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Development of a combinational drug delivery system for synergistic lung cancer treatment.

By

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Dedication

I dedicate this dissertation to my husband Tito, who always believed in me and prayed for me. To my son, who encouraged me just with a smile. To my parents for their love and support. And lastly, to my exceptional mentors, family, and friends for their hard work, words of advice, and encouragement.

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µg	microgram
µl	microliters
A549	human lung adenocarcinoma cells line
A549	Human lung cancer cell line
ADCC	antibody cellular cytotoxicity
Ag5	antigen 5
ALL	Acute Lymphocytic Leukemia
AML	Acute Myeloid Leukemia
ANOVA	analysis of variance
APL	acute promyelocytic leukemia
ASK1	apoptosis signal-regulated kinase 1
ATM	ataxia telangiectasia mutated
ATRA-ATO	All-trans retinoic acid-arsenic trioxide
BCL-2	B-cell lymphoma-2
BeA	Betulinic acid
BRCA	Breast cancer gene
BSA	Bovine serum albumin
CD	Circular dichroism
CD44	cluster of differentiation 44
CDK	Cycling dependent kinase
CDKN1A/p21	Cyclin-dependent kinase inhibitor 1
CHD4	Chromodomain-helicase-DNA-binding protein 4
CI	concentration index
CML	Chronic Myelogenous Leukemia
CRISPS	cysteine-rich secretory proteins
Cyt c	Cytochrome C
Da	Daltons
DAPI	4’,6-diamidino-2-phenylindole
DDS	drug delivery systems
DLS	Dynamic light scattering
DMEM	Dulbecco's Modified Eagle's Medium
DMF	N, N-Dimethylformamide
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNMTs	DNA methyltransferases
DOPE	di-oleoyl phosphatidyl ethanolamine
DOTAP	1,2-dioleoyl-3-trimethylammonium propane
Dox	Doxorubicin
e.g.	For example
EGFR	epidermal growth factor receptor
EMA	European Medicines Agency
EPR	enhanced permeability and retention

ER	endoplasmic reticulum
FAS	Fas cell surface death receptor
FBS	fetal bovine serum
FDA	United States Food and Drug Administration
FITC	fluorescein isothiocyanate
GBM	glioblastoma multiforme
GEM	gemcitabine
GIST	Gastrointestinal Stromal Tumors
GLIPR1	Glioma pathogenesis-related protein 1
GmbH	“Gesellschaft mit beschränkter Haftung”, company with limited liability
Gp60	albumin-binding glycoprotein
h	hours
H2A.X	histone variant
HDAC	histone deacetylases
HeLa	ovarian cancer cells
HER2	Human epidermal growth factor receptor-2
HIFs	Hypoxia-inducible factors
HIV	human immunodeficiency virus
Hsc70	heat shock cognate protein 70
i.e.	in other words
IC50	half maximal inhibitory concentration
IL-17	interleukin 17
JNK	Jun N-terminal kinase
kD	kilodalton
LPPC	liposome-polyethylene-glycol–polyethyleneimine complex
MCF7	breast cancer cells
MDR	multidrug resistance
MEK	mitogen-activated protein-extracellular signal-regulated kinase
ml	milliliter
mM	Millimolar
MPM	malignant pleural mesothelioma
MtCK	mitochondria creatine kinase
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
NDRG1	N-myc downstream-regulated gene
nm	nanometers
NP	nanoparticles
NSCLC	non-small cell lung cancer
NuRD	nucleosome-remodeling and histone-deacetylation
ºC	Celsius degree
OCLN	Occludin
p53	TP53, tumor suppressor gene
PARP	Poly (ADP-ribose) polymerase
PARP	poly ADP ribose polymerase

PARP	poly ADP ribose polymerase
PBS	Phosphate buffer solution
PD-1	programmed death receptor-1
PDI	Polydispersity index
PE	phosphatidylethanolamine
PEG:	Poly(ethylene glycol)
PEI:	Polyetherimide
P-gp	P-glycoprotein
PLGA	polylactic-co-glycolic acid
Pr-1	pathogenesis-related 1 protein
RNA	Ribonucleic acid
RNAi	RNA interference
ROS	reactive oxygen species
SD	Standard deviation
siRNA	small interfering RNA
siRNA	Small interfering RNA
SP factor	Special transcription factors
Tf	Transferrin
TKI	tyrosine kinase inhibitor
TPGS	d-α-tocopherol polyethylene glycol succinate
TPGS	d-α-tocopherol polyethylene glycol succinate,
UK	United Kingdom
UV-VIS	Ultraviolet-Visible
VEGF	vascular endothelial growth factor

Acknowledgements

This doctoral dissertation has been a long journey for me. Hence, I feel thankful to many people, and I would like to express my gratitude to all professors, colleagues, and co-authors for their contribution to the process.

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Abstract

Proteins are a key component in cell regulation while playing various cell roles. Among their many roles, transporter functions are considered a vital part of cell functionality. Thus, the incorporation of proteins in pharmaceutical drug design has increased over the years. The principal benefits of protein therapeutics are the specificity to complex functions, low tendency to side effects, and high tolerance in the body. These benefits are mainly due to the body's wide variety of protein production, so it is not detected as a foreign agent. Some therapeutic formulations are composed of protein-drug conjugates and protein-based drug delivery systems (including drug encapsulation) to improve treatment for targeted tissues. In the case of cancer therapy, several protein-based nanoparticles are FDA-approved and show excellent pharmacological results in delivering their drug component. Unfortunately, cancer tissue can develop resistance to anticancer drugs delivered. Thereby, that tissue becomes tolerant to cancer treatment.

Doxorubicin is one of the most common anticancer drugs reported to induce chemoresistance in cancer cells. The development of drug resistance is a cellular response which uses differential gene expression to enable adaptation and survival of the cell to diverse threatening environmental agents. Otherwise, betulinic acid, a pentacyclic triterpene (plant-derived compound), has shown great cytotoxic activity against different cancer types. In chapter 3, the term Chemoresistance will be introduced, and some key genes involved in cancer therapy resistance meanwhile mentioning some drug delivery systems that may improve the efficiency of chemotherapy. Chapter 4 investigates a protein-based drug delivery system (DDS) nanoparticles from serum albumin (BSA) as the drug's carrier combining two compounds. Due to

their synergistic cytotoxicity demonstrated against non-small cell lung carcinoma A549 cells. The chemotherapy drug doxorubicin (Dox) and the triterpene betulinic acid (BeA) were encapsulated using an oil-water-like micro-emulsion method. In addition, the BSA (Dox+BeA) DDS demonstrated cytotoxic activity after 24h incubation. The mechanism of action studies confirmed S-phase cell cycle arrest, ATM-dependent DNA damage, multi-caspase pathways activation, and a reduction in the epidermal growth factor receptor (EGFR) expression compared to the drugs alone.

Chapter 1

Introduction

1.1 Protein-based therapeutics

Proteins are macromolecules with various roles in the body. Among their many roles are the catalytic, regulator, and transporters functions. As a result of all these functions, proteins have become an important research focus for investigating a wide range of diseases. The United States Food and Drug Administration have approved more than 170 different proteins and peptides for treatment, while many other are in development.[1] The principal benefits of protein therapeutics are the specificity to complex functions, low tendency to side effects, and high tolerance in the body. These benefits are mainly due to the body's wide variety of protein production, so it is not detected as a foreign agent to combat.[1]

In 1982, the FDA approved a genetically engineered insulin, a recombinant therapeutic protein, by Eli Lilly & Company for diabetes disease treatment. An essential part of the recombinant technology is strategically producing a large quantity of protein throughout an expression system, such as *Escherichia Coli*.[2] The recombinant protein market for biomedical purposes has been tremendously developing. Accordingly, *Markets and Markets Research*, the recombinant protein market will reach $1.7 billion by 2026. This market is predicted to be generally focused on treating inflammatory diseases such as cancer.[3]

Protein-based therapeutics have been evolving throughout the last 40 years. First, more naturally occurring proteins were dominant in the field, but later on, modifications were implemented to enhance the protein benefits such as specificity, biodistribution, and efficiency.

An example of this evolving technology is another product from Eli Lilly, Humalog (modified insulin), where the protein was modified to enhance fast-acting features.[4]

Since the long-term goal of Medicine is to develop individualized treatments, protein therapeutic development plays an important role. This can be confirmed as more treatments directly applying protein therapeutics are developed, such as monoclonal antibodies, vaccines, and various cell therapies. Another protein-based therapeutic benefit is that it can be used in combination with molecule drugs to achieve an additive or synergistic effect on patients.[1]

1.1.1 Proteins in cancer therapy

Cancer is a set of diseases characterized by invasiveness and uncontrolled cell division of abnormal cells that permeate and damage normal body tissues. Considering that cancer is the second leading cause of death globally, researchers have increased efforts to investigate and detect cancer risk factors and treatment options.[5] Although cancer diseases have been studied for many years, they still are challenging to eradicate due to their different behavior and origin from patient to patient. Currently, cancer treatment includes, the most common: surgery, radiation, and chemotherapy, and the recent novel strategies from targeted drugs to a more personalized therapy.[6] Chemotherapeutic agents can cause toxicity to cancerous cells, but also to normal cells, triggering many side effects due to their lack of specificity. To this extent, proteins have been incorporated into the development of cancer therapies.

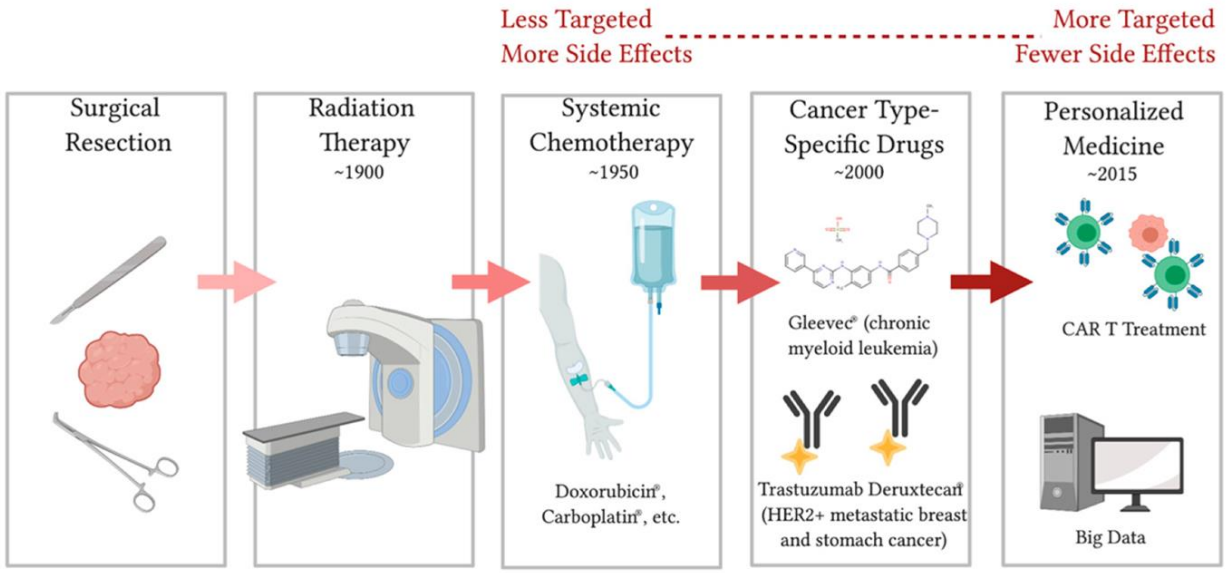


Figure 1.1: Illustrated diagram of the clinical management progress of cancer. Initially, surgical resection of the solid tumor was the treatment available. Then, radiotherapy became effective for localized tumors. Afterwards, chemotherapies, targeted therapies and personalized medicine emerged. The last three options' primary focus is to improve the efficacy of the treatment to reduce patients' side effects.[6]

Several proteins have been used in Medicine to fight against cancer due to their capacity to induce cytotoxic effects. Some of the many ways that proteins are used to engineer therapeutics are: as treatment, carrier, and enzyme, among others.[7] The most prominent protein characteristics justifying their use in cancer therapy are: (1) natural in biological origin and high availability, (2) excellent biocompatibility and biodegradability, (3) abundance, (4) three-dimensional conformation (5) biological function ability, (6) versatility to interact with molecules and solvents, (7) and availability for chemical conjugation.[8] Therefore, most proteins show high solubility and an amphiphilic property allowing interactions with hydrophobic and hydrophilic moieties. This amphiphilic property makes them an excellent material for nanomedicine drug formulation.[9] Thus, incorporating proteins into drug delivery systems (DDS), such as nanoparticles, has been a natural, feasible and versatile alternative for a more specific and efficient treatment for cancer.

1.1.2 Protein nanoparticles

Many attempts have been made to comprehend the scenario and precisely design efficient therapies to focus on improving cancer treatment.[10] Nanocarriers of drug molecules, and nanoscale-based delivery systems, emerged to innovate therapeutics while overcoming pharmaceutical limitations.

