBC is associated with poor prognosis and a low survival rate, which were found to be associated with tumor size and lymph node status. In addition to that, TNBC tumor is highly proliferative and larger (N. U. Lin, Vanderplas, et al. 2012). Markers that are a high expression of EGFR and cytokeratin CK5/6, CK14, and CK7 are found in the basal cell layer of TNBC (Valentin et al. 2012). Most basal-like subtypes have a mutation in the TP53 gene and BRCA1 germline (Carey et al. 2006; Foulkes et al. 2003).

Treatment for the early diagnosed patients including chemotherapy, in addition to, a promising inhibitor for the enzyme poly (ADP-ribose) polymerase (PARP) has an essential role of this enzyme in DNA repair. It makes it possible to target treatment of TNBC. It acts by targeting the mutated BRCA1 and BRCA2 in TNBC. It can combine with immune target therapy and checkpoint inhibitors, programmed death 1 (PD-1),

checkpoint inhibitors such as pembrolizumab were found to be effective in TNBC patients, in addition to, an antibody-drug conjugate (Lyons 2019; Dent et al. 2007).

1.3.4. Claudin Low

Claudin-low subtype is named due to the low expression of the claudin protein. It has been identified in 2007. The claudin-low is a subgroup of the basal-like subtype. It has a poor prognosis. The low expressed genes including, occludin, E-cadherin, claudin (3,4 and 7) are involved in cell-cell adhesion and the tight junction while luminal protein and HER2 are not expressed. (Herschkowitz et al. 2007) For invasive breast cancer claudin-low accounts for around 7-14%. In this subtype, the expression of the genes that are involved in epithelial-mesenchymal transition (EMT), the tumor tends to be progressed and develop metastasis (Prat et al. 2010; Dias et al. 2017).

1.3.5. Normal Breast

This subtype rarely occurs in some people. It accounts for around 5-10% of BC. It is also negative for (ER, PR, and HER2 negative) similar to the basal-like subtype. However, in basal-like EGFR and cytokeratin CK5 are expressed, while the situation is opposite in normal-like. The term normal-like came due to some genes expressed are also found in fibroadenomas and normal breast tissue samples. They express an adipose tissue gene, the prognosis of this subtype is intermediate which is between luminal and basal-like subtype (Yersal and Barutca 2014; T. O. Nielsen et al. 2004).

1.3.6. HER2 Enriched

Human epidermal growth factor receptor 2 (HER2) subtype characterized by HER2 overexpression. This gene encodes for the HER2 receptor that is located in the 17q12, HER2 found to be low expressed in normal tissue. However, the amplification of HER2 develops oncogenic activity leading to tumor development (Figure1.2). Hormones (ER and PR) are found to be negative in this subtype. HER-2 receptor overexpression cause consistent signaling pathway which control cell survival, differentiation, and

metastasis, those pathways (PI3/Akt, MAPK, MEK, RAF, and RAS), HER-2 subtype account for around 15-20% of breast cancer (Arteaga et al. 2012; Schettini et al. 2020), and the of this subtype had poor prognosis (Prat et al. 2014).

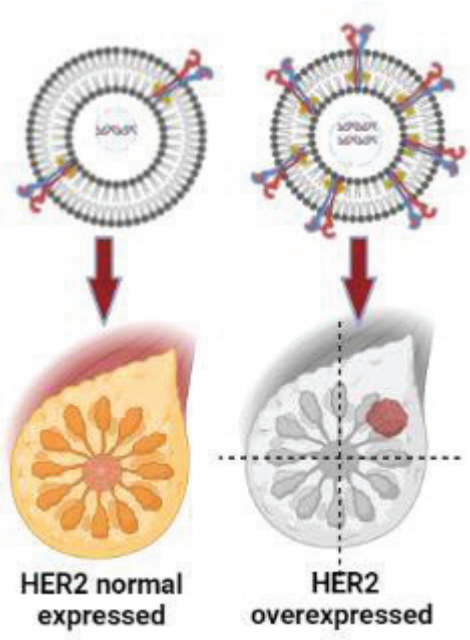

Figure 1.2. HER2 status in normal breast and HER2 breast cancer subtype.
(Figure drawn by BioRender).

Patient with HER2 enriched has a unique clinical characteristic. They show sensitivity to doxorubicin (DOX), which can block topoisomerase II that is found to be coamplified and located on chromosome 17 (Villman et al. 2006) that can provide a better response. Also, monoclonal antibody trastuzumab is used to target HER2 receptors, in addition to, lapatinib which can inhibit tyrosine kinase of HER2 activity and cause inactive HER2 accumulation on the cell surface (Scaltriti et al. 2009). Many methods are used to detect HER2 overexpression those includes immunohistochemistry (IHC) which is based on using mono or polyclonal antibody, chromogenic in situ hybridization (CISH) (Sauter et al. 2009), and fluorescence in situ hybridization (FISH) (Lambros, Natrajan, and Reis-Filho 2007). HER2 subtype is an aggressive and poor prognosis, despite many treatments approaches some patients can have resistance to the treatment, and many side effects can be developed which affect patient life quality.

1.4. Major Breast Cancer Signaling Pathway

In the cell membrane, receptors or ion channels can activate many signaling pathways that involve in BC progression. The pathways can be activated by many stimuli, which could be cytokines, growth factors, and antibodies, or influenced by extracellular ions. The breast cancer complexity arises from the activation of many signaling pathways, which causes many obstacles in curing breast cancer when tumor suppressor and proto-oncogene undergo mutations, including genes that encode for cell proliferation, survival, mutated cell pathway. The upregulated protein expression is involved in those pathways, as a result, constitutive cell signaling occurs which leads to breast tumorigenesis (Chial 2008).

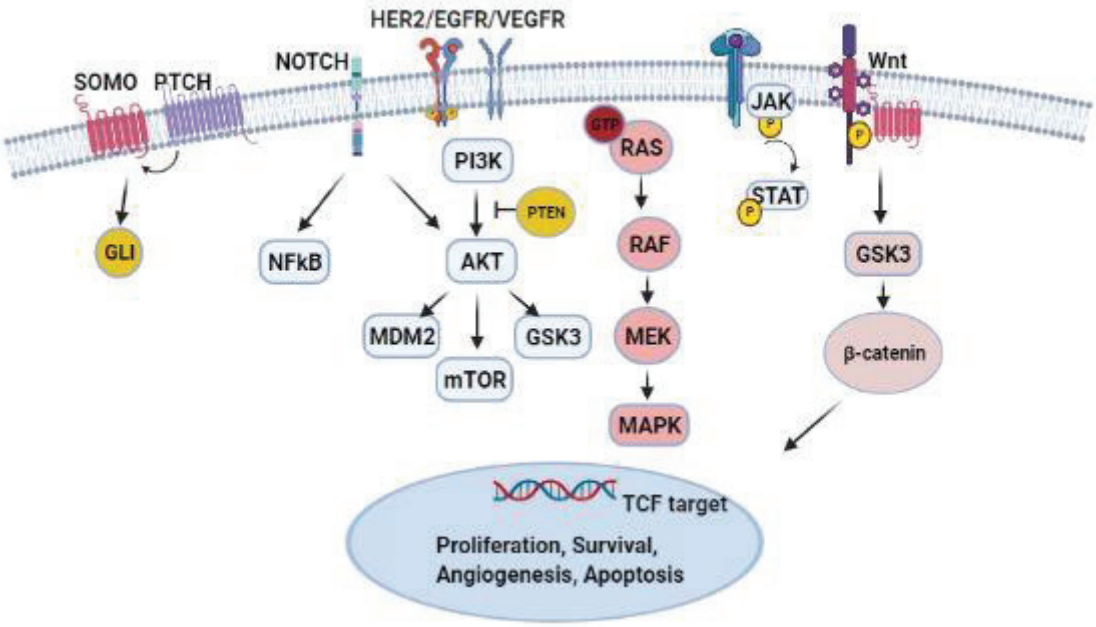

Figure 1.3. Major signaling pathway implemented in breast cancer development. (Figure drawn by BioRender).

1.4.1. PI3/AKT pathway

The protein kinase B is an important regulator for many cellular activities, including cell proliferation, survival, apoptosis, in addition to, glycogen metabolism and drug resistance. Phosphoinositide 3-kinase (PI3K) connects signals between cell

receptors and AKT. Three isoforms of AKT are found in mammals, AKT1, AKT2, and AKT3, each isoform has a distinct function. 70% of BC had a mutation in this pathway. PI3K is activated when a ligand binds to tyrosine kinase receptors that transfer the signal to AKT by phosphorylation and the mTOR signaling pathway. The role of AKT in cell cycle regulation by inhibiting FOXO transcriptional activity and its target proteins including P27 and retinoblastoma like2 (RBL-2). The survival regulatory of AKT includes forkhead box O (FOXO) target which is Fas ligand and Bim (Wickenden and Watson 2010). A study shows that targeting AKT by nanoparticle combine with chemotherapy (paclitaxel), and, loaded with siRNA to knockdown AKT in BC cell line MCF-7 and animal model. These nanoparticles had successfully reduced the AKT protein expression by increase drug efficiency, and decrease tumor size. Also, it can overcome drug resistance (Fatemian, Moghimi, and Chowdhury 2019).

1.4.2. Mitogen-Activated Protein Kinase Pathway (MAPK)

The protein of this pathway is responsible for delivering and amplifying the extracellular signals, there are six groups of MAPKs have been identified. The extracellular signal-regulated kinases (ERK) including ERK (1/2,3/4,5,7/8), Jun N-terminal kinase (JNK)1/2/3, and P38 mitogen-activated protein kinases ERK6/δ, MAPK pathway can be activated by ERK pathway through binding between ligand and the tyrosine kinase receptor, to promote downstream signaling pathway. The downstream pathway includes Ras activation, which stimulates ERK1/2 signaling proteins, the signals transmitted by the nucleus for further gene transcription and gene expression. These genes determine cell fate such as proliferation, differentiation, survival, and apoptosis (Cargnello and Roux 2011). In BC, MAPK is found to be complex, due to its interaction with several pathways, which regulate different cellular responses. MAPK pathway overexpression is found to be influenced by ER and HER2, using a large panel of MAPK genes. MAPK correlates with ER expression in BC. MAPK can be used as a prognostic indicator, better outcome observed in ER+ patients in which pan-ERK1/2 and p-ERK1/2. In addition, MAPK was positively associated with ER and BCL-2 (Ahmad et al. 2016). migration and proliferation in BC were shown to be decreased by MAPK downregulation (Meng et al. 2011).

1.4.3. Notch Signaling Pathway

The notch pathway regulates many cellular processes including proliferation, apoptosis, angiogenesis, and metastasis. It can be activated by the interaction of ligand (Delta Serrate, LAG-2) from one cell and notch receptor to neighbor cell, in mammalian four notch receptor are found (notch1-4), notch receptor is overexpressed in BC. In this pathway, cell proliferation in BC occurs due to the upregulation of Cyclin (A, B, and D1). In addition, it prevents BC apoptosis through AKT activation. The notch pathway also regulates stem cell self-renewal in BC. BC cell line experimentally overexpresses notch ligand jagged1 were metastasize to bone, due to the role of notch pathway in BC progression and metastasis it represents an important therapeutic target for BC patients (Acar et al. 2016; Harrison et al. 2010).

1.4.4. Epidermal Growth Factor Receptor (EGFR)

Epidermal Growth Factor Receptor (EGFR) which is known as the ErbB family, is one of the transmembrane proteins in the tyrosine kinase family. This family acts as a ligand to activate the downstream signaling pathway (PI3K/AKT, Ras/Raf and, MAPK). EGFR pathway overexpression leads to poor prognosis and worse BC patient outcomes. Approximately half of TNBC tumors have EGFR overexpression. This pathway enhances cancer cell migration by EMT upregulation, in an aggressive subtype of BC. Targeting EGFR can enhance chemosensitivity. The downstream signaling of EGFR, ERK2 was involved in EMT by regulating the transcriptional activity of fra1, which was associated with regulating the expression level of ZEB1/2 a marker for EMT (Masuda et al. 2012; Dent et al. 2007).

JAK/STAT, calcium pathway, hedgehog signaling pathway, insulin-like growth factor 1 receptor (IGF-1R), transforming growth factor receptor (TGF-βR), Wnt/ß-Catenin signaling pathway, and VEGFR activate BC cellular processes. These pathways contribute to BC tumorigenesis. The JAK/STAT signaling pathway also contributes to cellular proliferation, apoptosis. This pathway can be activated by cytokinesis and interleukin. Both proteins are corporates to transfer the signals from the transmembrane protein receptor to the nucleus for cellular DNA gene transcription and expression. A

study showed 69.2% of BC tumors overexpress STAT3 protein and contribute to breast tumor pathogenesis (Thomas et al. 2015; Dolled-Filhart et al. 2003).

The calcium signaling pathway is important for cellular signaling, Ca^{2+} initiates many protein phosphorylations and activation in MDA-MB-231. The cell proliferation and migration in BC are observed when intracellular calcium level elevated after a GTP binding protein Rap2B overexpression, which phosphorylates ERK1/2. In addition to that, Ca^{2+} contributes to program cell death after it is released from ER and being uptake by mitochondria. Another study demonstrated Ca^{2+} leads to MCF-7, proliferation in the BC cell line due to MAPK activation by17ß-estradiol (Improta-Brears et al. 1999; Di et al. 2015).

The hedgehog signaling pathway contributes to many cellular processes. It also induces BC progression and metastasis. The activation of this pathway in human mammary stem cells leads to glioma-associated oncogene activation. In addition, GLI1 overexpression has been correlated with worse outcomes in BC. Also, it can be used as a prognostic indicator based on BC subtype, in basal-like breast cancer, the hedgehog pathway was found to be activated by forkhead-box transcription factor C1. Some endocrine resistance cell lines show a high hedgehog signaling pathway which was activated by PI3/AKT (Han et al. 2015; Bhateja et al. 2019).

Insulin-like growth factor 1 receptor (IGF-1R) is also associated with cancer progression. IGF-1R overexpression activates PI3K/AKT which induces resistance to apoptosis. It had been noticed with the resistance of BC (Voudouri et al. 2015). The transforming growth factor receptor (TGF-βR) is a protein-ligand located in the extracellular matrix. It initiates the intracellular signaling pathway in BC. The TGF-βR receptor can promote tumor metastasis by EMT activation and expression of chemokine receptor type 7 through P38, MAPK. Also, the TGF-βR receptor is found to be elevated in BC plasma patients (Pang et al. 2016; Chod et al. 2008).

Wnt secreted protein in the Wnt/ß-Catenin signaling pathway also contributes to cellular fate, stem cell self-renewal, cell tumorigenesis, and metastasis as EMT dependent. Wnt gene also is expressed in the breast tissue of BC with an elevation level of catenin. Wnt pathway is found to be overexpressed in over 50% of BC patients. It is correlated with patients' low survival rate. In TNBC and BLBC, both canonical and noncanonical Wnt receptors are elevated. The releasing of ß-Catenin acts as a transcriptional coactivator after it has been released to the cytoplasm then translocated to the nucleus, an overexpression of EMT transcriptional factor that was noticed in invasive

BC. The direct target examples of the Wnt signaling pathway are Snail, Slug, ZEB1, ZEB2. Targeting of the Wnt pathway by a specific inhibitor such as porcupine shows an effective treatment approach for BC (Xu et al. 2020; Jiang et al. 2019; Wu et al. 2012).

Vascular endothelial growth factor receptor (VEGFR) which is a tyrosine kinase receptor, contributes to angiogenesis, migration, differentiation, and metastasis of BC by the activation of PI3K, mTOR, MEK pathway. In BC patient tissue, a higher expression of VEGFR was noticed compared to the normal patient. Also, worse patient outcomes were noticed in patients with high VEGFR (Srabovic et al. 2013). The targeting VEGFR-2 could be a potential and effective therapy in the treatment of TNBC patients (Zhu and Zhou 2015).

1.4.5. HER2 Signaling Pathway

A HER2 signaling pathway is a complex network, compromise of membrane receptors, the receptors consist of two domain extracellular domain for the ligand binding and intracellular domain for the residue protein kinases (Arteaga and Engelman 2014). To regulate cellular functions, the HER2 receptor which is 185 KD, can be activated via homo- or heterodimerization with one of another family members, including, HER1, HER3, and HER4. HER2 can make a complex with insulin-like growth factor receptors (Nahta et al. 2005). After HER2 dimer being phosphorylated, it can activate the downstream signaling pathway, which is found to be associated with BC progression. This pathway regulates cell survival and growth of BC including PI3K, mTOR, and MAPK which regulate cell proliferation, the amplification of the HER2 signaling pathway results in HER2 protein overexpression which causes tumor progression. Targeting the HER2 pathway by specific molecule has been used to treat HER2 enriched subtype (Mayer and Arteaga 2016; Feng et al. 2018).

1.5. Breast Cancer Treatment

Breast cancer treatment option is made based on intrinsic gene expression. For this purpose, determining the patient subtype is important. This is achieved by microarray technology, alongside understating the regulatory signaling pathway for each subtype that contributes to the development of new targeted drugs. Immunohistochemistry (IHC) is an

important technique, it is used for the determination of specific proteins overexpressed in breast tumor tissue. Another technique is fluorescence in situ hybridization (FISH) that is used for the determination of the presence or absence of specific DNA sequences on chromosomes. Treatment choice will be decided based on BC subtype and progression. In general, patient age, gender, and comorbidities are taken into consideration. Surgery was used for the localized BC, followed by adjuvant therapy. Hormone receptors can be targeted for the treatment of BC by endocrine therapy. Such as tamoxifen, or aromatase inhibitors, or combined with chemotherapy. However, some patients can experience resistance, due to cyclin D1 overexpression (Butt et al. 2005).

