

**TURKISH REPUBLIC
ERCIYES UNIVERSITY
SCHOOL OF NATURAL AND APPLIED SCIENCES
DEPARTMENT OF BIOMEDICAL ENGINEERING**

**THE EFFECT of LC3 siRNA and DOXORUBICIN DUAL
TREATMENT AGAINST BREAST CANCER**

**Prepared by
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Master Thesis

**September 2022
KAYSERİ**

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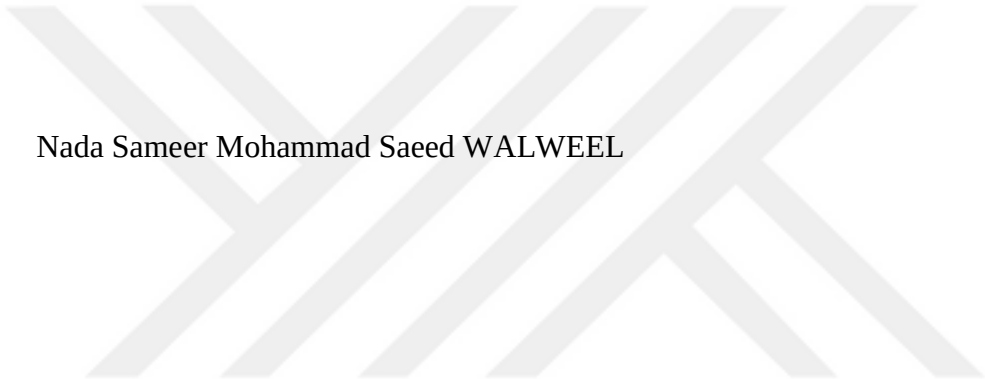
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**This study was supported by Erciyes University Scientific Research Projects
Coordination Unit with project number FYL-2022-11565.**

**September 2022
KAYSERİ**

COMPLIANCE WITH SCIENTIFIC ETHICS

I declare that all information in this study has been obtained in accordance with academic and ethical rules. At the same time, as required by these rules and conduct, I declare that I have fully cited and referenced all materials and results that are not at the core of this work.



Nada Sameer Mohammad Saeed WALWEEL

APPROVAL OF COMPLIANCE WITH THE DIRECTIVE

The Master's Thesis titled "The Effect of LC3 siRNA and Doxorubicin Dual Treatment Against Breast Cancer" was prepared in accordance with Erciyes University Graduate Thesis Proposal and Thesis Writing Directive.

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ACKNOWLEDGMENT

First of all, without the encouragement and guidance of Asst. Prof. Ömer Aydın, this work would not have been possible. I owe him a huge debt of gratitude for his oversight, guidance, inspiration, knowledge, and expertise that he shared with us. And without a doubt, I also want to express my great appreciation to my second adviser, Dr. Cansu Ümran Tunç, who has consistently remained by our side, offered her assistance, and supervised us with the utmost care. Additionally, I would like to express my sincere gratitude to the sponsors who helped make this work feasible, which include Turkish Scientific and Technological Research Council (TUBITAK) COST (CA17103) 2515 and Erciyes University Scientific Research Project Coordination Unit (Grant No: 118Z952). And of course, I am extremely grateful to Turkey Scholarships (YTB) Postgraduate Scholarship Program which without I could not have undertaken this journey.

A special thanks should also be extended to Professor Zühal Hamurcu and Professor Yasemin Yüksel Durmaz for their unwavering cooperation and support in completing this work. Special thanks to Dilek Kanarya and Venhar Cinar for offering their experimental help. I would be remiss if I did not express my gratitude for being given the opportunity to be a member in NanoTheraLab, where every colleague was more like a sibling than a workmate, sharing our laughter, tea, grumbles, tears, and—most importantly—support.

I am also grateful for being the daughter of my dad, Dr. Sameer Walweel, as he has always been a role model for me in terms of perseverance, diligence, patience, and hard work. Without his constant encouragement and support, I wouldn't be who I am today. And for sure, I would like to thank my lovely mother, Nadia Walweel, for her tolerance in allowing me to be away for the past three years, as well as for her ongoing prayers and blessings. Last but not least, I would want to express my sincere gratitude to my husband, Abdulrahman Shehada, for providing me with all kinds of support, from emotional support to helping me draw my own figures and modify my tables. I sincerely appreciate the belief you instilled in me and all of your ambitions for my prospective academic pursuits.

Nada Sameer Mohammad Saeed Walweel

August, 2022, KAYSERİ

LC3 siRNA ve DOKSORUBİSİN İKİLİ TEDAVİSİNİN MEME KANSERİNE KARŞI ETKİSİNİN İNCELENMESİ

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ÖZET

Otofaji, işlevini kaybeden sitoplazmik molekülleri parçalayarak ve ortadan kaldırarak beslenme geri dönüşümünü ve metabolik uyarlanabilirliği sağlayan kademeli bir lizozomal bozunma sürecidir. Otofaji, kanseri destekleyen bir süreç olarak bilinen hücrelerin stresten kaçmak için kullandığı sağlam bir aracı temsil eder. Buna göre otofajiyi inhibe etmek kanser hastalarında terapötik sonuçların iyileştirilmesini sağlayacaktır. Bu çalışmada, DOX ve siLC3 kombinasyonunun DOX'un etkilerini artıracağı yönündeki öncülümüzle kanser tedavisi için "akıllı" bir nanoparçacık sistemi tasarlanıp DOX ve siLC3'ün kombine tedavisi ile kullanıldı. Böylece siLC3'ü iletmek için "akıllı" bir nanoparçacık yaklaşımı kullanmanın, otofaji ile ilgili bir gen olan LC3'ü etkili bir şekilde bastırabileceği, hücresel otofajiyi engelleyebileceği ve gelişmiş antikanser etkileri sergileyebileceği gösterildi. Ayrıca, üçlü reseptör negatif meme kanseri hücre hattı MDA-MB-231 üzerinde, siLC3 ve DOX'un birlikte uygulanmasının meme kanseri tedavisinde her iki ajanın tek başına uygulanmasından daha etkili olduğu hücre proliferasyonu ve hücrelerde migrasyonun engellenmesi deneyleri ile gösterildi. siLC3 ve DOX birleştirildiğinde düşük dozda DOX uygulanması sonucu koloni oluşumunun %85'in üzerinde baskılandığı, buna karşın DOX'un tek başına yalnızca yaklaşık %50 oranında inhibisyona neden olduğu sonucuna ulaşıldı. Bununla birlikte, G1 alt fazındaki hücrelerin oranı, tek başına DOX tedavisinde kombine tedavi durumuna göre 4.8 kat arttı. Bunların yanı sıra, otofaji ve pro-apoptoz ile ilişkili proteinlerin ekspresyon seviyelerini incelemek için western blot kullanıldı, böylece ilgili proteinlerin hepsinde önemli bir düşüş olduğu ortaya konuldu. Son olarak otofajiye bağlı kesecikleri tespit etmek için kullanılan akridin turuncusu ile boyanan otofagozomların sayısının tedavi gruplarında azalması otofaji baskılanmasını doğruladı. Birlikte ele alındığında bulgularımız, meme kanseri tedavisi için "akıllı" nanopartiküller tarafından verilen kemoterapi ve otofaji baskılamanın birleştirilmesinin sinerjik bir etkiye sahip olduğunu gösterdi.

Anahtar Kelimeler: Üçlü reseptör negatif meme kanseri, Otofaji, LC3 siRNA, Doksorubisin, Sinerjistik etkinlik.

THE EFFECT OF LC3 siRNA AND DOXORUBICIN DUAL TREATMENT AGAINST BREAST CANCER

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Master Thesis, August 2022
Supervisor: Asst. Prof. Ömer AYDIN

ABSTRACT

Autophagy is a stepwise lysosomal degradation process that enables nutrition recycling and metabolic adaptability by degrading and eliminating malfunctioning cytoplasmic molecules. Autophagy represents a solid tool that cells employ in order to escape stress, being known as a process that supports cancer. Accordingly, inhibiting autophagy would result in improving therapeutic outcomes in cancer patients. In recent study, a combined treatment of DOX and siLC3 was utilized for cancer therapy using a “smart” nanoparticle system, based on our premise that this combination would increase DOX's effects. We demonstrated that using a "smart" nanoparticle approach to deliver siLC3 can efficiently suppress the autophagy-related gene LC3, inhibit cellular autophagy and exhibit improved anticancer effects. Furthermore, in the TNBC cell line, MDA-MB-231, co-administration of siLC3 and DOX was more effective than either agent alone in treating breast cancer. The inhibition of cell growth and migration in the cells demonstrated this. Besides, it has been demonstrated that when siLC3 and DOX are combined, colony formation was suppressed by over 85% under the conditions of low-dose DOX, whereas DOX alone only caused inhibition of around 50%. Nevertheless, this combination increased apoptosis, as the proportion of cells in the sub-G1 phase showed 4.8-fold greater apoptosis in the case of the co-treatment than DOX treatment alone. Furthermore, western blotting was used to examine the expression levels of autophagy and pro-apoptosis-associated proteins, which revealed a significant decrease in all of them. Finally, the results of acridine orange staining, which is used to detect autophagy-related vesicles; autophagosomes, confirm that autophagy suppression is evident. Taken together, our findings showed that combining autophagy suppression with chemotherapy delivered by "smart" nanoparticles for breast cancer therapy has a synergistic effect.

Keywords: Triple-negative breast cancer, Autophagy, LC3 siRNA, Doxorubicin, Synergistic efficacy

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ABBREVIATIONS

3-MA	3- methyladenine
ATCC	American Type Culture Collection
ATGs	Autophagy-related genes
CDs	Cyclodextrins
CP	Copolymer
CQ	Chloroquine
DLS	Dynamic light scattering
DMAEMA	2-(dimethylamino) ethyl methacrylate
DMEM	Dulbecco's Modified Eagle's Medium
DOX	Doxorubicin
ER	Endoplasmic reticulum
FBS	Fetal bovine serum
HCC	Hepatocellular carcinoma
HCQ	Hydroxychloroquine
HMA	Hexyl methacrylate
LC3	Microtubule-associated light chain 3
miRNAs	MicroRNAs
mPTP	Mitochondrial permeability transition pore
mRNA	Messenger mRNA
N/P ratio	Nitrogen (N)/phosphate (P) ratios
NP	Nanoparticle
PARP1	Poly (ADP-ribose) polymerase-1
PDAC	Pancreatic ductal adenocarcinoma
PE	Phosphatidylethanolamine
PEG	Polyethylene glycol
PI3K	Phosphoinositol 3 phosphate kinase
RAFT	Reversible addition-fragmentation chain transfer
RISC	RNA-induced silencing complex
RNAi	RNA interference
ROP	Ring-opening polymerization

siLC3	Microtubule-associated light chain 3 siRNA
siRNAs	Small interfering RNAs
TAE Buffer	Tris-acetate-EDTA Buffer
TMAEMA	Trimethylaminoethyl methacrylate iodide
TNBC	Triple-negative breast cancer



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INTRODUCTION

Breast cancer is the most prevalent diagnosed cancer in women and the major cause of cancer-related fatalities [1]. With a median survival time of less than three years, 30% of patients acquire an incurable metastatic illness [2]. Triple-negative breast cancer (TNBC) that accounts for 12–18% of all breast cancer cases is defined by the absence of estrogen (ER), progesterone (PR), and human epidermal growth factor receptor 2 (HER2) expression. Indeed these three receptors represent the biological objectives for disease treatment, therefore no effective targeted therapy could be utilized in TNBC [3]. Consequently, aggressive chemotherapy remain as the only mainstay treatment.

Doxorubicin (DOX) is the most frequently used drug to treat breast cancer. Its unique properties made its use desirable since its anti-tumor effect is both robust and predictable, and it is inexpensive [4]. DOX therapy, on the other hand, can cause drug resistance in breast cancer cells [2]. As a result, new studies are urgently needed to overcome DOX resistance and improve its efficacy in order to improve patient outcomes.

Autophagy is a complex stepwise lysosomal degradation process that is responsible for degrading and removing dysfunctional cytoplasmic materials and damaged organelles, which supports nutrient recycling and metabolic adaptation. Autophagy represents a solid tool that cells employ in order to escape stress, being known as a process that regulates cancer [5]. There are three different kinds of autophagy classified as microautophagy, chaperone-mediated autophagy, and macroautophagy, with all focus being centered on macroautophagy [6]. Autophagy represents an intricate pathway controlled by the regulation of almost twenty autophagy-related genes (ATGs) [7], consisting of five distinguished phases.

Among the ATGs, microtubule-associated light chain 3 (LC3) protein represents a crucial function in mammalian autophagy [7], possesses uniqueness by being the only protein to

exist on autophagosomes, which made it seen as a quite particular marker of autophagosome formation [8]. There are two forms of LC3, the cytosolic LC3-I and the membrane-conjugated form, LC3-II [9]. Under varied stresses, a modification of LC3-I to LC3-II by conjugating LC3-I with phosphatidylethanolamine (PE) is being stimulated [10].

Autophagy's effect on tumor progression and development is controversial and still not completely understood, but generally its influences changes according to the type and stage of cancer and genomic context [11]. For instance, in the earliest stages of tumorigenesis, it works as tumor inhibitor through maintaining homeostasis by achieving a clearance of old, nonfunctional proteins and deteriorated organelles [12]. Whereas in established cancers, plurality of data sources suggest the ability of autophagy in enabling tumor spread and promoting its growth [13].

Autophagy has been shown to have tumor boosting [14–16] and tumor prohibiting [7,17,18] functions in breast cancer. Several investigations, however, have shown that inhibiting autophagy boosts the lethal effects of chemotherapeutic drugs, specifically DOX, emphasizing the importance of autophagy in stress tolerance and drug resistance [19–21]. In MDA-MB-231 breast cancer cells, for example, retroviral shRNA suppression of ELK3, a signaling protein involved in autophagy activation, decreased autophagy and improved DOX responsiveness [22]. In a recent study, downregulation of LC3 gene significantly reduced cell viability and blocked the main cell mechanisms; proliferation, invasion, migration, and resistance to apoptosis in breast cancer cells [23]. As a result, we believe that combining microtubule-associated light chain 3 siRNA (siLC3) with DOX might improve breast cancer treatment efficacy.

The current study used a "smart" nanoparticle approach as the carrier of siLC3 to deliver a combination of DOX and siLC3 for cancer therapy. The “smart” nanoparticle system is based on the use of FDA -approved β -cyclodextrin (β -CD) that consists of two sides: a primary side and a secondary side. Half of the primary side consists of three different moieties: 2-(dimethylamino) ethyl methacrylate (DMAEMA) monomers; which are pH sensitive, hydrophobic hexyl methacrylate (HMA), and cationic trimethylaminoethyl methacrylate iodide (TMAEMA) monomers, while the other half is polyethylene glycol (PEG) bound. This polymer may detect changes in the environmental pH, disrupt the

endosomal membrane, and achieve electrostatic complexation between its cationic amine groups and the anionic phosphate groups of siLC3 (24). Over MDA-MB-231 cell line, we investigated whether combining DOX and siLC3 may inhibit cell proliferation, colony formation, and migration compared with free DOX or "smart"NP/LC3 siRNA. In the meanwhile, we studied the effect of this combination on the expression of a set of cancer-related proteins, cell cycle phases, and apoptosis rate.

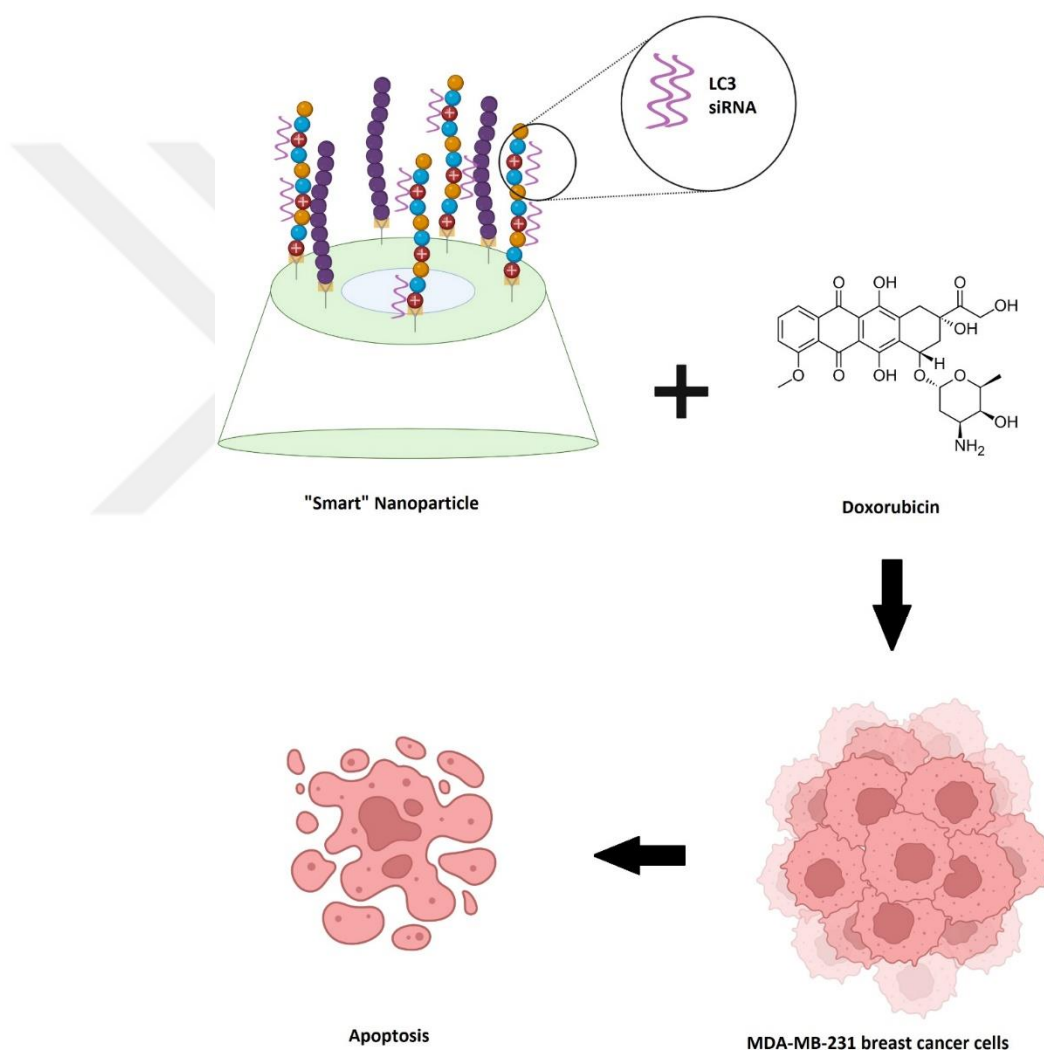


Figure 1. The effect of combined LC3 "smart" NP and DOX on MDA-MB-231 breast cancer cells.