A nanoparticle (NP) is a unit with dimensions within de nanometer range (1-1000 nm). The composition of an NP can vary depending on its elements but these are generally composed of a surface layer, a shell layer, and a core (central portion of the NP).[5] NPs have gained interest as a therapeutic strategy due to their remarkable features, including high surface area and manipulative characteristics. As a result, NPs have been implemented as drug delivery systems alternative methods to some drug molecules as an option for modification and improvement to drug stability and solubility. [11] [12]

The FDA-approved drug Doxil was the first nanoparticle-based chemotherapeutic agent in 1995 as a treatment for various types of cancer. Since then, the FDA and the European Medicines Agency (EMA) have approved many other drug nanocarriers for cancer treatment. These nanoparticle-based therapeutics include lipid-based nanoparticles, protein-drug conjugates, and metallic nanoparticles. Some of these approved by the FDA and EMA are listed in table 1.1. [13] [14] Hence, nanomedicine has constantly evolved to improve chemotherapeutic agents delivery using nanoparticles as carriers.

Table 1.1 Some FDA and EMA-approved nanoparticle-based therapeutic agents for cancer treatment. [13] [14]

Brand name (Company)	Nano-platform (Nanostructure)	Approved for the treatment of:
Doxil (Ortho Biotech)	Liposomal-polyethylene glycol doxorubicin (Lipid-based nanoparticle)	HIV-related Kaposi's sarcoma, metastatic breast cancer, and metastatic ovarian cancer
DaunoXome (Galen)	Liposomal daunorubicin (Lipid-based nanoparticle)	HIV-related Kaposi's sarcoma
Myocet (Teva UK)	Liposomal doxorubicin (Lipid-based nanoparticle)	Metastatic breast cancer (In combination with cyclophosphamide)
Margibo (Spectrum)	Liposomal vincristine non-pegylated (Lipid-based nanoparticle)	Philadelphia chromosome-negative acute lymphoblastic leukemia
Onivyde (Merrimack)	Liposomal irinotecan pegylated (Lipid-based nanoparticle)	Metastatic pancreatic cancer
Oncaspar (Enzon)	Polyethylene glycol-L-Asparaginase (Protein-based nanoparticle)	Acute lymphoblastic leukemia
Abraxane (Antra-Zeneca)	Albumin-bound paclitaxel (Protein-based nanoparticle)	Metastatic breast cancer
Pazenir (Ratiopharma GmbH)	Albumin-paclitaxel conjugate nanoparticle (Protein-based nanoparticle)	Metastatic breast cancer, metastatic adenocarcinoma of the pancreas and non-small cell lung cancer
Nanoterm (Magforce)	iron oxide nanoparticle coated with amino silane (Metallic nanoparticle)	Glioblastoma, prostate, and pancreatic cancer

The primary effect of anti-cancer drugs is to inhibit the activities of target molecules, thereby triggering various cellular signal transduction pathways, leading to cell death or cell cycle arrest. The secondary effects result in apoptosis or other types of cell death, including autophagy, mitotic catastrophe, necrosis, and senescence.[15] Over the years, proteins have shown the ability to be drug carriers for a better-targeted delivery, while others have demonstrated anti-cancer

properties.[16] Considering protein abundance and diversity, these molecules can function as anticancer drugs or as drug carriers for the development of novel treatments.

1.1.2.1 Proteins as nanoparticle-sized drugs

Proteins can be a therapeutic agent in the formulation of anti-cancer drugs. Considering the biocompatibility and biodegradability of proteins, they are an attractive substitute for cytotoxic medicines. In cancer, therapeutic proteins are superior to chemotherapy drugs, which may cause additional mutations and lead to multidrug resistance.[17] For instance, L-Asparaginase, an FDA-approved enzyme protein drug, is used for leukemia treatment. It can selectively hydrolyze the extracellular L-asparagine into L-aspartate and ammonia to promote apoptosis in lymphoblastic cells.[18] Cytochrome c is an example of one of the proteins under study against cancer due to its innate pro-apoptotic function. Normally, cytochrome cis in the inner membrane of the mitochondria. One of the cytochrome c roles is to participate in cellular respiration, supporting ATP synthesis. While the other is to promote apoptosis. This occurs when the cell receives an apoptotic stimulus, cytochrome c is released into the cytosol and promotes apoptosis.[19] However, studies in cancer cells have shown that cytochrome c can be delivered directly into the cytoplasm to induce apoptosis as a potential treatment. [20] [21] [22] [23]

1.1.2.2 Proteins as carriers

Protein-based nanoparticles have been used as carriers to deliver chemicals, biomolecules, and drugs to the cell's interior as drug delivery systems. There are some technical advantages for protein-based nanoparticles as the available methods to synthesize them and the feasibility of characterizing the proteins and other components in the system, e.g., concentration. The main advantages of using proteins as carriers include their three-dimensional structure and

the hydrophobic pockets where non-covalent interactions can load drug moieties. Besides drug stability and drug protection from enzymatic degradation and renal clearance, a drug's half-life can be extended based on it's carrier's protection.[24] A valuable property of proteins as transporters is their unique features, such as their amphiphilicity. This property allows them to hold hydrophilic and lipophilic interactions, and expands the possibility of being used with many types of drugs.[25] Amphiphilicity is the capacity of a molecule to have both hydrophobic (nonpolar) and hydrophilic (polar) regions.[26] Some proteins used for drug delivery systems include gelatin, transferrin, lipoproteins, and serum albumin.[24] An important transport protein in the blood is albumin, which possesses specific sites for acidic and basic drug-binding while interacting in plasma.[27]

1.1.2.2.1 Albumin drug delivery systems

Albumin is the most abundant protein in blood plasma. Its molecular weight is about 67 kDa, highly water-soluble and has outstanding stability in the pH range of 4-9, and can be heated for up to 10 h at 60 °C. Albumin plays a determinant role in the osmotic balance and acts as a carrier for hydrophobic molecules, metabolic compounds, and drugs. These characteristics make Albumin an ideal candidate for drug delivery in addition to its abundance, non-toxicity, and ability to improve solubilization to loaded components. Additionally, albumin can interact with overexpressed receptors in cells and tissues, equipping this protein with valuable features and moving it toward an active targeting component without modification.[28] These exceptional characteristics have made researchers incorporate albumin as an ideal component for nanocarriers.

Several innovative techniques can be applied to prepare albumin nanoparticles. One must consider the component that needs to be loaded, adding versatility to the albumin nanoparticles. The common nano preparation techniques include desolvation, emulsification, thermal gelation, nano-spray drying, and self-assembly techniques. The first two techniques mentioned are the most widely used to prepare albumin nanoparticles. The desolvation technique is possible through the dropwise addition of solvent to albumin in an aqueous solution. This technique is mainly used for nanoparticles that need further modification to enhance drug stability or targetability. Meanwhile, the emulsion technique provides the flexibility to add oil-like combinations, increasing thermal stabilization of the system.[29]

Abraxane® is an FDA-approved an anti-cancer drug delivery system where the moieties of the chemotherapy paclitaxel is loaded by albumin as part of the nanoparticle formulation. This DDS take advantage of both passive and active targeting due to the accumulation through the irregular vasculature and the receptor-mediated cellular uptake, respectively. Once the tumor overexpresses the Gp60 receptors, Abraxane targets these receptors to increase and enhance intra-tumoral concentrations of paclitaxel drug concentration through transcytosis in endothelial cells. [30]

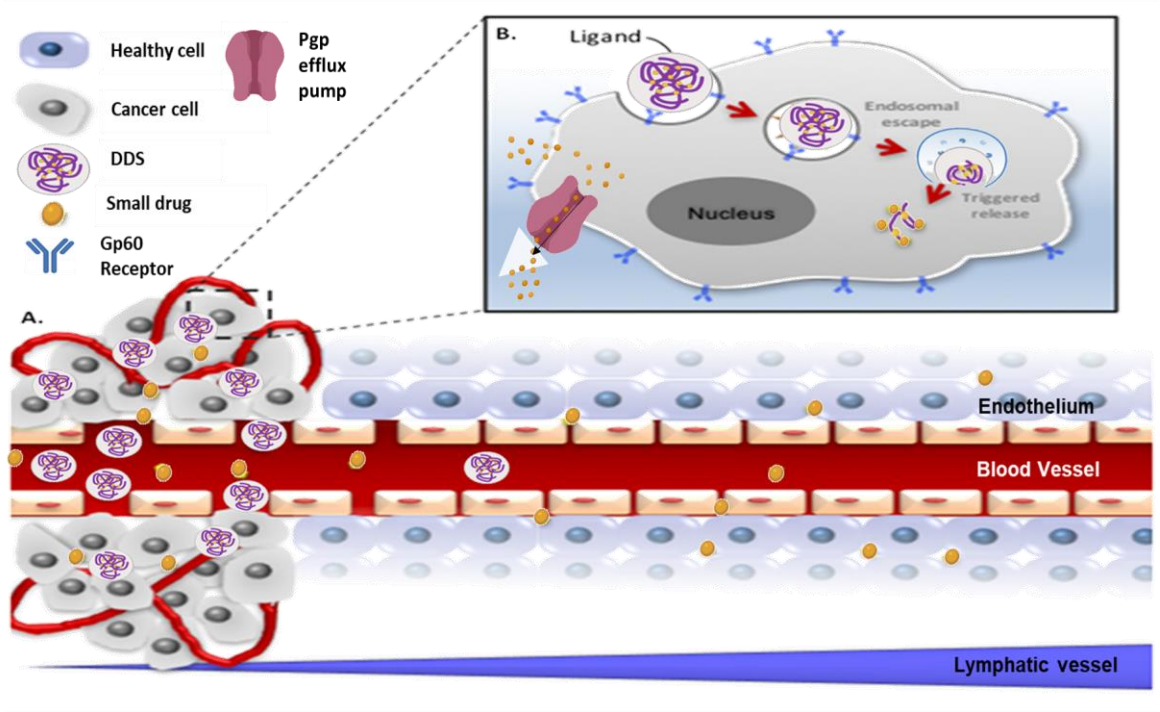


Figure 1.2 Scheme of A. *passive and B. active targeting* via enhanced permeability and retention (EPR) effect and receptor recognition, respectively. While small drugs can efficiently penetrate both healthy and tumor tissue, nanoparticle-based DDS permeate preferentially into tumor tissues through its leaky tumor vasculature. Additionally, they are retained due to the tumor's lack of efficient lymphatic drainage. Active targeting occurs when nanoparticles (or DDS) contain a component recognized by a particular receptor, allowing specific binding to the tumor cell's membrane (receptor-mediated endocytosis). Once the DDS is inside the cell, the therapeutic compound and /or drug can be released in the intracellular environment. This diagram was modified from Morales-Cruz et al.[31]

1.2 Naturally derived products against cancer

For centuries, humankind has turned to nature to heal and treat their health situations. Fortunately, nature has plenty of chemical diversity. Natural products are rich in bioactive compounds with therapeutic potential. For over 50 years, natural products have been incorporated into chemotherapeutics due to their biological activity. Several of them are established as anti-cancer agents. Such compounds can be unmodified (naturally occurring) or synthetically modified.[32] Those therapeutic agents can originate from plants (such as taxanes (e.g., taxol), vincristine, vinblastine, and the podophyllotoxin analogs),[33] bacteria (antibiotics, bacteriocins, non-ribosomal peptides, polyketides, and toxins),[34] and marine sources

(chondroitin, heparin, and fucoidan).[35] Some of the anti-cancer properties and mechanisms of action that natural-derived products exhibit are pro-apoptosis, pro-necrosis, reduce angiogenesis, inhibit translation and splicing, and obstruct essential signaling pathways to promote cancer cell death.[36]

1.2.1 Current chemotherapeutics from natural sources

Over 60% of the current anti-cancer drugs were derived or from a natural source.[37] Natural products and their derivates have contributed to developing pharmaceutical formulations used as chemotherapeutic agents. Anti-cancer therapies that originate from plants include irinotecan, vincristine, etoposide, and placlitaxel.[33] Meanwhile, some successful alternative from marine sources includes ribulin, trabectedin, cytarabine, and brentuximab vedotin. Several well-known antibiotics, from microbes, that exhibit potent anti-cancer properties are anthracycline (such as Doxorubicin), bleomycin, antimycin, and mitomycin C. [32] In the meantime, anti-cancer agents from natural sources and their derivates play a valuable role cancer therapeutics.[34]

1.2.1.1 Doxorubicin

Doxorubicin is an anthracycline anti-cancer drug isolated from *Streptomyces peucetius* bacteria pigments. Doxorubicin is mainly used to treat many cancer types, including soft tissue, bone sarcomas, breast, ovary, bladder, thyroids, leukemia, Hodgkin lymphoma, and small cell lung cancer. Doxorubicin can intercalate into a DNA helix and/or covalently bind the protein involved in DNA replication.[38] One of the mechanisms of action for doxorubicin is permeating the cancer cell membrane through diffusion and binding the proteasome in the cytoplasm, creating a Doxorubicin-proteasome complex that enters the nucleus via nuclear pores. Then, doxorubicin dissociates from the proteasome and binds DNA intercalating the double-strand helix due to

higher affinity. These interactions inhibit DNA, RNA, and protein synthesis, ultimately causing cell death. Other mechanisms of action for doxorubicin include interaction with mitochondria, blocking mitochondria creatine kinase, and increasing reactive oxide species (ROS) production in cancerous cells by increasing doxorubicin redox cycling (see figure 1.3).[39]

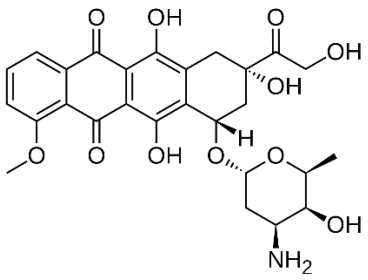


Figure 1.3 Doxorubicin structure

Regardless of Doxorubicin's wide range of actions, careful considerations in dosage are needed from case to case because of its many adverse effects. As an anthracycline compound, Doxorubicin exhibits high cellular uptake. This potent chemotherapy agent can produce dangerous side effects on vital organs such heart, brain, liver and kidney.[40] [39] However, despite their dangerous side effects, researchers have increased their efforts to develop new strategies for safer therapeutic alternatives incorporating doxorubicin and other anthracycline drugs against cancer cells.