The treatment of TNBC approach such as poly (ADP) Ribose Polymerase (PARP) inhibitors are generally used for an aggressive BC with BRCA1 and BRCA2 mutation (Ashworth 2008). Tyrosine kinase inhibitors also are preferred in BC treatment. In addition to mTOR inhibitors. Developing new targeted therapy to overcome many risks that are associated with the aggressiveness of BC. Lowering the side effects on the healthy cell is needed for this purpose, nanotechnology contributes significantly to the cancer-targeting therapy to deliver systemic therapy (Nounou et al. 2015).

1.5.1. Targeted Treatment in Breast Cancer

Targeted treatment had been developed by scientists to overcome the adverse side effect of current treatment, such as hair loss, immune suppression, gastrointestinal disturbance, besides, cancer metastasis to another body organ. In estrogen-positive BC patients, the key drivers in this subtype of BC progression are estrogen and estrogen receptor. By targeting them with specific inhibitors to inhibit the estrogen signaling pathway. The selective modulators of estrogen receptor such as tamoxifen was the first drug approved to target estrogen-positive BC. It has successfully inhibited tumor progression and metastasis. In addition to, aromatase inhibitors, such as anastrozole, letrozole (Den Hollander, Savage, and Brown 2013), their main mechanism is used to block androgen biosynthesis to reduce estrogen enzyme.

Herceptin is a recombinant antibody that had been firstly proven by FDA in 1998, this antibody can target the HER2 receptor, by binding a tyrosine kinase domain (juxtamembrane), result in HER2/HER3 heterodimer uncoupling, which blocks the HER2 downstream signaling pathway. However, some patients can develop resistance to

Herceptin due to HER2 gene amplification causing HER2 protein overexpression and continuous oncogenic activity, even with treating the patients with Herceptin, due to many reasons such as consistent signaling from receptor tyrosine kinase which increases the PI3K out ErbB family. Besides, some alternative forms of HER2 might not be detected by the antibody. However, combining Herceptin and chemotherapy is preferred for metastatic BC patients (Pegram et al. 1998; Ritter et al. 2007).

Many side effects can arise after treatment with trastuzumab (Herceptin). One of the most dangerous effects is heart failure. Since the HER2 receptor is overexpressed in the human myocardium, targeting HER2 by trastuzumab can develop heart failure. A recent clinical study showed that HER2 positive BC patients treated with trastuzumab had an adverse side effect including, 12.5% cardiotoxicity, 4.16% abdominal pain, and nausea, pulmonary thromboembolism, dysuria, odynophagia (Lima Cavalcanti, Silveira Cabral, and Dos Santos 2017). Another study found that around 32.9% of patients treated with trastuzumab caused cardiotoxicity. Besides other adverse side effects, for this purpose, a fundamental follow-up is needed for the patient under trastuzumab treatment and cardiological monitoring is necessary to recognize the possible side effects and foxing on reducing therapeutic drug toxicity (Ayres et al. 2015).

In breast cancer patients, over 70% of them activate the PI3/AKT/mTOR pathway. The large scale of this pathway and protein kinase regulates cellular fate. For this reason, it opens the way for another promising target for BC patients, silencing these pathways could be an effective treatment for patients who are resistant to another targeting strategy. It could be a combination of mTOR inhibitors and either HER2 inhibitor or an estrogen receptor inhibitor can be a promising strategy (W Grunt and L Mariani 2013). In BC, the hypoxia microenvironment contributes to VEGR upregulation. The vascular formation is another BC therapeutic target. However, a meta-analysis for BC patients by combing both chemotherapy and angiogenesis inhibitor paclitaxel and bevacizumab induce severe and fatal side effects. These side effects include stroke, gastrointestinal tract perforations, hemorrhage, artery blockage, and neutropenia (Ranpura, Hapani, and Wu 2011).

Immunotherapy might represent a promising targeting therapy for HER2 and TNBC. Since immune infiltration is found in these two subtypes, it can play a synergistic effect with other drugs used for some type of cancer including melanoma and lung cancer. A responsive immunotherapeutic agent is ipilimumab since the immune checkpoint receptor (PD-1) was found to be overexpressed on tumor-infiltrating lymphocytes. Its

main role is to inhibit T-cells, through binding activity between PD-1 ligand (PD-L1) causing local immune downregulation (Mittendorf et al. 2014). For this reason, the immune checkpoint inhibitor is effective to activate immune response toward cancer cells, such as PD1 antibody pembrolizumab and an anti-PD-L1 antibody atezolizumab, the combination of trastuzumab and lapatinib are found to be effective in luminal B subtype with HER2 positive. In addition to the previously mentioned therapeutic targeted approach, each BC subtype phenotype, representing a challenge for the researcher to investigate different cellular survival mechanisms (Masoud and Pagès 2017).

1.5.2. The Role of Nanocarriers in Breast Cancer Targeted Therapy

Nanotechnology is an advanced technology, refers to the interaction between cellular molecular components and engineered material. This technology represents an incredibly small NP between 1-100 nm called nanoparticles (NPs). Nanotechnology contributed to cancer treatment by providing a selective drug delivery through a specific nanocarrier system. In the last years, understanding cancer molecular biology and nanotechnology are both significantly contribute to BC's research and can increase drug safety and efficiency. The disadvantage of using the only chemotherapeutic is that it cannot stay a long time in the blood circulation and drug poor solubility. In recent years, several targeted therapies were developed including small molecules such as peptides and proteins. They have been approved to mimic chemotherapy, immunotherapy, and increase their drug circulation in the blood, and provide easily penetrating in the cellular membrane. Furthermore, the advantage of NPs is their targeting, solubility, and drug stability properties. Moreover, NPs have a high tumor vessel permeability when compared to the healthy vessel that allows drug entry. The pH of a cancer cell is acidic around 6.7 due to the high metabolism rate. For this reason, cancer passive targeting by nanotechnology provides both pH sensitivity drug-releasing into the cancer cell (Cho et al. 2008).

The main problem in tumor treatment is multidrug resistance such as p-glycoprotein and reflux drug that prevents its accumulation in tumors site. For this purpose, the researcher tried to use NPs with the chemotherapeutic agent to increase drug accumulation and efficiency. The physicochemical characterization of NPs such as type, size, shape, charge, target, and surface are all modified to improve cancer treatment. The

NPs can be synthesized surface using PEGylating or another coating; shape with a cube, rod, sphere, or plate; materials with organic and non-organic contract (Krishna and Mayer 2000).

The organic NPs includes polymeric NPs, ferrite, micelles, liposome, and dendrimer. Examples of non-organic NPs are quantum dots, gold NPs, iron NPs. The advantage of polymeric NPs is their hydrophilicity, nontoxicity, biocompatibility, drug release, and biodegradability. The liposome NPs are characterized by their biocompatibility. However, it has some disadvantages such as low delivery efficiency. The dendrimers system has a stable and well-defined structure. In addition to, surface functionalization capability, they can incorporate hydrophobic or hydrophilic molecules to provide the ability of drug distribution in the targeted area (Davis, Chen, and Shin 2010; Grigore 2017).

Non-organic NPs such as gold NPs are used in drug delivery and imaging since they have electric and optical properties. They are easy to synthesize and can diffuse into a tumor cell. A study demonstrated that gold NPs loaded with DOX showed higher anti-cancer activity on the Hela cell line when they compare to only DOX (Tomoaia et al. 2015). A study by (Papagiannaros, Aristarchos, et al.) shows that developing a dendrimer system (PAMAM G4) DOX loaded that had been incorporated into a liposome was tested against BC. Provides DOX releasing stability in vivo, and slower drug released was optimized, this achievement is important in terms of therapeutic index and reducing the toxicity on the healthy cell (Papagiannaros et al. 2005).

Among all drug delivery systems, polymeric micelle was the most type that attracts researchers and successfully reached the clinical trial. They are an amphiphilic block of copolymers called micelles. They are self-assembled to create a spherical shape. Micelles are with great biocompatibility. They consist of a hydrophobic core and hydrophilic shell to stabilize the hydrophobic core, which can be aspartic acid, L-lysine, and propylene oxide. The drug is loaded in the hydrophobic core, the hydrophilic shell, such as (PEG). Loading drugs in micelles can be achieved by either physical encapsulation or chemical covalent attachment. Several studies reported delivering drugs to tumor cells via the PEG polymeric micelles. The role of hydrophilic PEG provides higher circulation time, through coupling with the liposome surface. It is important to decreases the clearance by the phagocytosis because PEG can create a steric barrier and decrease the protein absorption (Z. G. Gao et al. 2005; W. Lin and Kim 2011).

The development of stimuli micelles is at great of manner. It can be sensitive to endogenous or exogenous stimuli. The most important stimuli are the pH-sensitive micelles. They can control drug release. Generally, they are stable at physiological pH, while in acidic pH they undergo a structural destabilization, which provides drug-releasing at the targeted tumor site due to pH difference, the pH near tumor cell is around (6.5), while the pH of tumor intracellular environment at endosome and lysosome is around (4.5-5.5). On the other hand, the pH of a healthy cell is around 7.4 (Z. Wang et al. 2018). To achieve increase the micelles circulation time, scientist stabilizes them by crosslinked, examples are covalent bond, hydrogen bond. Crosslinked micelles can improve micelle's performance in vivo from many different aspects such as biodistribution, more accumulation in the target side, drug efficiency, and tolerability (Talelli et al. 2015).

1.5.3. The Role of Antibody Conjugated Nanoparticles in Breast Cancer Targeted Therapy

Multifunctional polymeric micelles drug-loaded are coupled with antibodies or peptides to provide targeting tumor site, for example, micelles bearing the monoclonal antibody. Herceptin is used to target of BC or gastric tumor tissue that have overexpressed HER2 receptor. The mechanism of mAb in the cancer cell is varying from direct or indirect activity. It can either inhibit signaling by targeting cytokine and inhibit their binding to the receptors or inhibit growth through their binding to overexpressed membrane receptors. The antibody is conjugated to NPs by a specific linker for targeted treatment of BC. Trastuzumab has been explored to generate a novel nanocarrier targeting system. In addition, antigen-binding fragments also had been studied and conjugated to NPs to obtain a higher tumor uptake. They also increase the diffusion rate, the internalization of NP occurs by the process of receptor-mediated endocytosis (Bareford and Swaan 2007; Xenaki, Oliveira, and van Bergen En Henegouwen 2017).

For targeting HER2 positive subtype, a study done by (Lin, Yu-Ling, et al.) developed lipoplex NPs and loaded them with curcumin and DOX, it was also conjugated with trastuzumab. Showed a better drug targeting and efficacy on HER2 positive *in vitro* and *in vivo* study (Y.-L. Lin et al. 2019).

The development of nanocarrier systems, in the field of cancer-targeting in general and targeting breast cancer specifically, is one of the most important strategies, it has attracted scientists and researchers to develop a novel nanocarrier system, which can target a specific breast cancer subtype, one of which is the aggressive HER2 positive subtype.

1.5.4. HER2 Therapeutic Targeted Treatment

The HER2 subtype status should be accurately determined to provide the best targeted treatment option for HER2-positive patients. Some of the HER2 receptor-targeted inhibitors have been approved by FDA, such as trastuzumab, pertuzumab, and lapatinib. As we mention, trastuzumab is a monoclonal Ab, IgG1 against HER2 extracellular domain, despite several studies for trastuzumab, the mechanism of action still needs further studies, in general, it can inhibit the cellular proliferation, angiogenesis, in addition, trastuzumab contributes to immune response towards tumor cell through antibody-dependent cell-mediated cytotoxicity.

Trastuzumab accelerates cellular HER2 degradation and internalization, which leads to HER2 receptor downregulation and prevents the homodimerization of truncated HER2 receptors by cleavage inhibition of metalloproteinases. In addition, it contributes to the disruption of HER2/Src interaction (D. L. Nielsen et al. 2013). A synergetic effect can be obtained by combining trastuzumab with DOX. However, the cardiotoxicity rate is correlated with the concurrent usage of both combined. The trastuzumab combined with chemotherapy increase patient survival, reduce the percentage of recurrence (Nahta et al. 2006; Orphanos and Kountourakis 2012).

Lapatinib is a tyrosine kinase inhibitor (TKI), this small molecule can block the HER2 downstream signaling pathway, by blocking the kinase activity of HER1 and HER2, by inhibiting the pathway that regulates cell processes including, proliferation, survival, In addition, it induces cellular apoptosis. These inhibitors are used to target HER2 overexpression in BC cells. The mutation of HER2 causes the loss of their extracellular domain that causes a problem of trastuzumab binding. Lapatinib can still be effective in blocking tyrosine kinase activity. The advantage of lapatinib is its ability to cross the blood-brain barrier, which is effective in patients with brain metastasis. (Moy et al. 2007).

Several anti-HER2 agents are known as monoclonal Ab such as pertuzumab is a humanized antibody, its mechanism by blocking the HER2 dimerization with other HER family member. It targets different extracellular domain that is targeted by trastuzumab. It also inhibits HER2 dimerization, which shows a promising drug targeted efficiency since dual HER2 blockage was found to be beneficial. However, the cardiotoxicity of combined trastuzumab and pertuzumab had been noticed in most patient who receives it. For this purpose, determining the severe risks should be taken into consideration (Portera et al. 2008).

Another trastuzumab emtansine is a T-DM1, this molecule is an antibody-drug conjugated, consist of trastuzumab conjugated to a highly toxic agent maytansinoid. For HER2 positive metastatic BC, it was more effective and had better safety than lapatinib and capecitabine, the T-DM1 was tested on patients who failed to respond to trastuzumab. However, common side effects were noticed such as elevation in liver enzyme, and thrombocytopenia (Verma et al. 2012). The resistance to T-DM1 had been noticed in the initial responder. The intrinsic and acquired resistance was noticed in patients treated with T-DM1. Understanding the resistance mechanism such as drug efflux transporter and another resistance pathway including multidrug resistance-associated protein MDR1,2 is important to develop an alternative combination that can overcome the drug resistance (Hunter et al. 2020).

Neratinib is a tyrosine kinase inhibitor, it is irreversible pan HER for HER1, HER2, and HER4 in 2017 was approved by the FDA as extended for the adjuvant therapy. The initial study of solid tumor patients who were administrated to neratinib, confirmed by (IHC) to be HER2 or EGFR positive revealed the maximum tolerated dose as 320 mg. The common several side effect on patients was diarrhea (Wong et al. 2009; Kunte, Abraham, and Montero 2020)**.** A clinical phase 2 trial study about early-stage BC patients with hormone receptor-negative and HER2 positive, received neratinib. The patients showed a better pathological complete response when compared to standard combined chemotherapy and trastuzumab. However, diarrhea is the most problem noticed in a patient treated with neratinib (Park et al. 2016).

Afatinib is a small molecule and a TKI, it is also an HER family irreversible blocker. A phase II study has investigated the effect of afatinib in a patient with HER2 positive metastatic BC. After administration to trastuzumab, the study showed a promising benefit (41%) for the patient who progresses after trastuzumab treatment (N. U. Lin, Winer, et al. 2012).

Comparing to antibody conjugated NPs, peptides also represented a good tool for targeting the tumor. Some of them have been approved by the FDA to be used in cancer research or cardiovascular disorder. Examples are LTVSPWY, KCCYSL and, FCDGFACTMDV. They are used to target the HER2 receptor. The peptide can also be conjugated to organic or inorganic nanocarrier. A dual drug (DOX and Metformine) is conjugated with NPs that showed higher efficiency and contribute to overcome MDR in the MCF-7 cell line (Shafiei-Irannejad et al. 2018). A study demonstrated the development of a peptide-specific for matrix metalloproteinases. Two peptides in this study were used, GPLGV and GPLGVRG, a PEGylated with DOX-loaded micelles show a higher DOX in plasma patients when it was compared to DOX free and increase the drug efficiency, it also reduces toxicity and inhibits the tumor growth (Lee et al. 2007).

Several HER2 inhibitors and therapeutic approaches are still being developed by scientists today. Despite a landscape of treatment many alternations are still followed to achieve a better treatment option and lower the toxicity on the healthy tissue. The reason for that is not all patient benefits from the available treatment, such as chemotherapy, monoclonal antibody. However, the usage of nanocarrier targeting system in the development of treating HER2 positive of BC nowadays controls the most attention by the researcher to overcome several problems and possible risks associated with current treatment.

1.6. Aim of The Study

HER2 enriched breast cancer subtype characterized by HER2 overexpression, which leads to an aggressive subtype, through activating many oncogenic proteins. The overexpression of HER2 induces upregulating of cellular pathways that control cell proliferation, survival, and angiogenesis. In the development of cancer treatment, targeted therapy had shown to be a more effective treatment option. The nanocarrier system is the most reliable technology that had been implemented recently to increase drug efficiency and lower extra toxicity.

In this study, we determined and compared the therapeutic potential of antibody (Herceptin) conjugated DOX-loaded micelles (DMA) and peptide (LTVSPWY) conjugated Dox-loaded micelles (DMP) on HER2 positive cell line as well as HER2 negative healthy breast cell line for negative control. We compared the targeted efficiency

of DMA and DMP. We aimed to increase DOX efficiency by loading into polymeric micelles. DOX is a chemotherapeutic agent and its main mechanism is to inhibit the topoisomerase II that arrest DNA replication. In our study, a breast cancer cell line SKBR-3 is a HER2 positive was used to show the targeting efficacy of our NCs system. We also checked the potential chemotherapeutic effect of a multifunctional drug-conjugated carrier system with enhanced stability, and double moiety pH sensitivity to understand their cytotoxic, apoptotic, cytostatic, and genotoxic effect on HER2-positive breast cancer cells. Micelles carrier system had several advantages in targeting therapy, it can increase drug stability, efficiency, and circulation in the blood (Lu et al. 2018). In this study, using crosslinked micelles can increase the dynamic structure and stability of micelles, in addition to that, increase micelles circulation time in the blood.