1. CHAPTER

GENERAL INFORMATION AND LITERATURE STUDY

1.1. Breast Cancer

Breast cancer is the most prevalently diagnosed malignancy and a leading cause of cancer-related deaths among women worldwide [1]. Being known as a highly heterogeneous disease [24], consist of several subtypes with patent pathological attributes and clinical indications [25]. Several factors affect the chance of disease occurrence, including obesity, endogenous hormones, alcohol consumption, race, smoking and ancestry (Figure 1.1) [26].

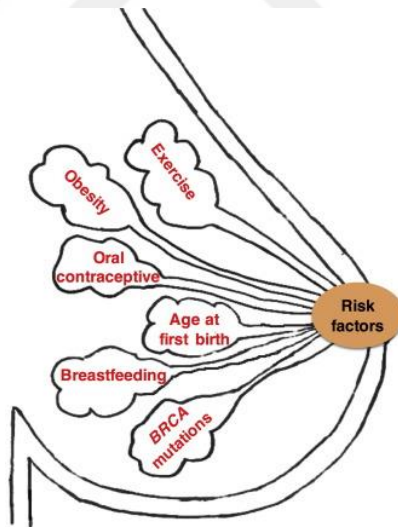


Figure 0.1. The most important breast cancer risk factors [27].

Thirty percent of patients ultimately develop an incurable metastatic disease with an average life expectancy not exceeding 3 years [2]. In which bones, brain, lungs, soft tissue and adrenal glands represent the main sites for the cancer spread [28]. Surgery, hormonal treatment, immunotherapy, chemotherapy, radiotherapy, or a mix of these are currently available therapies treating breast cancer [29].

1.1.1. Triple Negative Breast Cancer

TNBC, which approximately comprises 12–18% of breast cancer cases, is characterized with lack expression of estrogen (ER), progesterone (PR), and human epidermal growth factor receptor 2 (HER2) (Figure 1.2) [30]. Indeed these three receptors represent the molecular targets for treating breast cancer, therefore no effective targeted therapy could be utilized in TNBC [3]. TNBC, with its aggressive nature, is typically seen in young and obese women, those beneath forties, with most incidence in African-American women (Figure 1.3) [31].

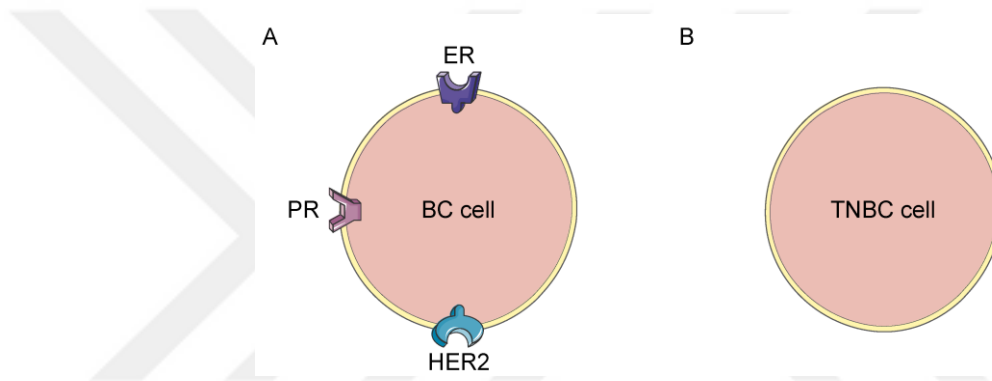


Figure 0.2. In terms of receptors' expression, this figure shows the difference between most breast cancer cells (A) and TNBC cells (B) [32].

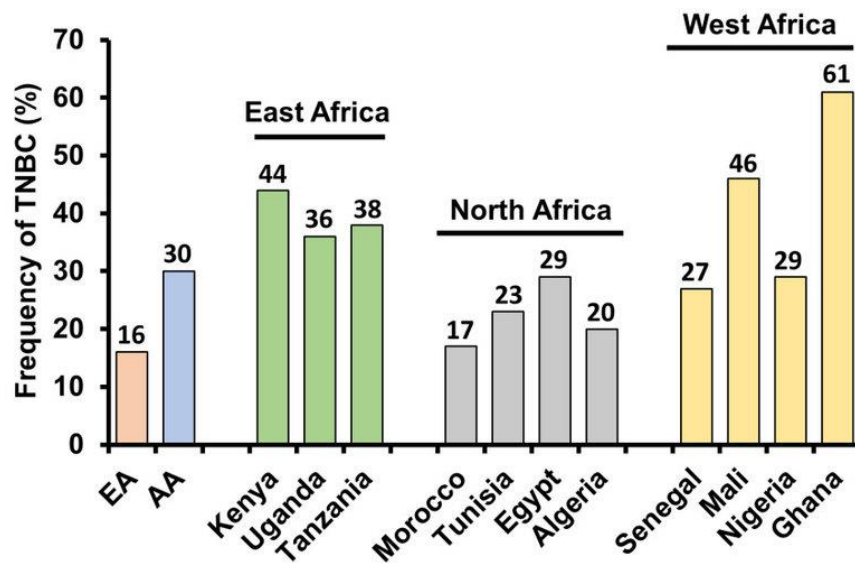


Figure 1.3. TNBC is seen to be more common in women of African heritage [31].

It is frequently affiliated with early recurrence (with rates up to 25% after surgery), distant metastatic progression (developed in nearly 46% of TNBC patients) distinguished by early relapse and a predominance of hepatic, pulmonary and central nervous system metastasis, as in most cases the brain and the lung are the main destinations for this cancer phenotype [33,34]. Unfortunately, patients with this type of breast cancer come with poor outcome as well as poor overall survival with quite high mortality rate as 40% within the first years after diagnosis [35]. Consequently, aggressive chemotherapy remain as the only mainstay treatment.

1.2. Doxorubicin

DOX, which is an elemental member of the anthracycline antibiotic drugs family, has been utilized in chemotherapy since 1960s [36]. The unique characteristics of DOX made its use demandable since its anti-tumor activity is highly robust as well as reliable and its price is frugal [4]. Although it is widely used in the treatment of different types of cancers, such as malignant lymphoma, ovary, bladder, lung and gastrointestinal cancers, it has been approved to be the most chemotherapeutic active agent in treating breast cancer, especially TNBC [37]. DOX output manifests its effect by getting involved in a set of molecular mechanisms. Starting from the moment it enters the cytoplasm, it generates free radicals, oxidative stresses and damages in mitochondria's enzymes. These occurrences activate the mitochondrial permeability transition pore (mPTP), which ruptures and swells the outer mitochondrial membrane, releasing cytochrome C into the cytoplasm and ultimately leading to cellular apoptosis [38] (Figure 1.4). Doxorubicin can also cause the permeabilization of the mitochondrial outer membrane, which allows the diffusion of a number of pro-apoptotic proteins into the cytoplasm [39]. All of which leads to cell proliferation inhibition, apoptosis induction and blockage in cell cycle progression [25].

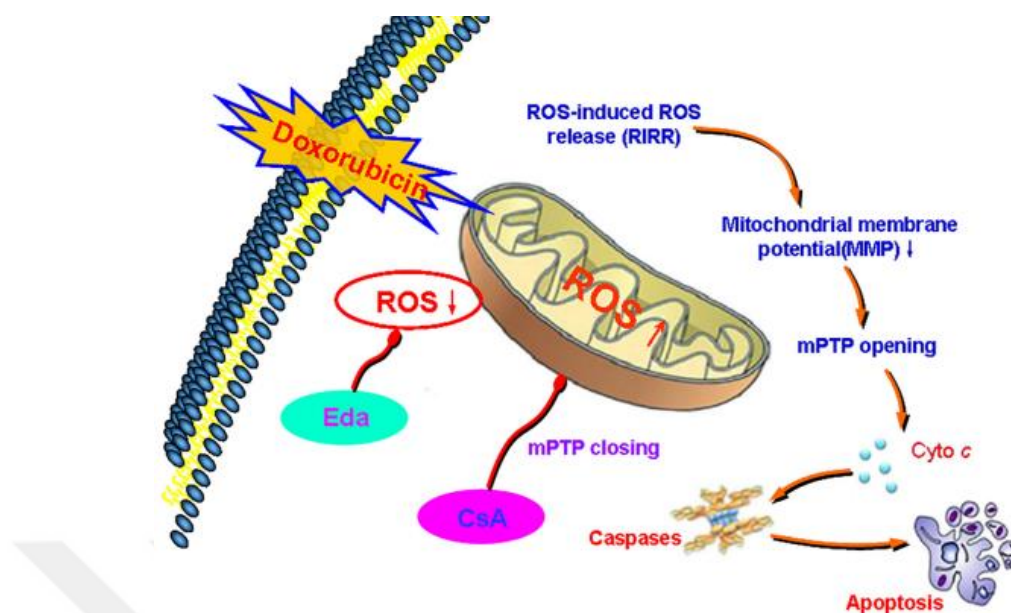


Figure 1.4. This graphic shows the process via which DOX exerts its effect on cellular mitochondria, ultimately leading to apoptosis [40].

However, the therapeutic application of DOX can bring on drug resistance in breast cancer cells [2]. And once drug resistance takes place, cancer cells aggressiveness and metastasis became inevitable which brings about failure in the treatment and eventual motility [41]. Therefore, new researches are highly required to overcome DOX resistance and increase its efficiency in order to improve patient outcome.

In fact, cells may undergo multiple ways that can be imputable to the acquired drug resistance [42], particularly apoptosis and autophagy [43]. Autophagy has been recently proposed as a potential cause for the evolution and progression of resistance toward several anticancer agents headed by DOX [15,44,45]. The ability of autophagy to trigger mitophagy—the selective autophagy of mitochondria—which prevents cellular apoptosis—is the most plausible explanation.

1.3. Autophagy

Autophagy is a complex stepwise lysosomal degradation process that is responsible for degrading and removing dysfunctional cytoplasmic materials and damaged organelles, which supports nutrient recycling and metabolic adaptation. Autophagy represents a solid tool that cells employ in order to escape stress, being known as a process that regulates

cancer [5]. There are three different kinds of autophagy classified as: microautophagy, chaperone-mediated autophagy and macroautophagy (Figure 1.5), with all focus being centered on macroautophagy [6]. The difference between these subtypes of autophagy mainly depends on the way the cargos are transferred in to lysosomes [46]. In microautophagy, cargos are simply engulfed and internalized directly into lysosomes as a consequence of lysosomal membrane invaginations [47]. Whereas, chaperone-mediated autophagy requires a specific recognition of the cargos (proteins) that are going to be delivered to lysosomes, usually being marked with the amino acid motif (KFERQ), by another set of heat shock proteins (HSC70) for the degradation to be processed [48].

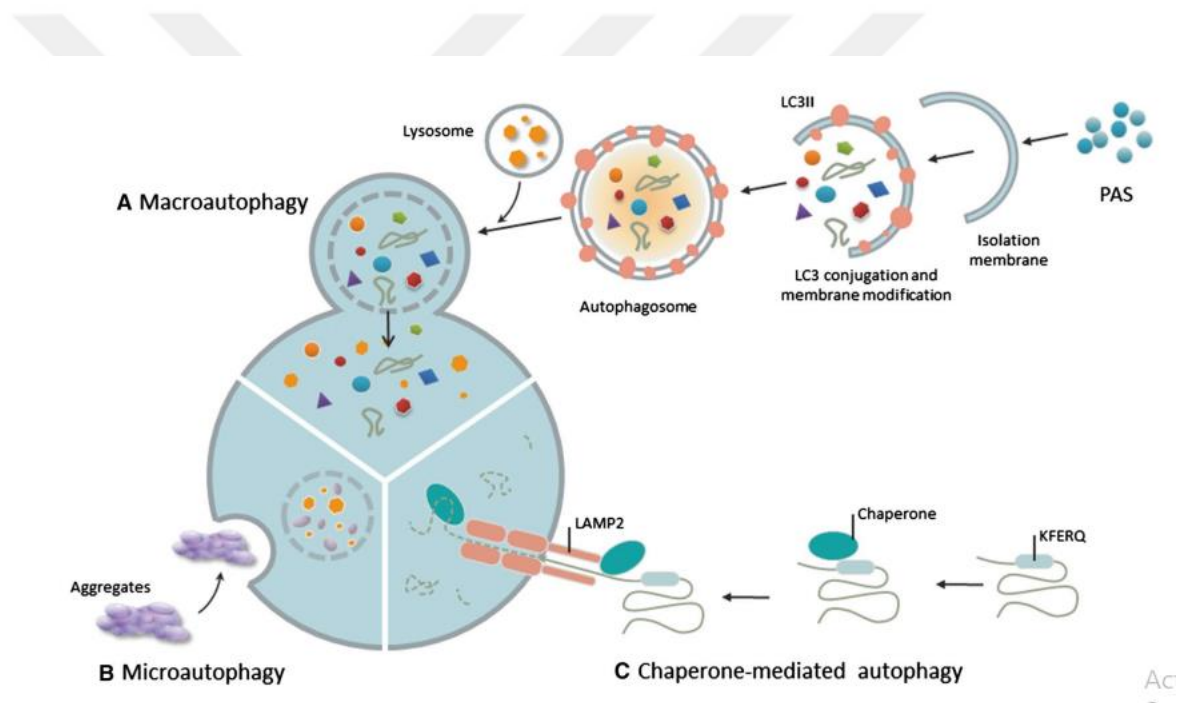


Figure 1.5. This figure illustrates the three different kinds of autophagy [49].

1.3.1. Macroautophagy

Macroautophagy, which would be referred to as autophagy throughout the context, the main common pathway of autophagy, is characterized by the formation of multiple membrane structures, starting with phagophores formation that are derived mainly from Golgi complex, endosomes, the endoplasmic reticulum (ER), mitochondria and the plasma membrane [50]. Phagophores mature into autophagosomes which thoroughly enclose the cargo within and then fuse with lysosomes to form structures called

autolysosomes. Inside the autolysosomes, cargos got degraded completely by lysosomal enzymes [48] (Figure 1.6).

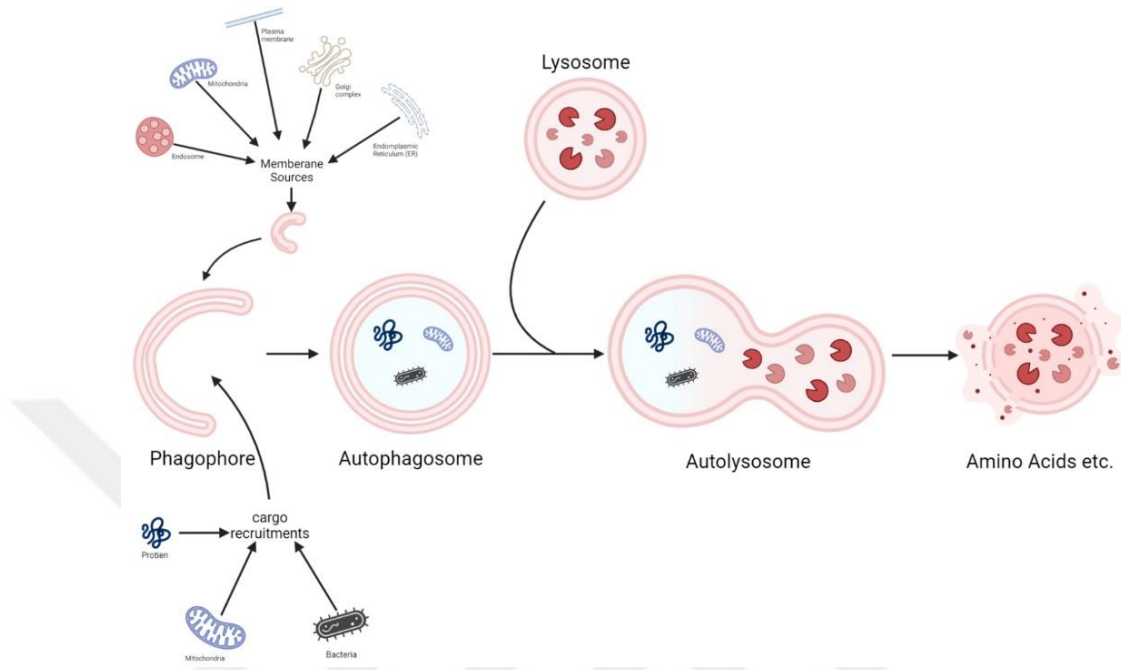


Figure 1.6. This figure shows the multiple membrane structures involved in the macroautophagy (This figure was created using BioRender).

Autophagy represents an intricate pathway controlled by the expression of almost twenty ATGs [7], consisting of five distinguished phases; initiation, elongation, closure, maturation, and degradation [51]. Among the ATGs, which have been first specified in yeast *Saccharomyces cerevisiae* some time ago [52], LC3 protein represents a crucial function in mammalian autophagy [7], possesses uniqueness by being the only protein to exist on autophagosomes, which made it seen as a quite particular marker of autophagosome formation [8]. There are two forms of LC3, the cytosolic LC3-I and the membrane-conjugated form, LC3-II [9]. Under varied stresses, a modification of LC3-I to LC3-II by conjugating LC3-I with phosphatidylethanolamine (PE) is being stimulated [10].

In normal conditions, autophagy represents a quality control tool in cells that help maintain homeostasis and redox balance by digesting non-functional proteins and damaged organelles, collaborating with the adaptive immune system [53,54], and surviving malnutrition situations by recycling cellular elements to regenerate energy [51]. As a result of the failure of autophagy in immunologic ailments and neurodegenerative illnesses like Alzheimer's and Parkinson's, aberrant, malfunctioning proteins and

organelles accumulate, causing the disease to develop [55,56]. However, autophagy's function in cancer is uncertain because, mostly based on the stage of the tumor, it could either suppress or accelerate the disease. [57,58].

1.4. Autophagy and Cancer

Autophagy's effect on tumor progression and development is controversial and still not completely understood, but generally its influences changes according to the type and stage of cancer and genomic context [11]. For instance, in the earliest stages of tumorigenesis, it works as a tumor inhibitor by maintaining homeostasis by achieving clearance of old, nonfunctional proteins and deteriorated organelles [12] and inhibiting malignant necrosis and inflammatory responses [60]. However, in established cancers, a plurality of data sources suggest the ability of autophagy in promoting tumor growth, survival, and colonization through reducing the damage in DNA, maintaining functional mitochondria, dealing with cellular stresses including hypoxia, nutritional lack, and anticancer drug treatments [13,61–63] and meeting the high metabolic demands of the tumor in terms of nutritional supply [64,65]. Accordingly several studies have been conducted showing both the promotive and suppressive effect of autophagy on different types of cancers (Figure 1.7).