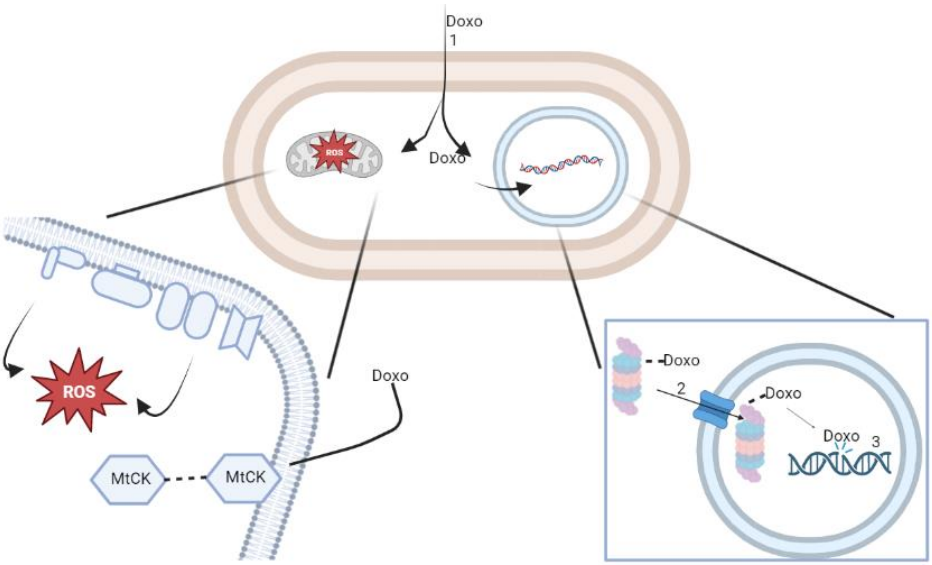


Figure 1.4 Doxorubicin (Dox) mechanism of actions. Doxo principal role is DNA intercalator. Dox enters cancer cells by diffusion and binds to the proteasomes in the cytoplasm, forming a complex (step 1). The complex translocates via nuclear pores to the cell nucleus (step 2). Once inside the nucleus, Dox dissociates from the proteasome and binds DNA with higher affinity (step 3). Additionally, Doxo can interact with mitochondria and blocks mitochondria creatine kinase (MtCK) from binding to the mitochondrial membrane. Furthermore, ROS production increases due to the redox cycling of Dox by complex I of the mitochondrial respiratory chain. This diagram was modified from Carvalho et al.[39]

The FDA and EMA have approved several chemotherapeutic drugs made of nanoparticles for cancer chemotherapy, including Doxil and Myocet (table 1.1). Both pharmaceuticals stably retain doxorubicin encapsulated. A safety improvement reduced the side effects of this drug compared to free doxorubicin, encouraging larger acceptance of the drug dose and increasing tumor uptake, becoming a more effective treatment.[13 14] Efforts have been implemented to continue developing better nanoparticles with the idea of increasing targetability, efficiency, and specificity in cancer treatment.

1.2.1.2 Potential cancer therapeutics from natural sources

Although cancer is still a leading cause of mortality worldwide for many years, a specific and efficient treatment is not available. Therefore, the interest in finding novel potential therapies has increased, specifically from natural sources, so treatment becomes more efficient.

Special attention has been focused towards natural products, including polyphenols (e.g., curcumin, resveratrol), cardiotonic steroids (e.g., bufalin and digoxin), terpenoids (e.g., paclitaxel and artemisinin), and polysaccharide extracts (e.g., lentinan).[41] Many cancer treatment advances have been directly correlated with natural product drugs. Nevertheless, a critical need for more effective anti-cancer strategies is still present. Another contemplated strategy is related to chemoprevention agents in combination with chemotherapeutic agent.[37]

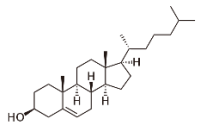
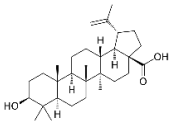
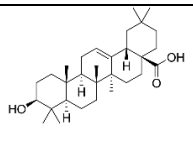
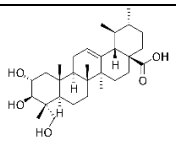
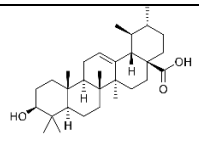
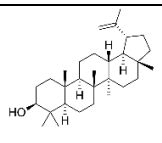
Phytochemicals (compounds found in plants) have developed special interest due to their anti-cancer activity shown. Mainly, the phytochemical mechanism of action includes anti-inflammatory activity and growth modulation through cellular signaling. [42] The largest subgroup of phytochemicals that have shown pharmacological activities, low toxicity, and have sparked interest towards human health and disease are triterpenoids.[43]

1.2.1.2.1 Triterpenoids

Triterpenoids are natural organic compounds with a common basic backbone present in more than 20,000 natural varieties of plants with a wide range of biological effects. Several pentacyclic triterpenoids exhibit antitumor and anti-inflammatory properties.[44] Besides the triterpenoids' capacity to inhibit cancerous cells' viability, originating apoptosis and other cell death mechanisms, they exhibit selectivity towards cancer cells instead of normal cells. Such particularity boosts research interest in developing alternatives to cancer treatment and prevention.[45] One of the simplest and most important triterpenoids is cholesterol. Studies with natural cholesterol analogs (i.e., betulinic acid, oleanolic acid, asiatic acid, ursolic acid, and lupeol) have shown cytotoxicity at micromolar range against the human non-small cell lung adenocarcinoma A549 cell line.[46] Modifications to triterpenoids through acetylation and

conversion into several amides has been reported to improve their cytotoxicity.[47] Studies of triterpenoids being incorporated in conjugates also report improvements in cytotoxicity because they can be dual-action agents, both therapeutic and preventive.[48] Therefore, many studies using triterpenoids have been conducted due to their promising anti-cancer properties.

Table 1.2 Cholesterol and the structure of triterpenoids

					
Cholesterol	Betulinic Acid	Oleanolic Acid	Asiatic Acid	Ursolic Acid	Lupeol

1.2.1.2.1.1 Betulinic acid

Betulinic acid (BeA), a lupine-type pentacyclic triterpene mainly from Betula species plants, retains various biomedical applications, as part of the triterpenoid family. Some multifunctional aspects include antimicrobial, antiviral, anti-inflammatory, and anti-cancer activities. [49,50] The anti-cancer properties of BeA have shown cytotoxic activity against various types of cancer while causing tumor growth inhibition in xenograft mouse models. [51] An important disadvantage of BeA, which limits its biomedical application to a greater extent, is its poor water solubility (0.02 µg/ml at room temperature).[50] Therefore, researchers have reported encapsulation methods, including self-assembling properties, to enhance water solubility and improve its biological activity for treatment. Researchers have reported BeA antitumor mechanism through mitochondrial oxidative stress induction, regulation of SP transcription factors, and mediates

tumor cells due to proliferation inhibitor effects.[51] Hence, combining BeA with other drugs is a great alternative for treatment.

1.3 Lung cancer

Lungs are complex but fragile organs composed of several cell types that support gas exchange in the body.[52] The National Cancer Institute defines lung cancer as a disease of uncontrollable cell growth formed in lung tissues and the cells that line the air passages. The American Cancer Society stipulates that there are two types of lung cancer: small-cell lung cancer (a more aggressive type of disease) and non-small-cell lung cancer (the most common type of disease) (Figure) .

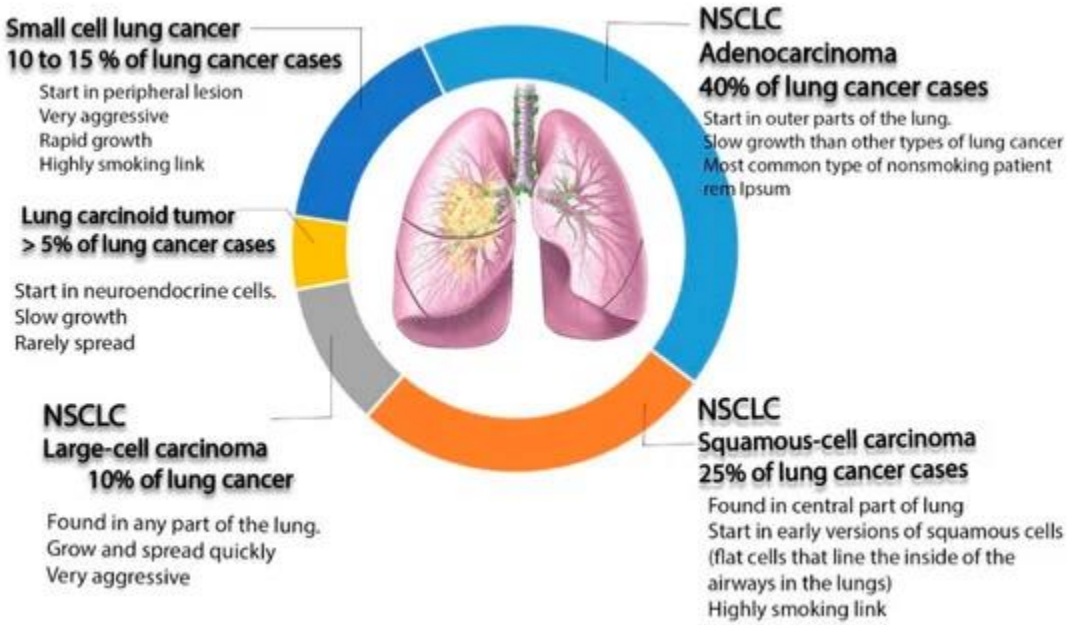


Figure 1.5: Schematic diagram illustrating different lung cancer types; non-small cell lung cancer (NSCLC), small cell lung cancer, and lung carcinoid tumors.[53]

1.3.1 Non-small cell lung cancer (NSCLC)

The American cancer society informs that 80-85% of all diagnosed lung cancer cases are NSCLC. NSCLC is a heterogeneous class of tumor, where the most common subtypes are adenocarcinoma, squamous cell carcinoma, and large cell carcinoma, among other less frequent subtypes. NSCLC class usually is less sensitive to chemo- and radiotherapy. Thus, the curative tendencies are low due to its tendency to develop drug resistance. [52] Nevertheless, patients diagnosed with NSCLC can survive and improve survival with chemotherapy, targeted agents, and other supporting measures.

1.4 Cancer resistance

Drug resistance and inefficient cancer therapy account for up to 90% of cancer-related deaths.[17] Resistance occurs when a cancer cell develops the ability to keep the chemotherapy drug out of it or reduces the amount that can enter a non-damage level. Cancer drug resistance is mainly led by some key genes involved in DNA repair and apoptosis pathways. Also, drug resistance is generated as a cellular response and adaptation to diverse environmental agents.

Drug resistance can be developed due to intrinsic genetic causes or acquired upon exposition to chemo drugs.[54] Tumors with intrinsic resistance exhibit cell heterogeneity and inherent decreased responsiveness to chemotherapy.[17] On the other hand, acquired resistance is generated by most cancer patients under chemotherapy as a gradual decrease in drug efficiency.[54] Furthermore, in this type of resistance, mutations can affect the expression level of the drug target, affecting the structure of the protein (mostly receptors) and the target of the therapy.