The pH-sensitive micelles provide drug-releasing at the tumor site. Furthermore, we used a specific HER2 targeted peptide (LTVSPWY) conjugated to micelles to ensure the drug accumulation at HER2 positive breast cancer cells since the peptide size is smaller than the antibody, it might provide a better drug penetrating and targeting to the tumor site. To sum up, all, comparing the targeting properties of multifunctional nanocarrier DOX loaded system with pH sensitivity and double moiety, conjugated to HER2 peptide (LTVSPWY), or mAb (Herceptin) was investigated. Firstly, the cytotoxic effect of free micelles, only DOX and DOX loaded micelles which are DOX-micelles (DM), Antibody conjugated DOX-micelles (DMA), and peptide conjugated DOX-micelles (DMP). They are newly synthesized as novel targeted strategies for this project. The cytotoxic effects in SKBR-3 a HER2 positive cell line, and MCF-10A a HER2 negative cell line were determined by cell proliferation MTT assay. After that, the apoptotic, cytostatic, and genotoxic effects of DM, DMA, and DMP were investigated by using Annexin V/PI double staining, JC-1 staining, determining pro-apoptotic and anti-apoptotic proteins with Western Blotting, cell cycle assay, comet assay, respectively. The main purpose of our study is to increase (DOX) efficiency by using polymeric micelles to increases drug stability in blood and target HER2 positive breast cancer cell line this novel approach can lead to a better treatment option for the patient who is diagnosed with HER2 positive BC subtype.

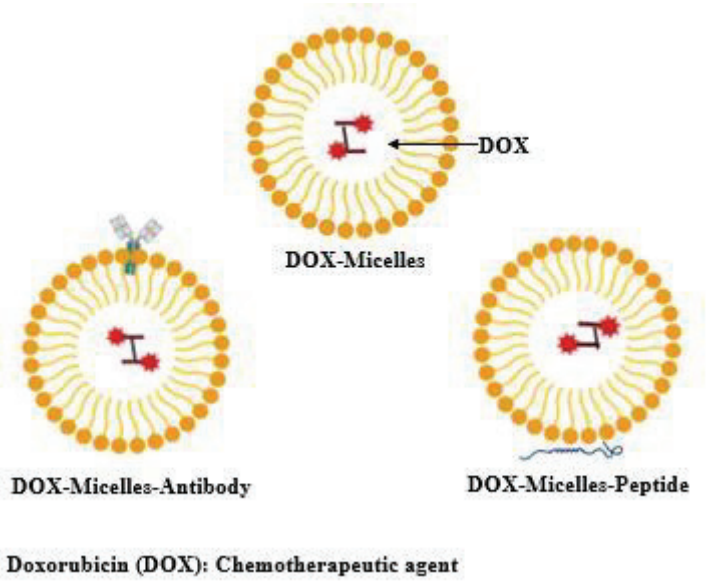

Figure 1.4. The structure of synthesized DOX-loaded-Micelles used for the study. (Figure drawn by BioRender).

CHAPTER 2

MATERIALS AND METHODS

2.2. Materials

In this study, different experiments were used to investigate the effect of our newly synthesized nanocarriers system on two different cell lines which are HER2 positive breast cancer cell line (SKBR-3), and HER2 negative, non-tumorigenic cell line (MCF-10A).

2.3. Cell Lines

In this project, SKBR-3 and MCF-10A cell lines were used to determine the effect of different types of nanocarriers (DOX-loaded micelles).

SKBR-3 cell line was kindly provided by Prof. Dr. Sevil Dinçer İşoğlu, AGU-Kayseri, MCF-10A was kindly provided by Prof. Dr. Ayşe Elif Erson Bensan, METU, Ankara.

Table 2.1. The cell line types used in this study.

Cell line	Type
SKBR-3	Adherent cell, HER2 enriched breast cancer subtype, derived from human mammary gland/breast adenocarcinoma cell line.
MCF-10A	Adherent cell, HER2 negative, non-tumorigenic breast cell line, derived from human mammary gland/breast non-tumorigenic cell lines.

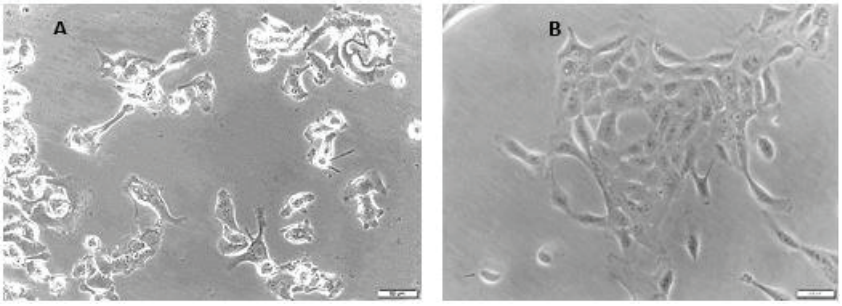

Figure 2.1. HER2 positive, SKBR-3 (A), HER2 negative, MCF-10A (B), magnification (20X) brightfield.

2.4. Chemicals

All chemicals used in this study to investigate the effect of the nanocarriers on breast cancer cell lines and to show the cytotoxic, apoptotic, cytostatic, and genotoxic effects of the applied nanocarriers (NCs).

2.4.1. Nanocarriers

The nanocarriers (NCs) were synthesized by Prof. Dr. Sevil Dinçer İşoğlu from Abdullah Gül University with her Ph.D. candidate student Nazende Nur Akşit, they analyze the physicochemical characteristic of NCs. A total of Six different NCs were applied which consists of Free micelles and Dox-loaded micelles. Three different DOX-free and DOX-loaded NCs were compared which are shown in table 2.2.

Table 2.2. The type of NCs with their different size.

Nanocarriers	Size
Free-Micelles.	70 nm
Free-Micelles-Antibody.	134 nm
Free-Micelles-Peptide.	141 nm
DOX-loaded-Micelles (DM).	
Antibody (Herceptin®) Conjugated DOX-loaded Micelles (DMA).	
Peptide (LTVSPWY) Conjugated Dox-Loaded Micelles (DMP).	

2.4.2. Cell Culture Chemicals

The culture mediums of the different cell lines and their additional ingredients used including DMEM high glucose provided with L-glutamine and sodium pyruvate, trypsin-EDTA obtained from Sigma-Aldrich, Dulbecco's MEM Nutrient (DMEM/F12) provided with 25mM HEPES and L-glutamine, non-essential amino acid (100X) were obtained from Euroclone, penicillin-streptomycin, fetal bovine serum (FBS) and horse serum were obtained from Gibco. Dulbecco's phosphate-buffered saline 1X (PBS) from Biowest, epidermal growth factor, insulin, and hydrocortisone were obtained from Sigma-Aldrich, also, the cholera toxin (the usage permission has been approved) from Sigma-Aldrich. Trypan blue dye was obtained from SIGMA-ALDRICH.

2.4.3. Cell Viability Assay

The MTT tetrazolium (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay kit was obtained from Invitrogen Thermo Fisher Scientific, dimethyl sulfoxide (DMSO) was obtained from the Advanced Diagnostic Research group.

2.4.4. Fluorescence Imaging

For the microscopy study, the Olympus-IX83 Fluorescence microscopy was used. Image J program was used for image processes. For the staining, the 4',6- Diamidino-2-Phenylindole Dihydrochloride (DAPI) powder dye was obtained from InvitrogenTM and Paraformaldehyde (PFA) powder was obtained from Sigma-Aldrich for fixation of the cell.

2.4.5. Apoptosis Assays

2.4.5.1. JC-1 Assay

JC-1 assay kit obtained from Cayman Chemical was used to determine the mitochondrial membrane potential. The staining buffer was prepared by dissolving 1

tablet 100 ml of dH2O. 1X of JC-1 dye was diluted in a culture medium. For the analysis, the recommended protocol was followed from the kit. The Thermo ELECTRON with Multiskan Spectrum was used for monitoring the mitochondrial membrane potential.

2.4.5.2. Annexin-V

FITC annexin-v (90 µg/ml) from BioLegend, PI from AppliChem, and Annexin Binding Buffer (ABB) were obtained from life technologies^{TM}, experimental samples were analyzed by flow cytometry in IZTECH BIOMER by BD FACSCanto software after following recommended protocols from the kit.

2.4.6. Western Blotting

Western blotting was used in this study to determine the expression level of apoptotic and anti-apoptotic related proteins, antibody (Ab) used which are Bcl-2, PARP1, Pro-Caspase-3, and Bax, Bak, and Bcl-xL.

2.4.6.1. Protein Lysis for Western Blotting

Tris lysis buffer was used for the cell lysis. It was prepared by mixing (10mM Tris-HCL, 1mM EDTA, and 0.1% of Triton-x).

2.4.6.2. Determine the Protein Concentration by BCA Assay

The SMART ^{TM} BCA Protein Assay Kit from thermo scientific was used to determine the protein concentration.

2.4.6.3. Polyacrylamide Gel Electrophoresis, Sodium Dodecyl Sulfate (SDS-page)

Acrylamide Kit (10%) from BIO-RAD, TGX Stain-FreeTM FastCastTM, used for gel preparation, running buffer Tris-Glycine-SDS (TGS) 10X, Ammonium persulfate

(APS) obtained from BIO-RAD, TEMED was obtained from SIGMA, for protein loading, Laemmli buffer kit, 2-mercaptoethanol obtained from BIO-RAD, Prestained Protein SHARPMASSTM VI Protein MW marker from Euro Clone with lot: 025S1903.

2.4.6.4. Protein Transfer from Gel to Membrane

For the transfer process, the materials were obtained from BIO-RAD, a Trans-Blot Turbo ™ Transfer System was used including, a 5X Transfer Buffer, PVDF membrane, and transfer stacks, Methanol obtained from SIGMA-ALDRICH.

2.4.6.5. Protein Detection by Antibodies

Primary antibodies were obtained from Abcam are Anti-Bcl-2 antibody ab32124 (E17), Anti-PARP1 antibody ab32138, Anti-Caspase-3 antibody ab184787, Anti-Bax antibody ab32509 (E63). Both Anti-BAK antibody (D4E4) and Anti-Bcl-xl antibody (54H6) were kindly provided by Dr.Ayten NALBANT. For secondary antibody the Goat Anti-Rabbit IgG ab250718 (HRP), loading control, Anti-GAPDH antibody ab9485 membrane washing buffer, Tris Buffer Saline 10X (TBS), nonfat dry milk, Clarity™ ECL kit, were all obtained from BIO-RAD, Tween20 obtained from SIGMA-ALDRICH, Bovine Serum Albumin (BSA), obtained from Chem Cruz™, to observe protein band an imaging system USION SL VILBER LOURMAT was used.

2.4.7. Cell Cycle Assay

Propidium iodide (PI), 1mg/ml was obtained from AppliChem, RNase was obtained from Thermo scientific (10mg/ml), Triton X-100 from AppliChem, ethanol 100% (EtOH) were obtained from MERCK, Dulbecco's phosphate-buffered saline 1X (PBS) from Biowest. The flow cytometry in IZTECH BIOMER was used to determine the cell cycle phase using BD FACSCanto software.

2.4.8. Genotoxic Assay (Comet Assay)

Alkaline Electrophoresis Solution pH >13 with (300 mM NaOH, 1 mM EDTA), a one-liter stock solution of 500mM EDTA with pH 8 was prepared, which contain (0.3 M final concentration of NaOH (12 g), 500 mM EDTA (2ml), completed with dH_2O to 1 liter. for slide coating normal melting agarose is used, low melting agarose.

2.5. Methods

2.5.1. Cell Culture

The culture medium was prepared to maintain the cell line and stored at +4 to be used for the next cell passage.

2.5.2. Sterilization of The Materials

In this study, the materials that were used for the cell passage were sterilized by autoclave. The tips and glassware were sterilized at 120 °C for 20 minutes, dH_2O was sterilized at 120 °C for 15 minutes.

2.5.3. Cell Culture Preparation

The culture mediums were prepared to maintain the cell line. The culture conditions of SKBR-3 and MCF-10A cell lines were different. The medium content with their additional ingredient is explained for SKBR-3 and MCF-10A in (table 2.3, 2.4) respectively. The SKBR-3 cell line was grown in DMEM high glucose, provided with both L-glutamine sodium pyruvate (Table 2.3).

Table 2.3. The ingredient of DMEM high glucose for SKBR-3 cell line.

The Medium Content	Final Concentration
Penicillin/Streptomycin	1%
Fetal Bovine Serum	10%

MCF-10A was grown in Dulbecco's Mem Nutrient Mix F12 (1:1) with 25mM HEPES and L-glutamine, which are listed in (Table 2.4).

Table 2.4. The ingredient of Dulbecco's Mem Nutrient Mix F12 (1:1) for MCF-10A cell line

Medium ingredient	Final Concentration
Penicillin/Streptomycin	2mM
Hydrocortisone	0.5 µg/ml
Epidermal Growth Factor (EGF)	20 ng/ml
Horse Serum	5%
Insulin	10 µg/ml
Choleratoxin	100 ng/ml

2.5.4. Maintenance of The Cell Lines

In this study, SKBR-3 and MCF-10A were used as a HER2 positive and a HER2 negative, respectively. The cells were grown in a different medium. The medium contents are referred to in table 2.3 and 2.4. For all cell lines, a T25 or T75 cell culture flask was used. The cell lines were passaged when they reached 80% confluency. The cell was passaged to continue their growth. Firstly, the old medium was removed. The cells were washed with 4 ml of 1X PBS. Then PBS, 2 ml of trypsin-EDTA was added. The cell was then incubated at 37°C, 5% CO_2 for 1-7 minutes. After the cell detaches from the flask, 4ml of the medium was used to collect the cell. The cells were centrifuged for 5 minutes at 800 RPM for SKBR-3 and MCF-10A. The supernatant was removed, then the cell pellet was resuspended in 1 ml fresh medium. The number of the cell was counted by trypan blue (2.4.5), then cells were seeded based on their number, in 25T or 75T to continue the experiment 20k cells per cm for SKBR-3 and 10k cell for MCF-10A.

2.5.5. Trypan Blue

The trypan blue staining was used to determine the required cell number to be seeded for the next passage or the experiment, the needed cell number was counted by mixing a 90 µl of trypan blue, and a 10 µl from the cell (1ml of the dissolved pellet), then by gentle mixing, a 10 µl of the mixture was put in Neubauer hematocytometer from MARIEN$_{\text{SUPERIOR GERMANY}}$, the cell was counted under light microscopy at 10X magnification of (Carl Zeiss-12V DC) microscopy by following the down equation:

$$\text{Number of cells} = 1\ \text{ml}$$

$$\text{Number of needed cells} = X$$

X: The cell volume that should be taken. After the number of cells in 1 ml was determined, the desire cell number in volume was taken, and the total volume was completed with a cell culture medium.

2.5.6. Thawing The Frozen Cell Lines

When the cell lines were needed for the experiment, the cryogenic vials (contain the cells) were taken quickly from -86°C refrigerator, when one drop of melted ice was recognized, the cell was dissolved with 500 ul of fresh culture medium, and collected in falcon tubes with 4 ml of medium, to ensure obtaining the highest amount of viable cell, the falcon centrifuged at 600 RPM, for 5 minutes, the supernatant was removed, cell pellet dissolved in 1 ml medium, the cell put into 25 cm^2 tissue flask culture, completed to a total of 5 ml medium, the cell placed in an incubator at 37°C, 5% CO2, to continue their passage, the experiment was started after the third passage.

2.5.7. Cell Line Freezing

SKBR-3 and MCF-10A cell lines were frozen in a cryogenic vials tube, before the cell was frozen, two solutions for cell freezing were prepared, solution details explained in (Table 2.5).

Table 2.5. The content and percentage of cell freezing mix1 and mix2.

Mix Number	Ingredients
Mix1	6 ml of pure culture medium (60%) + 4 ml FBS (40%)
Mix2	8 ml of pure culture medium (80%) + 2 ml DMSO (20%)

The cell was frozen to continue the further experiment, for this purpose, the cell in T-75 flask was washed with 4 ml of 1X PBS, then 2 ml trypsin-EDTA added and incubated at 37°C, 5% CO2, after 1-7 minutes, 4 ml of culture medium used to collect the cell in a falcon tube, the tube was centrifuged at 800 RPM for (SKBR-3 and MCF-10A), for 5 minutes, after that, the supernatant was removed, and cells were dissolved in 500 µl of mix 1, then the cell number was counted by trypan blue dye, see (2.4.5) the desired cell number $2*10^6$ cell per 500 µl of mix1 was taken and transferred into cryogenic vial tube, then 500 µl of mix 2, was added slowly drop by drop to the cell, the cryogenic tube was taken quickly to -86 °C refrigerator to be stored.

2.5.8. Determining The Cytotoxic Effect of NCs on The Cell Line

The cytotoxic effect of the NCs on SBBR-3, MCF10-A was determined by MTT assay. This assay was used to evaluate cell proliferation and cytotoxic activity.

2.5.9. MTT Cell Proliferation Assay

In this assay, an MTT assay kit was used, to evaluate the cytotoxic of NCs on the cell line, MTT dye is reduced by metabolically active cells to form an insoluble purple formazan product that is quantifiable by spectrophotometry.

The cell viability assay was applied to determine the IC50 concentration of the used nanocarriers. This assay depends on the crystal formation which occurs when the live cell releases the NADH enzyme, in their metabolic activity. It can convert the tetrazolium dye 3-(4, 5- dimethylthiazol-2-yl)-2, 5 diphenyltetrazolium bromide from yellow color to the purple one, due to oxidative reaction, the higher crystal is formed from the live cell. Then, a dissolvent (DMSO) is used to dissolve the crystal. Each of the cell lines was seeded differently, due to their growth property and doubling time. For this

purpose, a 96-well plate was used, and 5000 cells of SKBR-3 per well, and a 2000 cell of MCF-10A, were seeded in a total 100µl medium, in 4 replicate for each different drug concentration. Two different plates were prepared to take the result after 48- and 72-hours. The next day, the old medium was removed and 100 µl of each drug prepared was applied, below detail concentration of the only DOX, DOX-free micelles, and micelles DOX-loaded, DM, DMA, and DMP are clarified below.