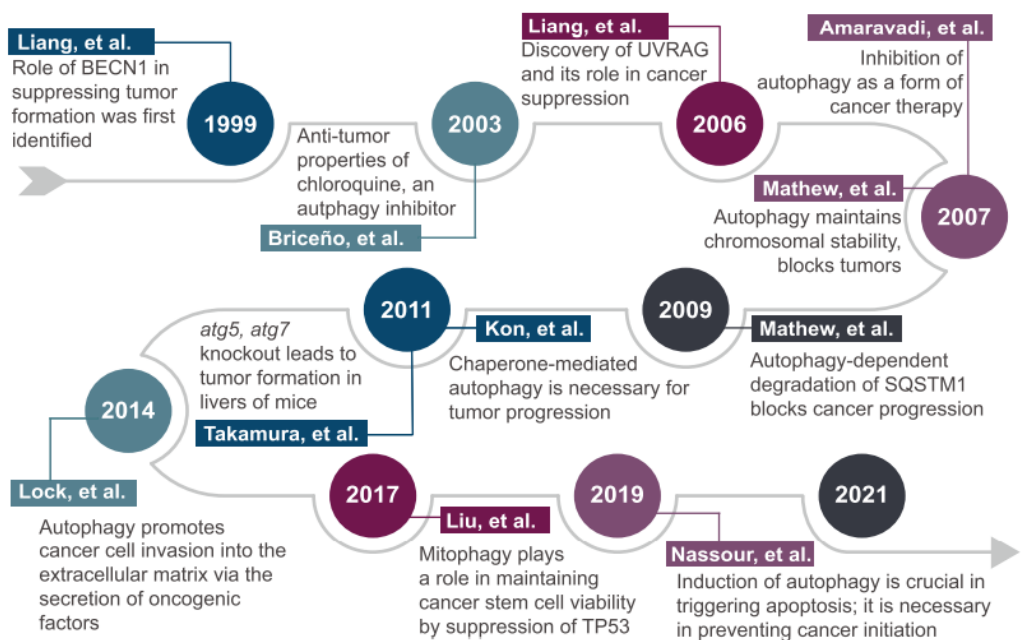


Figure 1.7. The diagram shows a selection of significant breakthroughs in cancer research involving autophagy [59].

Altogether, while autophagy seems to be critically important in protecting normal cells from tumorigenesis initiation, inhibiting it is in favor when dealing with established, developed cancers such as TNBC [66,67].

1.5. Autophagy Inhibition

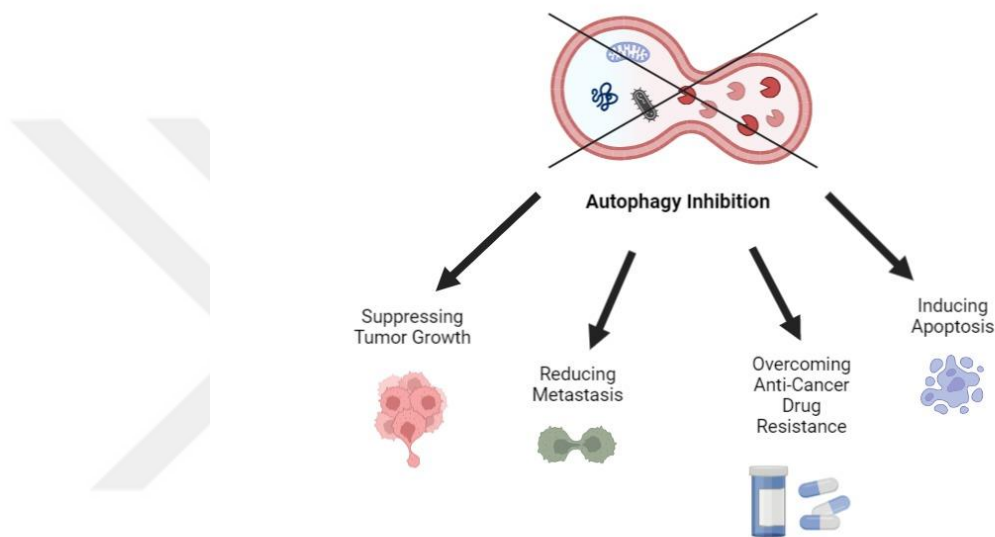


Figure 1.8. This diagram depicts a summary of the key impacts of autophagy inhibition on cancer cells, as represented by tumor growth suppression, tumor metastasis reduction, overcoming anticancer treatment resistance, and apoptosis activation (This figure was created using BioRender).

Multiple sensors, including LC3, Beclin-1, phosphoinositol 3 phosphate kinase (PI3K), Atg5 Atg7, BECN1, and microRNAs could be detected in analyzing autophagy suppression. Recently, autophagy inhibition effect over cancer cells has been studied via conducting mice models with deleted genes responsible in autophagic activity. As an example, in a polyoma middle T-driven model of breast cancer, by knocking down Fip200 autophagy gene, a considerable delay in both tumor initiation and progression has been noticed [68]. Nevertheless, in a set of studies mouse models with deleted Atg5 or Atg7 autophagy genes have been established, in which a decrement/ prevention in tumors growth have been observed [69–71]. It has also been shown that downregulation of LC3 gene significantly reduced cell viability and blocked the main cell mechanisms;

proliferation, invasion, migration, and resistance to apoptosis in TNBC [23]. Tumor growth in pancreatic ductal adenocarcinoma (PDAC) was slowed in one study by suppressing autophagy with autophagy inhibitors and gene silencing tools [69].

Besides, many studies have been published indicating that high levels of LC3 and other ATGs are correlated to cancer progression, implying the critical role autophagy plays in metastasis [14,16,72,73]. Autophagy also causes inflammation in the tumor microenvironment and cancer-adjacent cells, which promote tumorigenesis, as evidenced by the presence of inflammatory regulators at high levels in these areas [74].

Nevertheless, autophagy has also been recently proposed as potential cause for evolution and progression of resistance towards several anticancer agents [15,44,45,75,76]. Several investigations have shown that inhibiting autophagy boosts the lethal effects of chemotherapeutic drugs, emphasizing the importance of autophagy in stress tolerance and drug resistance [19–21,77,78]. For example, retroviral shRNA suppression of ELK3, a signaling protein involved in autophagy activation, decreased autophagy and improved DOX responsiveness [79]. In another study, miR-520b significantly improved DOX sensitivity in hepatocellular carcinoma (HCC) via suppressing ATG7-dependent autophagy [80]. However, inhibiting autophagy, as shown by the cell surface protein CD24, led to a large rise in the sensitivity to the drug sorafenib, which is used to treat HCC [81]. More importantly, autophagy can also induce apoptosis. It has been demonstrated that inhibiting autophagy reduces glycolytic capability, oxidative phosphorylation, and cell proliferation, while also increasing apoptosis [82,83]. One study found that utilizing the lucanthone drug, a possible sensitizer to chemotherapy and radiation, caused lysosomal dysfunction, autophagy suppression, and apoptosis in cancer cells [84]. Nonetheless, suppressing autophagy in cancer cells increased the apoptotic effect of paclitaxel, an apoptosis inducer used to treat lung adenocarcinoma [85]. Accordingly, great efforts have been made in order to inhibit autophagy as a mechanism for enhancing cancer treatment therapies [86] using different methods in order to accomplish the inhibition including the use of autophagy inhibitors (such as 3-methyladenine and chloroquine), knockdown critical ATG by RNA interference (RNAi) or a combination of them.

1.5.1. Autophagic Drugs

Nowadays, there are a variety of drug agents that block autophagy at its various stages, ranging from autophagy induction and autophagosome formation through lysosomal degradation [87], headed by chloroquine (CQ), hydroxychloroquine (HCQ), 3-methyladenine (3-MA) and Bafilomycin A1 (BafA1), with CQ and HCQ being the most investigated in clinics [88] (Figure 1.9).

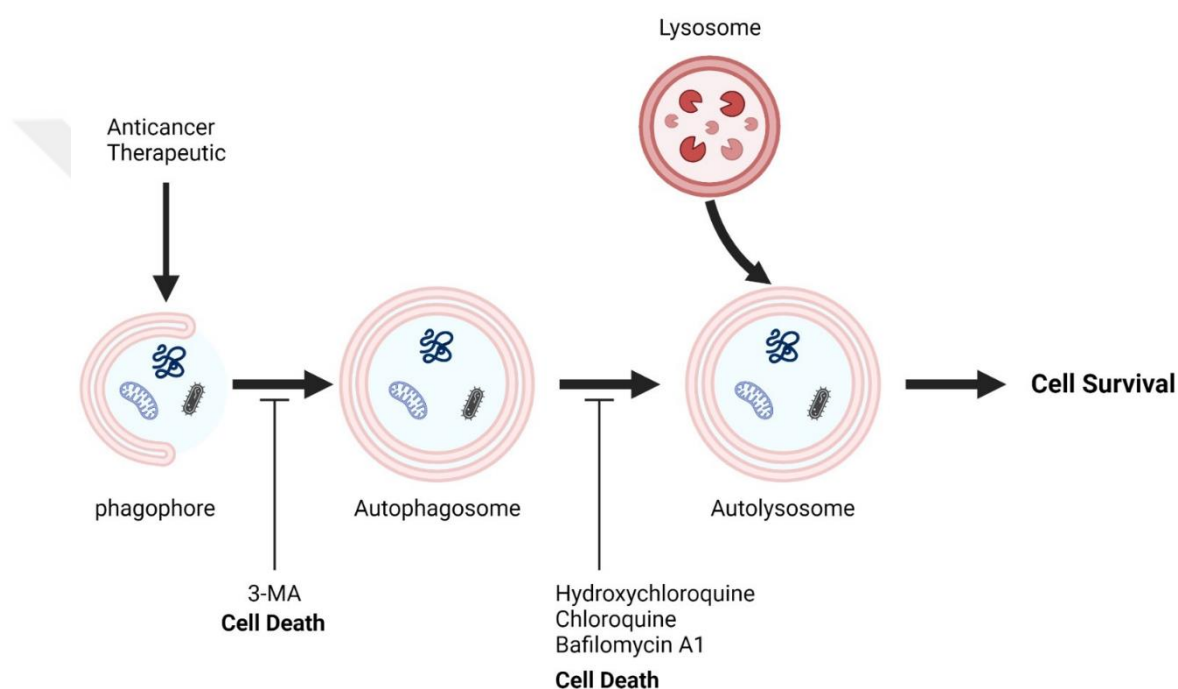


Figure 1.9. The points that the most common autophagy inhibitors work on are depicted (This figure was created using BioRender).

CQ was developed in 1934 and has primarily been used to treat malaria [89]. It has been recently employed as an autophagy inhibitor since it has been shown to impede the functionality of lysosomes by causing a stoppage in the fusion of autophagosomes and lysosomes [90]. HCQ, a CQ derivative (Figure 1.10), suppresses autophagy by manipulating the pH of lysosomes, which prevents autophagosome destruction [91,92]. Because HCQ is less hazardous than CQ at peak doses, it has been frequently utilized in clinical trials over CQ [91,93]. CQ has been utilized in glioma cells to see whether there are any synergistic effects that may be produced by mixing temozolomide, a medicine used to treat brain malignancies, with autophagy inhibitory agents, and the results reveal

that inhibiting autophagy with CQ can help to prevent cancer growth [94]. Furthermore, by inhibiting autophagy, CQ suppressed tumor growth in PDAC mice models [71]. The combination of Thymoquinone, a major bioactive ingredient isolated from black cumin [95], and CQ triggered apoptosis in cancer cells in glioblastoma [96]. In another study, treatment with CQ coupled with artemisinin, an element derived from sweet wormwood [97], induced lung cancer cells to apoptosis in a synergistic manner [98]. In a recent study, HCC cells were treated with a combined treatment of HCQ and sorafenib, which reduced tumor growth, cellular proliferation, migration, and invasiveness by suppressing autophagy and regulating apoptosis, overcoming drug resistance [99].

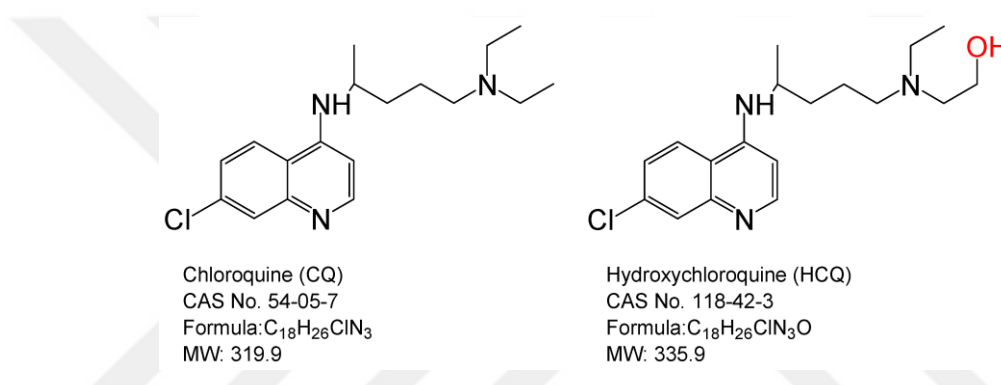


Figure 1.10. The difference between CQ and HCQ chemical structures [100].

3-MA is an autophagy inhibitor that inhibits the development of autophagosomes by altering PI3K, a critical autophagy's arranger [101]. It's been proven that suppressing autophagy with 3-MA improves chemotherapeutic medication efficacy in laryngeal cancer [102,103]. In a recent study, treating HCC cells, HepG2, with sorafenib and 3-MA at the same time inhibited cell proliferation while also triggering apoptosis. Thus, by suppressing autophagy, MA may be able to reduce drug resistance and improve drug efficiency [104]. 3-MA enhanced Tocomin apoptosis, an available commercial drug with antitumor characteristics made up of vitamin E components, in breast cancer cells [105].

BafA1, a *Streptomyces gresius*-derived antibiotic, suppresses autophagy by blocking the acidification of endosomes and lysosomes, which prevents their union [106]. By suppressing autophagy in colorectal cancer cells, BafA1 may be able to prevent cell cycle transition and increase cell death [107,108]. BafA1 also stopped prostate cancer cells from spreading by blocking autophagy [109]. In HCC, BafA1 treatment inhibited cellular proliferation, induced cell cycle arrest, prompted Cyclin D1 turnover, and caused caspase-independent apoptosis in both 2-dimensional and 3-dimensional cultures. All of this was

caused by autophagy suppression, which was demonstrated by an increase in LC3 conversion [110].

1.5.2. Gene Therapy

RNAi, which was first reported in 2006 and involves the use of microRNAs (miRNAs) or small interfering RNAs (siRNAs) to inhibit the expression of certain genes, is swiftly gaining traction as a powerful technique for gene silencing [111]. Both of them are short RNA duplexes that contain an average of 21–23 nucleotides [112]. Despite the structural and functional similarities between miRNAs and siRNAs, there are some significant distinctions between them. The primary distinction between the two molecules is found in their modes of action [113]. While both act by promoting the inhibition of messenger RNA (mRNA) expression, siRNAs work by cleaving mRNAs, whilst miRNAs work by blocking mRNA translation into proposed proteins (Figure 1.11). In addition to their modes of action, they differ in the way they recognize target mRNA. To have an effect, siRNA must be completely complementary to its target mRNA, allowing it to be used to silence a single target gene. For miRNA, however, no such thing is required; a partial interaction between miRNA and its target is sufficient to cause it to act (Figure 1.12). As a result, it might be utilized to block several genes [113].

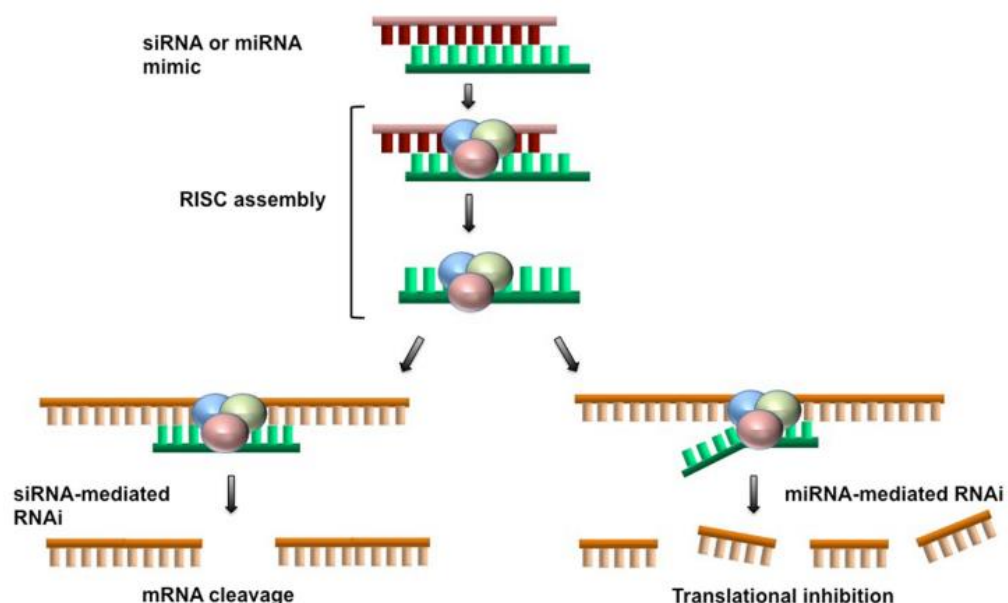


Figure 1.11. The difference in the mechanism of action of siRNA and miRNA [111].

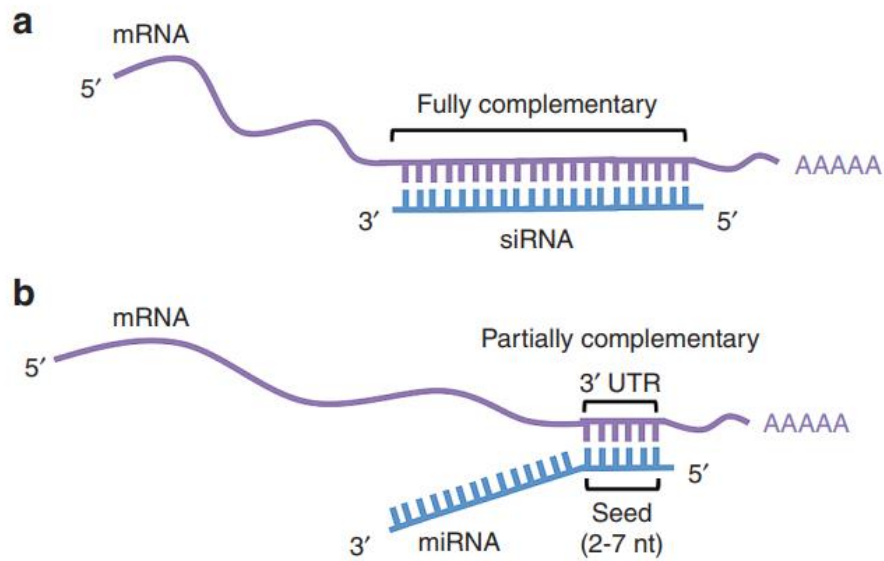


Figure 1.12. siRNA and miRNA target recognizing ways [114].