In chemotherapy, cytotoxic agents target metabolic pathways, mainly the apoptotic machinery. Cancer cells can become resistant to these cytotoxic agents and several cancer treatments due to the dysregulation of cell signaling pathways. One of the principal causes leading to drug resistance is the genetic predisposition of the patient, where the individual inherits genetic characteristics leading to ineffective drug response. Another cause leading to the development of tumor drug resistance can arise after cancer cells grow exposed to the drug. In this last scenario, exposure of tumor cells to the drug leads to resistance as a genetic adaptation to the tumor microenvironment.[55]

Although many cancers initially respond successfully to chemotherapy, the development of drug resistance occurs in most patients (Figure 1.6).[56] The initial solution to the resistance problem to single-agent chemotherapy is the combined administration of agents with non-overlapping mechanisms of action. Another strategy adopted by researchers to improve the effectiveness of the chemotherapeutic drug against tumors is the design of nanocarriers. Those nanocarriers (improving targetability to the tumor and irregular vasculature) must include characteristics that allow the drug delivery system to be absorbed across the cell membrane while carrying the anti-cancer agent. The two major categories of chemotherapeutic nanocarriers are inorganic (metallic core) and organic carriers (polymer- and lipids-based nanoparticles).[57]

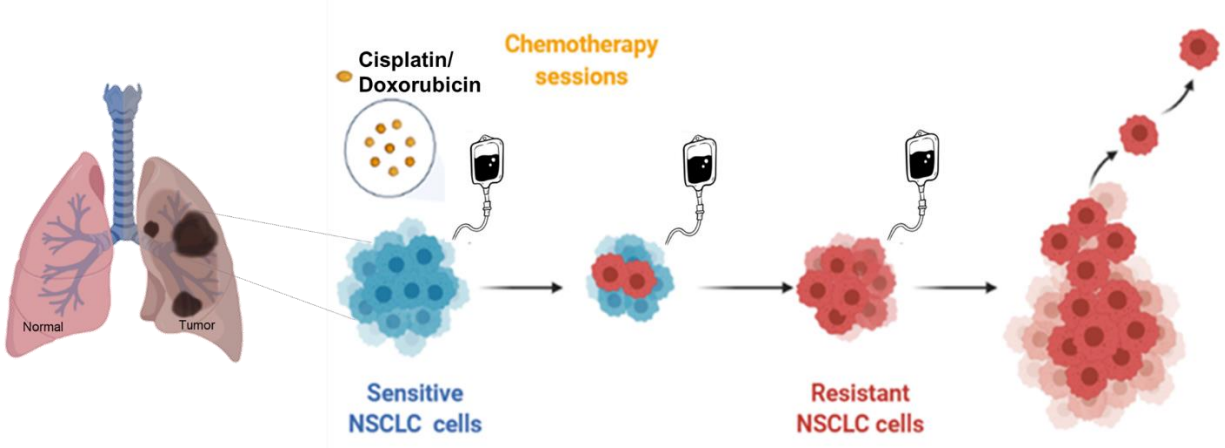


Figure 1.6: Schematic illustration of cancer cell acquiring resistance to chemo-drug after exposition to chemotherapy.

1.5 Synergy

Considering that cancer disease varies from patient to patient, more personalized treatment is needed. Clinical results reflect that drug combinations have brought positive effects and responses than each administrated drug.[58] Synergy in cancer is when a drug combination treatment simultaneously affects unrelated mechanisms acting on independent processes. The interaction of the drugs produces greater combined effects than the sum of each drug alone. Therefore, drug combination in cancer therapy usually is more effective because when one drug fails, the other compensates by providing synergistic or additive effects. Researchers have focused their studies on combining therapies for more synergistic effects in drug combination to certain cancers due to their positive results.[59] Although personalized cancer therapy with synergistic drug combinations is the desired goal, it is highly challenging.

Tumor cells depend on a restricted number of molecular mechanisms for proliferation and survival, so a drug combination to intervene in those specific mechanisms is needed to target and diminish cancer resistance. Drugs combined with synergistic purposes are expected to aim at

cancer type, where other chemotherapy has failed or the cancer has developed therapy resistance. Another advantage of synergistic drug combinations is minimizing drug dose and side effects. Several studies have reported that drug combination where doxorubicin is combined with another anti-cancer agent, synergistic effects are observed in several cancer types. [60] [61]

1.6 Specific Aims

The following specific aims were designed to test the viability of protein-based nanoparticles with a drug combination of Doxorubicinm (Dox) and Betulinic acid (BeA) as therapeutic agents for cancer treatment:

Specific Aim #1: Determine the effect of the co-incubation of doxorubicin and betulinic acid against NSCLC

The anti-cancer drug Dox was selected to be combined with a triterpenoid from natural sources, BeA , which exhibits anti-cancer properties. Since Dox cannot completely erradicate non-small cell lung cancer (NSCLC), a combination with BeA, a potent bioactive compound, improves anti-cancer properties. A drug ratio of Dox and cytotoxic agent BeA was determined when Dox is effective in the lung cancer cell at a lower concentration. We hypothesized that Dox and BeA combination would work to enhance the anti-cancer effect. This has been successfully tested by in vitro experiments. Results showed synergism of the drug combination while improving the efficacy at a lower Dox dose in comparison to the drug alone. The advantage of reducing Dox dosage but still have the anti-cancer effect is that it reduces the chances of cancer drug resistance.

Specific Aim #2: Develop a protein-based drug delivery system combining Doxorubicin and Betulinic acid to promote synergy while preventing chemoresistance in NSCLC

Bovine serum albumin (BSA) nanoparticles containing a drug combination of Dox and BeA were prepared by oil-in-water-like emulsion technique. These protein-based nanoparticles increase targetability and drug accumulation in cancerous cells. Using the concept proven in Specific Aim #1, the delivery system designed results in synergistic effects in NSCLC. Besides, drug accumulation, the delivery system decreases drug inactivation and toxicity. BSA (Dox + BeA) DDS demonstrate cytotoxic activity on in vitro studies. Cytotoxic mechanism and effects of this delivery system containing a combination of drugs that were evaluated as a potential therapy for cancer.

Chapter 2

Materials and methods

2.1 Methodology -Chapter 3

For this literature evidence review, sources for the database were identified for use. Initially, PubMed and Google Scholar were utilized to assess articles about *Genes involved in chemoresistance*. Regarding the database searches, the articles selected were peer-reviewed and primary sources. The articles utilized from the search were selected based on their appropriateness and relevance to our topic of interest. In general, the information selected was related to gene characteristic profile, cancer type involved in the gene regulation and when chemoresistance was developed by certain drug therapy. Additionally, drug-delivery system nanoparticles were presented as an alternative to current chemotherapy to improve the effectiveness of resistant tumors.

2.2 Experimental procedures – Chapter 4

2.2.1 Chemicals and Reagents

Fatty acid-free bovine serum albumin (BSA), betulinic acid (BeA), and doxorubicin (Dox) were purchased from Sigma-Aldrich (St. Louis, MO). The cell lines A549 (human lung adenocarcinoma; ATCC® CCL-185™) and MRC5 (human fibroblast-like; ATCC CCL-171™) were from m the American Type Culture Collection (Manassas, VA). CellTiter 96 aqueous non-radioactive cell proliferation assay (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) reagent) was from Promega Corporation (Madison, WI). NucBlue® Fixed Cell

Ready Probes® Reagent (4',6-diamidino-2- phenylindole, dihydrochloride, DAPI), fluorescein (FITC), and Vybrant™ DiO Cell-Labeling Solution were purchased from Thermo-Fisher Scientific (Waltham, MA). Cell cycle, caspase-3, DNA damage and ROS assay were from Luminex Corporation (Austin, TX). All other chemical rea-gents (analytical grade) were purchased from various suppliers and used without further purification.

2.2.2 Preparation of the DDS NPs

Protein-based DDS NPs were designed using an oil-in-water-like emulsion using a nanoprecipitation principle. Protein NPs were obtained following a procedure with several adaptations described by Molina et al. 2016, and Delgado et. al. 2015,[23, 62] The DDS's optimal preparation was achieved by adjusting several parameters and evaluating their effect on physical properties. Briefly, for the aqueous phase, BSA was dissolved in a 1X phosphate buffer solution, pH 7.4, to achieve a final 20 mg/ml concentration at 40 ºC. Meanwhile, for the oily (organic) phase, the two drugs, BeA and Dox, were dissolved in N, N-Dimethylformamide (DMF) and then, the drugs' mixture in DMF was added with a syringe needle at a constant flow of 120 ml/h to achieve a final concentration ratio of 1:5 protein: drug. The dispersed emulsion was left stirring at a constant rate for 4 h. Afterward, this preparation was centrifuged thrice with nanopure water using a 10 kDa filter unit at 5,000 rpm for 10 min to remove the unattached free drug moieties. The final DDS solution was freeze-dried for 48h and stored at -20°C until further use.

2.2.3 Characterization of the DDS NPs

2.2.3.1 Quantification of DDS components

Spectrophotometric measurements were implemented to determine the concentration of each component in the DDS. The standard protocol using the Bradford Coomassie reagent was utilized

to determine BSA protein concentration in the DDS measuring at 570 nm [63]. Dox concentration was determined using its intrinsic absorbance at 485 nm [64]. For the determination of the BeA, we selected to use the vanillin-sulfuric acid assay [65]. In brief, we prepared several dilutions from a BeA stock solution in ethanol (5 mg/ml) for a calibration curve. The dilutions and the DDS samples were heated at 85 ºC for solvent evaporation. Afterwards, 250 µL of Vanillin stock (50 mg/ml) were added followed by 500 µl of sulfuric acid to each sample. Then, the solutions were heated at 60 ºC for 30 minutes. Subsequently, the solutions were transferred into an ice bath. Then, 2 ml of acetic acid (99.7%) were added and incubated for 20 minutes. Once room temperature is reached, the DDS samples and triterpene dilutions were measured at 548 nm. For these colorimetric assays, we used the Thermofisher Scientific Multiskan FC microplate reader spectrophotometer.

2.2.3.2 Encapsulation efficiency (EE)

An aliquot of 20 µl of the DDS was collected to determine the concentration of each drug. The final amount of each drug in the DDS was obtained as explained before for the quantification of Dox and BeA. The equation used to calculate this parameter is [20]:

% EE= ((final drug weight in DDS)/ (Initial weight of drug for DDS preparation)) x 100

2.2.3.3 Particle size, polydispersity and zeta potential

The DDS's size, polydispersity index and zeta potential properties were measured using the dynamic light scattering (DLS) instrument (Mobius, Wyatt Technology, California, USA). Samples were dissolved in nanopure water and placed in a quartz cuvette for size, polydispersity and zeta potential measurements. Each value is an average of three runs of around 13 measures each.

2.2.3.4 In vitro drug release studies

Drug release were conducted as described by Morales-Cruz et al.[66]. Briefly, one ml of 5 mg/ml of the DDS dissolved in 25 mM sodium bicarbonate at pH 6.8 and 7.4, was incubated at 37 ºC under stirring. At pre-determined time intervals (typically 24 h), the DDS sample was filtered through a centrifugal filter device at 12,500 rpm for 15 min. The filtrated solution (with the released drug) was measured at 470 nm (Dox intrinsic absorbance) after the filtration through the centrifugal filter device. Also, from the filtered solution the BeA component was quantified using the vanillin-sulfuric acid assay previously described. To maintain the conditions, the DDS concentrated was re-suspended in 1 mL of bicarbonate buffer. These data are presented as the accumulative release of an average triplicate and standard deviation calculated.

2.2.4 *Cell culture experiments*

2.2.4.1 Cell culture conditions

NSCLC A549 and human normal lung MRC5 cell lines were maintained following ATCC protocols. These cells were cultured in supplemented Dulbecco's Modified Ea-gle's Medium (DMEM) (containing 1% L-glutamine, 10% fetal bovine serum (FBS), and 1% penicillin/streptomycin). Cells were kept in a humidified incubator under 5% CO2 and 95% air at 37°C. Experiments were conducted before cells reached 25 passages. For cell viability experiments, A549 and MRC5 cells were seeded in 96-well plates for 24 h.