- DOX on SKBR-3 ranging concentration from (0-6 µM), MCF-10A ranging from (0-2 µM) was applied.
- Free micelles without DOX for SKBR-3, MCF-10A, ranging concentration from (0-100 µg) were applied as (0, 1µg, 5 µg, 10 µg, 50 µg, 100 µg).
- Micelles DOX-loaded, DM, DMA, and DMP concentration on SKBR-3 was (0, 0.25 µM, 0.5 µM, 1 µM, 2 µM and 4 µM), for MCF-10A micelles DOX-loaded DM, DMA, and DMP concentrations were (0, 0.125 µM, 0.25 µM, 0.5 µM and 1 µM).

After drug concentration was applied. The cells are incubated at 37 ^{o}C, 5% CO2, after 48 hours and 72 hours, a 10 µl of MTT dye (5mg/ml dissolved in 1X PBS) was added to each well, the cells incubated for 4 hours at 37 ^{o}C, 5% CO_2, next, the plate was centrifuged at 1400 RPM for 10 minutes, the supernatant was removed, and a 100 µl of DMSO was added to each well to dissolve the crystal, the plate was put on a shaker for 10 minutes, finally, the absorbance value was detected by the spectrophotometer at 570 nm.

2.5.10. Fluorescence Image to Examine Drug Uptake

The fluorescence imaging was applied to understand the drug uptake and release in both cell lines, SKBR-3 and MCF-10A. This experiment can explain drug intensity inside the cell that can be achieved by cell staining followed by analyzing the fluorescence intensity. Firstly, a 6-well plate was taken and a sterile slide was placed inside the well. For SKBR-3, $250x10^3$/2ml cells were seeded, for MCF-10A, a $150x10^3$/2ml cells were seeded in plate. After the next day, the medium was discarded and drug IC_{50} (0.34 µM) of SKBR-3 was used for also for all micelles drug-loaded (DM and DMA, and DMP), for MCF-10A the IC_{50} of 0.12 µM was applied for all micelles drug-loaded, DM, DMA and

DMP, after 48 hours the drug was removed, the cell washed with 2 ml of 1X PBS for two times, nextly, we follow the steps in cell fixation (2.4.10.1) and cell staining (2.4.10.2).

2.5.10.1. Cell Fixation

For the cell fixation, 2 ml of paraformaldehyde (PFA) 3.7% was added to each well, followed by 20 minutes of incubation, next PFA was removed, cell was washed 3 times with 2 ml of 1X PBS.

2.5.10.2. Cell Staining by DAPI

Cell staining was applied by using DAPI, a (5 mg/ml) of DAPI stock was prepared at (1:500) dilution rate, 4 ul of DAPI was added to the cell and incubated for 30-45 minutes, wash 3 times then a coverslip was placed on the slides, the image was taken as 10 different images, at Alexa flour 594 to understand DOX intensity, DOX is a red fluorescence, DOX emission is 595 and, to understand its distribution in SKBR-3 and MCF-10A, the image was taken in red, blue, phases then DAPI and DOX image was merged to understand the fluorescence intensity, by using the image-J program.

2.5.11. Apoptosis assay

In this study, the apoptotic effect of the NCs was determined by JC-1 assay, a mitochondrial membrane potential assay, JC-1 assay evaluates the loss of mitochondrial membrane, also the apoptotic assay, Annexin-V/PI double staining was used to determine the apoptosis, necrosis percentage after NCs application.

2.5.11.1. Annexin-V/PI Double Staining

Annexin-V assay was applied to determine the effect of NCs in terms of cell apoptosis or necrosis on the SKBR-3 cell line. Two dyes were used in this experiment, PI is DNA fragmentation detection dye, Annexin-V (FITC) is a phosphatidylserine binding dye, for this purpose, a $5x10^5$ cells/2ml were seeded in a 6-well plate, two wells for experimental control and cell control, and three well for drug application. The cell was

incubated at 37 ^{o}C, 5% CO_2, next day, the IC50 value of 0.34 µM was applied for DM, DMP, and DMA, to understand the effect of NCs on cell apoptosis, after 48 hours, the cell medium was collected in falcon tubes, and 1 ml of trypsin-EDTA was added and incubated at 37 ^{o}C ,5% CO_2, then cells were collected with 4ml medium, falcon tubes were centrifuged at 800 RPM for 5 minutes, then the supernatant was removed, and the pellet washed with 5 ml of 1X PBS.

The cells were re-centrifuged at 1000 RPM for 10 minutes. The supernatant was removed, and all tubes dissolved in 200 µl of Annexin Binding Buffer (ABB), experimental control tubes were dissolved in 600ul of (ABB), to be separated into 3 tubes, (Figure 2.2). the first tube is non-stain, the second was stained with 2 µl of PI, the third tube was stained with FITC, all other tubes were stained with 2 µl of both FITC and PI, then the cell was incubated for 15 minutes in dark, finally, flow cytometry used to detect apoptotic and necrosis percentage. In the alive cells, phosphatidylserine is located in the inner membrane of the cell, while under apoptosis effect, the phosphatidylserine is located in the outer membrane of the cell, the FITC binding can bind under apoptosis effect.

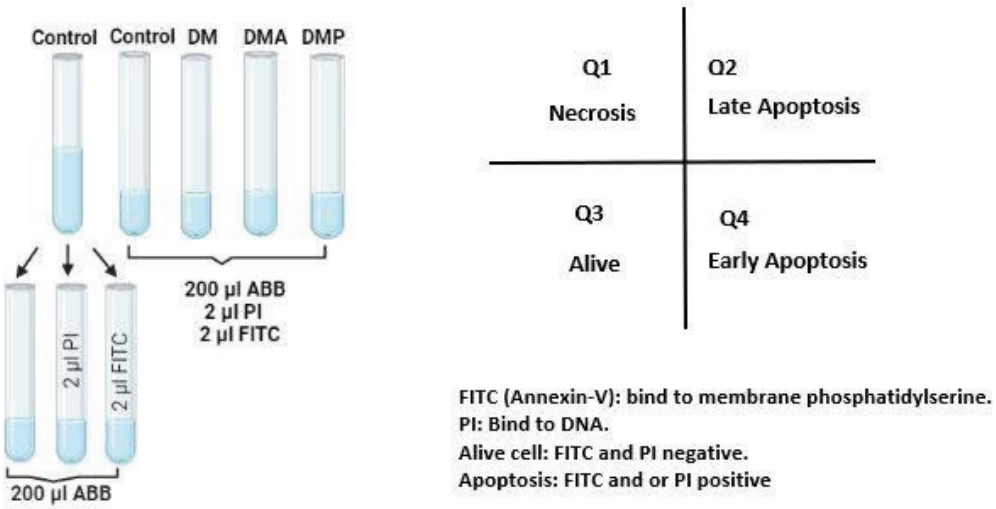

Figure 2.2 Annexin-V/PI experimental cell staining steps and flow cytometry quadrants. (Figure drawn by BioRender).

2.5.11.2. JC-1 assay

JC-1 assay kit obtained from Cayman, mitochondrial membrane potential assay was used to detect the effect of NCs on the loss of mitochondrial membrane potential. The JC-1 dye is a fluorescent dye it can bind to mitochondria.

Firstly, the JC-1 buffer was prepared by dissolving 1 tab in 100 ml of dH_2O, also a 1X JC-1 dye staining solution was prepared with a culture medium.

$5x10^5$/2ml of SKBR-3 cells were seeded in a 6-well plate, the next day a 0.34 µM/2ml of DM, DMP, and DMA was applied. After 48 hours of incubation at 37 ºC, %5 CO_2, 500 µl of Trypsin-EDTA was added and incubated at 37 ºC, %5 CO_2, the cell was collected with 4 ml medium, and centrifuged at 1000 RPM for 5 minutes. The supernatant was removed, then pellet washed with 1 ml of 1X PBS. Then, a 1 µl of staining solution was added, after 5 minutes of incubation with the buffer at 37 ºC, A 10 µl of JC-1 staining solution was added, followed by another incubation for 20 minutes at 37 ºC, then a 100 µl of the solution was added to each black well plate, finally, the result was examined by spectrometry at Excitation-Emission 550-600 nm and 485-535 nm, from Thermo ELECTRON CORPORATION Multiskan Spectrum.

2.5.12. Western Blotting

Western blotting was used to show the apoptotic, anti-apoptotic, and proapoptotic protein levels after the cell was treated with NCs.

2.5.12.1. Protein Isolation, Cell Lysis

$5x10^5$ cells/2 ml medium cells of the SKBR-3 cell line was seeded in 6 well-plate After 1 day, the old medium was removed, drugs were added, IC50 value 0.34 was applied for DM, DMA, DMP as 2 ml/well, the cell incubated at 37 ºC, %5 CO_2 for 48h, after 48 hours, cell medium was collected in tubes, and 1ml of Trypsin-EDTA was added and incubated at 37 ºC, %5 CO, the cell collected with 4ml of the medium, and centrifuged at 800 RPM for 5 minutes. The supernatant was removed, then pellets were dissolved in 150 µl of Tris buffer, the tubes were re-centrifuge at 14000 rpm for 20

minutes at +4 ºC, finally, the supernatant was collected (they contain the proteins) in a new Eppendorf tube, and stored at -86 ºC to be used.

2.5.12.2. Determination of the Protein Concentration by BCA Assay

The protein sample was prepared by BCA assay, which adjusts their concentration, The BCA stands for the Bicinchoninic acid, it produces a purple color based on the amount of protein concentration, then a standard curve used as a reference for the calculation, there are two steps, first one is the preparation of (standard and a working) solutions, the standard solution was prepared from 2.0 mg/ml BSA, and prepared in different concentrations to be used as a reference in determining the protein concentration, based on (Table 2.6). The working solutions were prepared by mixing solution A and solutions B in a ratio of 50:1, then in a 96-well plate, a 25 µl of the standards and unknown sample were put in the microplate, then a 200 µl of the working solution was added to the samples. For proper mix, the plate was put on a shaker for 30 seconds, the plate was incubated at 37°C for 30 minutes. Finally, the protein concentration absorbance value was measured at 562 nm by spectrometry.

Table 2.6. The preparation of standard BSA protein concentrations.

BSA	Diluent volume dH_2O	Final concentration
0	400 µl	0 µg/ml
40 µl from 125 µg/ml	160 µl	25 µg/ml
100 µl from 250 µg/ml	100 µl	125 µg/ml
100 µl from 500 µg/ml	100 µl	250 µg/ml
100 µl from 1,000 µg/ml	100 µl	500 µg/ml
100 µl from 1500 µg/ml	100 µl	750 µg/ml
100 µl from 2000 µg/ml	100 µl	1,000 µg/ml
150 µl from 2,000 µg/ml	50 µl	1,500 µg/ml
200 µl from 2,000 µg/ml	0	2,000 µg/ml

2.5.12.3. Acrylamide Gel Preparation

Acrylamide Gel was prepared by using TGX Stain-FreeTM FastCastTM, Acrylamide Kit (10%). First, the resolver solution was prepared based on (Table 2.7) and loaded between the two glasses, then a stacker solution was prepared based on (Table 2.8) and loaded between the two glasses. Polymerization is occurred for around 30 minutes, the sample protein was loaded. Before loading the protein, they were mixed with 2-mercaptoethanol and Laemmle buffer (4X), to denature the protein. The mixture of protein samples and buffer was incubated at 95 ºC for 5 minutes. The well separator was added and 1X of TGS of running buffer was added in the tank, then a 4 µl of Standard marker was loaded and a 25 µg of protein sample loaded. The samples were run until we observe the dye at the gel bottom. The running process was set for 1.5 hours at 90 Volt and 19 milliamperes.

Table 2.7. The SDS page resolver solution ingredient and concentration

Resolver Solution Ingredient	Volume
Resolver A	3 ml
Resolver B	3 ml
Ammonium persulfate (APS), 10%	30 µl
TEMED	5 µl

Table 2.8. The SDS page stacker solution ingredient and concentration.

Stacker Solution ingredient	Volume
Stacker A	1 ml
Stacker B	1 ml
Ammonium persulfate (APS), 10%	10 µl
TEMED	3 µl

2.5.12.4. Protein Transfer to The Membrane

The proteins were transferred from acrylamide gel to polyvinylidene fluoride (PVDF) membrane. The PVDF firstly were immersed with methanol for 10 minutes to facilitate the effective transfer. Then, it is immersed in a 1X transfer Buffer, as a two-set piece of stacks immersed with 1X transfer buffer. Then, all were assembled as, one set piece of stacks, PVDF, gel, then another set piece of stacks, and put in a semi-dry transfer machine by using Trans-Blot® TurboTM at voltage 25 and 1.3 amperes for 22 minutes, After transfer, the membrane was incubated for the blocking in a 5% of BSA for 1 hour at room temperature. Membrane washed with 5 ml of 1X TBST, 3-times for 5 minutes. The membrane is coated with primary antibody (Table 2.9) for overnight incubation at 4 ^{o}C, next day. The membrane was washed with 5 ml of 1X TBST buffer, 3-time for 10 minutes. Next, it was incubated with a secondary antibody (Table 2.9) in 5% BSA for 1 hour at room temperature. The membrane was washed again with 5 ml of 1X TBST buffer 3 times/10 minutes, to visualize the band. A Clarity TM Western ECL, Enhanced Chemiluminescence was applied on the membrane for 2 minutes incubation. Next, the imaging process was applied by the automated program imaging system with FUSION SL VILBER LOURMAT to observe the bands.

Table 2.9. Antibody used for protein level detection.

Antibody	Diluent solution	Dilution
GAPDH: loading control	5% BSA in TBS-Tween	1:2500
Bax: primary Ab	5% BSA in TBS-Tween	1:1000
PARP1: primary Ab	5% BSA in TBS-Tween	1:2000
Pro-Caspase-3 primary Ab	5% BSA in TBS-Tween	1:2000
Bcl-2: Primary Ab	5% BSA in TBS-Tween	1:1000
IgG: Secondary Ab	5% BSA in TBS-Tween	1:3000
Bak: primary Ab	5% Milk in TBS-Tween	1:5000
Bcl-xL primary Ab	5% Milk in TBS-Tween	1:1000

2.5.13. Determination of The Cytostatic Effects of NCs on SKBR-3

The cytostatic effect of NCs can be determined by cell cycle assay. This assay was used to understand the cytostatic effect of the NCs on SKBR-3 by using flow cytometry to measure the DNA content and evaluating the cell growth, survival, or arrest.

2.5.13.1. Cell Cycle Analysis

In this assay, propidium iodide (PI) which binds to DNA allows for the measurement of DNA content in G0/G1, G2/M, and S phases, depending on the fluorescence intensity.

For this purpose, $6x10^5$/2 ml cells of SKBR-3 were seeded in a 6-well plate and incubated at 37 ºC 5% CO2, after 24 hours the IC50 value 0.34 μM/2 ml was applied for DM, DMA, DMP, to compare the effect of these NCs on cell cycle phases. After 48 hours of incubation time, the cell was collected in tubes, and 1 ml of trypsin-EDTA was added, after 1-7 minutes at 37 ºC 5% CO_2, cells were collected with 4 ml medium. Then cells were centrifuged at 800 RPM for 5 minutes. The supernatant was removed, and the pellet was dissolved with 1 ml of cold PBS. The tubes were placed on ice for 15 minutes. The cells were fixed with 4ml of 100% ethanol from -20 ºC added gently. The cells were stored at -20 ºC overnight. The next day, the cell was centrifuged at 800 RPM for 5 minutes, supernatants were removed, and the pellet dissolved with 5 ml of cold PBS. Then, the cell was centrifuged at 1200 RPM for 10 minutes, the pellet was dissolved in 200 ul of 1x PBS and 0.1% of triton, 20 μl of RNase-A final concentration of 200 ug/ml was added, followed by 30 minutes incubation at 37 ºC. Then 20ul of PI final concentration (1 mg/ml) was added, was followed by 10 minutes of dark incubation at room temperature. Finally, the fluorescence intensity of the cell cycle phases was detected by flow cytometry in IZTECH-BIOMER.

2.5.14. Genotoxic Analysis by Comet Assay

Comet assay is a sensitive DNA damage assay, it can detect DNA damage. The effect of NCs (DM, DMA, DMP) on DNA damage in SKBR-3 cell line, was investigated by this assay. Firstly, 1 gram of low melting agarose and 1ml of 1X PBS was mixed and

heated until it dissolved. For the coating slide, normal melting agarose was prepared by mixing 1g of agarose and 100 ml of dH_2O. The slide was covered with normal agarose, the slide was dipped from one side and incubated at +4°C for 1 hour, in 6 well plates as 500k cells/well then the IC_{50} value of 0.34 µM was applied on cells. After 48 hours, under alkaline conditions, cells were mixed with low melting agarose, in the water bath, 40 µl of the cell, and 140 µl of 1% low melting agarose. Then, 75 µl of the sample was taken and put on the slide, around two drops, and covered with a coverslip. Then, slides were incubated in +4°C for 30 minutes, the coverslip was removed and the slides were immersed in lysis solution for 1 hour at +4°C. After lysis, the slides were rinsed with enzyme buffer or PBS buffer 3 times, then slides were put in the tank for the electrophoresis, and were covered with buffer, alkaline electrophoresis solution, for 40 minutes at 25V 300 mA. The slide waited for the neutralizing buffer for 10 minutes, then immersed in water for 10 minutes. After they dried, 10 µg/ml of PI was added, the slide was examined by fluorescence microscopy. Finally, the comet area calculation was evaluated by comet2.0 Score software.