Nowadays, these technologies are employed to inhibit autophagy by focusing on key ATGs implicated in the process as a successful method to boost the antitumor response while lowering cancer toxicity [5,115]. For instance, HCC have been shown to be more sensitized to DOX and fluorouracil therapy when autophagy is inhibited by miR-101 [116]. Another study found that inhibiting autophagy via miR-101 can improve cisplatin cytotoxicity in HCC cells and hence increase apoptosis population [117]. In one study, it was demonstrated that by reducing autophagy activity by suppressing ATG12 through miR-23b, radiotherapy effectiveness was improved in pancreatic cancer cells [118]. Additionally, miR-29c inhibited autophagy and elevated gemcitabine-induced apoptosis, a cytotoxic drug used to treat some cancers, in pancreatic cancer by regulating the expression of USP22 [119]. In another study, miR-373 was used to target the autophagy-upregulated proteins CD44 and TGFBR2 in glioblastoma multiforme (GBM), which prevented the cancer cells' migration and invasion [120]. In one study, a co-delivery of siATG7 and docetaxel, a drug utilized in treating breast cancer, enhanced drug efficiency and boosted apoptotic rate by inhibiting autophagy [121]. In a recent study, Beclin siRNA significantly improved paclitaxel sensitivity, chemotherapeutic medication, in lung cancer cells via suppressing autophagy [122]. Additionally, downregulation of LC3 gene by delivering siLC3 significantly reduced cell viability and blocked the main cell

mechanisms; proliferation, invasion, migration, and resistance to apoptosis in the metastatic MDA-MB-231 cell line, which represents a simulation of TNBC [23].

1.6. Delivery Systems for Gene therapy

Genetic therapeutic agents must be successfully introduced into cells in order to inhibit autophagy utilizing gene therapy. The quick degradation of siRNAs once they enter the circulation, as well as their negative charge, which prevents them from entering the cell membrane, are two of the challenges they face. Viral-based delivery systems were developed and employed for this purpose, but they were shown to be unsafe [123]. In order to transport genetic materials and meet the many obstacles in the area, a variety of non-viral gene vectors have been used. A summary of the most commonly used vectors is shown in (Figure 1.13). In which cyclodextrins (CDs) are one of the components utilized to make a variety of vectors [123].

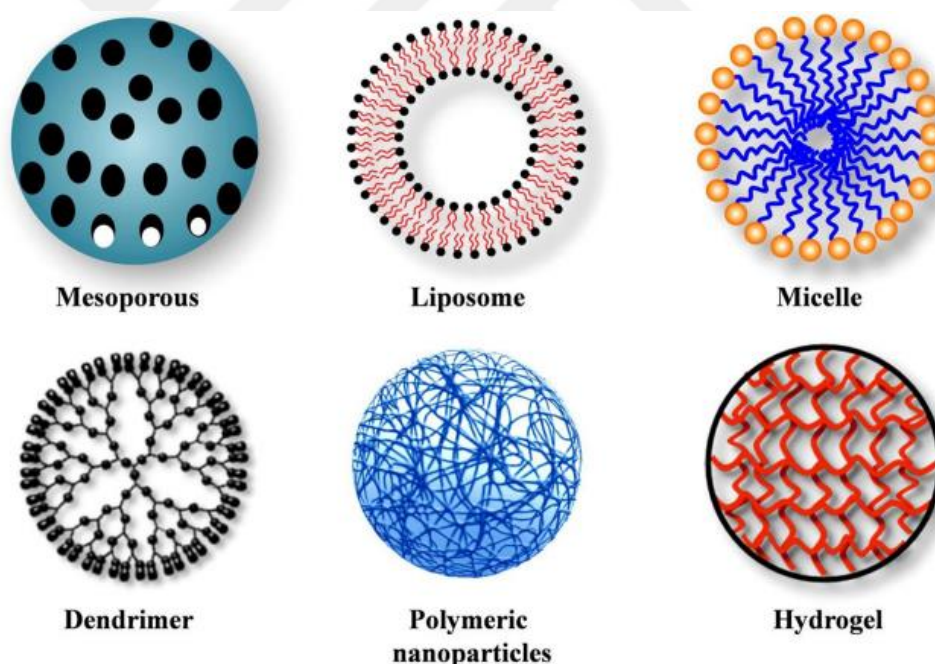


Figure 1.13. Some of the non-viral gene vectors utilized in transporting genetic materials [1].

1.7. Cyclodextrins

CDs represent a type of cyclic oligosaccharide made up of six or more glucose subunits linked together by α -1, 4 glycosidic linkages. CDs are divided into three groups based on the amount of glucose subunits they contain: α -CD, β -CD, and γ -CD (Figure 1.14) [124].

CDs have a hydrophobic interior cavity with numerous hydroxyl groups on the outside, giving the cavity's outside part a hydrophilic property. This structure allows CDs to encapsulate hydrophobic compounds within, increasing their solubility and biological availability while lowering related non-specific toxicity [125]. CDs are being applied in a variety of applications including pharmaceuticals [124,126,127], food [128,129] and textile finishing [130,131].

1.7.1. Cyclodextrin Nanoparticles and Polymers

Primary and secondary hydroxyl groups make up the outside surface of CDs [123]. The availability of these groups affords reactive groups for chemical modification or polymerization of the substance, which opens up new avenues for constructing a variety of cyclodextrin-based nanostructures [132]. These changes enable cyclodextrins to be used in a precise applications, including gene therapy, cell therapy, nanomedicine therapy, immunotherapy, and chemotherapy.

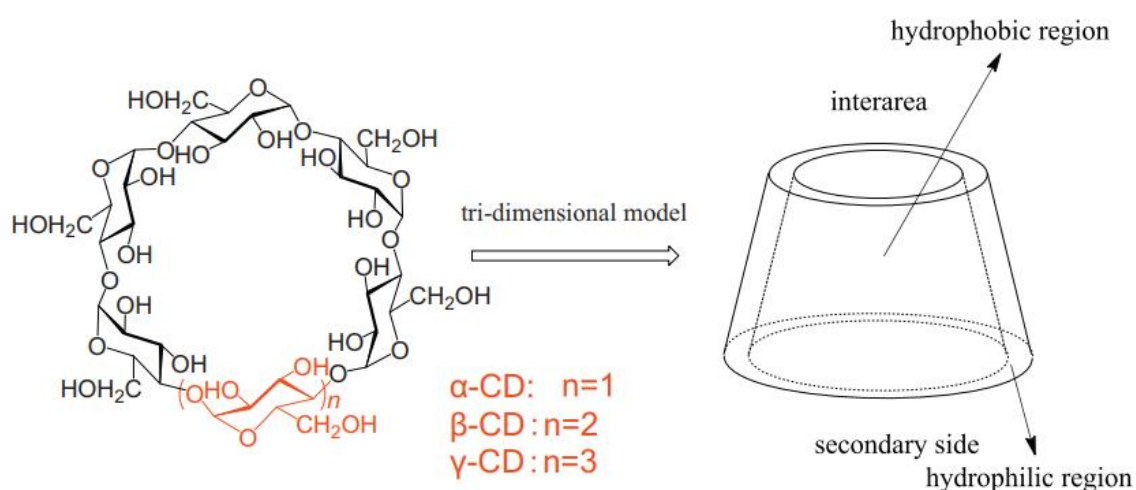


Figure 1.14. The structure of CD and its most common forms: α-CD, β-CD, and γ-CD [123].

Generally, the CDs used in the construction of nanoparticles for siRNA delivery operate as polycation, allowing different sizes and types of nucleic acids to be assembled through electrostatic interactions. In one study, nanoparticle system has been generated, in which it consisted of CD, transferrin, and polyethylene glycol and successfully accomplished a delivery of siRNA to targeted cancer cells [133].

Reversible addition-fragmentation procedures such as chain transfer (RAFT), ring opening polymerization (ROP), and free radical polymerization all use CDs or their derivatives as reactants [132]. CD-based polymers can be broadly classified into three kinds based on the role of CDs in polymer architecture as seen in (Figure 1.15).

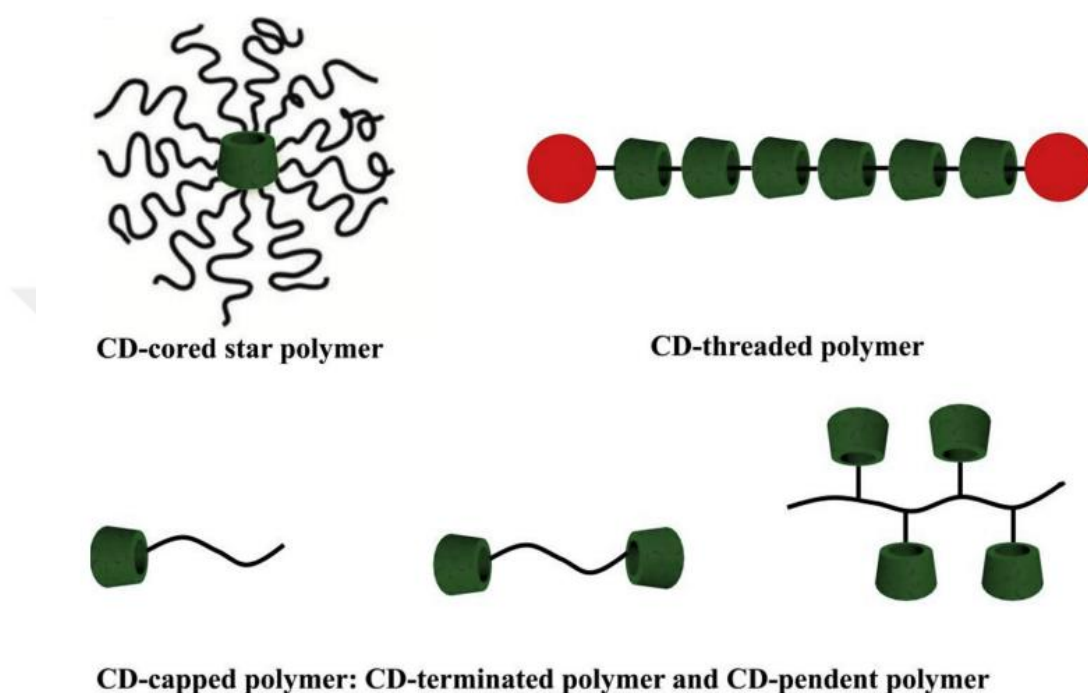


Figure 1.15. The CD polymer varieties are depicted [132].

Additionally, a number of anticancer drug carriers have been developed using CDs. Table 1.1 provides a summary of several of these carriers.

Table 1. 1. Anticancer medication carriers based on CDs.

Drug	Cyclodextrin	Nanocarrier prepared	Ref
DOX	γ -CD	Liposomes	[134]
Curcumin	HP - γ -CD	Liposomes	[135]
Methotrexate	β -CD	Niosomes	[136]
Camptothecin	β -CD	Nanosponges	[137]
Paclitaxel	β -CD	Nanosponges	[138]
DOX	β -cyclodextrin -based star copolymers	Micelles	[139]
B -lapachone	α -CD	Polymeric millirods	[140]
Camptothecin	Amphiphilic β -CD	Nanoparticles	[141]
Paclitaxel	Amphiphilic β -CD	Nanoparticles	[142]
Docetaxel	Amphiphilic β -CD	Nanoparticles	[143]

2. CHAPTER

MATERIALS AND METHODS

2.1. Materials

1. Dulbecco's Modified Eagle's Medium (DMEM 12-604F, Fisher Scientific)
2. Trypsin EDTA Solution A (03-050-1B, Biological Industries (BI))
3. FBS (04-127-1A, Bio-Trend)
4. L-Glutamine (03-020-1B, Bio-Trend)
5. Penicillin and Streptomycin (DE17-602E, Lonza)
6. Phosphate Buffered Saline (Nutri Culture PBS 1x (PBS500))
7. DOX
8. Resazurin
9. HMA: Hexyl methacrylate
10. DMAEMA: 2-(dimethylamino) ethyl methacrylate
11. TMAEMA: N , N , N -trimethylaminoethyl methacrylate iodide
12. LC3 siRNA (Sigma-Aldrich)
13. eBioscience™ Annexin V-FITC Apoptosis Detection Kit
14. Propidium Iodide (P4170-250MG, Sigma-Aldrich)
15. RNase Cocktail ™ Enzyme Mix (AM2288, Thermo Fisher Scientific)
16. AVOs acridine orange staining
17. Crystal Violet
18. Anti-LC3B Abbkine (Abp57204-2)
19. Anti-Integrin beta 1 Proteintech (12594-1-AP)
20. Anti-Src Proteintech (11097-1-AP)
21. Anti-phospho-Src Cell Signaling Technology (12432S)
22. Anti-Cleaved-Caspase3 Elabscience (E-AB-30003)
23. Anti-Caspase3 Elabscience (E-AB-63602)

24. Anti- β -Actin Proteintech (60008-1-IG)
25. Anti-cyclin D1 Proteintech (55004-1-AP)
26. Anti-PARP Cell Signaling Technology (9542S)
27. Anti-p62 Cell Signaling Technology (5114S)

2.2. Devices

1. Zetasizer Nano series (Nano-ZS90) device (Malvern Instruments, United Kingdom).
2. Elisa plate reader (Promega Glomax Multi Detection System).
3. Phase-contrast microscopy (Zeiss Microscope)
4. Flow cytometry (Guava easyCyte, Merck Millipore).
5. ECL Western Blotting Substrate (Bio-Rad, Hercules, CA).
6. Fluorescent microscope (Nikon Eclipse Ti)
7. Centrifuge device 5810R
8. Incubator ICO105 MED
9. Shaker UNIMAX 1010
10. Ice Maker EF156
11. Vortex Mixer SA8

The devices used in this thesis work belong to Erciyes University Nanotechnology Research Center (ERNAM), Erciyes University Betül Ziya Eren Genome and Stem Cell Research Center (GENKÖK), Erciyes University Ziya Eren Pharmaceutical Application and Research Center (ERFARMA).

2.3. Methods

2.3.1. Cell Culture

MDA-MB-231 cell line (ATTC) was used for this study. The cells were cultured in DMEM with 10% heat-inactivated FBS and 1% streptomycin and penicillin antibiotics. When cells attained a density of 80-85%, they were routinely passaged and cultivated in an incubator at 37 °C in a humidified environment of 5% CO₂ and 95% air [144].

2.3.2. Utilized “Smart” Nanoparticles

The "smart" nanoparticle system employed was developed using the Lin et al. [145] group's protocol. It is based on the use of β -CD, that is FDA approved, and consists of two sides: a primary side and a secondary side. The major side is made up of three distinct moieties: pH-sensitive DMAEMA monomers, hydrophobic HMA, and cationic TMAEMA monomers, and the other half is PEG-bound (Figure 2.1). This polymer may detect changes in pH in the environment, break the endosomal membrane, and form an electrostatic combination with the negatively charged siLC3. Whereby it accomplishes its endosomal escape in a dual form, combining the hydrophobic disrupting effect of HMA units with the proton sponge buffering capacity of DMAEMA monomers in a single membrane-active nanoplatform, which increases the effectiveness of its escape [146].

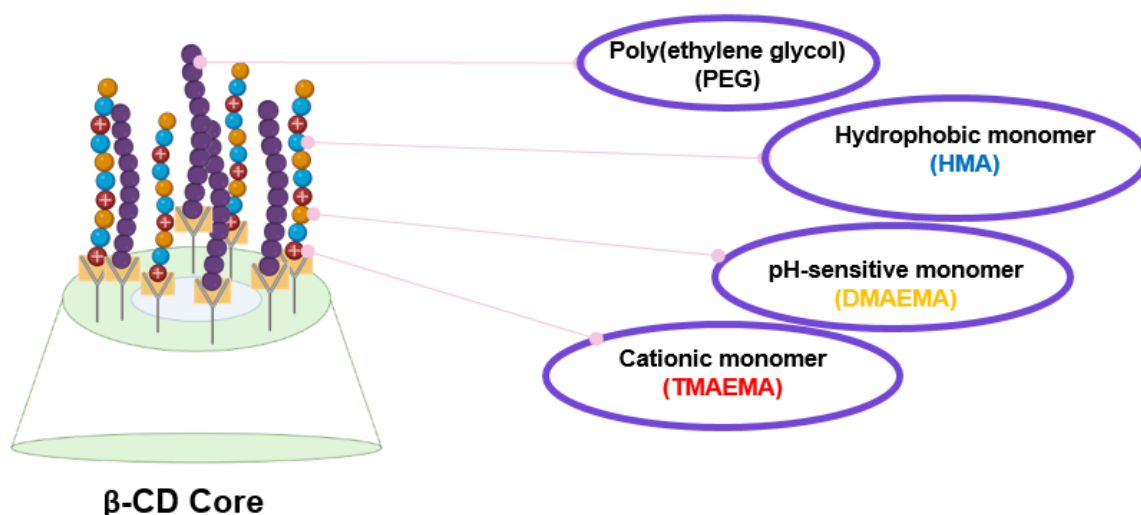


Figure 2.1. Representation of “smart” nanoparticles

2.3.3. Characterization of “Smart” Nanoparticles

Agarose gel electrophoresis was used to test the ability of the "smart" polymer in compacting LC3 siRNA completely. Polymer/siRNA mixtures with various N/P ratios were prepared, as shown in (Table 2.1). After that, an amount of 4 μ l of 6X loading dye was added to each mixture. 5 μ l of ethidium bromide was used in preparing 1% w/v agarose gel, which get then immersed in TAE solution. Finally, the samples were loaded to the gel before being exposed to 60 V for 60 minutes. After the running period, the gel was treated with UV light to obtain results [147].

Table 2. 1. Each specimen's components are represented.

Well No	Treatment (N/P)	LC3 siRNA (μ l)	β CD-PEG-L (μ l)	Loading Dye (6x) (μ l)	N.F. Water (μ l)
1	DNA Ladder				
2	Free siRNA	1.07	_____	4	14.93
3	1_1	1.07	2.15	4	12.78
4	2_1	1.07	4.30	4	10.63
5	4_1	1.07	8.60	4	6.33
6	6_1	1.07	12.90	4	2.03
7	8_1	1.07	17.20	4	0
8	Free Polymer	_____	17.20	4	0

Using a Nano-ZS90 device, particles' diameters and surface charges were evaluated in triplicate for 2/1, 3/1 and 4/1 ratios using DLS analyses. At a temperature of 25°C and a scattering angle of 90°, the data were taken [148].