2.2.4.2 Cells viability

A549 and MRC5 cells were seeded into 96-well plates at a density of 1 x105 cells/well in 0.1 ml supplemented DMEM medium. After 24 h, cells were treated with various con-centrations (10, 25, 50, 75, 100 mg/ml) of BSA-(Dox+BeA) DDS and controls (BSA DDS, BSA-BeA, BSA-Dox) and

incubated for 24 h. Then, 10 ul of the MTS reagent from CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay (Promega G5421) was added to each well. Then, the plate was incubated at 37 ºC and 5% CO2 atmosphere for 1 h. After incubation, absorbance was measured at 492 nm in a microplate reader (Mean ± SD, n=8). The inhibitory concentration at 50% value (IC50) was calculated by Graphpad Prism 9 software

2.2.5 Synergism, additive or antagonism drug interactions

To evaluate the cytotoxic interactions of the BeA and Dox combination, A549 cell viability was measured with different concentrations of Dox and BeA by the MTS reagent. The absorbance was measured at 492 nm after 1 h of incubation. The cell viability was calculated from a quadruplicate sample group. The concentration index (CI) was calculated by CompuSyn software based on the Chou and Talalay [67] equation.

$$CI=D1/Dx1+D2/Dx2$$

In the formula, Dx1 or Dx2 is the drug dose alone with the inhibition on x%, and D1 or the D2 is the portion of the drug in combination with the same inhibition on x%. After the interaction of different drugs is determined, the CI value can be obtained. Depending on the CI and combination of drugs can be stated as synergism, antagonism, or additive effect within the drug combination. A CI=1 indicates additive effects in the drug combination studied, differentially if C<1 indicates synergism; meanwhile, CI>1 indicates antagonism.

Otherwise, Synergy Finder 2.0 software was used to determine a synergy score for combination therapy [68]. Synergy Finder 2.0 is an open and free software that implements efficient synergy estimation for multi-drug combinations, novel visualization, and statistical treatment of replicate measurements among other features to determine synergism in drug combinations. When the

synergy score is larger than 10 indicates synergistic effect, differently if the score is less than -10 indicates an antagonism effect, meanwhile, if the score between is -10 to 10 the interaction between the drugs is additive.

2.2.6 Cellular internalization of DDS

The DDS were labeled with fluorescein isothiocyanate (FITC), to visualize cellular internalization using confocal microscopy.

2.2.6.1 DDS-FITC labelling

Co Controls (BSA) and samples (BSA-(Dox+BeA) DDS) were labeled with FITC by 24 h of incubation. . In brief, we prepared the DDS at 2 mg/ml BSA concentration in 0.1 mM sodium bicarbonate (pH 7.4) buffer and then, we added 50 µl of FITC (1 mg/ml FITC stock in DMSO) gently per 1 ml of protein solution. Let the reaction occur in the dark-ness under continuous stirring for 8 h at 4 ºC. To remove the excess of the FITC, we centrifuged the reaction mixture using a filter device (10 kDa cut off) at 5,000 rpm for 10 minutes. We repeated the centrifugation against nanopure water for up to five washes. The labeled DDS solution was freeze-dried for further use.

2.2.6.2 DDS uptake visualization

A549 cells were seeded in 8-well cover slip plates with a density of 10,000 cells/well and incubated for 24 h in supplemented culture media (DMEM). Then, A549 cells were incubated with DDS and controls for 24 h. Afterwards, the A549 cells were washed with PBS 1X and fixed for 15 minutes with a 3.7% formaldehyde solution at 37 ºC. Af-ter removing the fixing solution, wells were washed twice with PBS 1X followed by in-cubation at room temperature for 15 min of nuclear counterstain DAPI and Vybrant DiO dye solutions. Plates were stored at 4 ºC until further analysis. The plates were observed by confocal microscopy using a Nikon Eclipse Ti

microscope and 60X objec-tive. FITC was excited at 487 nm and monitored at 525 nm, DAPI was excited at 402 nm, and the emission was observed at 420-480 nm, and Vybrant DiO was excited at 484 nm, and the emission was observed at 501 nm. Fluorescence intensity was ana-lyzed using the NIS-Elements Viewer program (version 5.21 64-bit).

2.2.7 Flow cytometry analysis

Flow cytometry was implemented to analyze several cellular events involving metabolic pathways. In general, cells were seeded in a 6-well plate at a density of 10,000 cells/well and incubated for 24 h in DMEM medium containing L-glutamine, 10% FBS, and 1% penicillin/streptomycin at 37 ºC in 5% CO2. After 24 h, A549 cells were treated with DDS samples and controls, followed by each manufacturer protocol. The flow cytometer instrument used was Guava® Muse® Cell Analyzer.

2.2.7.1 Cell cycle arrest

For the cell cycle assay (Luminex MCH100106), we followed the manufacturer's pro-tocol. Briefly, after A549 cells were incubated with treatments for 24 h, the medium was discarded and cells were scrapped, and centrifuged. The pellet was mixed with cold 70% ethanol and centrifuged for fixing. Afterwards, the pellet was washed, sus-pended in the cell cycle reagent, incubated for 30 min, and measured after

2.2.7.2 Multi caspase activation

The multi caspase assay (Luminex MCH100109) was performed following manufac-turer protocol. After the treatment incubation, A549 cells were scrapped, and centri-fuged. The pellet was dissolved in the assay buffer provided by the kit, and the working solution was incubated for

30 min at 37 ºC in a 5% CO2. Afterwards, a caspase working solution was added and incubated for 5 min at room temperature for measuring.

2.2.7.3 DNA damage induction

The DNA damage assay (Luminex MCH200107) was performed following the manu-facturer's protocol. After the treatment incubation, A549 cells were scrapped, and cen-trifuged. The pellet was dissolved in the assay buffer provided by the kit. Later, fixa-tion buffer was added and incubated for 10 min. Afterwards, the centrifuged pellet was dissolved in permeabilization buffer (also provided by the kit) and incubated for another 10 min. Then, the permeabilization buffer was discarded after centrifugation, and the antibody cocktail was added and incubated for 30 min. Next, the solution was washed with assay buffer and suspended in fresh assay buffer for measuring.

2.2.7.4 Oxidative stress production

The oxidative stress assay (Luminex MCH100111) was performed following manufac-turer protocol. After A549 cells were treated for 24 h, the cells were scrapped and cen-trifuged to add the reagents from the kit, incubated for 30 minutes and washed to be measured on the Cell Analyzer.

2.2.7.5 EGFR expression

The EGFR assay (Luminex MCH200102) was performed following the manufacturer's protocol. After the treatment incubation, A549 cells were scrapped, and centrifuged. The pellet was dissolved in the assay buffer provided in the kit. Later, the fixation buffer was added and incubated for 5 min. Afterwards, the centrifuged pellet was dis-solved in permeabilization buffer (also provided by the kit) and incubated for another 5 min. The permeabilization buffer was

discarded after centrifugation and the anti-body cocktail was added and incubated for 30 min. Next, the solution was washed with assay buffer and suspended in fresh assay buffer for measuring

2.2.8 Statistical Analysis

All experiments were performed three times (at least). All data were expressed by plot-ting values with an average of four to eight measurements for each treatment condi-tion as mean ± standard deviation (SD). Quantitative data were analyzed with the sta-tistical software GraphPad Prism 9. Statistical analysis was performed using a one-way analysis of variance (ANOVA).

Chapter 3

Cancer drug resistance: Key genes and drug delivery systems to improve the effectiveness of chemotherapy

3.1 Background

Drug resistance and inefficient cancer therapy account for up to 90% of cancer-related deaths [17]. Resistance occurs when a cancer cell develops the ability to keep the chemotherapy drug out of it or reduces the amount that can enter to non-damage level. Current cancer management programs include surgery, radiation therapy, immunotherapy, and chemotherapy (including toxic, non-targeted, and targeted therapy). The Intrinsic and extrinsic factors highly sustain the problem of drug resistance through any cancer that initially responds successfully to chemotherapy; the development of drug resistance occurs in most patients [56]. The initial solution to the resistance problem to single-agent chemotherapy is the combined administration of agents with non-overlapping mechanisms of action.

Cancer cells have many growth mechanisms, releasing proliferative signals while avoiding growth suppressor molecules to resist cell death. Anticancer agents have different strategies to cause DNA damage by targeting cellular replication and signaling molecules of rapidly dividing cells [69]. Usually, cytotoxic drugs are cell-cycle specific to target a phase of the process. In this way, they typically induce mitochondria-mediated caspase-dependent apoptosis. For example, tamoxifen hormone targets the stages, antimetabolites target the S-phase, podophyllotoxins target the G2 phase, and taxanes target M-phase [70].

In contrast, there are anticancer agents which modulate cellular processes that are cell cycle-independent [71]. For example, alkylating and platinum-based agents can disrupt DNA at any cell cycle stage. Anthracyclines, such as doxorubicin, interfere with DNA replication and mainly generate reactive oxygen species (ROS) [71-72]. However, anticancer drugs affect healthy cells producing severe side effects. Depending on their mechanism, anticancer drugs can be divided into a) alkylating agents, b) antimetabolites, c) mitotic spindle inhibitors, d) topoisomerase inhibitors, e) anthracyclines, and others [73] (Table 1).

Table 3.1. Common classes of anticancer drugs

Classification	Mechanism of action	Type of Drug	Examples of drugs	Cancer Type	Ref.
Alkylating agents	Add alkyl groups to guanine on DNA; create crisscrosslinks inn the DNA	Platinum-based agents	Cisplatin Carboplatin Oxaliplatin	Breast, Leukemia, Lymphoma, Multiple Myeloma, Sarcoma, Brain Cancer, Ovary, Lung	[74]
		Nitrogen mustards	Chlorambucil Cyclophosphamide		
		Alkylsulfonates	Busulfan		
Antimetabolites	Interfere with vital metabolic pathways by acting as a false substrate duringcelle cycle synthesis phase	Pyrimidine antagonists	5-Fluorouracil Gemcitabine	Leukaemia, Breast, Ovary, Intestinal Tract, Pancreatic, Colorectal	[75]
		Purine antagonists	Fludarabine		
		Purine analogs antifolates	6-Mercaptopurine Methotrexate		
		Ribonucleotide reductase inhibitors	Hydroxyurea		
Mitotic spindle inhibitors	Inhibit microtubule polymerization disrupting mitotic spindle formation	Taxanes	Paclitaxel Docetaxel	ALL, Burkitt lymphoma, Hodgkin lymphoma, Neuroblastoma, Rhabdomyosarcoma, Wilms tumor, NSCLC, Ovarian, Head and neck	[75d, 76]
		Vinca alkaloids	Vincristine Vinblastine		
Topoisomerase inhibitors	Prevents resealing of DNA breaks	Topoisomerase inhibitors I and II	Topotecan Etoposide	Leukemia, Lung, ovarian, gastrointestinal, and other cancers	[75d, 77]

anti-tumor antibiotics	Different mechanisms (free radical formation, lipid peroxidation, direct membrane effects, and enzyme interactions)	Anthracyclines	Doxorubicin Daunorubicin	ALL, AML, Hodgkin's and non-Hodgkin's lymphoma, Bladder, Breast, Metastatic cancers, Esophageal	[75d, 78]
Tyrosine kinase inhibitors	Blocks the action of tyrosine kinases	Small molecules inhibitors	Erlotinib Lapatinib Ripretinib	Breast, CML, NSCLC, Lung, Renal, Hepatocellular, Prostate, Renal, Colorectal, ALL, GIST	[79]

Abbreviations: ALL: Acute Lymphocytic Leukemia, AML: Acute Myeloid Leukemia, CML: Chronic Myelogenous Leukemia, NSCLC: Non-small-cell lung carcinoma, GIST: Gastrointestinal Stromal Tumors

Table 2 lists the most well-known anticancer drugs used in chemotherapy for over 20 years. Unfortunately, these anticancer drugs are not entirely compelling and lead to the development of drug resistance to cancer treatment. Most genes presented in this review have developed resistance to one or more of these drugs.

Table 3.2. Common chemotherapeutic drugs associated with cancer cell resistance[#]

Chemotherapeutics	**Metabolic pathways disrupted**	**Metabolic pathways activated**	**Main resistance-related genes**	**Ref.**
Cisplatin	DNA repair / Any phase of the cell cycle	-DNA damage DNA-platinum adducts leading to apoptosis	BRCA (1 and 2)- DNA damage repair	[80]
Doxorubicin	DNA replication and DNA repair	-DNA structure changes --Formation of free radicals and oxidative damage	BCL-2- Cardiotoxicity to non-cancer cells	[81]
Paclitaxel	Mitosis	-Cytoplasmic microtubule-assembling disruptor	MDR1- Overexpression of P-gp, drug target alteration	[73, 82]
5-Fluorouracil	DNA synthesis	-Cell replication inhibitor -Cell growth inhibition leading to apoptosis	BCL-2, Bcl-XL and P-53 Overexpression-	[83]

	-DNA and RNA damage	drug influx/efflux alteration, drug inactivation, and drug target alteration.

[#]This is not an extensive list of all the drugs in chemotherapy that have shown acquired resistance. This table lists the most well-known anticancer drugs used in chemotherapy for over 20 years.

Cancer-stem cells are essential in resistance to cancer treatment, promoting uncontrolled cell growth and generating tumors. Cancer-stem cells can process self-renewal and differentiation into multiple cell types. Such cells can persist in tumors as a distinct population causing relapse and metastasis by giving rise to new tumors [84]. Furthermore, the tumor microenvironment can contribute to the cancer drug resistance, decreasing therapy effectiveness [85].