CHAPTER 3

RESULTS AND DISCUSSION

3.2. DOX-Free Micelles Show No Anti-Proliferative Effects on SKBR-3 and MCF-10A

In the first step, the cytotoxic effect of DOX free micelles was examined to test *in vitro* effect of Nanocarriers (NCs) on SKBR-3 and MCF-10A that was achieved by step-wide increasing the concentration for all NCs (0,1-100 µg / ml), at 48 hours and 72 hours.

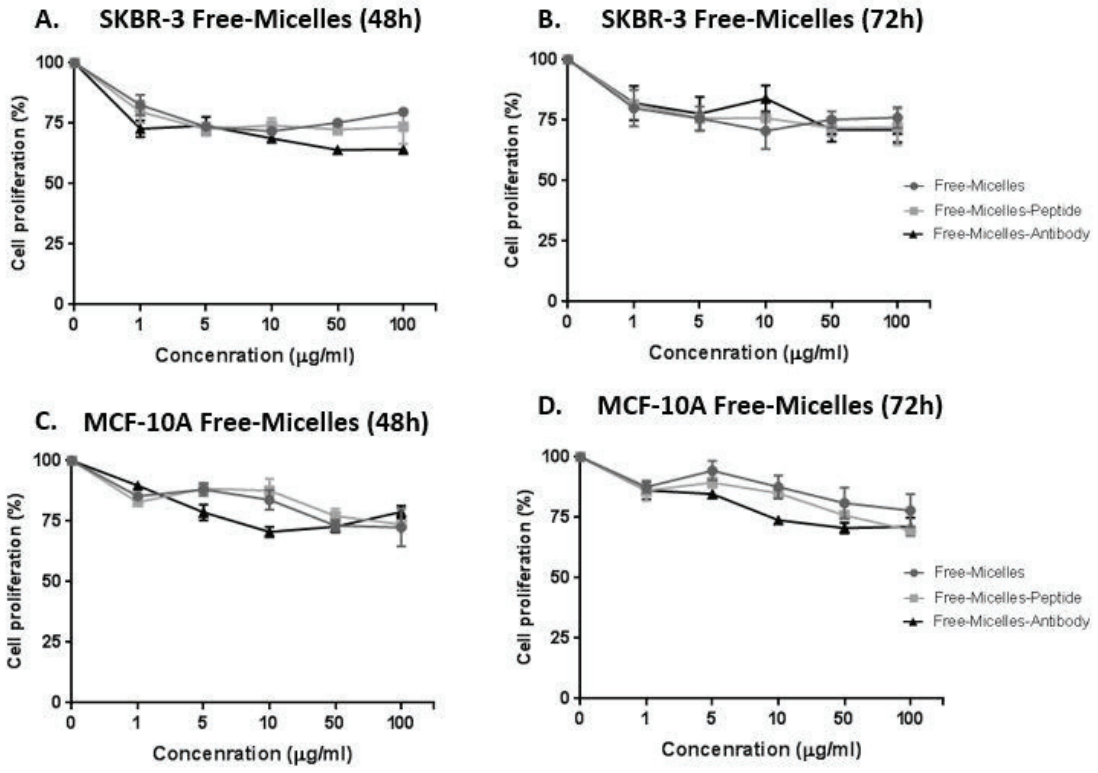

Figure 3.1. Cytotoxic effect of free micelles (0-100 µg / ml) at 48- and 72-hours.

SKBR-3 (A-B) and MCF-10A (C-D). The study consists of three dependent replicates. Statical analysis done by using paired t-test, NS: not significant, $p < 0.05$: *; $p < 0.01$: **; $p < 0.001$: *** was considered significant. Error bars represent standard deviation. Error bars are not visible when graphics are smaller than the thickness of the lines.

The cytotoxic effects were examined by cell proliferation assay (MTT), as it is shown in (Figure 3.1). The three categories of DOX-free-NCs which are free-micelles, free-micelles-peptide, and free-micelles-antibody were determined on both cell lines, SKBR-3 and MCF-10A cells at a concentration ranging from (0.1-100 µg / ml). Our result showed that there was no cytotoxic effect of all DOX-free-NCs (0-100 µg / ml) on SKBR-3 breast cancer cells and MCF-10A healthy breast epithelial cells. When cell viability percentages were compared, it was noticed to be between 100-75%.

Figure 3.1A showed a continuous proliferation of SKBR-3 cell line upon the exposure to DOX-free-NCs after 48 hours of incubation time, also, at 72 hours (Figure3.1B). Cell proliferation was at around 75% which represents no cytotoxic effects. Figure 3.1C, D showed that upon exposure to DOX-free-NCs on MCF-10A cells, at 48 and 72-hours respectively. The cell proliferation was also at around 100-75%. At the end of this step, our result showed that in the three categories, DOX-free-NCs had no cytotoxic effect on the two cell lines.

3.3. Determination of IC_{50} Value of DOX on Breast Cancer and Healthy Breast Cell Lines

The cytotoxic effect of DOX molecules on SKBR-3 cells and MCF-10A cells was investigated by MTT assay. The main purpose was the determination of the concentrations of the only DOX on breast cancer and health cell line. This determination was used for DOX-loaded-NCs to be carried out like determined doses. This step was important to determine DOX-loaded-NCs concentration. For this study, the doses that were used were based on the previous studies of literature.

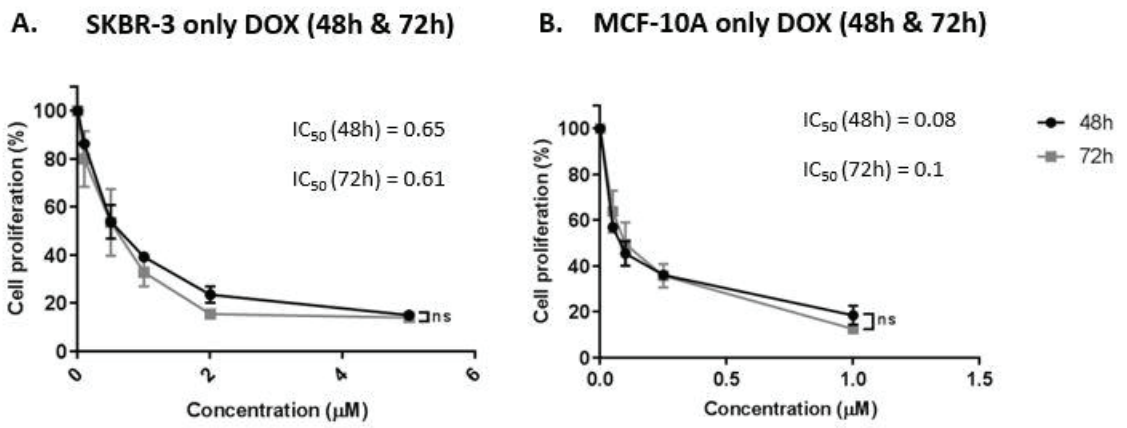

Figure 3.2. The cytotoxic effect of DOX molecule (0.5-5μM) on SKBR-3 and MCF-10A cells (0.05-1μM) at 48- and 72- hours.

The study consists of three dependent replicates. Statical analysis done by using paired t-test, NS: not significant, p <0.05: *; p <0.01: **; p <0.001: *** was considered significant. Error bars represent standard deviation. Error bars are not visible when graphics are smaller than the thickness of the lines.

Figure 3.2A showed the IC50 value of DOX molecule on SKBR-3 cell line at 48hr and 72hr that were 0.65 μM, 0.61 μM, respectively. The IC_{50} of DOX on MCF-10A at 48hr and 72hr is 0.08 μM, 0.1 μM, respectively, shown in Figure 3.2B. After this study, the specified concentrations will be used when determining the cytotoxic effect of all DOX-loaded micelles.

3.4. Drug-Loaded Micelles Increase The Cytotoxic Effect of DOX in SKBR-3 Cell Line at 48 hr

In this part of the study, an MTT assay was applied to determine the IC50 concentration for DOX-Micelles (DM), HER2 targeting peptide (LTVSPWY)-conjugated-DOX-loaded micelles (DMP), and monoclonal antibody (Herceptin)-conjugated DOX-loaded micelles (DMA) at 48 hours. The concentration of DOX-loaded micelles for SKBR-3 was (0-5 μM) and for MCF-10A (0-1 μM) were applied.

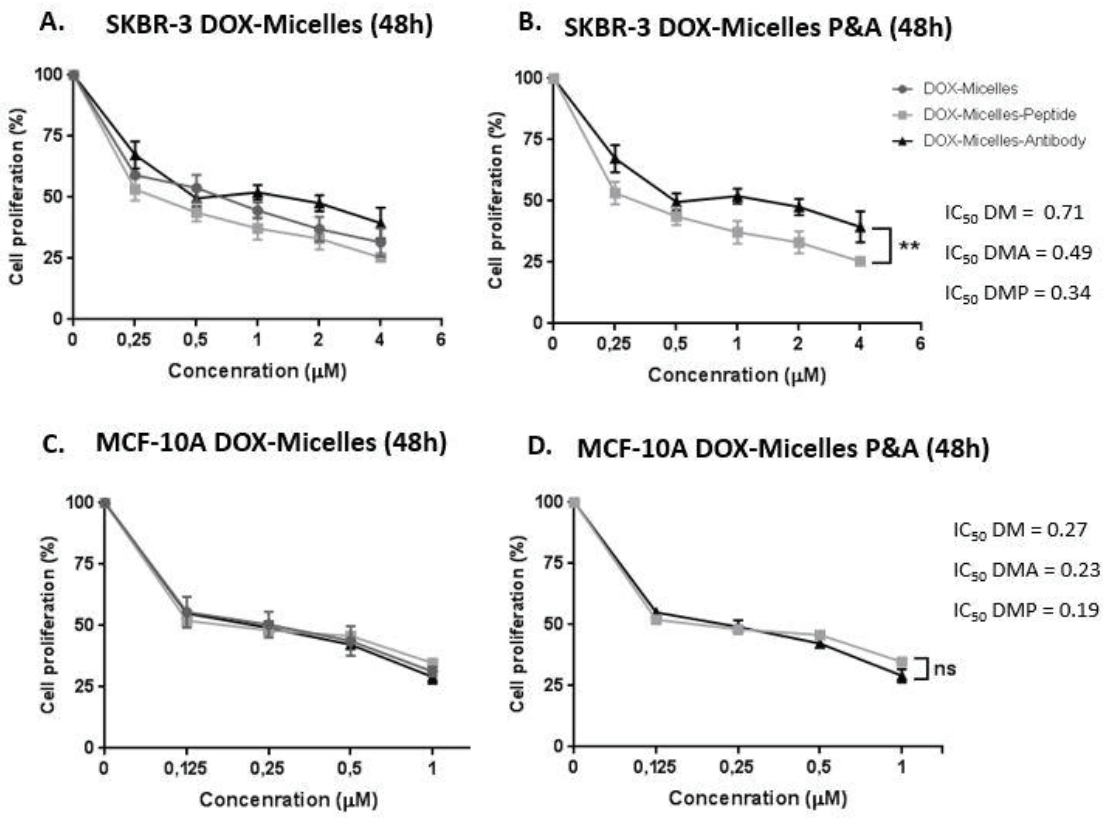

Figure 3.3. The Cytotoxic effect of DOX-loaded micelles (0-5 μM) on SKBR-3 (A-B) and (0-1 μM) MCF-10A (C-D) cells at 48 hours.

The study consists of three dependent replicates. Statical analysis done by using paired t-test, NS: not significant, $p < 0.05$: *; $p < 0.01$: **; $p < 0.001$: *** was considered significant. Error bars represent standard deviation. Error bars are not visible when graphics are smaller than the thickness of the lines.

IC_{50} value of SKBR-3 with (0-5 μM) DOX-loaded micelles result was 0.71 for DM, 0.34 μM for DMP, and 0.49 μM for DMA (Figure 3.3A). While MCF-10A with (0-1 μM) of DOX-loaded micelles, result was 0,27, 0.19 μM for DMP and 0.23 μM for DMA. Based on these results, a clear difference was observed on the SKBR-3 cell line after applying DM, DMP, and DMA by IC_{50} of (0.71, 0.34 μM, 0.49 μM) respectively, (Figure 3.3A). The effect of DMP and DMA on cell proliferation of SKBR-3 at 48 hours was demonstrated the statistical difference between DMA and DMP (Figure3.3B).

On the other hand, for the MCF-10A cell line, the IC_{50} of DM, DMA, and DMP was 0.27 μM, 0.23 μM, 0.19μM respectively, as is shown in (Figure 3.3C). When we compare the result of DMP to DMA in MCF-10A cells, we did not see an observable statistical difference between them (Figure3.3D).

The effect of DMP was more effective on the SKBR-3 cell line, also DMP can increase drug efficiency from 0.65 μM to 0.34 μM to compare only DOX application. A study done by (Khondee, Supang, et al.) showed that TNBC BT549 Luc and T47D cell lines which overexpress human mucin1 protein (MUC1), were treated with peptide DOX-loaded micelles had increased the effects of the drug in terms of targeting, efficiency, and uptake to compare their result to free DOX and only DOX itself (Khondee et al. 2018). Another study (Lee, Gee Young, et al.) had investigated DOX circulation by using micelles, showed that DOX circulation increased in blood circulation when they used DOX-loaded PEGylated peptide–DOX conjugate micelles. In their study, they use peptides that are cleaved by matrix metalloproteinases (MMP), which are overexpressed in lewis lung carcinoma LLC, *in vitro* study, and *in vivo*. DOX-loaded PEGylated peptide–DOX conjugate micelles showed more targeting efficiency. In addition to that, the drug released around the tumor site that is more than DOX itself and evaluation of DOX in serum were maintained longer in the plasma (Lee et al. 2007).

3.5. The Cytotoxic Effect of Drug-Loaded Micelles on SKBR-3 and MCF-10A at 72 hr

At this step of the study, IC_{50} concentration of DM, DMP, and DMA at 72 hours was determined on SKBR-3 and MCF-10A as applying concentrations ranging from 0-5 μM and, 0-1 μM respectively. The IC50 value of DM, DMP, and DMA showed 0.70-, 0.24- and 0.53- μM on SKBR-3, respectively. The IC50 value of DM, DMP, and DMA was 0.12-, 0.14- and 0,11- μM on MCF-10A, respectively. The effect of DMP is greater on HER2 positive SKBR-3 cell line to compare DM and DMA application, (Figure3.3A). However, there was no difference between them on MCF-10A, (Figure 3.3B) that showed the effect of DM, DMP, and DMA on cell proliferation of MCF-10A after 72 hours. Considering this situation, the effect of the micelles applied to the SKBR-3 cell compared to the MCF-10A, the DMP have high specificity and stability effects on targeted cells (Figure 3.3C) that demonstrated doses distribution of DOX loaded micelles at IC_{50} on SKBR-3 cells 48- and 72-hour. However, when examined in MCF-10A cells, stability decreases as it has a low specific effect (Figure3.3D) that showed dose distribution of DOX loaded micelles at the IC_{50} on MCF-10A cells 48- and 72-hour. Consequently, the

use of synthesized DOX-loaded HER2 targeting peptide micelles (DMP) is much suitable and effective for targeting therapy on HER2 positive SKBR-3 cell lines.

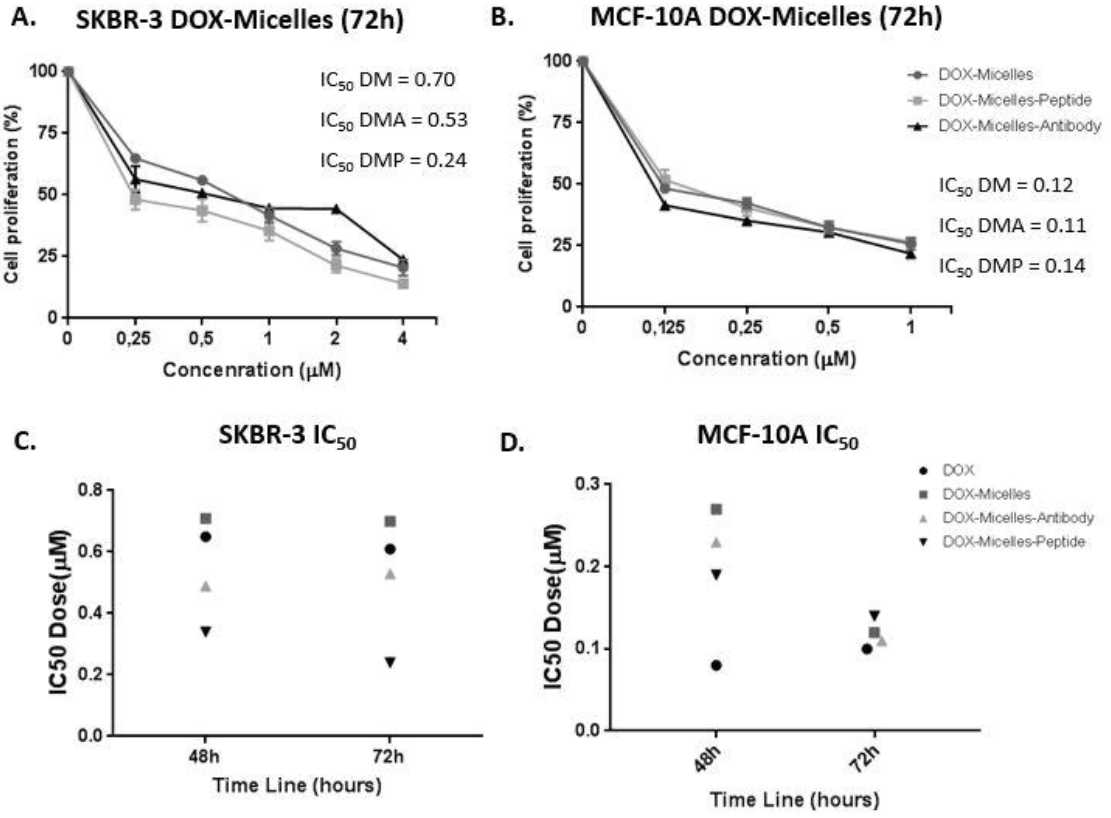

Figure 3.4. Cytotoxic effect of DOX loaded micelles at 72 hours for SKBR-3 (A) and MCF-10A (B), IC50 dose distribution of cells at 48- and 72 hours SKBR-3 (C) and MCF-10A (D).