2.3.4. Effect of DOX Concentration on Cancer Cell Survival

Before we investigate the therapeutic effects of our complexes on cell viability, we must figure out the suitable DOX dosage to use. A total of 10,000 cells per well were seeded in 60 of the 96 well plate's wells. Following a 24-hour incubation phase, cells were incubated with various concentrations of DOX for 48 hours using a serial dilution experiment, starting with a high concentration of 500 nM. Following that, DMEM was withdrawn from the wells and Resazurin dye was added to each of them at a concentration of 10% (v/v), following our published protocols [149]. The plate was cultured in an incubator for 3 hours in the dark. Fluorescence measurements were carried out at two different wavelengths (570–590 nm).

2.3.5. Therapeutic Effect of “Smart” NP/LC3 and DOX

Cell viability was investigated when cells were treated with DOX alone (at its IC₅₀ dose) or with DOX and "smart" nanoparticles containing 47.5 nM or 95 nM of siLC3 1 and siLC3 2. In 60 of the 96 well plate's wells, 1.25×10^3 cells per well were seeded. Cells were treated in the same way as they were in the "effect of DOX on cancer cell survival" section, only instead of Dox concentrations, they were treated with the following groups:

1. Free Polymer
2. IC₅₀ value of DOX (110 nM DOX)
3. β CD-PEG-CP– siLC3 1 (50 nM polymer, 47.5 nM LC3 siRNA)
4. β CD-PEG-CP– siLC3 1 (50 nM polymer, 47.5 nM LC3 siRNA)+ 110 nM DOX
5. β CD-PEG-CP– siLC3 2 (50 nM polymer, 47.5 nM LC3 siRNA)
6. β CD-PEG-CP – siLC3 2 (50 nM polymer, 47.5 nM LC3 siRNA)+ 110 nM DOX
7. β CD-PEG-CP– siLC3 1 (100 nM polymer, 95 nM LC3 siRNA)
8. β CD-PEG-CP – siLC3 1 (100 nM polymer, 95 nM LC3 siRNA)+ 110 nM DOX
9. β CD-PEG-CP– siLC3 2 (100 nM polymer, 95 nM LC3 siRNA)
10. β CD-PEG-CP– siLC3 2 (100 nM polymer, 95 nM LC3 siRNA)+ 110 nM DOX

And in the same way, Resazurin dye has been utilized to order to take fluorescence measurements.

2.3.6. Wound Healing Assay

This experiment was used to determine the ability of cells to migrate across a distance. The cells were seeded in six-well plates (500,000 cells per well). After 24 h incubation, a scratch was formed with a 200- μ l sterile pipette tip in two directions (vertically and horizontally), creating a "wound" across the center of each well. The unattached cells were then rinsed away with 1 ml PBS in each well. Following that, monolayers of cells were treated with DOX alone (at its IC₂₀ dose) or DOX plus 47.5 nM LC3 siRNAs. Finally, after particular lengths of time, we collected a set of photos (at a magnification of 4) that will show the wound region at t=0, t=24, and t=48h. Wounds were processed at various times using ImageJ software [150].

2.3.7. Colony Formation Assay

Colony formation assay was set up in accordance with our earlier protocol [23]. We seeded cells with 1.5×10^3 cells/well population in 1.5 ml/well of medium in six-well plates and let them grow for two nights. The cells were then incubated with the relevant

groups (same in previous section) for 4 hours before replacing the media with fresh DMEM and allow the cells to be cultivated for 2 weeks. Subsequently, the cells were rinsed in PBS before being stained with 1% crystal violet. The colonies were counted by eye and a microscope was used to ensure that each colony contained at least 50 cells.

1% crystal violet preparation: 5 mL methanol and 1g of crystal violet powder were added into 45 mL of distilled water. The stock should be wrapped with aluminum foil to protect it from light and stored at room temperature.

2.3.8. Cell Cycle and Apoptosis Assays

Overnight, we seeded cells in 24-well plates (60,000 cells/well) in 300 μ l /well of media, then treated them with the indicated groups for 48 hours. Cells were collected using trypsin enzyme and centrifuged at (2000 rpm, 5 mins, 20°C) conditions after treatment. The cells were then dissolved in 150 μ l of ice cold PBS while being carefully pipetted up and down. 350 μ l of pure ethanol (total ethanol concentration: 70%) was added drop by drop to each sample. Following that, 200 μ l of RNase mixture was added to each sample, in which samples were then placed in an incubator for 30 minutes (for each 20 ml PBS, we added 20 μ l RNase). After the 30 minutes of incubation, we centrifuged the cells again (2000 rpm, 10 minutes) and added 250 μ l of a solution containing propidium iodide (PI) and PBS to each sample and waited a few minutes (0.5 l PI stain and 250 l PBS for each sample). Flow cytometry was used to evaluate the samples right away [150]. FlowJo software was used to process the data.

For apoptosis analysis, cells were seeded as well as treated in the same way as in the cell cycle assay. After 48 h incubation, cells were harvested, rinsed in ice-cold PBS, and dyed with Annexin V–fluorescein isothiocyanate (FITC) and PI, according to the manufacturer's protocol. Apoptosis was analyzed with flow cytometer.

2.3.9. Western Blotting Assay

Western blotting was performed as previously described [23]. Briefly, t-25 flasks were used to cultivate the cells (350,000 cells in 4 ml DMEM). Cells were collected and lysed for 30 minutes after treatment. To quantify protein concentration, the Bio-Rad Protein Assay Standard 2 kit was utilized, and then similar amounts of total protein were separated by SDS-PAGE and electrotransferred to PVDF membranes. Membranes were

blocked with 5% milk for 90 minutes before being incubated overnight at 4 °C with primary antibodies (table 2.2). The membranes were then treated for 60 minutes at room temperature with anti-rabbit or anti-mouse secondary antibodies coupled with horseradish peroxidase. Finally, ECL Western Blotting Substrate was used to view and evaluate membranes. The quantities of all proteins were standardized to β -Actin as an internal control.

Table 2. 2. List of primary antibodies used in western blotting

Antibody name	Company (Catalog No.)	Dilution
Anti-LC3B	Abbkine (Abp57204-2)	1 : 1000
Anti-Integrin beta 1	Proteintech (12594-1-AP)	1 : 1000
Anti-Src	Proteintech (11097-1-AP)	1 : 1000
Anti-phospho-Src	Cell Signaling Technology (12432S)	1 : 1000
Anti-Cleaved-Caspase3	Elabscience (E-AB-30003)	1 : 1000
Anti-Caspase3	Elabscience (E-AB-63602)	1 : 1000
Anti- β -Actin	Proteintech (60008-1-IG)	1 : 2000
Anti-cyclin D1	Proteintech (55004-1-AP)	1 : 1000
Anti-PARP	Cell Signaling Technology (9542S)	1 : 1000

2.3.10. Acidic Vesicular Organelles (AVO) Detection Assay

The formation of autophagosomes, which merge with lysosomes to form acidic vesicular organelles (AVO), is a hallmark of autophagy [151]. To identify these AVOs, cells were planted in 6-well plates (100,000 cells/2 ml medium). After treatment, cells were stained with acridine orange (AO) at a final concentration of 1 μ l/ml for 30 minutes, as described previously [152]. The cells were then washed in PBS before being viewed under a fluorescence microscope. The presence of AVOs in acidic compartments leads to the formation of red-colored cells [153].

2.3.11. Statistical Analysis

The results are presented as mean values for several replicates from at least three different experiments. Directional Analysis of Variance (DAV) was used for statistical analysis

(Two-way ANOVA). The symbol "*" was used to indicate that statistical differences between groups existed. * $p < 0.05$, *** $p < 0.001$ are the statistical significance levels.



3. CHAPTER

RESULTS

3.1. Characterization of “Smart” Nanoparticles

3.1.1. Size and Zeta Measurements

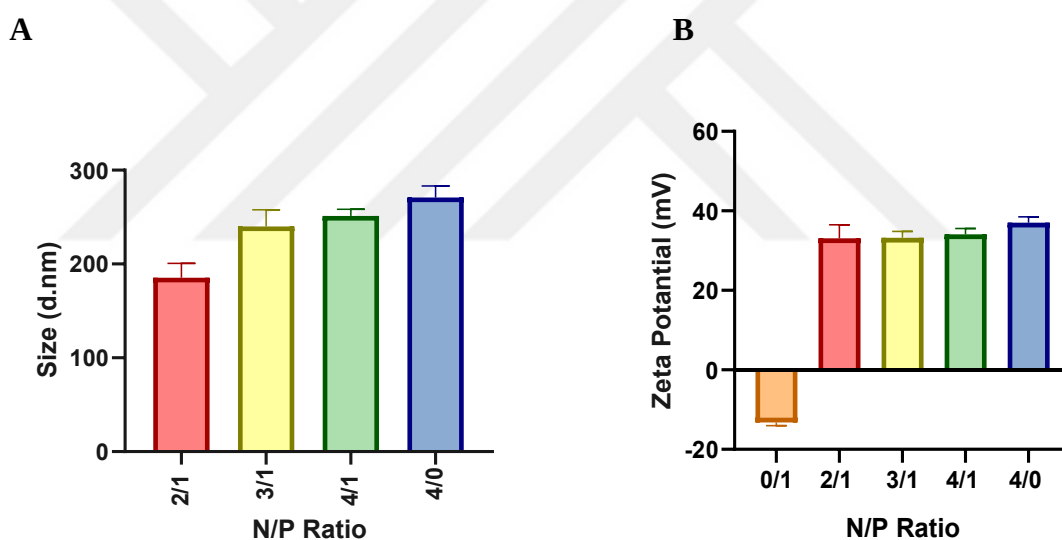


Figure 3.1. **A:** This figure represents the size (in nanometer) of siLC3 (47.5 nM) at 2/1, 3/1 and 4/1 N/P ratios. **B:** Zeta potential. Statistical analysis was performed with one-way ANOVA (Turkey) (* $p \leq 0.033$, ** $p \leq 0.02$, *** $p \leq 0.001$).

DLS has been utilized in measuring the particle sizes and zeta potentials of "smart" NPs at different N/P ratios. As illustrated in (Figure 3.1), the diameter of the complex particles increases as the N/P ratio increases. All "smart" NPs with varying N/P ratios had particle sizes of less than 270 nm (2/1: 184.4 nm, 3/1: 235.9 nm, 4/1: 259.4 nm). The charge of "smart" NP is positive, whereas the charge of siLC3 is negative, while all complexes had a positive surface charge of 30.0 ± 5.5 mV.

3.1.2. Agarose Gel Electrophoresis

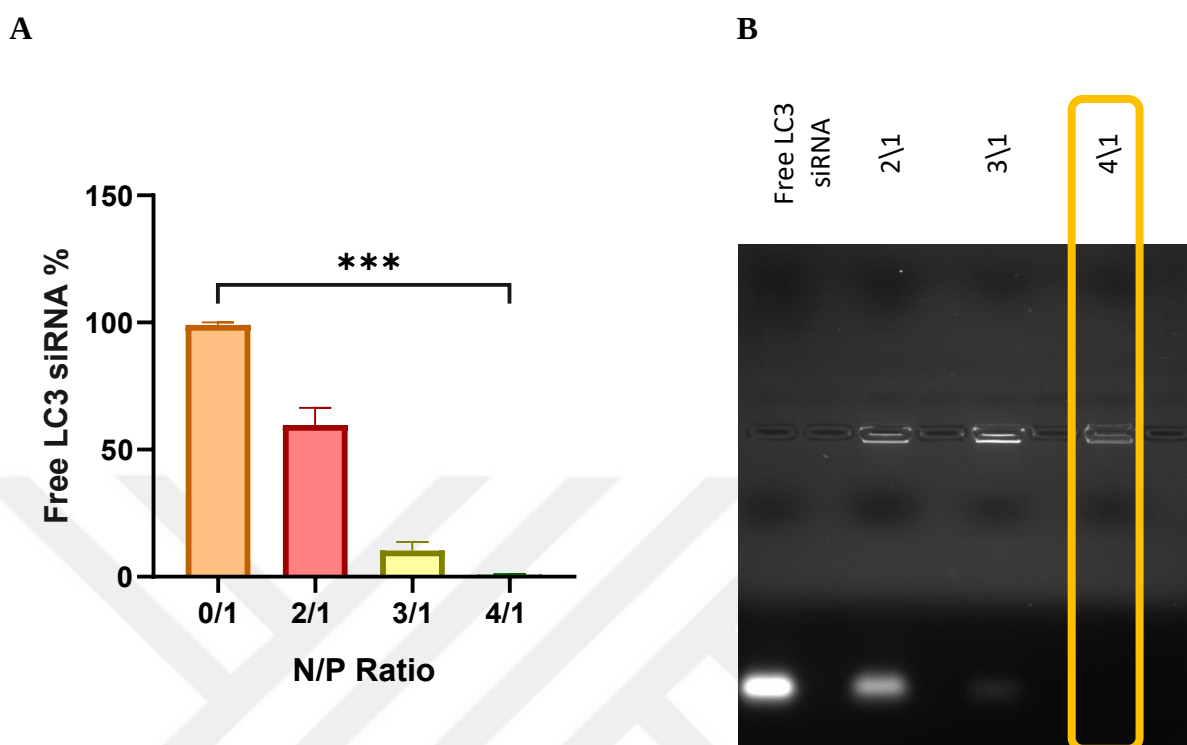
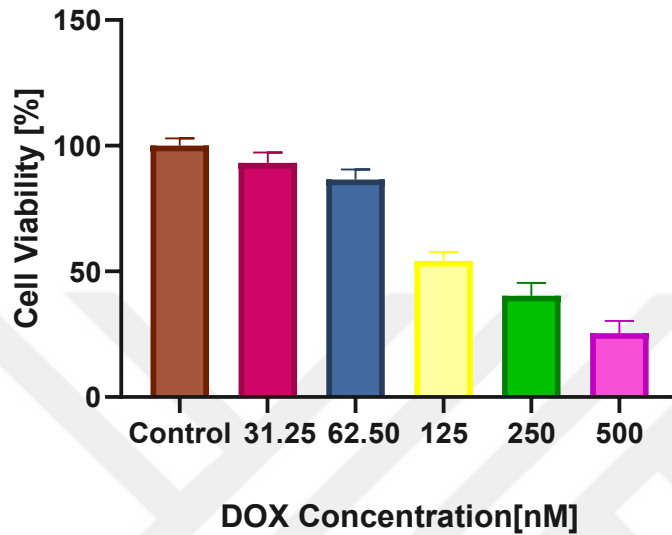


Figure 3.2. **A:** Images demonstrating the electrophoretic activity of the complexes at different N/P ratios (LC3 at 47.5 nM) ran through 1% w/v agarose gel containing ethidium bromide. **B:** Graphical representation of free LC3 ran through the gel.

The capacity of the complexes to condense siLC3 was tested using a conventional gel retardation technique. The number of moles of phosphate groups in siRNA is denoted by P, while the number of moles of nitrogen groups in the nanoparticle is denoted by N. Figure 3.2 shows that no radiation was generated at the 4/1 ratio, indicating that siLC3 was entirely condensed with our utilized polymers at this ratio, preventing free siRNA from moving across the gel.

3.2. Effect of DOX Concentration on Cancer Cell Survival

A



B

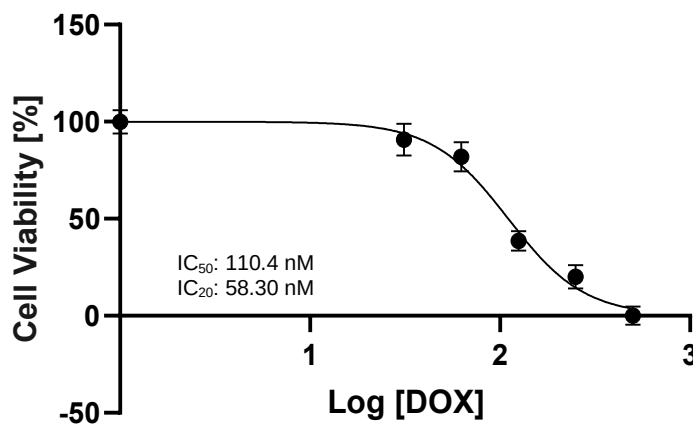


Figure 3.3.

A: Cells were incubated with DOX concentrations (31.25, 62.50, 125, 250, 500 nM) and after 48 h of incubation cell viability was measured. Results are in triplicate and mean $\pm$ standard error (SD). **B:** This figure represents cell viability percentage of the cells of the normalized logarithmic values of different molar concentrations of DOX.

Before examining the therapeutic effect of siLC3 and DOX co-treatment on cellular viability, the inhibitory concentrations of DOX (IC₂₀ and IC₅₀) that cause a 20% and 50% inhibition of cellular vitality, have been determined to be 58.3 and 110.4 nM, respectively.

3.3. Therapeutic Effect of Combination Therapy in TNBC Cells

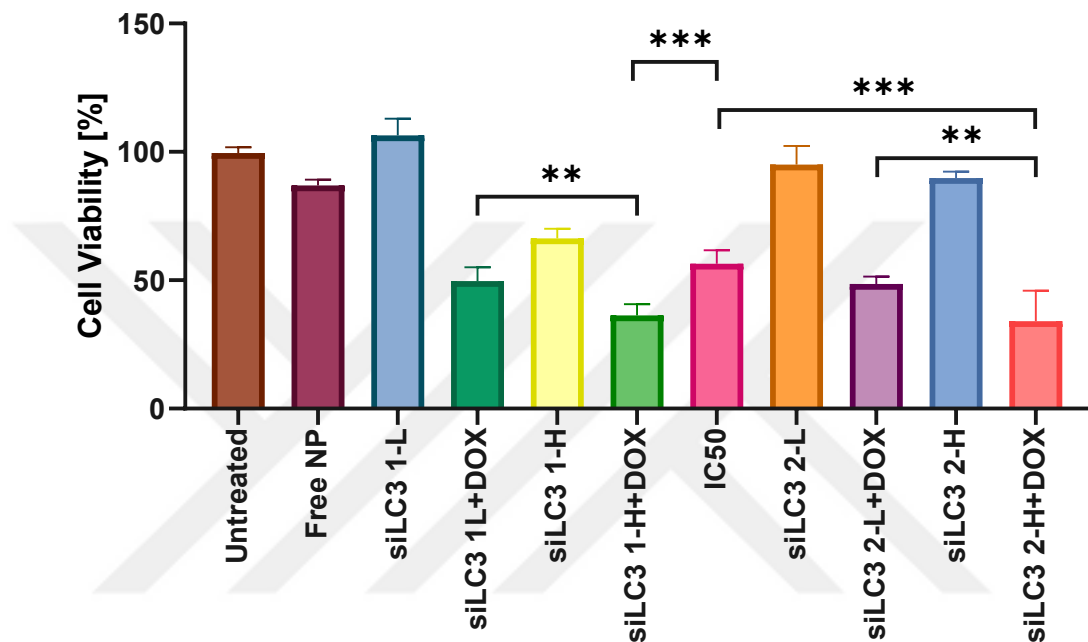


Figure 3.4. Cell viability of MDA-MB-231 cells utilizing siLC3 combination therapy in the presence of 110 nM DOX detected by proliferation assay using Resazurin staining for two different LC3 siRNA combinations (LC3 1 and LC3 2) at L: low concentration 47.5 and H: High concentration 95 nM, delivered by “smart” nanoparticles (***) $p < 0.001$.