Drug resistance can be developed due to intrinsic genetic causes or acquired upon exposition to chemo drugs [54]. Tumors with intrinsic resistance exhibit cell heterogeneity and inherent decreased responsiveness to chemotherapy [17]. On the other hand, acquired resistance is generated by most cancer patients under chemotherapy as a gradual decrease in drug efficiency [54]. Furthermore, in this type of resistance, mutations can affect the expression level of the drug target, affecting the structure of the protein (mostly receptors). Mutations can also affect other proteins within the cancer cells, which can become an oncogene, also known as a second proto-oncogenesis.

Cancer stem cells are also a result of mutations that turn them into a subset of cells within the tumor with the potential for self-renewal, differentiation, and tumorigenicity, making the tumor resistant to chemotherapy. Finally, chemotherapeutic drugs can also cause DNA damage in

cancer cells and might increase the probability of the emergence of new mutations, including, for example, the activation of cell growth factors and cell defense systems [17].

The multidrug resistance (MDR) syndrome impedes the efficiency of cancer treatments, and it can occur during or after the cancer treatment. MDR can result from a difference in the structure or mechanism of the anticancer drugs. The principal causes of MDR include: increases in the efflux activity of drug pumps and a decrease in drug transporters within the membrane [86]. MDR is common in cancers such as ovarian, breast, cervical, lung, prostate, and melanoma [87]. The development of MDR is the main cause leading to failure of the most widely used chemotherapeutic drugs (paclitaxel, cisplatin, docetaxel, vincristine, epirubicin, 5-fluorouracil, and oxaliplatin), and leads to recurrence after one or more years of treatment[88].

Some of the well-studied mechanisms in cancer drug resistance include drug inactivation, alteration of drug target, efflux pump, DNA damage repair, cell death inhibition, cancer cell heterogeneity, and epigenetics (explained in Table 3).

Table 3.3: Mechanisms of anticancer drug resistance

Mechanism	Short description	Ref
Drug inactivation	Cancer cells generate an alternative mechanism that inactivates the drug once inside the cell, contributing to modification, degradation, or complex formation. This inactivation decreases the drug's toxicity levels and reduces the damage and activity of the drug in cancer cells.	[89]
Alteration of drug target	Altered or unrecognized protein structure in the drug's transporter protein due to accumulated mutations can prevent the proper attachment of the drug on its binding site. As a consequence, cancer cells become unable to internalize the cytotoxic drug, leading to their survival.	[73, 90]

Enhanced efflux pumps	The anticancer drug is pumped out of the cell through a transmembrane protein (efflux pump), preventing the accumulation of the effective drug concentration from causing toxicity in the cell, sabotaging the therapy.	[89, 91]
DNA-damage repair	Cancer cells may gain the ability to repair the DNA damage/breakage caused by anticancer drugs as a response to promote cell survival.	[73, 91]
Cell death inhibition	When proteins that induce cell death pathways (apoptosis, necrosis, or autophagy) are mutated or altered, they are unable to induce cell death.	[92]
Tumor cell heterogeneity	Cancer cells multiply at an uncontrolled rate, accumulating genetic mutations and epigenetic changes that lead to resistance and affect their sensitivity to cancer drugs. The generation of cell heterogeneity leads to the development of stem cell-like properties on the new growing cells. The stemness effect is common in cancer cells that are in circulation.	[93]
Genetic factors	Include gene mutations, amplifications, and epigenetic alterations. Epigenetic events such as methylation and acetylation affect genetic expression leading to the silencing, overexpression, or amplification of oncogenes or tumor suppressor genes, resulting in the development of cancer drug resistance.	[94]

Researchers have suggested an alternative to reduce the possibility of developing acquired cancer resistance. A patient's biopsy sample of cancerous tissue can be screened to identify genetic anomalies that could lead to cancer treatment resistance [95]. This can contribute to determining the best suitable treatment, lowering the chances for the patient to acquire resistance after general chemotherapy sessions, and prevent the failure or risks of subsequent more toxic treatments. In addition to the standard pathological analysis, several clinicians have included these genetic studies as part of the diagnostic to guide the selection of drug combinations on different types of cancers [95]. This approach could lead to a personalized therapeutic alternative based on the patient's genetic pattern.

Investigations have been focusing on alternative drug delivery systems (DDS) designed to overcome cancer drug resistance. Efficiency in delivery and target specificity are the characteristics in consideration for drug delivery vehicle designs. DDS could increase

bioavailability, diminish side effects, and improve therapeutic indexes when compared to the current clinical drugs used for treatments [57, 96]. As a consequence, DDS could also help to overcome acquired resistance induced by chemotherapy or radiotherapy [97].

This work summarizes the most significant genes that contribute to drug resistance up to date, discuss anticancer drug inefficacy, and present DDS as an alternative to overcome this clinical challenge.

3.2 Cancer drug resistance related genes

Cancer cells can grow, develop, and survive in defiance of anticancer treatment due to intrinsic or acquired causes. Genes are key players to resistance to many common cytotoxic anticancer drugs. Strong evidences are pointing that most of these resistance-related genes are involved in DNA repair and apoptosis pathways [98]. In this way, the most well-known and significant genes that contribute to anticancer drug resistance, based on our understanding, are outlined in this review. We present in the list of genes below their general information, cancer types affected by drug resistance, how these genes are regulated in general, and recent research studies that incorporate drug delivery system techniques to combat cancer drug resistance

3.2.1 B-cell lymphoma-2 (BCL-2) family proteins

Evasion of apoptosis supports the cancer development, and it is an important resistance mechanism for cancer cells against chemotherapy. Two established pathways characterized apoptosis: an extrinsic pathway mediated by death receptors at the cell membrane, and an

intrinsic pathway mediated by the mitochondria. Gene products that influence apoptosis include Bcl-2 family proteins. This large multigene family encodes proteins that are capable of inhibiting apoptosis (BCL-2, BCL-XL, BCL-W, BFL-1, BRAG-1, MCL-1, and A1) or promoting it (BAX, BAD, BAK, BCL-XS, BID, BIK, BIM, and HRK). In mammalian cells, Bcl-2, Bcl-xL, Bcl-w, Mcl-1, and A1, are the Bcl-2 proteins that block the apoptosis promoting proteins Bak and Bax, inhibiting their action by interacting with them [99]. The cellular outcome of undergoing intrinsic apoptosis or living depends on the balance and interaction between the pro- and anti-apoptotic proteins inside the cell. These Bcl-2 proteins show in their sequence four homologous domains (BH1, BH2, BH3, and BH4) called the BCL-2 homology motifs [100] except the BH3-only proteins; Bim, Bid, and Bad. Genetic alterations associated with cancer and tumor growth often affect programmed cell death regulation in a way that favors cell proliferation [101]. These genetic changes are either inherited or acquired during the cell cycle. These include substitutions, insertions, or deletions of small or large fragments of DNA, genomic amplification, and rearrangements [102]. For example, chromosomal translocation t (14;18) activate BCL-2 gene in most non-Hodgkin's lymphomas [103], nucleotide substitution and a frameshift mutation [104] inactivates BAX gene in some colon, hematological, and stomach malignancy [105], retrovirus gene insertion activate BCL-XL gene in murine leukemia [106], and these BCL-2 family gene alterations result to an overexpression of either apoptosis-suppressing or apoptosis-inducing proteins of the Bcl-2 family. Similarly, the Bcl-2 protein is overexpressed in numerous breast and prostate cancers [107]. Other studies have shown that mutations found on the coding sequence of BCL-2 gene in patients at the time of diagnosis were associated with shortened time to its transformation into an aggressive lymphoma, and earlier death due to lymphoma [108].

Additionally, it was found that that the Bcl-2 protein induces cell migration and invasion in a breast cancer cell line and also promotes metastasis to the lungs in a mouse model [109]. BCL-2, MCL-1, and BCL-XL are also overexpressed in several non-small cell lung carcinomas (NSCLC) [110]. Amplification of the apoptotic inhibitors Bcl-2-like genes, MCL-1 and BCL-XL, and deletion of apoptotic-promoter genes BOK and PUMA are presented in the somatic copy number variations in over 3000 cancer specimens across 26 human cancer types [111].

Besides malignancy, the imbalance ratio between apoptosis-suppressing and apoptosis-inducing proteins of the Bcl-2 family often makes cancer cells more resistant to a number of cell death inducers, including chemotherapeutic drugs, by impeding drug-induced damage from successfully translating into cell death [112]. Multidrug resistance (MDR) is reported to be associated with the overexpression of specific proteins such as P-glycoprotein and the anti-apoptotic genes of the BCL-2 family, where the former plays the role of expelling the drug out of the cells while the latter induces their proliferation [113]. Bcl-2 affects cancer drug resistance by inhibiting the apoptotic effect on cancer cells through dimerizing with Bax and Bad, proapoptotic members of the Bcl-2 family. Also, overexpression of Bcl-2 can prevent chemotherapy treatment by blocking paclitaxel-induced apoptosis, preventing the translocation of nuclear factor of activated T lymphocytes. Additionally, BCL-2 antagonizes apoptosis induced by drugs through inhibition of calcineurin protein activation preventing the activation of T cells from the immune system [114].

In breast cancer cells, overexpression of Bcl-2 has been correlated to the formation of polyploid cells, which confer MDR properties to cancer cells [88b]. In colorectal cancer, it has been shown that the cytokine interleukin 17 (IL-17) plays an important role in promoting the development of

resistance to cisplatin by inhibiting the expression of several pro-apoptotic proteins, including those from the Bcl-2 family such as Bax [115]. In breast cancer, IL-17 has also been shown to promote the resistance to paclitaxel through activation of the ERK1/2 pathway [116]. Defects in splicing events lead to resistance against selected therapy agents. Studies provide evidence that BIM alternative splicing products play a key role in drug resistance. In one study, the inhibition of the three major protein products (BimEL, BimS, and BimL) resulted in different levels of resistance to glucocorticoid treatment in acute lymphoblastic leukemia cells [106]. Using paired-end DNA sequencing, Ng et al. (2012) discovered an intronic deletion polymorphism in BIM that was sufficient to confer intrinsic resistance to the tyrosine kinase inhibitors, imatinib and gefitinib in chronic myeloid leukemia (CML) and epidermal growth factor receptor–mutated non–small-cell lung cancer (EGFR NSCLC) [117]. In summary, these studies point to the targeting of the anti-apoptotic members of the Bcl-2 family as a strategy to prevent MDR.

3.2.2 Chromodomain-helicase-DNA-binding protein 4 (CHD4)

Chromodomain-helicase-DNA-binding protein 4 is the main component of the nucleosome-remodeling and histone-deacetylation (NuRD) complex. The NuRD complex's primary function is to regulate gene expression and promote DNA repair. This complex is expressed throughout all tissues, and it is composed of multiple subunits, including ATP-dependent chromatin remodeling helicases CHD3/CHD4. The NuRD complex contributes to several cellular processes such as stem cell differentiation, cell cycle regulation, genome integrity maintenance, DNA damage repair, and development of the immune system [118]. NuRD subunits contribute to oncogenesis and cancer progression through DNA-damage repair, impacting the tumor’s microenvironment [119].

The CHD4 gene plays a critical role in epigenetic transcriptional repression [119]. This gene has been associated with oncogenic effects such as promoting cancer cell stemness, renewal, altering cell-cycle [120], and poor prognosis of advanced-stage cancer [119]. In collaboration with the histone deacetylases (HDACs), which allow the histones to wrap the DNA more tightly, and DNA methyl transferases, which mostly repress genes, CHD4 contributes to silencing as well as reducing and blocking the transcription of tumor suppressor genes. One of the main reasons for the recurrence of tumors is resistance to DNA damage, and genes such as CHD4 enable this repair in cancer cells. CHD4 promotes DNA repair from insults such as oxidative damage in cancer cells [121].

Drug resistance is promoted in cancer associated with BRCA, which are sensitive to DNA-damaging agents, once the CHD4 expression decreases. Furthermore, CHD4 expression reduction affects cancer cell's autophagy process as well as ERBB2 gene, an epithermal growth factor member, resulting in a drug resistance effect [121a]. Expression of CHD4 can increase stem-cell characteristics in cancer cells, stimulating anticancer drug resistance to DNA-damaging drugs [84b]. CHD4 can regulate cancer cell behavior through post-transcriptional modifications. CHD4 is associated with transcriptional repression of genes involved in the repair of double-strand break DNA-damage, and it has been considered a potential biomarker present in biopsies of patients (with significant upregulation) in cancers such as: liver, renal, osteosarcoma, breast, and ovarian. [121a].