The study consists of three dependent replicates. Statical analysis done by using paired t-test, NS: not significant, $p < 0.05$: *; $p < 0.01$: **; $p < 0.001$: *** was considered significant. Error bars represent standard deviation. Error bars are not visible when graphics are smaller than the thickness of the lines.

3.6. Doxorubicin Uptake Was Higher in SKBR-3 cell line Treated with DMP as Compared to DMA

In this study, fluorescence microscopy was used to define DOX uptake and compare all applied micelles. The IC_{50} value at 48 hours, 0.34 µM of micelles on SKBR-3, and 0.19 µM of micelles for MCF-10A were applied in all synthesized micelles DM, DMP, and DMA. The purpose is to understand the fluorescence intensity of the DM, DMP, and DMA, as it is shown in (Figure 3.5 and 3.6). In this step, the amount of DOX molecule in the cell was analyzed based on the fluorescence intensity in the cell using the image Image J program.

According to analysis results, it was observed that the DOX amount when DMP was applied in SKBR-3 cells, is more than the DOX amount when DM and DMA were applied. In addition to that, a significant difference was analyzed when it was statistically evaluated by t-test as it is shown in (Figure3.5).

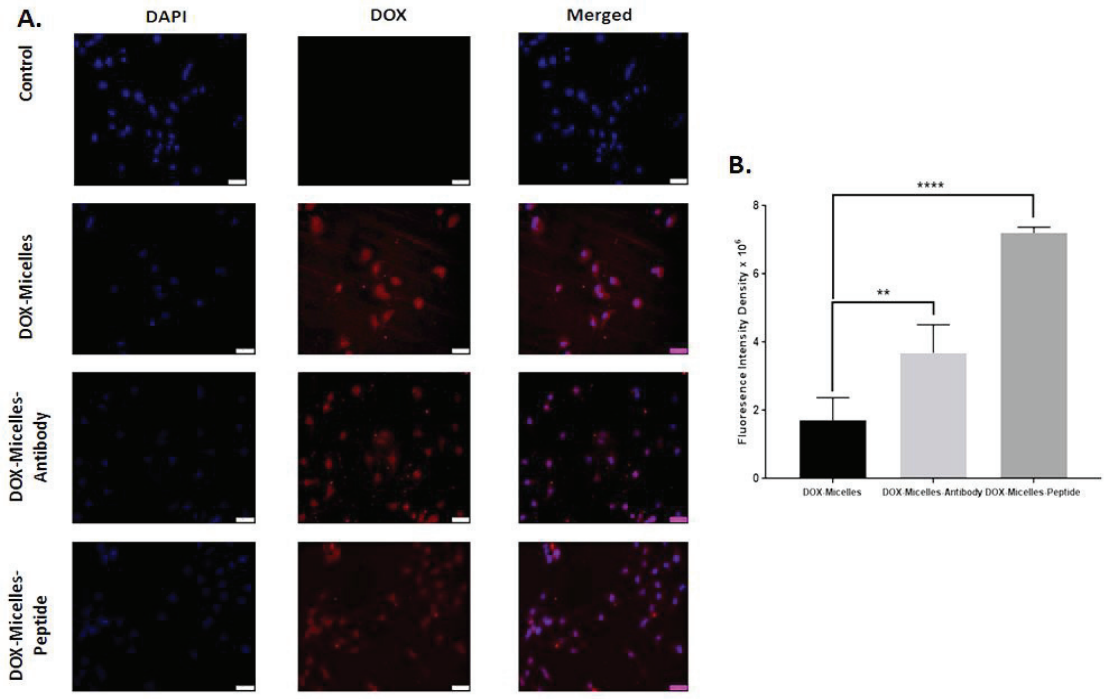

Figure 3.5. Analysis of the DOX fluorescence intensity of DM, DMA, and DMP IC50 in SKBR-3 cells at 48h.

DAPI: blue; DOX: red. SKBR-3 was grown in 6-well plate (250x10^3 cell/well) and treated with 0.34 µM at 48h. This study consists of the analysis of 10 images. Paired t-test, NS: not significant, p <0.05: *; p <0.01: **; p <0.001: *** was considered significant. Error bars represent standard deviation. When these graphics are smaller than the thickness of the lines, error bars are not visible.

3.7. DOX Uptake Was Similar in MCF-10A Treated With DM, DMA, and DMP When Examined By Fluorescence Microscope

According to analyzing DOX uptake by fluorescence microscope in MCF-10A, There was no significant difference between the three different applied micelles DM, DMP, and DMA as shown in (Figure 3.6).

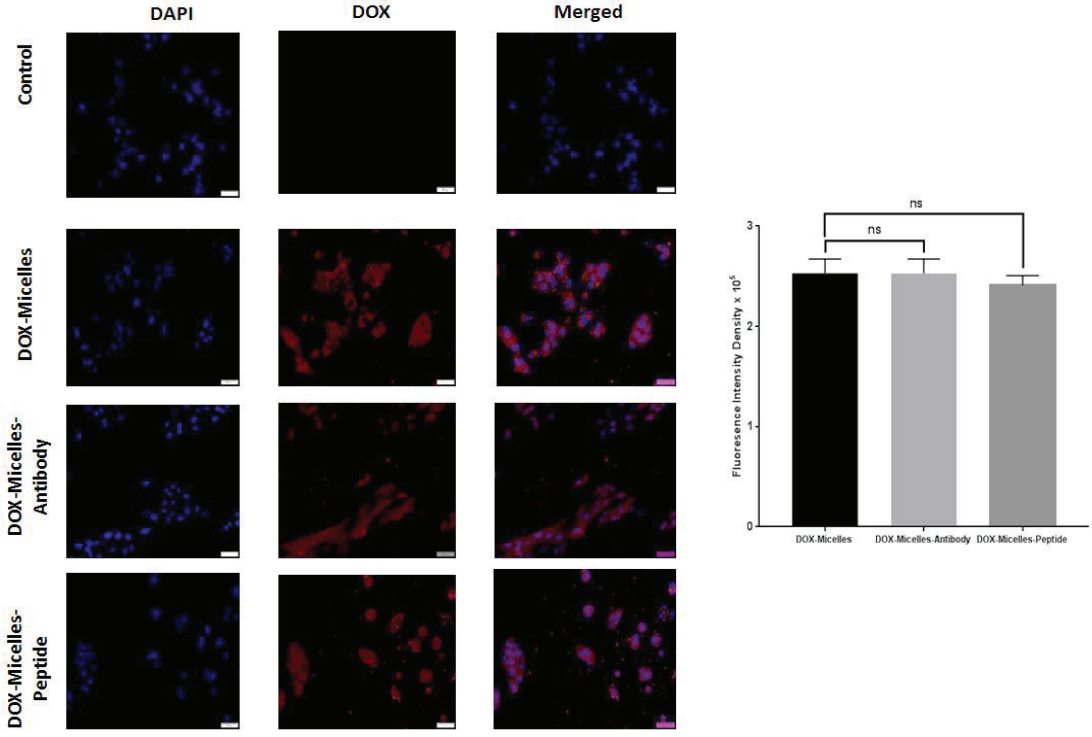

Figure 3.6. Analysis of DOX fluorescence intensity of DM, DMA, and DMP IC50 in MCF-10A cells at 48 h.

DAPI: blue; DOX: red. MCF-10A were grown in 6-well plate (150x10^3 cell/well) and treated with 0.23 µM at 48h. This study consists of the analysis of 10 images. Paired t-test, NS: not significant, p <0.05: *; p <0.01: **; p <0.001: *** was considered

significant. Error bars represent standard deviation. When these graphics are smaller than the thickness of the lines, error bars are not visible.

Our study showed uptaken DOX amount by SKBR-3 cell line was higher when DMP was applied that also confirmed the targeted effect of DMP including DOX release and uptaken amount by HER2 positive SKBR-3 cells. The uptaken DOX amount by MCF-10 was lower than by SKBR-3 This result represented an important drug feature, to lower the cytotoxic effect on healthy cells in addition to increase drug targeting and efficiency. A study showed cell-penetrating peptide (the HIV transactivator of transcription protein) TAT conjugated to DOX, increase drug uptake, and overcome cervical cancer cell line drug resistance (P. Zhang et al. 2013).

3.8. Mitochondrial Membrane Potential Decreased in SKBR-3 cells Treated with DMP as Compared to DM and DMA

The mitochondrial cells membrane potential analysis at 48 hours was determined by JC-1 assay. The loss of mitochondrial membrane potential (MMP) in SKBR-3 cells treated with 0.34 μM of micelles, after 48 hours of incubation time, cells were analyzed using 485/535-nm and 550/600-nm fluorescence spectroscopy using JC-1 dye. To show the role of apoptosis in response to change in cell membrane potential with this method, the decrease in mitochondrial potential was observed significantly and compared with the control group (Figure 3.7).

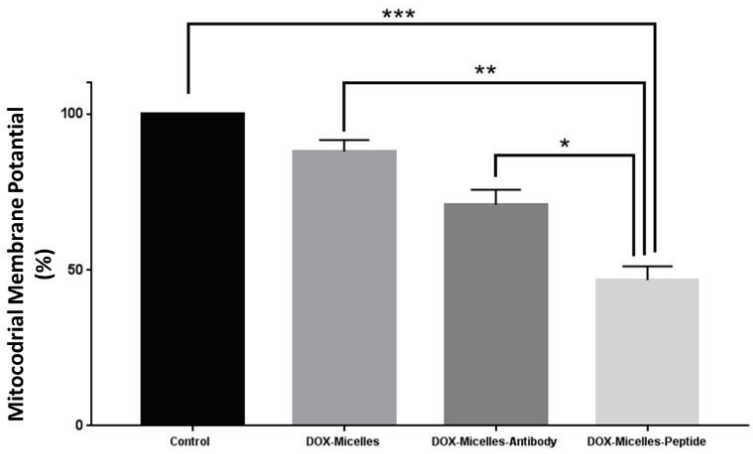

Figure 3.7. Apoptotic effects of mitochondrial membrane potential on SKBR-3 cells in response to DM, DMA, and DMP IC_{50} value at 48 h.

SKBR-3 cell grown in 6-well plate ($5x10^5$/2ml) treated with 0.34 μM. The study consists of three dependent replicates. Statical analysis done by using paired t-test, NS: not significant, $p < 0.05$: *; $p < 0.01$: **; $p < 0.001$: *** was considered significant. Error bars represent standard deviation. Error bars are not visible when graphics are smaller than the thickness of the lines.

The result of the JC-1 assay showed that the mitochondrial membrane potential of SKBR-3 changed in response to DM, DMA, and DMP. These results were analyzed and showed 87.9%, 70.8%, and 46.7% of MMP percentages, respectively. The apoptotic effect of DMP was higher on the SKBR-3 cell line (Figure 3.7). Previous research demonstrated that mitochondria were one of the first targets of DOX, to induce mitochondrial-mediated apoptosis pathways. These effects can occur due to modulation of Bcl-2 family protein, or reactive oxygen species (ROS). DOX can induce loss in MMP via modulation of Bcl-2 family protein such as Bax protein. Bax activation leads to apoptosis by evoking the release of cytochromes c which also can be released due to change in mitochondrial outer membrane pore formation (Petronilli et al. 2001; Kuznetsov et al. 2011).

3.9. DMP Induces Higher Apoptosis and Necrosis Rate in SKBR-3 cells at 48hr

In this step, the apoptosis and necrosis of SKBR-3 cells in response to NCs application at 48h were examined by Annexin-V/PI double stain assay. The percentage of apoptotic, necrotic, and alive cells was analyzed by flow cytometry.

In this experiment, Annexin-V/PI double staining was used to determine the four different quadrants, that show (Q1: Necrosis, Q2: Late apoptosis, Q3, Live cell, Q4: Early apoptosis). During this step, it was important to obtain a single cell stain to show fluorescence crosstalk for control by using (unstained cell, PI single labeling, Annexin-V single labeling, and Annexin-V/PI double stain).

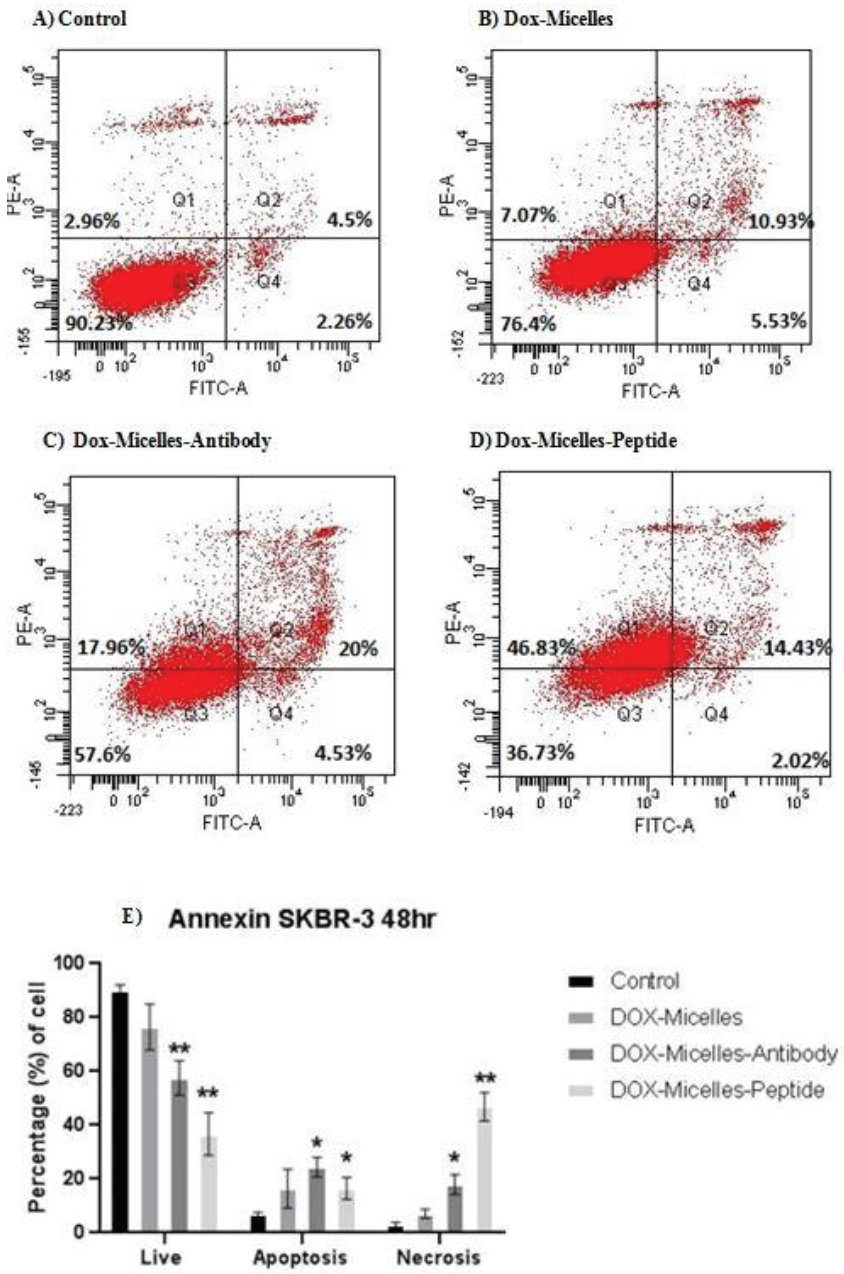

Figure 3.8. The apoptotic effect of DM, DMA, DMP on SKBR-3 cells at 48 h.

SKBR-3 cell grown in 6-well plate ($5x10^5$/2ml) treated with 0.34 µM. The study consists of three dependent replicates. Statical analysis done by using paired t-test, NS: not significant, $p < 0.05$: *; $p < 0.01$: **; $p < 0.001$: *** was considered significant. Error bars represent standard deviation. Error bars are not visible when graphics are smaller than the thickness of the lines.

The result of flow cytometry (Figure 3.8) shows that when treating SKBR-3 cell line with IC_{50} of 0.34 μM after 48h. Cells treated with DMP drug significantly increased in apoptosis at a percentage of 16.45% and necrosis at a percentage of 46.83% as shown in (Figure 3.8D) when the result was compared to the control group, which showed 90.23% of cells were alive, and 6.26% of cells went apoptosis (Figure3.8A). The differences were clear, in addition to that, when we compared SKBR-3 cells treated with DMP to cells treated with DMA (Figure 3.8C) we see that SKBR-3 cells treated with DMA also induced apoptosis and necrosis. However, 0.34 μM of DMP induced more apoptosis and necrosis effect on HER2 positive SKBR-3 cells. Moreover, the decrease in an alive cell in cells treated with DMA and DMP was 57.6% and 36.73%, respectively. The result of Annexin-V showed that DMP induced more apoptosis and necrosis in the SKBR-3 cell line. The drug is more effective on HER2 positive cells. Previous studies and literature had demonstrated that three types of cell death can occur in response to the DOX effect, those are apoptosis, necrosis, and senescence DOX-induced necrosis via PARP1 cleavage dependent (Shin et al. 2015; Christidi and Brunham 2021).