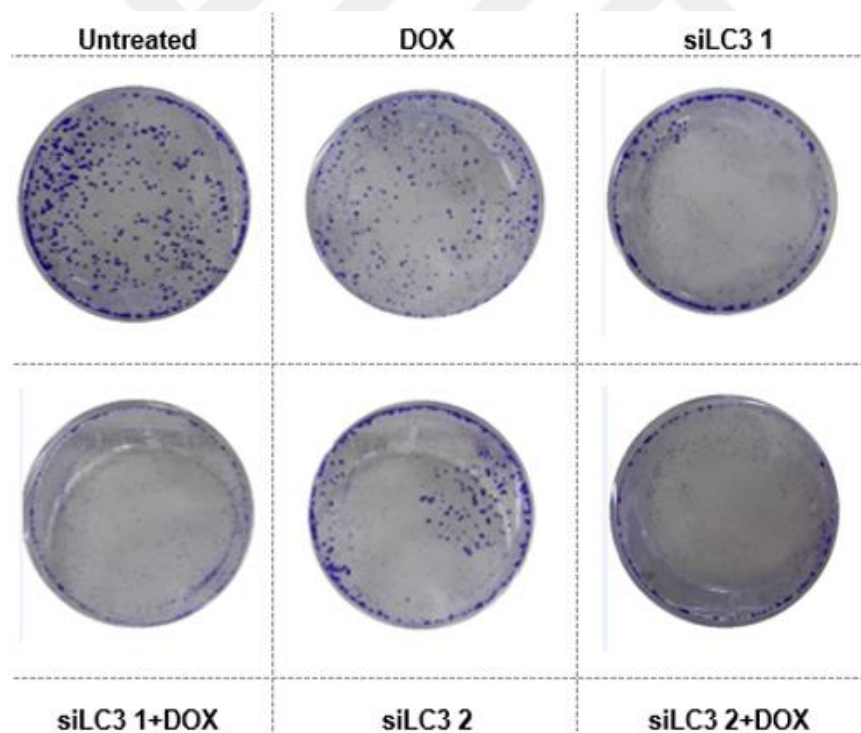
Cellular toxicity could be caused by the transport vehicle itself. Cell viability was tested 48 hours after being incubated with 100 nM empty “smart” nanoparticles to verify that there had been no cellular damage caused by the carrier (Figure 3.4).

We investigated whether treating the cells with only DOX or DOX in conjugation with “smart” nanoparticles containing two different concentrations of LC3 siRNAs (47.5 and 95 nM of LC3 1 and LC3 2 siRNAs) would lead to enhanced growth inhibition in the cells. Therefore, prior administering DOX and LC3 siRNAs, we ran serial dilution and proliferation assay to estimate the IC₂₀ and IC₅₀ concentrations of DOX (Figure 3.3). Subsequently we treated the cells with the identified groups for 48 hours (Figure 3.4). After 48 hours of incubation, the viability of DOX-treated cells (IC₅₀, 110 nM) was

reduced by half as compared with the control. Treating the cells with “smart” siLC3 2 nanoparticles at siRNA concentrations of 47.5 nM and 95 nM resulted in no change in cell viability compared to untreated cells. The same was observed when the cells were treated with “smart” siLC3 1 nanoparticles at siRNA concentration of 47.5 nM, but not while being treated with “smart” siLC3 1 nanoparticles at a siRNA concentration of 95 nM. In comparison, co-treatment with DOX (IC_{50}) and LC3 siRNAs at both concentrations enhanced the cytotoxicity of DOX in the cells, especially when 95 nM of LC3 siRNAs has been utilized, suggesting that the inhibition of autophagy by LC3 siRNAs leads to increase cells chemo-sensitivity to DOX.

3.4. Combinational Treatment Restrict Colony Formation

A



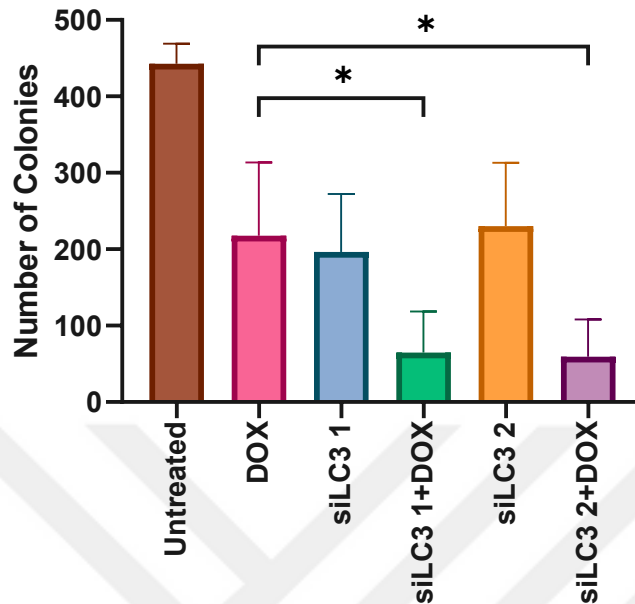
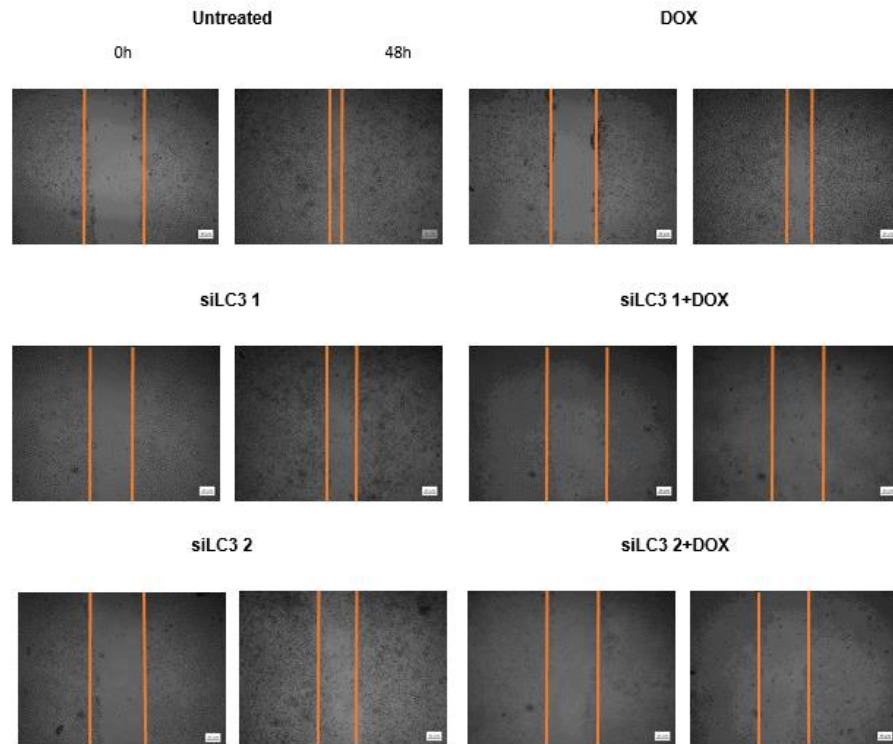
B

Figure 3.5. A: Cells were treated with 47.5 nM LC3 siRNAs and some groups were then co-administrated with DOX. Following that, the groups were stained with crystal violet to assess colony formation. **B:** Graphical representation of the number of colonies formed at the end of 2 weeks period. (* $P < 0.05$)

On the ability of cells to communicate with one another and form colonies, the effects of untreated, DOX, siLC3 1, siLC3 1 + DOX, siLC3 2, and siLC3 2 + DOX treatments were examined using a colony formation test. As shown in (Figure 3.5), DOX alone (IC_{20} , 58 nM) suppressed cell growth to a considerable extent, causing colony formation to be reduced by around 50%. In the same way, silencing LC3 gene using siLC3 1 and siLC3 2 “smart” nanoparticles significantly reduced the number of colonies formed by about 45% and 53%, respectively. In contrast, colony formation was significantly reduced—by more than 85%—when DOX (IC_{20}) was administered in combination with “smart” nanoparticles that were loaded with 47.5 nM siLC3 1 and siLC3 2. These results demonstrated that autophagy harbor DOX resistance.

3.5. Combined Treatment Inhibits Cells Migration

A



B

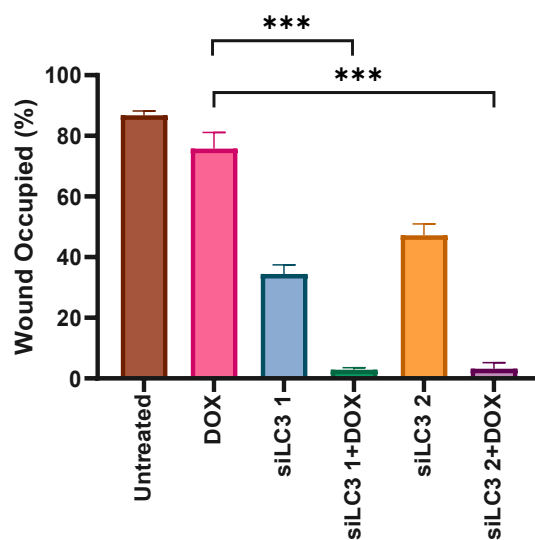


Figure 3.6.

A: Using 200- μ l sterile pipette, a straight scratch wound was generated in the monolayer in each well and cells were treated with the appropriate groups before being photographed at 0 and 48 hours at a magnification of 4. **B:** Graphical representation of the distance covered by cells after 48h treatment period. (***) $p < 0.001$).

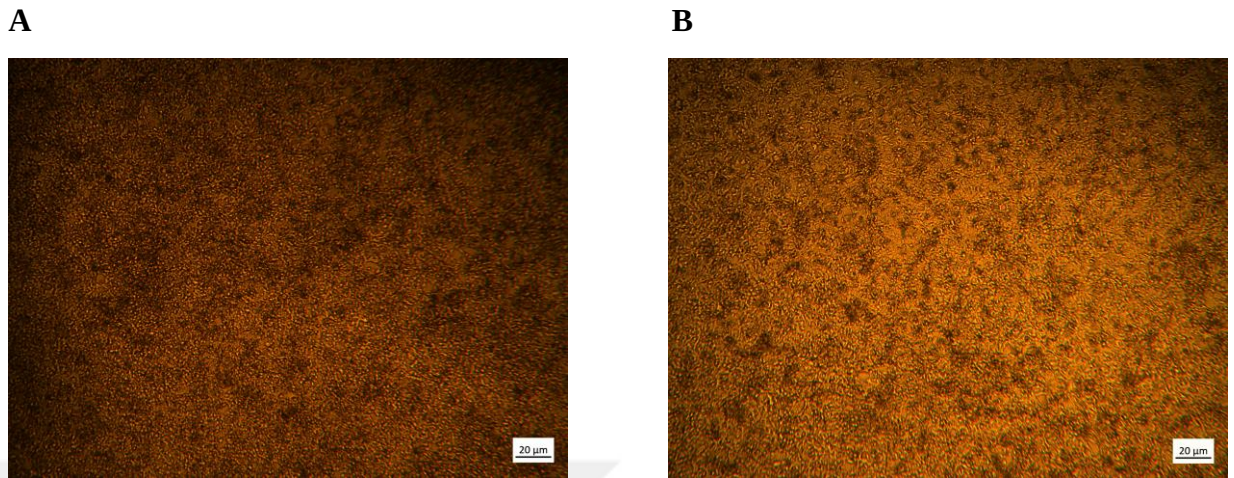
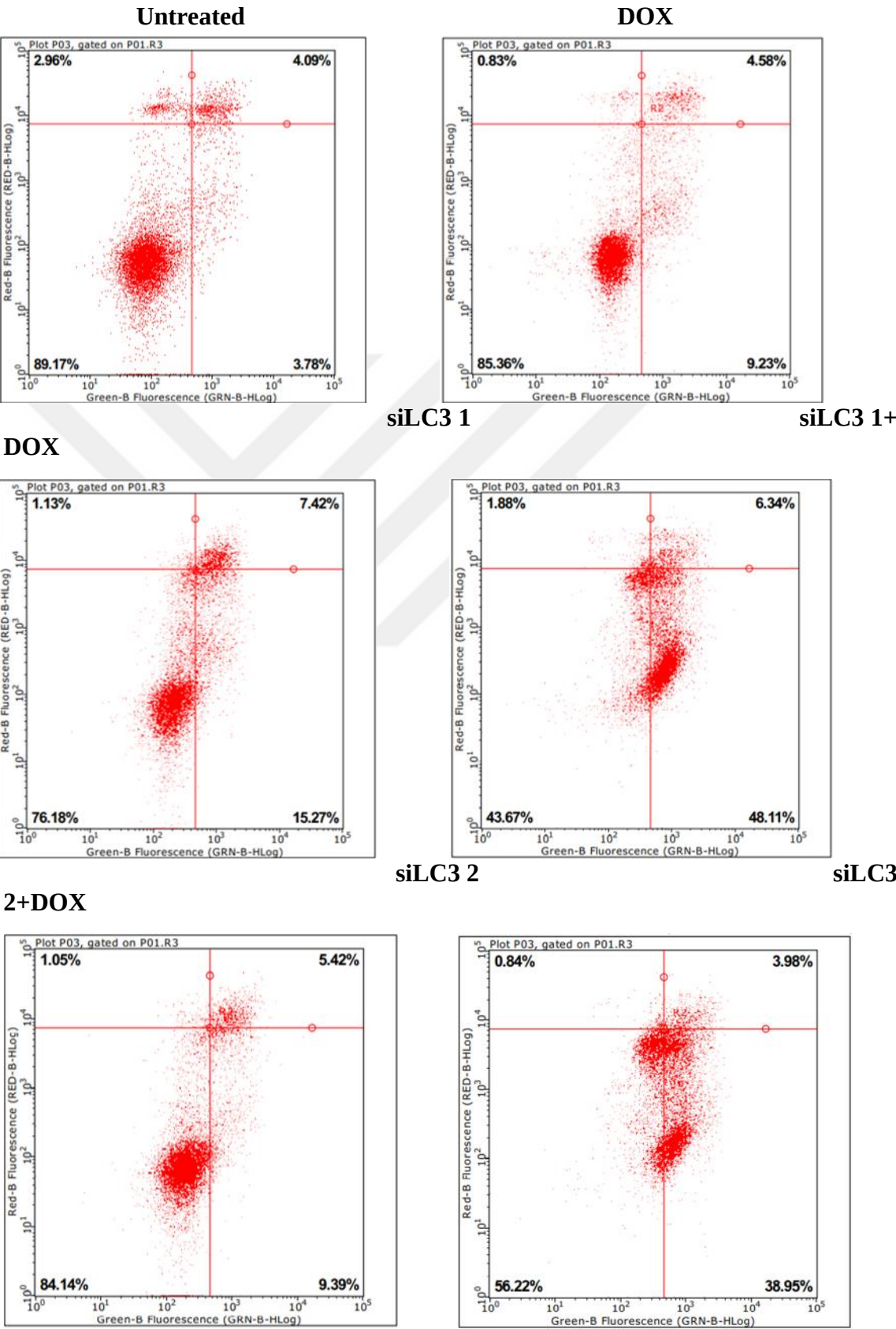


Figure 3.7. **A:** This figure represents the cells after being treated with 47.5 siLC3 1 for 48h.. **B:** The cells after being incubated with 47.5 siLC3 2 for 48h. The black spots represent entering the cells into apoptosis.

It has been previously reported that autophagy suppression influence tumor spread [154,155] [14,16]. In the current work, the effect of combining autophagy inhibition and chemotherapy on suppressing the ability of cells to migrate, which is implied by “healing” of the wounds, is examined using wound healing assay. Figure 3.6 reveals that as compared to the untreated cells, the cell migration rate of both 47.5 nM LC3 "smart" NPs groups was reduced. Nevertheless, comparing the images taken for these two groups at $t = 0$ h with those taken at $t = 48$ h, we could plainly observe entering the cells into apoptotic phases (Figure 3.7). However, the difference in migration between DOX-treated cells (IC_{20}) and untreated cells (control group) was modest.

In contrast with the other groups, cells treated with combined DOX and 47.5 nM siLC3 1 and siLC3 2 were unable to communicate with one another, resulting in a considerable fall in migration ($p \leq 0.001$). In (Figure 3.6), the images clearly show that the combined effect of these agents inhibits the migration of the cells by more than 90%. All of these highlights the crucial effect of autophagy inhibition in improving DOX efficacy.

3.6 LC3 "smart" NP and DOX Enhance Apoptosis



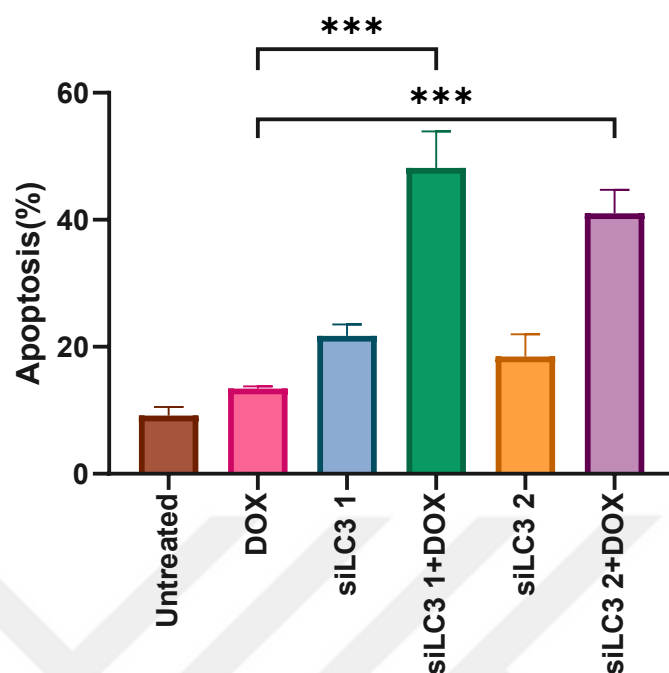


Figure 3.8. Apoptosis analysis of the cells after being dyed with Annexin/PI. Measurements were taken using flow cytometry ($p < 0.001$).