Wang *et al*. (2019) showed that CHD4-increased expression was associated with advanced tumor invasion during metastasis and increased vascularity, promoting a more aggressive cancer phenotype [121a]. This group also reported that increased expression of the CHD4 gene was proportional to cancer treatment resistance by suppressing the expression of the cell cycle

inhibitor and anti-proliferative effector, p21, which works together with the DNA-repair gene BRCA to cause an overall decrease in the sensitivity of cells to anti-cancer treatment. Furthermore, a decrease in the gene expression of CHD4 promotes radiotherapy sensitivity of head and neck cell carcinoma. CHD4 cooperates with DNA methyltransferases (DNMTs) in the silencing of many tumor suppressor genes; therefore, its decreased expression inhibits cell proliferation sensitizing cells to radiotherapy [121]. In $ERBB2^{+}$ breast cancer cells, which are resistant to Trastuzumab, a monoclonal antibody anti-cancer treatment, the depletion of CHD4 was shown to induce the cell's sensitivity to this antibody by reducing ERBB2 signaling, affecting the autophagy process, and decreasing cell proliferation [121a, 122].

The CHD4 gene has shown to have a crucial role in colorectal cancer, and it is important to consider the activity of this gene to establish a treatment for colorectal cancer patients [121a]. Overexpression of CHD4 led to pronounced radiotherapy-resistance by maintaining DNA hypermethylation transcription silencing on colorectal cancer patients [123]. In addition, CHD4 knockdown increased the chemosensitivity of breast cancer cells towards cisplatin [121b], and increased the sensitivity of hepatocellular carcinoma cells towards epirubicin, an antitumor antibiotic [84b].

The DNA-repair promoting gene CHD4 is responsible for the transcriptional activity of the anti-proliferative gene CDKN1A or p21; therefore, these genes have opposed functions regarding cell survival. CHD4 deficiency debilitates cell survival by not-suppressing and increasing p21 levels [121b]. Inhibition of CHD4 results in the restoration of p21 expression and recovery of breast cancer cell sensitivity to cisplatin and poly ADP ribose polymerase (PARP) inhibitors [121a].

Unfortunately, knockdown of CHD4 subunits can negatively affect the chromatin-remodeling ability of the NuRD complex, promoting cell proliferation, migration, and invasion, which represses apoptosis pathways and allows cancer cells to resist drugs that lead to DNA-damage [119]. Therefore, if CHD4 inhibitors are therapeutically tested, a targeted drug delivery system must be developed to direct this drug into the tumor, decreasing the chances of affecting healthy cells or other unwanted secondary effects. Many efforts have been made in the development of therapeutic strategies against cancer that are likely to develop resistance. The combination of radiotherapy, together with an inhibitor of the NuRD complex subunit CHD4, should be a viable alternative to treat colorectal and liver cancer [84b, 119, 121a].

3.2.3 p53 (TP53)

TP53 was the first tumor suppressor gene identified in 1979. Since then, this gene has been extensively studied. P53 works mainly as a transcription factor, and its most important function is to induce or suppress the transcription of effector genes that will inhibit cancer cell proliferation, promote apoptosis, and impede tumor development [124]. DNA integrity is maintained by p53 through activation of the transcription of genes inducing cell cycle arrest as a DNA damage response [83a]. Once DNA damage is detected in the cell, p53 favors the elimination of the affected cell, inducing pro-apoptotic genes such as FAS (Fas Cell Surface Death Receptor) and BAX (from the BCL-2 family), and downregulating anti-apoptotic gene such as BCL-2 [83a]. The activation of p53 occurs in response to cellular stress and can induce cell cycle arrest to ensure genomic integrity [124-125]. Once p53 is activated, several effectors and p53-responsive genes such as CDKN1A, GADD45α, p21, MDM2, and RIT42, among others, work to inactivate cyclin-dependent kinases on the cell cycle [125b]. Cancer drug resistance is influenced by the loss-of-

function p53 gene mutations affecting mainly its transcriptional activity. The function of p53 is lost through the modifications that the gene undergoes (i.e., single point mutations and some hotspot mutations), leading to sensitivity loss to cytotoxic agents [126]. In contrast, we also found studies that show gain-of-function p53 mutations by inducing new interactions with other transcription factors that further promote chemoresistance [127]. Mutations of the p53 gene will obstruct cancer treatment. Hypoxia promotes upregulation of p53 in cancer cells blocking the cell cycle, and this event leads to the downstream activation of the p21 gene, decreasing the cytotoxic effect of anticancer drugs like cisplatin [128]. In addition, an in vitro study reported that when p53 is mutated on cancer cells, anticancer drug 5-fluorouracil sensitivity is reduced as well [129]. The most common cancer types affected by the mutation of p53 are ovarian serous carcinoma, lung cancer, pancreatic cancer, head and neck squamous cell carcinoma, and breast carcinoma [130].

Studies have found small molecules that can restore the conformation and function of mutated p53 to prevent drug resistance. These include derivatives of the thiosemicarbazone family, PRIMA-1 and MIRA-1 [126]. In addition, treatment with blockers of p53-inhibitory proteins such as MDM2, could help restore p53's function in the cases where there is an under-expression of p53 or an overexpression of MDM2 [126]. To recover p53's decreased function leading to drug resistance, interventions with nanomedicine to deliver small molecules or MDM2 inhibitors, plus the specific treatment against the tumor, could help advance the battle against cancer drug resistance.

3.2.4 Cyclin-dependent kinase inhibitor 1 (CDKN1A or p21)

Cyclin-dependent kinase interacting protein 1, also known as p21, is encoded by the CDKN1A gene. p21 is capable of controlling cyclin complexes, including (Cycling dependent kinase 2) CDK2, a catalytic subunit that can restrict cell cycle and DNA replication. In healthy cells, p21 prevents proliferation, while in several cancer cells, this function is dysregulated. Among p21 functions, it is worth mentioning its role in maintaining genomic stability, DNA-damage repair, apoptosis, and tumor-suppressing functions [131]. In cancer cells, p21 functions as a tumor suppressor, anti-apoptotic protein, and its relationship with the tumor suppressor protein p53 have been under study due to its potential contribution to cancer therapy. Studies report various roles for p21 depending on its subcellular localization. P21 can be considered as an oncogenic protein in the cytoplasm, while it can operate as a tumor suppressor inside the nucleus [132]. As part of anti-apoptotic protein, p21 can promote cancer tumor evolution and growth through diminishing DNA damage accumulation [133]. A study incorporating human leukemia cells treated with SP600125, an anti-inflammatory and anti-cancer drug that inhibits c-Jun N-terminal kinase, generates an increase in p21 expression as well as p21 phosphorylation preventing its binding with proliferating cell nuclear antigen, a DNA polymerase cofactor, while inactivating caspase-3 and consequently apoptosis [122]. The anti-apoptotic role of p21 is inhibiting the ability of pro-apoptotic proteins to apoptosis. Differentially, p21 in the nucleus has a tumor suppressor role due to the regulations to the cell cycle on CDK/cyclin complexes suppression. In a study, p21 and p53 were introduced through a nanoparticle injection, and cells were introduced into a breast cancer mouse model resulting in a reduction in cell proliferation and tumor growth [134]. Once DNA is damaged, an increase in p53 levels leads to the activation of p21 transcription. Subsequently,

p21 can either inhibit cell cycle binding CDK/cyclin complexes or block DNA replication via its interaction with DNA polymerase cofactors [135] (Figure 1).

The p21 and p53 relationship has been under investigation to consider treatment for cancer cell drug resistance. P21 mediated p53-dependent apoptotic pathways and p53-independent [136] have been recently studied. These pathways lead to transcription induction of p21 and DNA-damage in cancer cells [137]. Reduced levels of p21 are associated with tumorigenesis on several cancers such as squamous cell carcinoma of the lung, colorectal, ovarian, cervical, and head and neck [138]. P21 is an important downstream target of p53 and is rarely mutated. This means that the resistance induced by P21 could be a consequence of different factors: deficiency of the P21 gene [139], or high expression of cytoplasmic p21, and as a result, the p21 binding to procaspase-3 blocking caspase cascade and apoptosis [140]. However, p21 overexpression is also correlated to the aggressiveness and invasiveness of different cancers (Figure 1) [135]. Other studies have reported that p21 collaborates with anticancer, DNA-damaging agents to promote cell cytotoxicity. DNA-damaging agents can be combined with the anti-apoptotic p21 function as a possible target for anticancer treatment [131]. Considering the controversy around p21's various responses, more research is needed to further understand its mechanism of action on specific cancer types. Research is being conducted to systemically study the regulation of p21's expression upstream and downstream at different levels (transcriptionally, post-transcriptionally, and post-translationally) and contribute with therapeutic approaches against cancer and drug resistance treatments [141].

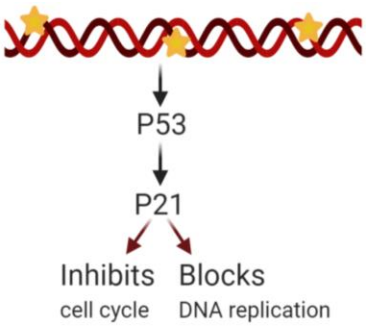


Figure 3.1: P21 overexpression effects after DNA damage. Excess p21 induces cell cycle inhibition or blockade of or DNA replication.

3.2.5 Multidrug resistance gene or P-glycoprotein-1 (MDR1)

The MDR1 gene is responsible for the expression of P-glycoprotein (P-gp), a transmembrane glycoprotein that mediates ATP-dependent efflux with permeability properties to expulse cytotoxic drugs into the extracellular space [142]. Multidrug resistance protein 1 is a member of the ATP-Binding Cassette (ABC) transporter protein family. ABC transporters are transmembrane proteins that move compounds into or out of the cell. These transporter proteins are composed of a pair of transmembrane domains and two nucleotide-binding domains. They are essential in the elimination of toxins from the human body. MDR1 is normally expressed in healthy tissue (usually on the liver, kidney, colon, pancreas, uterus, placenta, testis, and brain), although its overexpression has been associated with cancerous cells [143].

Before treatment, it is important to evaluate the presence of MDR1 mutations in cancer patients to predict the tumor's sensitivity to therapy. Patients with mutated MDR1 can be intrinsically resistant to drugs or could develop resistance over time [144]. It is worth mentioning that when P-gp is pharmacologically inhibited, thyroid hormones can promote its transcription and its function in the body [145]. MDR1/Pgp1 could cause resistance to chemotherapeutic agents that are

transported by this membrane pump (e.g., taxol and cisplatin). The overexpression of MDR1 contributes to drug resistance, particularly when genetic polymorphism variations are present. MDR1 G1199A variation exhibits a serine-to-asparagine transition in amino acid 400 in a Pgp cytoplasmic domain, producing an alteration on the efflux and transepithelial transport as well as drug sensitivity to chemotherapeutic agents [146].

Consequently, overexpression of P-gp decreases intracellular anticancer drug accumulation, which helps prevent the generation of MDR [144]. An increase in the expression of MDR1 by vitamin C has also been associated with the inhibition of the anti-tumor action of doxorubicin in ovarian and prostate cancer cells [78a]. Recently, a study defined the resistance mechanism of paclitaxel and olaparib (inhibitor of PARP1) in resistant ovarian cancer cells that was reversible with the MDR1 inhibitors verapamil and elacridar. They found that paclitaxel-resistant cells were cross-resistant to Olaparib, Rucaparib (PARP inhibitors), and doxorubicin, but not to the PARP inhibitors Veliparib or AZD2461 [147].

MDR1 gene expression can also be regulated through small interfering RNA (siRNA), which are lower in toxicity to healthy cells, and show higher specificity to the cells containing the mutated gene. This targeted siRNA therapy downregulates the MDR1 gene transcription, leading to a decreased amount of P-gp transporter proteins and a reduction of anticancer-drug expelled from the cell [143a]. Other strategies to decrease the efflux of anticancer drugs through P-gp consist of the development of compounds that either compete with anticancer drugs for transport or act as direct inhibitors of P-gp. Up to date, no P-gp blockers are being used in the clinic, possibly due to the toxic effects of such inhibition. Several alternative approaches could include the use of

nanotechnology to specifically target the cancer cells and deliver P-gp inhibitors, molecules that reduce the expression of P-gp in cancer cells, or anticancer drugs.

3.2.6 Glioma pathogenesis-related protein 1 (GLIPR1)

The glioma pathogenesis-related protein 1 (GLIPR1) is a member of the cysteine-rich secretory proteins (CRISPS), consisting of the following members: antigen 5 (Ag5), and pathogenesis-related 1 protein (Pr-1) CAP superfamily containing three core members, GLIPR1, GLIPR1-like 1 (GLIPR1L1), and GLIPR1-like 2 (GLIPR1L2) [148]. GLIPR1, a p53 target gene cluster found on human chromosome 12q21, is located in the endoplasmic reticulum (ER) membrane, and it is involved in the ER secretory pathway [149]. GLIPR1 is reported to contain an amino-terminal peptide sequence and a transmembrane domain that indicates its secretion or its location on the surface of the cell membrane [150].