3.10. Bcl-2 Protein Level Decreased in SKBR-3 Cells Treated with DMP as Compared to DM and DMA at 48hr

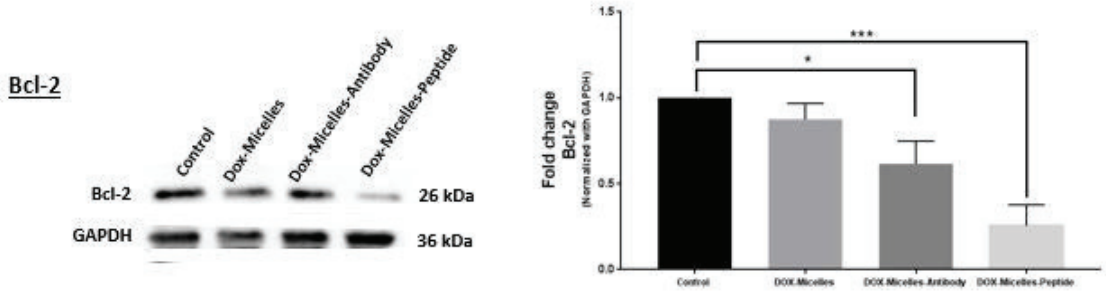

Figure 3.9. Effects of DM, DMA, and DMP on BCL-2 protein level for SKBR-3 cells at 48h.

GAPDH was used as an internal control. Cells are grown in a 6-well plate ($5x10^5$/2ml) treated with 0.34 μM of micelles. The study consists of three dependent replicates. Statical analysis done by using paired t-test, NS: not significant, $p < 0.05$: *; $p < 0.01$: **; $p < 0.001$: *** was considered significant. Error bars represent standard

deviation. Error bars are not visible when graphics are smaller than the thickness of the lines.

Bcl-2 protein is a family of proteins that regulates apoptotic, through either inducing or inhibiting apoptosis. In this study, the expression level of Bcl-2 was investigated by western blotting. GAPDH was used as internal control, to investigate the effect of NCs on Bcl-2 protein expression. Cells were treated with 0.34 μM of each DM, DMA and, DMP. The anti-apoptotic protein Bcl-2 was decreased in SKBR-3 cells treated with DMP, as it is shown in (Figure 3.9). The decreasing level of Bcl-2 in cells treated with DMA was less than DMP. These results showed that DMP with (LTVSPWY) peptide was more effective in terms of drug efficiency and decreasing the anti-apoptotic Bcl-2 compare to DMA, and DM.

3.11. Bax Protein Level Increased in SKBR-3 Cells Treated with DMP as Compared to DM and DMA at 48hr

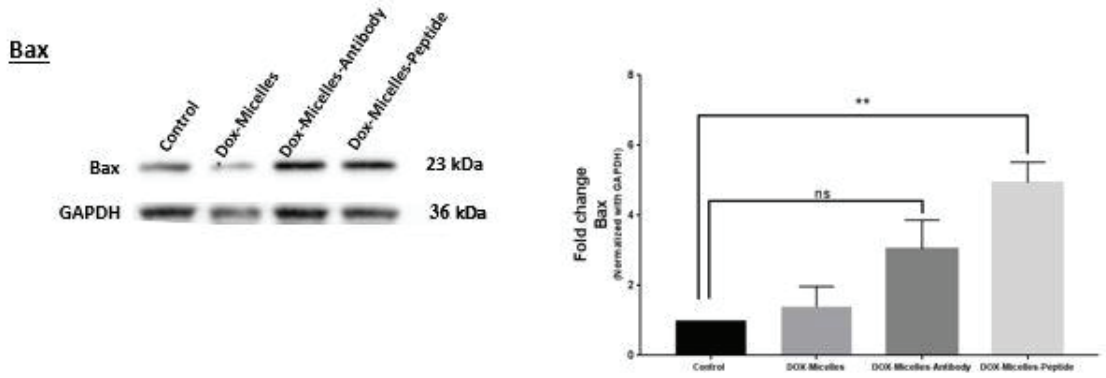

Figure 3.10. Effects of DM, DMA, and DMP on Bax protein level for SKBR-3 cells at 48h.

GAPDH is used as an internal control. SKBR-3 cells grown in a 6-well plate ($5x10^5$/2ml) treated with 0.34 μM. The study consists of three dependent replicates. Statical analysis done by using paired t-test, NS: not significant, $p < 0.05$: *; $p < 0.01$: **; $p < 0.001$: *** was considered significant. Error bars represent standard deviation. Error bars are not visible when graphics are smaller than the thickness of the lines.

Bax is a pro-apoptotic protein. The expression level of Bax protein was analyzed, GAPDH was used as an internal positive control. The result of the western blot showed a

significant increase in Bax protein level in SKBR-3 cells in response to DMP (Figure 3.10). On the other hand, the elevation of Bax protein level was not significant in SKBR-3 cells treated with DMA.

3.12. Pro-Caspase-3 and PARP1 Proteins Level Decreased in SKBR-3 Cells Treated With DMP as Compared to DM and DMA.

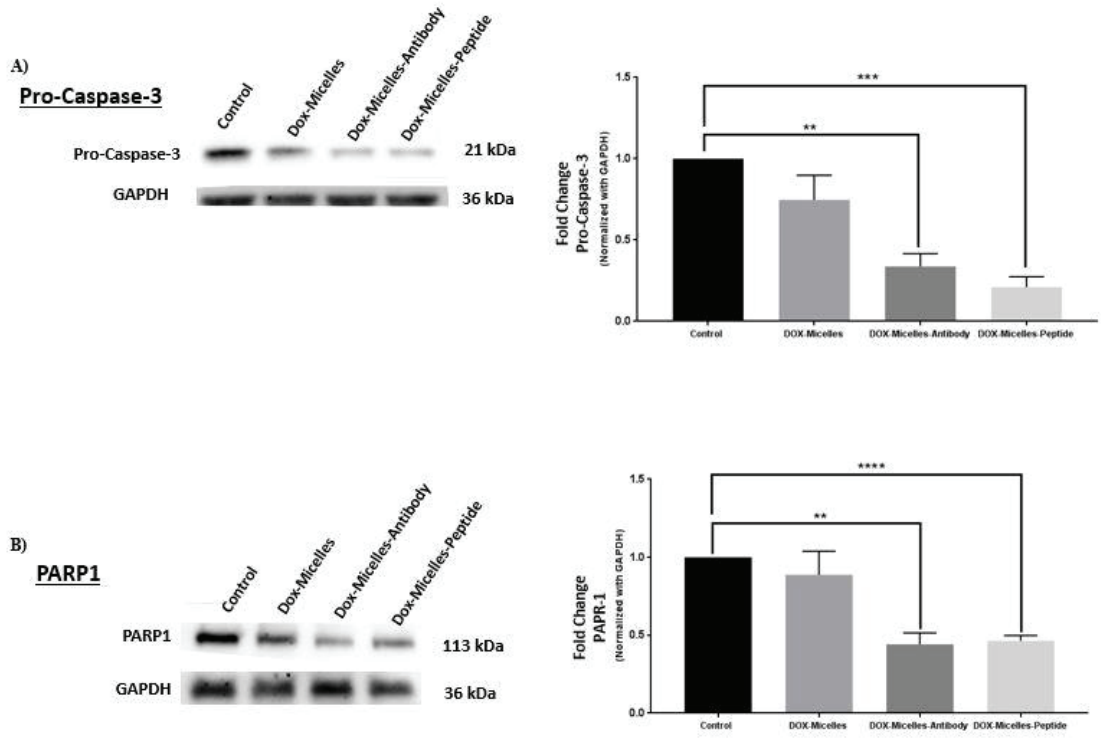

Figure 3.11. Effects of DM, DMA, and DMP on Pro-Caspase-3 (3.11A) and PARP1 proteins level (3.11B) for SKBR-3 cells at 48h.

GAPDH is used as an internal control. SKBR-3 cells grown in a 6-well plate ($5x10^5$/2ml) treated with 0.34 μM. The study consists of three dependent replicates. Statical analysis done by using paired t-test, NS: not significant, $p < 0.05$: *; $p < 0.01$: **; $p < 0.001$: *** was considered significant. Error bars represent standard deviation. Error bars are not visible when graphics are smaller than the thickness of the lines.

The apoptosis pathway is activated through the most common and specialized family of proteins. This pathway is regulated by cysteinyl-aspartate-protease (caspases). They are usually present in the cell as inactive zymogen form. Once the caspase is activated, it can initiate cell death by the mechanism of cleaving and activating caspase

effectors. Both Pro-Caspase-3 and PARP1 are known to be pro-apoptotic proteins. The cleavage of these two proteins is activated by the apoptosis intrinsic pathway. In our study, to determine Pro-Caspase-3 and PARP1 protein levels on SKBR-3 cells, GAPDH was used as an internal positive control. The pro-caspase-3 protein level decrease, which shows its cleavage rate, was decreased in the SKBR-3 cell treated with 0.34 μM of DMA, and DMP. However, data analysis shows that pro-caspase-3 decreased significantly in SKBR-3 cells treated with DMP, as it is shown in (Figure 3.11A). In addition to that, the study showed that PARP1 protein level decrease, which indicated its cleavage. DMP had more effect on increasing the cleavage rate of PARP1, compare the result to SKBR-3 cell treated with DMA which had a lower PARP1 cleavage rate (Figure 3.11B). The previous studies in the literature had shown that PARP1 is cleaved during apoptosis and necrosis (Shin et al. 2015).

3.13. Bak Protein Level Increased Significantly in SKBR-3 Cells Treated with DMP as Compared to DM and DMA

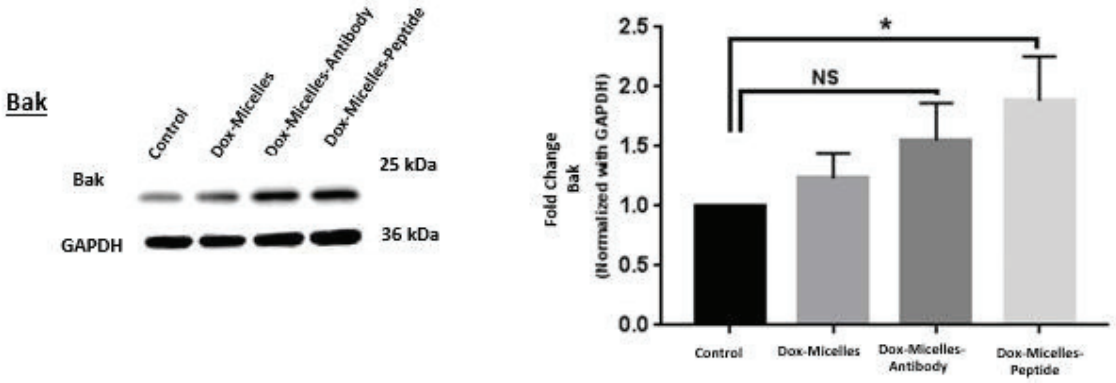

Figure 3.12. Effects of DM, DMA, and DMP on Bak protein level for SKBR-3 cells at 48h.

GAPDH is used as an internal control. SKBR-3 cells grown in a 6-well plate ($5x10^5$/2ml) treated with 0.34 μM. The study consists of three dependent replicates. Statical analysis done by using paired t-test, NS: not significant, $p < 0.05$: *; $p < 0.01$: **; $p < 0.001$: *** was considered significant. Error bars represent standard deviation. Error bars are not visible when graphics are smaller than the thickness of the lines.

The apoptosis pathway is regulated by Bcl-2 family protein, in parallel with the previous result, we investigated the expression level of Bak protein. Bak is a member of the Bcl-2 family protein. Under a normal condition in a healthy cell, both Bak and Bax are shuttled between the mitochondrial outer membrane (MOM) and the cytosol. The previous studies had shown that under apoptotic effect, Bak and Bax are activated and accumulated at the MOM. The oligomerization of both two protein causes the releasing of cytochrome c (pro-apoptotic factor) and induce apoptosis (Peña-Blanco and García-Sáez 2018).

In our study, to determine Bak protein expression level, GAPDH was used as an internal positive control, the result of a western blot for Bak protein, showed a significant increase in Bak protein level in SKBR-3 cells treated with 0.34 μM of DMP. On the other hand, when we compare the result to DMA and DM, the Bak protein level increase less significantly, which reveals that DMP was more effective.

3.14. Bcl-xL Protein Level Decreased in SKBR-3 Cells Treated with DMP as Compared to DM and DMA

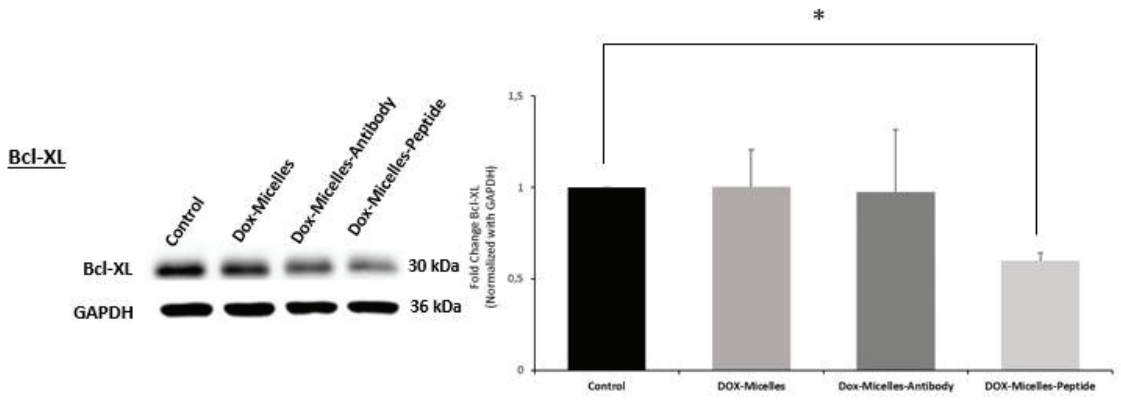

Figure 3.13. Effects of DM, DMA, and DMP on Bcl-xL protein level for SKBR-3 cells at 48h.

GAPDH used as an internal control. cell grown in 6-well plate ($5x10^5$/2ml) treated with 0.34 μM. The study consists of two dependent replicates. Statical analysis done by using paired t-test, NS: not significant, $p < 0.05$: *; $p < 0.01$: **; $p < 0.001$: *** was

considered significant. Error bars represent standard deviation. Error bars are not visible when graphics are smaller than the thickness of the lines.

Bcl-xl is an anti-apoptotic protein. In this study GAPDH was used as an internal positive control, the result of western blot show Bcl-xL protein level was significantly downregulated in SKBR-3 cell treated with 0.34 μM of DMP, while in cells treated with 0.34 μM of DM and DMA, Bcl-xl downregulation was not significant, previous studies had demonstrated that DOX decrease the expression level of Bcl-xL and upregulated Bax/Bak and caspase-8 which all involved in apoptosis induction (Sharifi et al. 2015).

3.15. Nanocarriers Application Changes Cell Cycle Profile and Induces G2/M Phase Arrest In Response to IC_{50} Value at 48 hr

In addition to determining the cytotoxic, apoptotic effects and DOX uptake of the NCs on SKBR-3 cancer cells, we investigate the cytostatic effect of the NCs on the SKBR-3 cell cycle profile (Figure 3.14.).

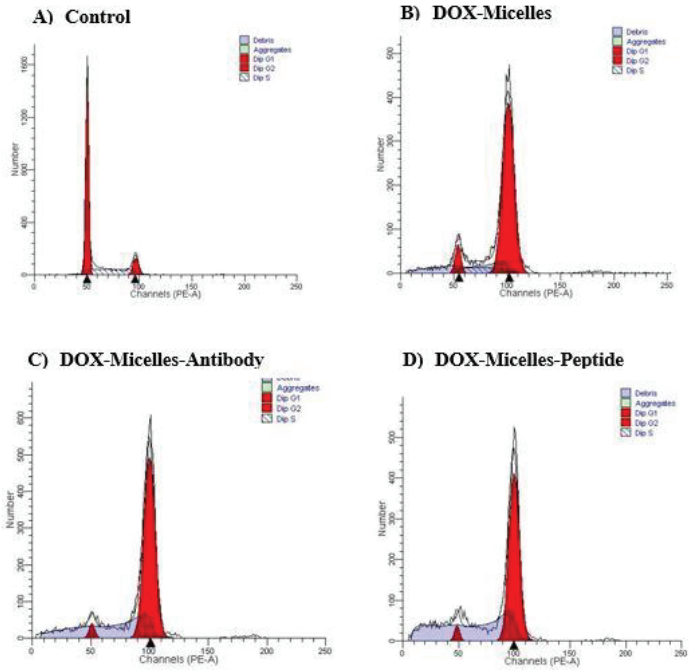

Figure 3.14. Effects of DM, DMA, and DMP on cell cycle phases of SKBR-3 cells at 48h.

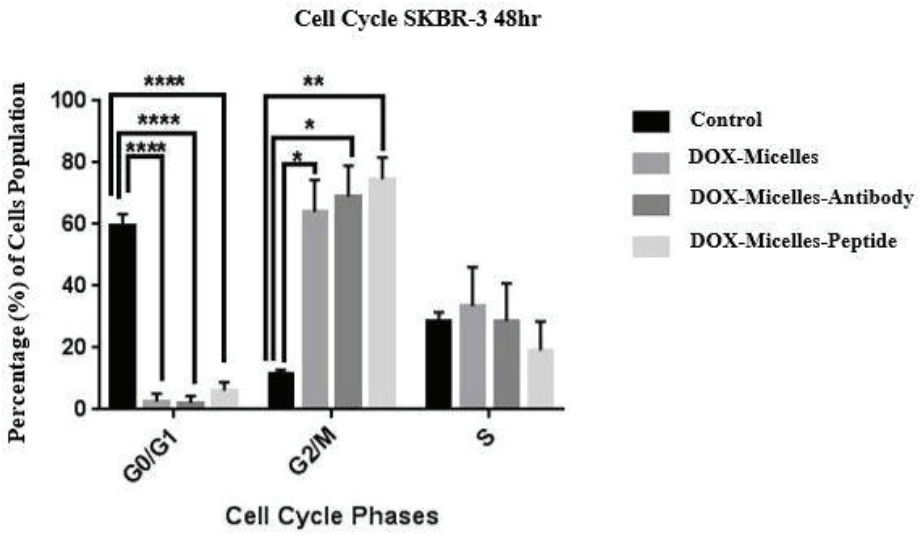

Figure 3.15. SKBR-3 cell cycle change in response to DM, DMA and, DMP at 48h.