Flow cytometry was used to detect cell apoptosis after Annexin V-FITC labeling. In comparison to untreated cells, cells exposed to only DOX (IC_{20}) did not demonstrate obvious apoptosis following 48 hours of incubation, as shown in (Figure 3.8). At the same time, treating the cells with 47.5 nM of LC3 siRNAs alone showed a non-significant difference compared with DOX. However, treating the cells with both DOX and 47.5 nM siLC3 1 resulted in a 48.10 percent increase in cell apoptosis, which was 3.58 times greater than the free DOX group. So same was observed while treating the cells with IC_{20} and 47.5 nM siLC3 2, in which an apoptosis percentage of 41.02 was obtained; which is 3 times greater than the free DOX group. These results emphasize the synergetic effect of combining LC3 siRNAs with DOX and affirm the high potential outcomes of the treatment.

3.7. Combined Treatment Induces Cell Cycle Arrest

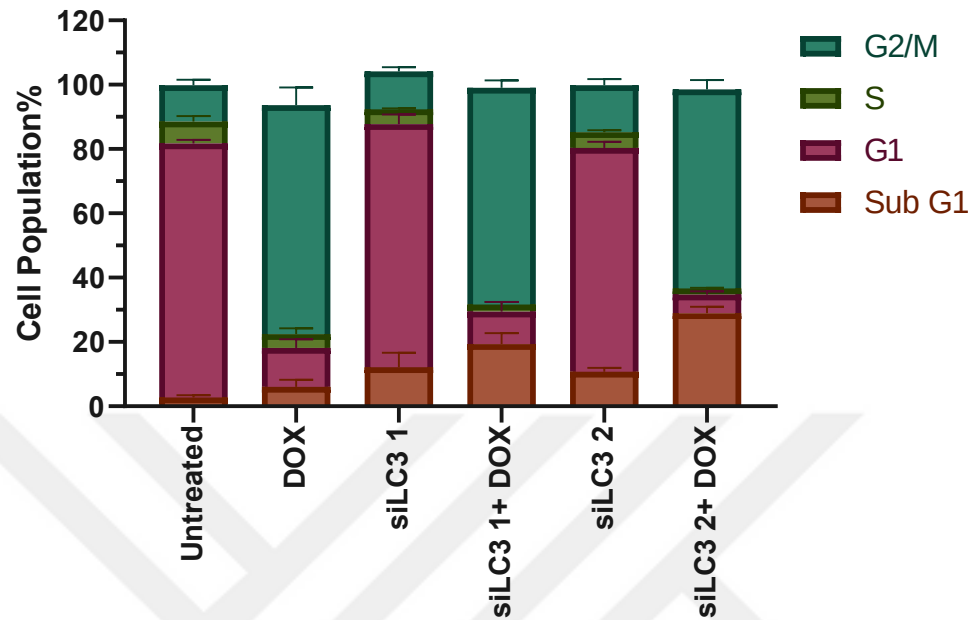


Figure 3.9. Cell cycle distribution in the cells after being stained with PI. The percentage of cells population in G2/M, S, G1 and Sub G1 is shown.

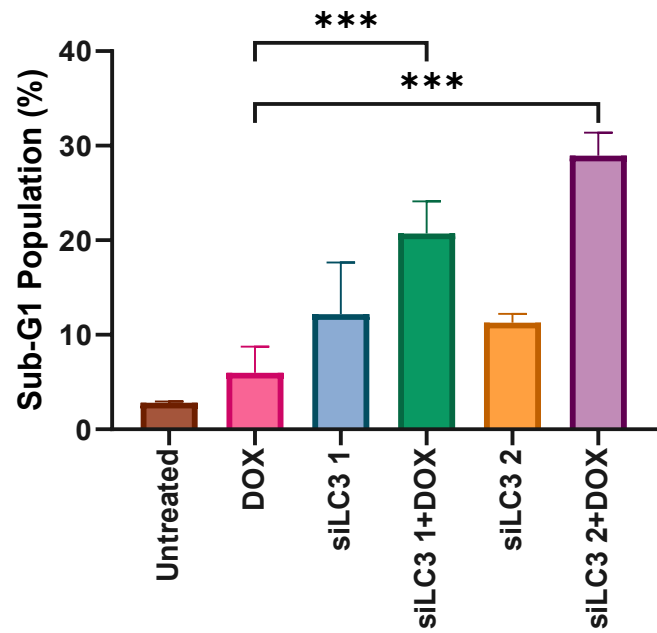
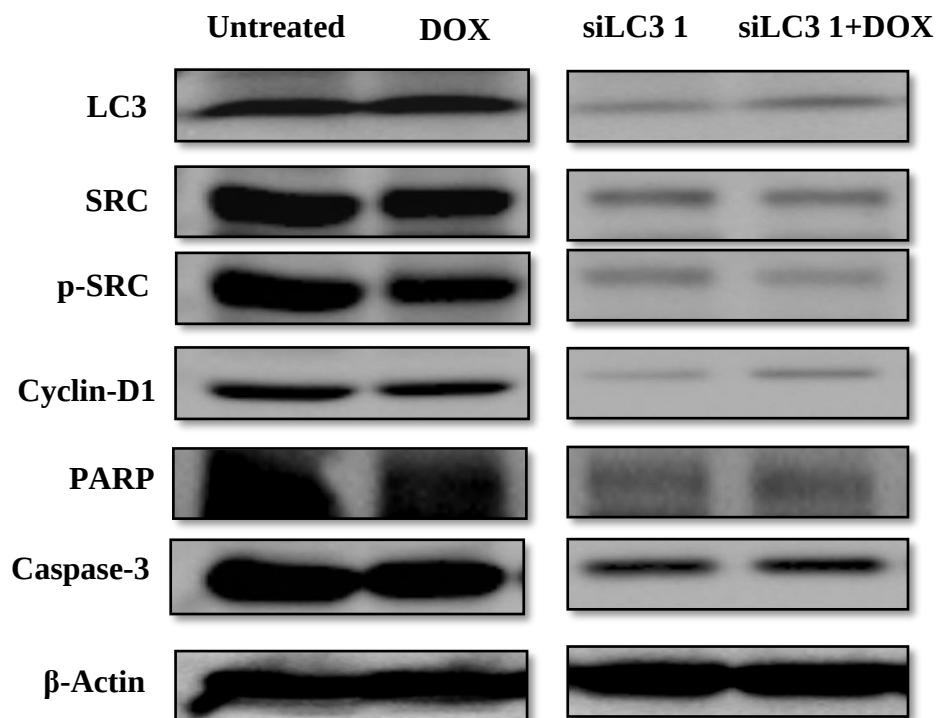


Figure 3.10. This figure represents the proportion of cells in the sub-G1 phase.

DOX suppresses cdc2/P34, causing G2/M arrest and death in cells [156]. Treatment of the cells using DOX alone, LC3 siRNAs and DOX combinations led in accumulating the cells at G2/M phase, according to flow cytometry analysis results (Figure 3.9). Moreover, our findings revealed that utilizing both DOX and LC3 siRNAs boosted the rate of apoptosis in the cells as compared to employing each agent alone. The fraction of cells in the sub-G1 phase demonstrated this (apoptotic cells). DOX alone showed a percentage of sub G1 arrest of 6 ± 2.24 %, while 47.5 nM LC3 1 and LC3 2 siRNAs results in sub G1 arrest of 12.17 ± 4.46 % and 11.27 ± 0.77 %, respectively. Nonetheless, treating the cells with both DOX and LC3 siRNAs increased the proportion of cells in sub-G1 phase to 20.71 ± 2.78 and 28.94 ± 1.99 , respectively (Figure 3.10), confirming our apoptotic assay results. Thus, we confirmed that treating cells with both LC3 siRNAs and DOX decreased cell cycle progression and promoted cell death, hence suppressing cell growth.

3.8 Western Blot Analysis

A



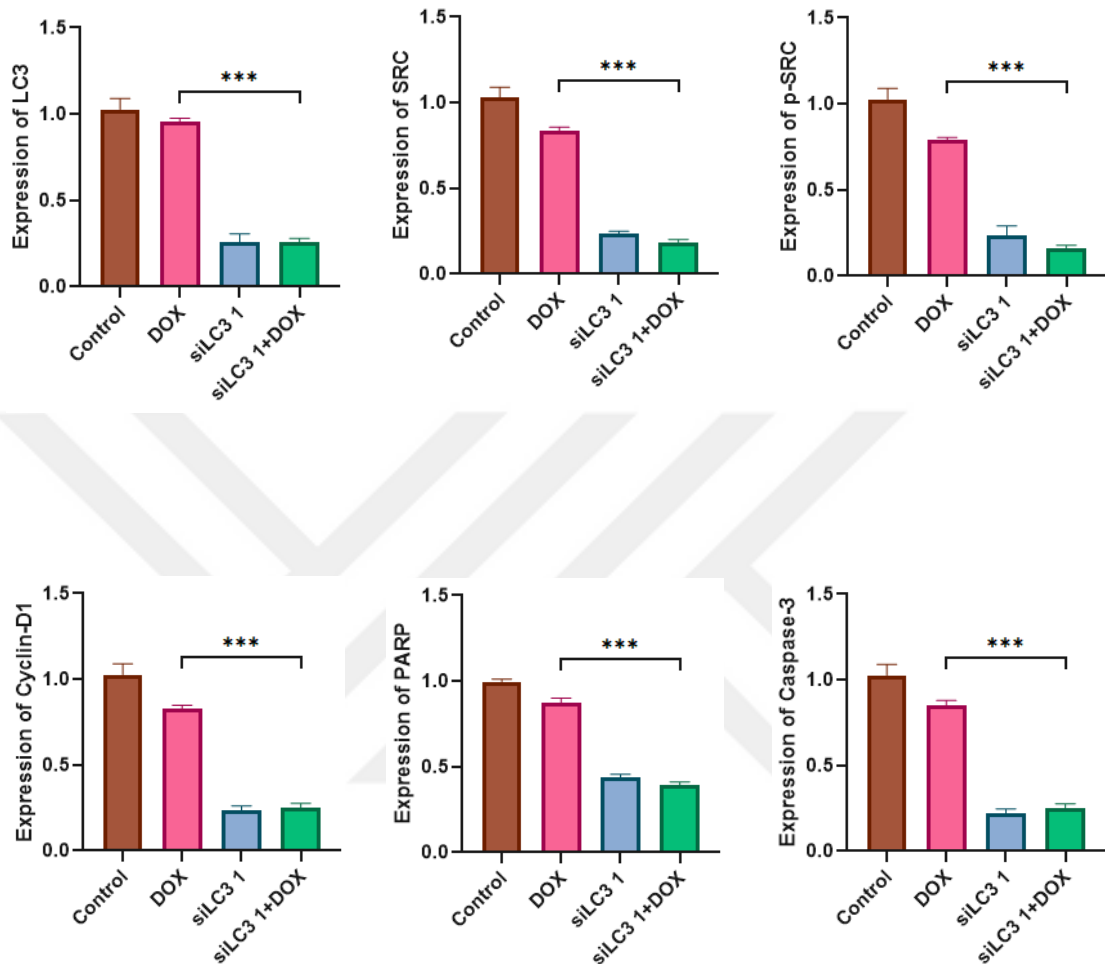
B

Figure 3.11. A: This figure shows the expression of LC3, p-src, src, cyclin D1, PARP, and caspase-3 after cells were treated with DOX alone, siLC3 1 as well as siLC3 1+DOX. As a loading control, β -actin was employed. **B:** LC3, src, p-src, PARP, Cyclin D, and Caspase-3 quantitative data analysis regarding the loading control β -actin (***) $p < 0.001$)

The link between Src activation and the progression of cancer appears to be considerable. Moreover, Src may influence the development of the metastatic phenotype [157]. The combined treatment (IC_{20} DOX+ 47.5 nM siLC3 1) reduced the expression levels of both Src proteins and the Src phosphorylation pathway, according to Western blot examination.

Furthermore, we assessed the expression of one of the most frequent pro-apoptotic proteins, caspase 3, by Western blot assay. Western blotting results showed that our combined treatment (DOX+ siLC3 1) dramatically decreased caspase 3 activations, suggesting the ability of our treatment in increasing cell apoptosis.

	Untreated	DOX	Scramble siRNA	Scramble siRNA+DOX	GAPDH	GAPDH+DOX	Free Polymer
LC3							
Cyclin-D1							
p-SRC							
SRC							
PARP							
Caspase-3							
β -actin							

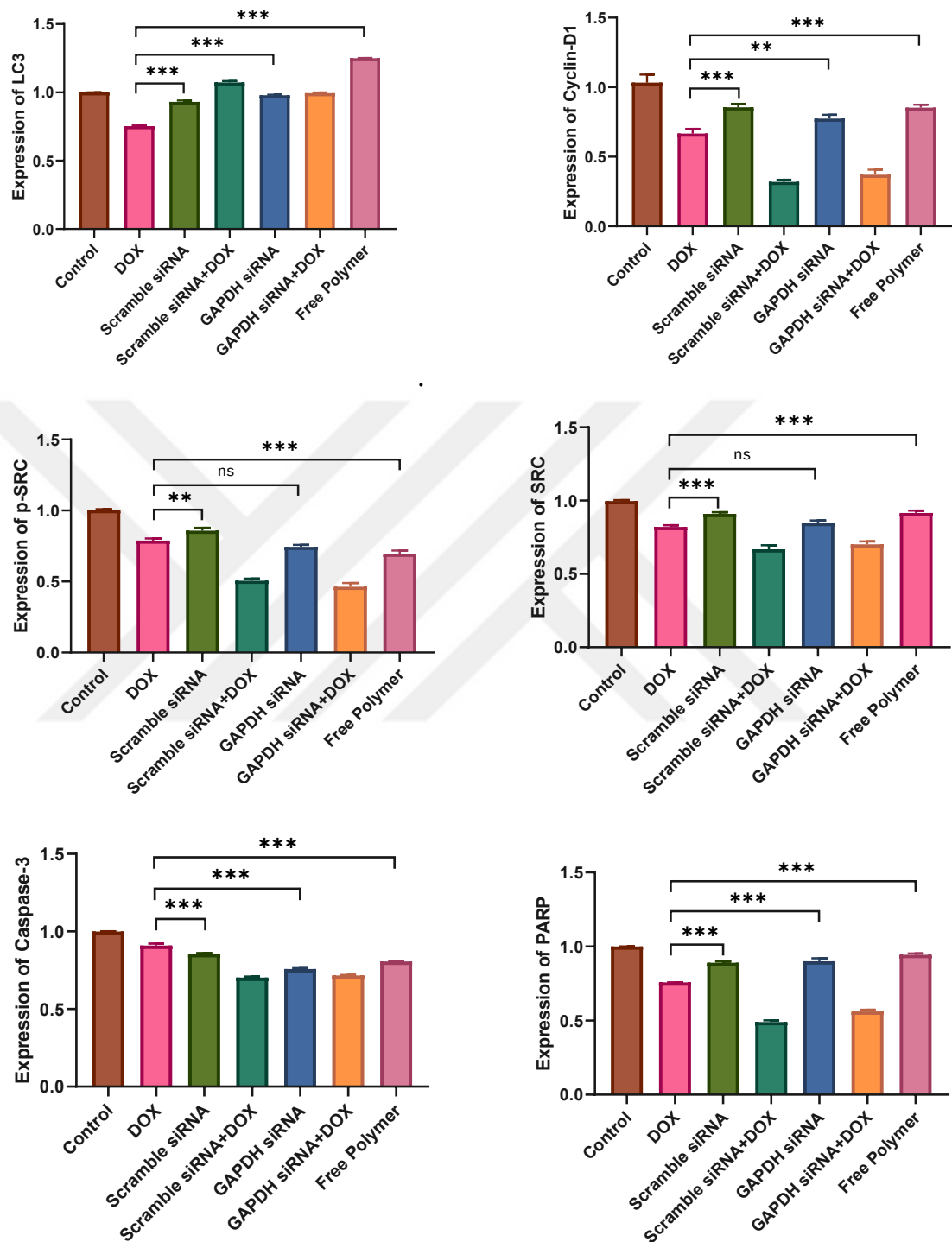
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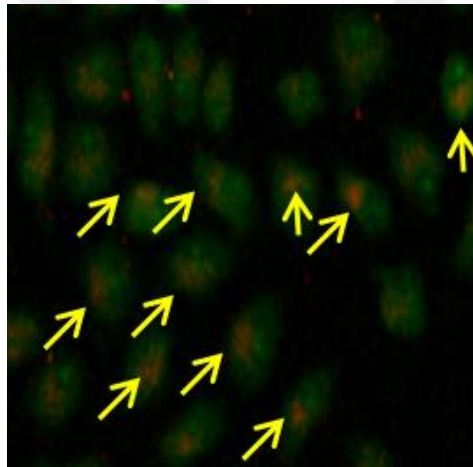
Figure 3.12. A: This figure shows the expression of LC3, p-src, src, cyclin D1, PARP, and caspase-3 after cells were treated with free DOX (IC_{20}), blanked polymer at 50 nM, Scramble siRNA (47.5 nM) and GAPDH siRNA(47.5 nM) as well as their combination with DOX. As a loading control, β -actin was employed. **B:** LC3, src, p-src, PARP, Cyclin D, and Caspase-3 quantitative data analysis regarding the loading control β -actin (***) $p < 0.001$)

We examined the expression of the same proteins (LC3, src, p-src, PARP, Cyclin D, and caspase-3) in a set of control groups involving blanked polymer at 50 nM, Scramble siRNA (47.5 nM), and GAPDH siRNA (47.5 nM) as well as their combination with DOX and compared them to untreated and DOX groups in order to confirm that the previous western blotting results came from our treatment. As shown in (Figure 3.12 B) minimal effect has been obtained in all proteins expressions relative to our treatment groups.

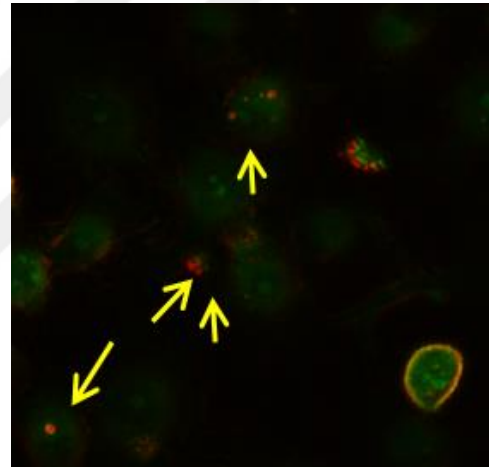
3.9 Acidic Vesicular Organelles Analysis

A

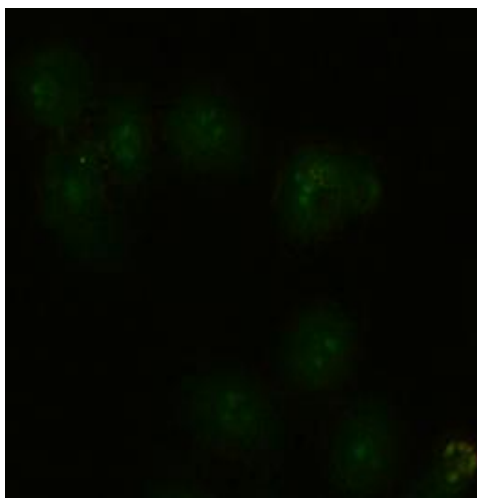
Untreated



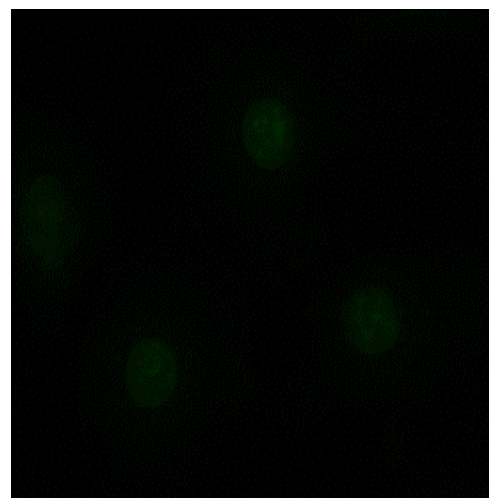
DOX



siLC3 1



siLC3 1 + DOX



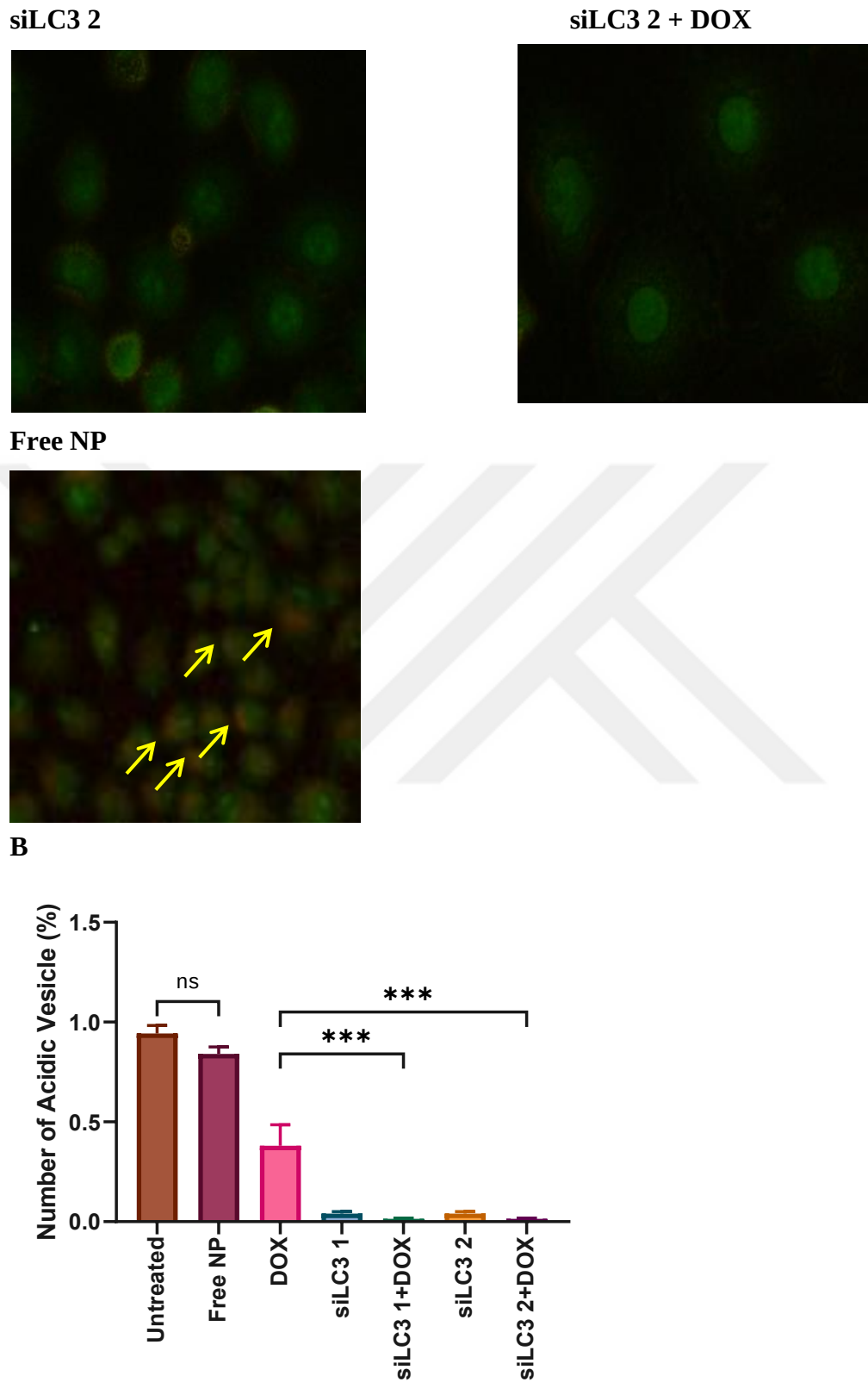


Figure 3.13. **A:** The different treatment groups' fluorescent microscopic photos of AVOs that exhibited red fluorescence. **B:** The percentage of AVOs is represented calorimetrically. To assess statistical differences, one-way ANOVA was used.

We measured the accumulation of AVOs to see how effective our treatment groups were at inhibiting autophagy. Figure 3.13 shows a clear green fluorescence in the control group with a maximum red fluorescence, indicating and confirming the link between autophagy activation and breast cancer development in MDA-MB-231 cells. In fact, AVOs were formed in every single cell in the control group. The DOX group has a noticeable difference in red fluorescence intensity compared to the control group (Figure 3.13). However, treatment of the cells with LC3 siRNAs caused a substantially greater decrease in red fluorescence intensity, indicating that they are capable of silencing LC3 and thereby suppressing autophagy, compared to DOX alone. Furthermore, there are almost no AVOs generated in cells co-treated with LC3 siRNAs and DOX. Nonetheless, compared to the other treatment groups, the number of cells treated with LC3 siRNAs and co-treated with LC3 siRNAs and DOX has drastically decreased, indicating that the aforesaid groups have a significant effect on cell proliferation.

4. CHAPTER

DISCUSSION-CONCLUSION AND RECOMMENDATIONS

In the field of gene delivery, a number of polymer nano-carriers have been created; however, they have primarily resulted in non-specific toxicity due to their non-degradable composition, which leads to long-term accumulation [159], low solubility and therapeutic utility due to the high content of hydrophobic motifs, as well as significant toxicity both in vitro and in vivo due to the use of excess polymer [160]. As a result, a "smart" NP system that has been studied and developed in other investigations [145,146] has been used in this study specifically as a nano-carrier system to carry siLC3s into TNBC cells, MDA-MB-231, as an advantageous system overcoming the other issues present in many other polymers. This system, which is based on the utilization of the FDA-approved β -CD, was able to detect changes in environmental pH levels, break the endosomal membrane in a dual effect, and interact electrostatically with the negatively charged LC3 siRNA.

The investigation of the zeta potentials and particle sizes of "smart" NP at various N/P ratios using DLS revealed that the particle size increased with increasing N/P ratio. This was consistent with previous research since a rise in the N/P ratio has increased the quantity of polymer that is accessible for particle formation [161,162]. Furthermore all "smart" NPs with varying N/P ratios had particle sizes of less than 270 nm and a net positive charge. The complexes' positive charge permits strong electrostatic interactions with cell membranes that boost absorption effectiveness and account for their uptake by the cellular process of adsorptive endocytosis [163]. While their sizes make them suitable candidates in delivering siRNA to solid tumors [164]. Nevertheless, considering the cationic content and the proportion of quaternized monomers in the polymeric matrix, the proportion of "smart" NP combined with siLC3 was estimated using gel retardation

assay [165]. Based on this, it has been demonstrated that siLC3 was completely condensed with the “smart” NP at a 4/1 ratio, which was evident in preventing free siRNA from moving across the gel.

The transport vehicle itself could cause cellular toxicity. Toxicity in polymers can be caused by a variety of ways, including immunostimulation, membrane instability, and changes in cellular signaling [166]. In this study, the therapeutic doses of LC3 siRNAs, siLC3 1 and siLC3 2, are accompanied by approximately four times their own weight of “smart” NPs, making vehicle-related toxicity an important consideration. Thus, the cellular vitality has been examined after the cells were incubated for 48 hours with blank “smart” NPs, which led to a negligible reduction in cell viability (approximately 15%). Before examining the therapeutic effect of siLC3 and DOX co-treatment on cellular viability, the inhibitory concentrations of DOX (IC_{20} and IC_{50}) that cause a 20% and 50% inhibition of cellular vitality, have been determined to be 58.3 and 110.4 nM, respectively.

According to the results of the resazurin-based cell viability test, it has been shown that the viability of the cells was not altered when treated with “smart” siLC3 1 and siLC3 2 NPs at siRNA dosage of 47.5 nM. Which agrees with earlier works that have been published [145,167,168]. Contrarily, when DOX (IC_{50}) and LC3 siRNAs (at both 47.5 nM and 95 nM) were administered together, the cytotoxicity of DOX was increased in the cells, compared it with DOX alone. This result suggests that LC3 siRNAs' inhibition of autophagy increases the chemo-sensitivity of the cells to DOX.

Autophagy has been correlated to tumor metastasis by promoting cancer's aggressiveness [154,169]. It has been identified and proven that autophagy plays a critical part in facilitating this type of adaptation because invasive cancer cells need to adjust to a different environment than the originating site [54] and overcome a number of challenges in order to develop colonies [170,171]. Many studies have been published showing a correlation between a group of ATGs and the development of cancer, implying the critical role autophagy plays in metastasis [14,16,72,73]. In one study, higher levels of LC3 expression in brain metastases was related to a considerably lower survival time in TNBC patients [172]. Accordingly in this study, we performed colony formation assay and wound healing assay to demonstrate the effectiveness of our combined treatment.

We examined the anti-proliferative effect of our treatment using a clonogenic assay, which assesses a single cell's capacity to overcome therapy and form colonies [173], reflecting the treatment's long-term impacts on the cells [174]. It has been demonstrated that when LC3 siRNAs and DOX are combined, colony formation was suppressed by over 85% under the conditions of low-dose DOX (IC_{20} value), whereas DOX alone only caused inhibition of around 50%, as compared to untreated cells. Additionally, using a wound healing assay, the impact of our combined treatments on inhibiting cell migration, which is inferred by "healing" of the wounds, was also investigated. According to the findings, there was only a small difference in migration between untreated cells and DOX-treated cells (IC_{20}), with wound occupation percentages of 85 and 78%, respectively. Cells treated with combined DOX and LC3 siRNAs, on the other hand, were unable to communicate with one another, which significantly inhibited cell migration by more than 90%. By overcoming the retention of cancer cells' proliferative capacity, these results highlight the innovation of our treatment in the field of cancer research. As the ability of cancer cells to retain this capacity typically results in medication failure as well as the growth and spread of cancer cells in patients.

Autophagy has the potential to slow apoptosis and is a crucial factor in cell death decisions. [175]. The most likely causes are autophagy's ability to activate mitophagy and diminish the amount of pro-apoptotic proteins in the cytosol [176]. For example, when autophagy is inhibited, the pro-apoptotic protein PUMA is elevated [177]. Anoikis, or the lack of extracellular matrix (ECM) attachment, causes cancer cells to die through apoptosis. Nevertheless, autophagy has been proven to help ECM-detached tumor cells in avoiding anoikis and surviving [178]. Here, we investigated whether inhibiting autophagy using LC3 siRNAs increased apoptosis and, in turn, raised the possibility of cancer cell death when combined with DOX. Results from flow cytometry after Annexin V-FITC labeling showed that cells exposed to only DOX (IC_{20}) did not exhibit apparent apoptosis in compared to untreated cells. However, treating the cells with both DOX and siLC3 1 resulted in a 48.10% increase in cell apoptosis, which was 3.58 times greater than the free DOX group. So same was observed while treating the cells with DOX and siLC3 2, in which an apoptosis percentage of 41.02 was obtained; which is 3 times greater than the free DOX group. These results emphasize the efficiency of combining LC3 siRNAs with DOX and affirm the high potential outcomes of the treatment (Figure 4).

Nevertheless, cell cycle assay has been conducted to confirm the results obtained. DOX suppresses cdc2/P34, causing G2/M arrest and death in cells [156]. Treatment of the cells using DOX alone and combined LC3 siRNAs and DOX led to accumulating the cells at G2/M phase, according to flow cytometry analysis results. However, our findings revealed that DOX and LC3 siRNAs co-treatment boosted the rate of apoptosis in the cells as compared to employing each agent alone. The fraction of cells in the sub-G1 phase demonstrated this (apoptotic cells). While treating the cells with DOX alone showed a percentage of sub G1 arrest of 6 ± 2.24 %, cells co-treated with DOX and LC3 siRNAs increased the proportion of cells in sub-G1 phase to 20.71 ± 2.78 and 28.94 ± 1.99 , respectively. All of which proving the ability of our combination in decreasing cell cycle progression and promoting cell death, hence suppressing cell growth.

Autophagy is characterized by the development of autophagosomes, which combine with lysosomes to generate acidic vesicular organelles (AVO) [151]. The buildup of AVOs was evaluated to assess the effectiveness of our therapy in suppressing autophagy. In the cytoplasm and nucleus, the AO stain emits green fluorescence, while acidic vesicles emit red fluorescence [179]. As a result, we could predict autophagy levels by measuring the red fluorescence intensity. The results demonstrated a clear green fluorescence in the control group (untreated) with a maximum red fluorescence, verifying and confirming the link between autophagy activation and breast cancer development in TNBC. Contrary to untreated and DOX groups, however, it was found that treatment with LC3 siRNAs resulted in a noticeably larger decline in red fluorescence intensity, demonstrating that they are capable of silencing LC3 and so reducing autophagy. Nevertheless, in cells treated simultaneously with LC3 siRNAs and DOX, little if any AVOs were produced.

We also used a Western blot analysis to demonstrate the decrease of LC3 and the inhibition of autophagy. As predicted, LC3 expression had significantly decreased in siLC3 1- and siLC3 1+DOX-treated cells compared to untreated and DOX-treated cells. In addition, the expression of several cancer-related proteins had been studied. In which our combined treatment showed an inhibition in PARP, Cyclin D1, Src, P-Src and caspase-3 signaling in tumor cells. These results confirmed the results obtained from the previous experiments. For instance, the link between Src activation and the progression of cancer is considerable, as Src influences the development of the metastatic phenotype [157]. The potential of our combination therapy to limit tumor metastasis, as shown in

both colony formation and wound healing experiments, is thus confirmed by the decrease in Src and Src phosphorylation pathway. Cyclin D1 is a crucial regulator of proliferation, serving as a bridge between the progression of the cell cycle and the extracellular signaling environment [158]. As a result, its suppression validated cell cycle findings. On the other side, the suppression of PARP and caspase-3 expression implied the induction of apoptosis. In the near future, In vivo tests will be conducted to assess the nanocarrier system and highlight the findings of the in vitro tests.

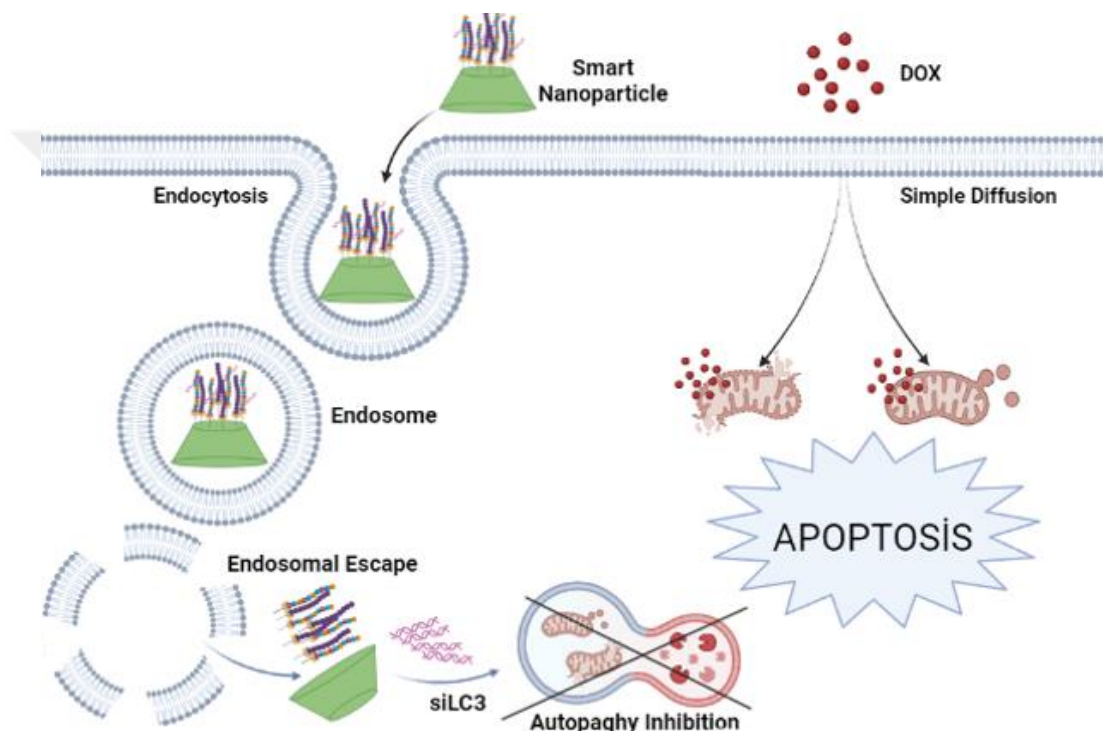


Figure 4. The dual effect of LC3 "smart" NP and DOX in inducing apoptosis in MDA-MB-231 breast cancer cells.

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RESUME

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WORK EXPERIENCES

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PUBLICATIONS

1. N.Walweel, C.U.Tunc, Z.Hamurcu, H.Ulutabanca, Y. Yüksel Durmaz, O.Aydin, "The Effect of LC3 siRNA and Doxorubicin Dual Treatment against Breast Cancer", 4th International Conference on Natural Products for Cancer Prevention and Therapy. Congress Abstract Book, page: 36.

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3. N.Walweel, A.U. Kutlu, U.Yilmaz, Z. Al-Shaebi, J.Charmi, O.Aydin "Review: mRNA delivery systems in vaccinations and COVID-19". Acceptance from: Journal of Research in Pharmacy.
4. N.Walweel, V.Cinar, D.Kanarya, U.Yilmaz, E.Demirel, E. Karaca,C.U.Tunc, Z.Hamurcu, H.Ulutabanca, Y. Yüksel Durmaz, O.Aydin, "The Effect of LC3 siRNA and Doxorubicin Dual Treatment against Breast Cancer", International Cell Death Society (ICDS)- Future of Cell Death in Basic Science and Translational Medicine meeting- Koç University School of Medicine, Istanbul, TURKEY .