SKBR-3 cell grown in 6-well plate ($6x10^5$/2ml) treated with 0.34 μM. The study consists of three dependent replicates. Statical analysis done by using paired t-test, NS: not significant, $p < 0.05$: *; $p < 0.01$: **; $p < 0.001$: *** was considered significant. Error bars represent standard deviation. Error bars are not visible when graphics are smaller than the thickness of the lines

SKBR-3 cells were treated with 0.34 μM of DM, DMA, and DMP that caused cell cycle arrest at the G2 phase, while the G1 phase was decreased significantly. These effects were caused by the DOX molecule. DOX was known as a topoisomerases-II inhibitor and the inhibition of topoisomerases-II causes cell cycle arrest at the G2 phase. However, cells treated with DMP had more effect on decreasing the G1 phase and increasing cell arrest at G2/M phases (Figure3.14). The result of the cell cycle study supported the role of DMP on targeting HER2 positive breast cancer cells and change in cycle profile leading to cell cycle arrest at the G2/M phase due to the DOX effect. Previous studies had shown the role of DOX in changing cell cycle profiles and causing cell cycle arrest at the G2/M phase (Kim, Lee, and Kim 2009).

3.16. DMP Induce Genotoxicity In SKBR-3 Cells Treated with DMP as Compared to DM and DMA.

For determination of the genotoxic effect of the NCs, SKBR-3 cells treated with 0.34 μM micelles, were carried out in alkaline electrophoresis buffer in the gel after 48

hours incubation time. The images were taken under fluorescence microscopy. Then, the color scale was determined by using the TriTek CometScore 2.0 program according to the visibility of the DNA amount as in (Figure 3.16) that demonstrated the Comet area and density.

Cells carrying undamaged DNA did not form tails while cells with damaged or broken DNA were observed to form the tail appearance, resulting from the speed differences in the electric field. Also, when we compared between micelles, (DM, DMA, and, DMP) it was observed that the effect of DMP was more effective on SKBR-3 cells compared to the same doses of DM and DMA on the SKBR-3 cell line.

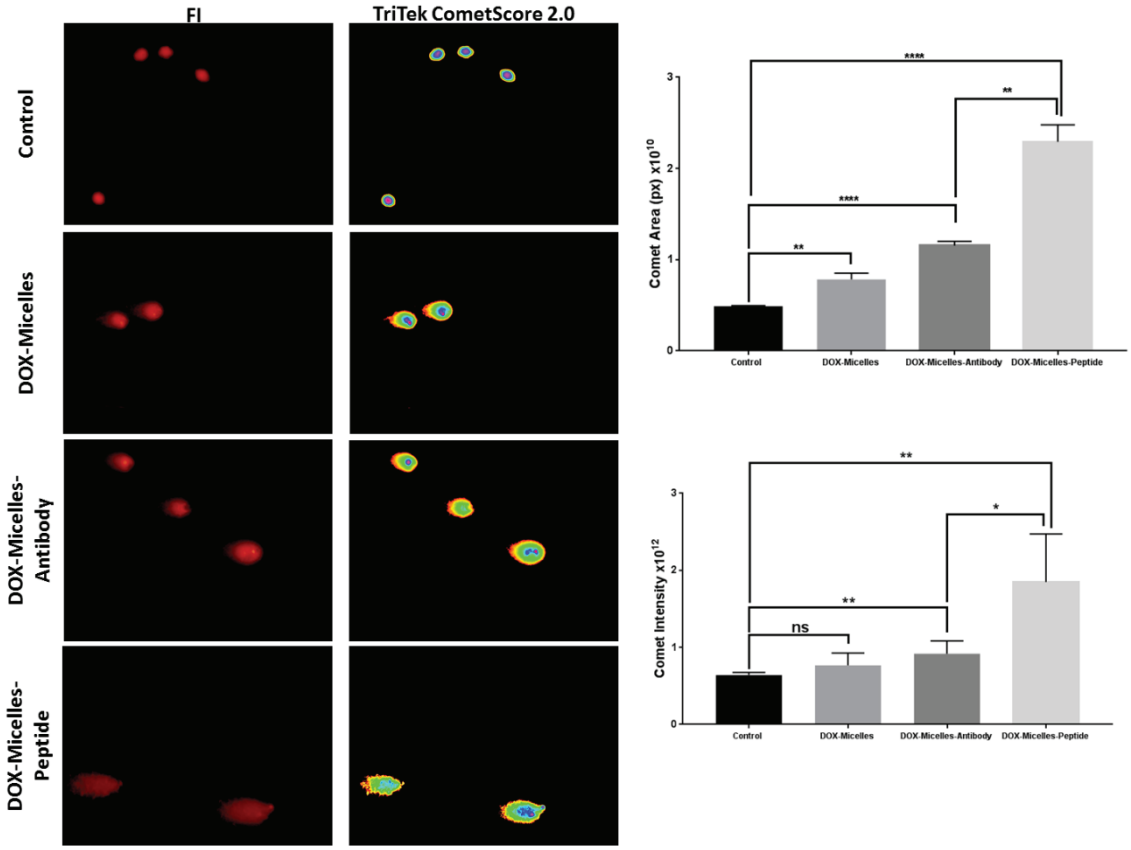

Figure 3.16. Genotoxicity analysis (comet assay) of SKBR-3 cells treated with DM, DMA, and DMP IC50 at 48 h.

FI: Propidium iodide (Red) TriTek CometScore 2.0: Fluorescence dye density (Multi-to-Pink; Pink-Blue-Green-Yellow-Red). This study consists of the analysis of 6 images. Paired t-test, NS: not significant, $p < 0.05$: *; $p < 0.01$: **; $p < 0.001$: *** was

considered significant. Error bars represent standard deviation. When these graphics are smaller than the thickness of the lines, error bars are not visible.

Analyzing the result of the comet assay, significant DNA damage was observed, in SKBR-3 cells treated with 0.34 μM of DMP. The comet intensity increased in cells treated with DMP. On the other hand, SKBR-3 cells treated with 0.34 μM of DMA and DM drugs showed lower DNA damage and, comet intensity was less significantly formed. DNA damage resulted from DOX effects, which is known to break double-strand DNA through either direct alkylation or intercalation.

CHAPTER 4

CONCLUSION

Breast cancer is an aggressive and heterogeneous disease. Based on the statical analysis, it remains the second cause of death worldwide. The estimated new case of breast cancer in 2020 was 2.3 million diagnosed and 685,000 deaths. The mortality rate accounts firstly for breast cancer in 110 countries. In addition to that, one in every four women is being diagnosed with breast cancer (Sung et al. 2021). Furthermore, statistics also showed that cancer incidence accounted firstly for breast cancer in a vast majority of many countries.

The treatment of breast cancer varies depending on breast cancer subtype, size, patient age, and tumor location. In the earliest stage of breast cancer, surgery with organ preservation is a standard approach, that had been widely accepted. It is also followed by radiation therapy. Other treatment options including hormonal therapy, immunotherapy, for advanced stages and aggressive subtypes of breast cancer chemotherapy with combined therapy are applied (Maughan, Lutterbie, and Ham 2010). However, many side effects are arising alongside the traditional treatment options. Most patients have severe side effects including bone marrow suppression, hair loss, nausea, diarrhea, vomiting, and fatigue (Partridge, Burstein, and Winer 2001). On the other hand, targeting therapy can provide better treatment options. It can precisely target specific tumors site, which can lower the toxicity on healthy tissue. Furthermore, it can increase drug efficiency and stability.

Targeted therapy provides delivering the chemotherapeutic agent into the tumor site through specific nanoparticles, that could be modified. Many nanoparticles are being used those including micelles, ferrite, liposome, and dendrimer, quantum dots, gold NPs and iron NPs. They have many advantages such as biocompatibility, hydrophilicity, nontoxicity, and providing drug release. Furthermore, multifunctional nanocarrier systems are being synthesized. Also, the different drugs can be loaded with the nanocarrier system. In addition to that, specific targets toward tumors are conjugated with

the nanocarrier system including mAb, proteins, and short peptide sequence (Sharma, Jain, and Sareen 2013).

HER2 breast cancer subtype is known as an aggressive subtype. This type is characterized by human epidermal growth factor receptor 2 (HER2) overexpression. This receptor activates several cell proliferation pathways. HER2 subtype is considered positive when it shows in more than 10% of tumor cells by IHC. Patients' response and survival outcomes are variable with HER2 enriched subtype (Schettini et al. 2020).

In this study, we investigated the therapeutic effects of the nanocarrier system that is consists of DOX-loaded micelles conjugated with HER2 targeting peptide (LTVSPWY) as a novel nanocarrier system. We also compared its effects to DOX-loaded micelles conjugated with (Herceptin) that were used for targeting HER2 receptors extracellular domain. We increase DOX efficiency and stability by DOX loading into polymeric micelles. The drug's effects were investigated on HER2 positive, SKBR-3 cell line and HER2 negative, MCF-10A cell line. For this purpose, the cytotoxic, apoptosis, cytostatic and genotoxic effects were determined on cancer cells.

HER2 enriched breast cancer subtype characterized by HER2 overexpression, which leads to an aggressive subtype, through activating many oncogenic proteins. The overexpression of HER2 induces upregulating of cellular pathways that control cell proliferation, survival, and angiogenesis. In the development of cancer treatment, targeted therapy had shown to be a more effective treatment option. The nanocarrier system is the most reliable technology that had been implemented recently to increase drug efficiency and lower extra toxicity.

In the first step, the cytotoxic effect of DOX-free NCs at 48hr and 72hr were determined by MTT cell proliferation assay. The three categories of DOX-free NCs are free micelles, free micelles antibody, and free micelles peptide. The result shows no antiproliferation effects on SKBR-3 HER2 positive cell line and MCF-10A HER2 negative cell line. In both cell lines, the proliferation rate was between 100-75%. After that, the cytotoxic effect of DOX in SKBR-3 and MCF-10A cell lines was determined, for loading micelles with the appropriate amount of DOX to be used for the micelles. The used concentration range was supported by the previous literature. The IC_{50} of DOX on HER2 positive SKBR-3 and HER2 negative MCF-10A cell lines was 0.65- and 0.08µM respectively at 48 hr. In the last step of the cytotoxic effect, the DOX-loaded micelles were determined to understand and compare the efficient delivery role of peptide (LTVSPWY) conjugated DOX-loaded micelles (DMP) with antibody DOX-loaded

micelles (DMA) to target HER2 receptors. DMP had a better cytotoxic effect by decrease cell proliferation significantly. DOX IC_{50} value was significantly different in HER2 positive SKBR-3 cell line treated with NCs, with an IC50 of 0.71 μM for DM, 0.34 μM for DMP, and IC_{50} of 0.49 μM for DMA. However, in HER2 negative MCF-10A cell line, no statistically significant difference in IC_{50} value in DOX-loaded-micelles with the IC_{50} of DM, DMA, and DMP was 0.27-,0.23- and 0.19 μM respectively. The study revealed that DMP had a more cytotoxic effect on the SKBR-3 cell line, the IC_{50} of DMP was 0.34 μM whereas the IC_{50} value of DMA was 0.49 μM. In addition to that, the DOX efficiency increased significantly in DMP from 0.65 μM to 0.34 μM. Furthermore, DMP showed selectivity and targeted effects on the SKBR-3 cell line compared to the MCF-10A cell line.

Apoptotic effects of NCs were investigated in this study. The mitochondrial membrane potential (MMP) was determined by the JC-1 assay. Applying IC_{50} of 0.34 μM of NCs caused a decrease in MMP in the SKBR-3 cell line. A significant decrease in MMP was noticed in cells treated with DMP. The percentage of MMP was 46.7% in DMP treated SKBR-3 cells while DMA decreased MMP to only 70.8%. Furthermore, Annexin-V/PI double staining apoptosis assay demonstrated the increase in cell apoptosis and necrosis of SKBR-3 cells treated with DMA and DMP. However, the flow cytometry statistical analysis showed a significant increase in cell apoptosis and necrosis 16.45% and 46.83% respectively, in SKBR-3 cells treated with DMP IC_{50} of 0.34 μM compare to DMA. Apoptosis assays result showed that DMP increases SKBR-3 cell apoptosis and necrosis, which is regulated by MMP and intrinsic apoptosis pathway. These pathways had been investigated by western blotting analysis. The protein expression level of Bax, Bak, Bcl-2, PARP1 (cleavage), Pro-Caspase-3, and Bcl-xL were determined. The internal positive control was GAPDH. The application of 0.34 μM of NCs showed that DMP increases Bak and Bax protein level significantly compare to DMA. Both Bak and Bax are responsible for mitochondrial outer membrane permeabilization, Bak and Bax are inactivated by the pro-survival Bcl-2 protein by direct interaction (Westphal, Kluck, and Dewson 2014). On the other hand, Pro-Caspase-3 and PARP1 proteins decrease in level which showed their cleavage to induce apoptosis. The cleavage of PARP1 is considered the hallmark of apoptosis (Kaufmann et al. 1993). Furthermore, the anti-apoptotic proteins Bcl-2 and Bcl-xL decreased in level which showed their role in inducing apoptosis in the SKBR-3 cell line.

DOX uptaken was demonstrated by our fluorescent microscope image studies. The fluorescent images were taken to test the difference between DOX fluorescent intensity density of the NCs, DM, DMA, and DMP in terms of penetrating and DOX releasing in the targeted cell. IC_{50} of 0.34 μM was applied for the HER2 positive SKBR-3 cell line. The IC_{50} NCs of 0.19 μM was applied for HER2 negative MCF-10A. The result showed a significant increase in DOX uptaken by SKBR-3 cell line treated with DMP compare to DMA. On the other hand, HER2 negative MCF-10A cell line showed no observable differences in DOX uptake in different applied NCs, DM, DMA and, DMP. These results revealed the role of drug penetrating in HER2 positive SKBR-3 cells. In addition to that, DOX releasing was observed significantly in SKBR-3 cells treated with DMP.

The cytostatic analysis demonstrated cell cycle profile changed. Treating SKBR-3 cells with 0.34 μM of NCs caused a decrease in the S phase under DMP to compare to DMA. On the other hand, the G2/M phase arrested on SKBR-3 cells which caused as a result of the DOX effect. DOX is known to cause cell cycle arrest at the G2/M phase due to topoisomerase II inhibition. In the last step of our study, genotoxicity analysis was investigated by comet assay. The application of 0.34 μM of NCs showed that SKBR-3 cells treated with DMP had more DNA fragmentation and genotoxicity induced significantly, compare to SKBR-3 cells treated with DM and DMA.

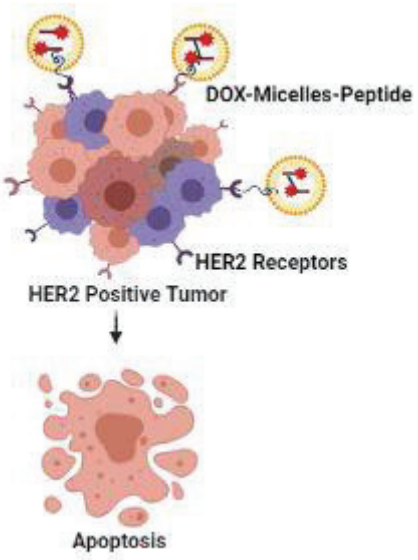

Figure 4.1. The effects of Doxorubicin micelles peptide (DMP) on HER2 enriched cancer cells.

In all previous results, the novel synthesized DMP with HER2 peptide (LTVSPWY) found to be more effective for the treatment of HER2 positive breast cancer cells. Furthermore, the efficiency of DOX was significantly better in DMP as compared to DMA with (Herceptin). In addition to that, the selectivity of DMP on HER2-positive breast cancer might provide a better treatment option for the treatment of HER2 positive subtype. Our study can contribute to the personilized medicine for HER2 enriched treatment. Many studies had demonstrated the effective role of micelles to overcome several problems in drug delivery and was successful in targeting specific cancer types. Micelles as innovative tools are used also to increase drug efficiency and stability. Our study had supported the effective role of the novel DOX-micelles-peptide (DMP) in HER2-positive breast cancer treatments. However, to continue our study, the *in vivo* study is necessary to continue the phase studies for drug approval.

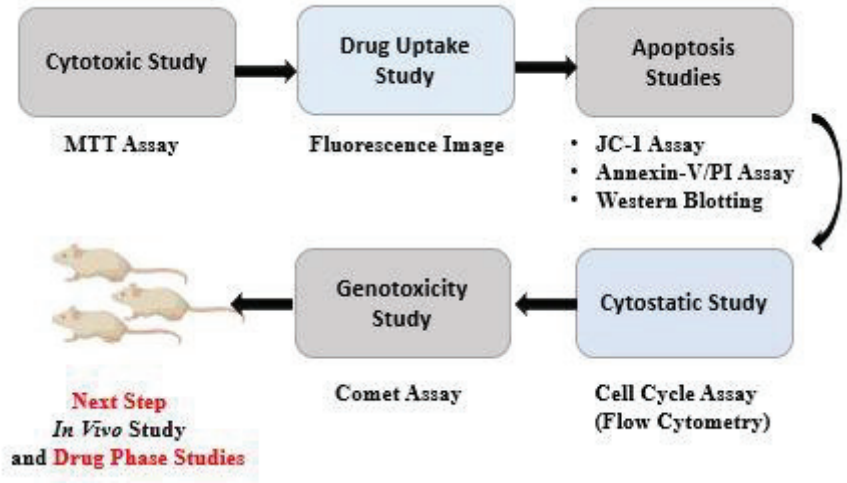

Figure 4.2. Flowchart of the study.

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