Downregulation of GLIPR1 in prostate cancer and other malignant cell lines has been observed, largely in part to the methylation of the human GLIPR1 promoter [151]. Initially identified as a tumor-suppressor gene with apoptosis-inducing activities in prostate cancer, GLIPR1 pleiotropic effects have been reported to be highly expressed, upregulated, and acts as an oncogene specifically in glioblastomas and gliomas, thus promoting cell proliferation [149b, 152]. The underlying mechanism of upregulated GLIPR1 cell growth stimulation has been studied in human lung adenocarcinoma A549 cells and correlates with inducing anti-apoptotic Bcl-2 protein expression [152a]. In glioma cells, GLIPR1 overexpression reduced c-Jun N-terminal kinase (JNK) phosphorylation and induced Bcl-2 expression, thus increasing cell survival and glioma cells' protective effect against apoptotic stimuli, for instance, Fas ligation, chemotherapy, and

radiation treatment [153]. Conversely, the mechanism contributing to GLIPR1-induced apoptosis is dependent on Bcl-2 downregulation of expression and phosphorylation at Thr56 and Ser70, supporting p53-induced apoptosis, and on the increase in ROS, signaling by apoptosis signal-regulated kinase 1 (ASK1), mitogen-activated protein-extracellular signal-regulated kinase (MEK), and the consequent activation of JNK. Thereby, GLIPR1 acts through the ROS-ASK1-MEK4/7-JNK signaling pathway [154]. Moreover, GLIPR1-mediated apoptosis through the Bcl-2 family proteins, caspases, may occur through caspase-dependent and caspase-independent pathways [154b].

Originally identified as a tumor-suppressor gene with apoptosis-inducing activities in prostate cancer, GLIPR1 has been reported to be upregulated in glioblastomas, enhancing cell proliferation [149b]. The mechanism contributing to GLIPR1-induced apoptosis is associated with an increase in ROS and consequent activation of the c-Jun N-terminal kinase (JNK) pathway [154a].

Downregulation of c-Myc protein and CK1α-mediated targeted destruction of c-Myc and β-catenin in prostate cancer cell lines contributes to apoptosis induction by GLIPR1. Also, serine/threonine-protein kinase AURKA, and Xenopus kinase-like TPX2 protein signaling pathway suppression by GLIPR1 interaction with heat shock cognate protein 70 (Hsc70) also contribute to apoptosis induction [155]. TPX2 has been associated with metastasis and prognosis of bladder cancer. New findings have identified GLIPR1 as part of a regulatory circuit composed of TPX2 and p53, which modulates cell proliferation, migration, invasion, and tumorigenicity of bladder cancer cells.

The GLIPR1 gene has been identified in different forms of human cancer, including prostate, lung, ovarian, Wilms' tumor, acute myeloid leukemia, and in the most aggressive types, brain cancer,

glioblastoma multiforme/astrocytoma, and within glioma cell lines [154a, 156]. Overexpression of GLIPR1 induces apoptosis in prostate and lung cancer cells. In contrast, GLIPR1 overexpression in glioma and osteosarcoma cells leads to an increase in the proliferation, survival, invasion, migration, and anchorage-independent growth [152, 157]. In Dong *et al.* study (2015), overexpression of GLIPR1 induced the differentiation of osteosarcoma cancer-initiating cells and upregulated miR-16, blocking anti-apoptotic BCL-2 genes [157]. GLIPR1 promotes an increase in Bcl-2 expression lowering apoptosis on A549/DDP lung cancer cells. The upregulation of GLIPR1 increases and affects drug resistance by promoting cell proliferation. Otherwise, if GLIPR1 is silenced in A549/DDP cells, caspase-3 dependent apoptosis is induced by mitochondrial signaling pathways through the decreased expression of the Bcl-2 protein [152a].

Downregulation of GLIPR1 and gene knockdown experiments in various leukemia cell lines treated with the small drug SB225002 (N-(2-hydroxy-4-nitrophenyl)-N'-(2-bromophenyl)urea) resulted in elevated production of ROS, a decrease in cell proliferation linked to an increased level of apoptosis due to GLIPR1 silencing, and amplified drug resistance [158]. In another study, the siRNA-mediated knockdown of GLIPR1 expression induced a reduction in the number of melanomas and glioma cell invasion and proliferation [156]. In human lung adenocarcinoma A549 cells, upregulation of GLIPR1 stimulated cell proliferation by inducing the increased expression of Bcl-2, thus increasing resistance to the chemotherapeutic drug cisplatin [152a]. To increase the apoptotic effects of docetaxel in prostate cancer cells and overcome resistance, synergistic treatment with recombinant GLIPR1 (GLIPR1-ΔTM) inhibited tumor growth, consequently enhancing the chemotherapy effect [159]. In summary, a decrease in GLIPR1 expression is another recommended strategy to diminish resistance to anticancer drugs such as cisplatin and docetaxel.

3.2.7 Human epidermal growth factor receptor-2 (HER2)

The ErbB family comprises four receptor tyrosine kinase members named EGFR, ErbB2 (HER2), ErbB3 (HER3), and ErbB4 (HER4), located at the cell surface. These four members share structure similarities, such as an extracellular binding domain, a transmembrane lipophilic segment, and an intracellular tyrosine kinase domain [160]. Of the ErbB family, HER2 is a proto-oncogene located on the long arm of chromosome 17, whose activation relies upon homodimerization when expressed at high levels and by hetero-dimerization with EGFR or kinase-inactive HER3] [160b, 161]. After ligand binding, intracellular cell signaling pathways result in the inhibition of apoptosis, promoting proliferation and tumorigenesis [162]. The molecular mechanisms of HER2-mediated tumorigenesis encompass various models, including the overexpression of HER2, which induces an increase in the HER2-containing dimers, maximizing and sustaining signaling activity [163]. Among the dimer complexes formed, HER2/HER3 is the most critical activator of the PI3K/Akt signaling pathway (crucial for cell survival) [164]. Transcript variants of HER2 manifest higher dimerization, increased ligand-independent signaling activity, and a significant presence in HER2 amplified tumors [163, 165]. Activation of src kinases, second messengers of HER2, exhibit increased src protein levels and protein kinase activity in many human tumor tissues when combined with EGFR, having a synergistic tumorigenic effect [163]. HER2 involvement in G1/S cell cycle checkpoint control is regulated by cyclin D1 and its cyclin-dependent kinases (CDK), which play a critical proliferative role in cell cycle progression, and the CDK inhibitor p27 as a cell-cycle regulator through the induction of G1 arrest, halting cell growth [166]. HER2 tumorigenic signaling also appears to be potentiated by a stable interaction via one of two EGF-like domains with the transmembrane mucin glycoprotein Muc4, known to frequently display an altered expression in

many cancer types, promoting tumor cell proliferation and metastasis [167]. Gene amplification of HER2 is known to occur in a variety of tumor types and in approximately 25% of human breast cancers, where it manifests as an early event [160b, 161c]. HER2 gene amplification is the primary mechanism prior to protein overexpression of HER2, consequently activating the HER2 signaling network leading to uncontrolled cell proliferation and poor prognosis [168]. In breast cancer, HER2 overexpression and activity drive a tumorigenic signaling cascade when homodimerization and HER2/HER3 heterodimerization events arise [169].

HER2 overexpression has been associated with resistance to chemotherapeutic agents [170]. It has been observed in malignancies other than breast cancer such as gastric, ovarian, colon, lung, cervical, pancreatic, and esophageal cancers presenting, in general, a more aggressive disease, a lower survival rate, and a higher recurrence risk [160b, 171]. In the case of HER2 knockdown, a reduced proliferation and apoptosis induction of HER2 overexpressing breast cancer tumors result *in vitro*, and tumor regression has been observed *in vivo* [163, 172]. The potential of HER2 as a target for cancer therapeutic strategies mostly involve the use of various antibody-based agents and tyrosine kinase inhibitors (TKIs) as single agents or in combination with other therapies.

The upregulation of HER2 in metastatic breast cancer to the uterus, in combination with tamoxifen therapy, stimulates aggressive growth and invasiveness of tumors, as HER2 overexpression is associated with relative resistance to tamoxifen, and increased sensitivity to anthracycline chemotherapy, usually 5-fluorouracil and doxorubicin [173]. In another study, treatment with gemcitabine (GEM) enhanced HER2 expression on low HER2 expression breast cancer cell lines, while paclitaxel treatment induced a low and moderate HER2 upregulation. Related studies in HER2-positive breast cancer cells demonstrated that overexpression of HER2

induced paclitaxel chemotherapy resistance [174]. The therapeutic outcome of the monoclonal anti-HER2 antibody-drug, Trastuzumab, is known to downregulate HER2 signaling PI3K/Akt and MAPK pathways and to exhibit primary resistance in HER2-positive tumors as a monotherapy [175]. Some breast cancers even contain an abnormal form of HER2 lacking the extracellular domain needed for Trastuzumab binding, thereby causing resistance to the drug [160b]. To overcome resistance, conjugation of Trastuzumab with the cytotoxic agent emtansine (T-DM1) requires elevated HER2 expression levels. Thus, pretreatment with GEM was used to increase HER2 upregulation, and T-DM1 binding to HER2 on breast cancer cell surface was used as a strategy to induce antiproliferative effects [176].

In malignant pleural mesothelioma (MPM) cancer cells, the TKIs, lapatinib, and afatinib, prevented cell proliferation, upregulating and downregulating HER2 expression, respectively. Furthermore, lapatinib enhanced the monoclonal anti-EGFR antibody drug cetuximab and Trastuzumab binding with MPM cancer cells. As a result of heightened cetuximab- and Trastuzumab treatment, antibody cellular cytotoxicity (ADCC) in MPM cell lines was observed. Likewise, lapatinib enhances Trastuzumab-mediated ADCC in HER2-positive breast cancer and esophageal and gastric cancer cell lines [164, 176b]. Cisplatin is the standard treatment for gastric cancer; however, high expression of HER2 is associated with resistance to cisplatin-based chemotherapy [177]. An improvement to HER2 downregulation, as well as an increased tumor cell binding and blockade of ligand-dependent and independent- tumor growth, was accomplished with the use of the antibody ZW25 [178]. In summary, several strategies can be used to target HER2's cancer drug resistance effects, from antibodies such as ZW25 to disulfide bond disrupting

agents such as RBF3 or a combination of drugs that allow HER2-overexpressing cells to regain their sensitivity to tamoxifen or cisplatin.

3.2.8 N-myc downstream-regulated gene (NDRG1)

Cancer metastasis is the process in which cancer cells from an organ disseminate to another through circulation [179]. The N-myc downstream-regulated gene (NDRG) family has been identified as one of several metastasis suppressors involved in cancer cell invasion. The NDRG family of proteins contains four members: NDRG 1-4. The family functions are not well known, but they are associated with tumor suppression, cell proliferation, and stress response [180]. NDRG1 has shown to be an iron-regulated growth suppressor and metastasis inhibitor, showing anti-oncogenic activity, decreased cell proliferation, migration, invasion, and angiogenesis [181]. NDRG1 is mainly located in the cytoplasm and translocates to the nucleus after DNA damage, hypoxia, and cell differentiation signals [182]. This protein is a downstream target of p53, and it is involved in cancer cell resistance to hypoxia and retinoic acid (anticancer activity and chemo-preventive properties) [183]. Nevertheless, it has not been established if NDRG1's expression is inversely related to the survival of cancer cells [184]. In one study, NDRG1 demonstrated its capacity to suppress metastasis progress without altering tumor progression in an in vivo model of prostate cancer [185]. NDRG1 has a pleiotropic behavior, considering similar effects were observed on colon and pancreatic cancer [186].

NDRG1 can associate with other genes and proteins such as KAI1 and ATF3. NDRG1 expression is elevated in non-small cell lung carcinoma and contributes to cancer growth while having a variety of functions. NDRG1's overexpression reduces anticancer drug-induced cytotoxicity in lung

cancer by downregulating the stress-inducible gene ATF3. The ATF3 protein, located in the cytoplasm and nucleus, promotes apoptosis and inhibits cisplatin-induced cytotoxicity in lung cancer A549 cells [187]. Thus, by inhibiting the ATF3's cisplatin-induced cytotoxicity, NDRG1 can also regulate anticancer drug sensitivity to cisplatin. On the other hand, suppression of the NDRG1-mediated metastasis occurred upon the loss of KAI1 expression in vitro and in vivo, demonstrating that KAI1 is a functional downstream target of the NDRG1 pathway on prostate cancer [185]. These results suggest that inhibition or suppression of KAI1 could also be a target to decrease NDRG1's mediated anticancer drug resistance.

3.2.9 Hypoxia-inducible factors (HIFs)

Hypoxia induces chemoresistance by two major factors: 1) low drug concentration in hypoxic cells and 2) impaired cell proliferation of hypoxic cells by starvation [188]. When hypoxia is induced through carcinogenic pathways, the cellular response is mediated by hypoxia-inducible factors (HIF-1α, -2α -3α, and -β). HIFs are transcription factors that form heterodimers whereas α -subunit implies degradation and sensitivity to oxygen while the β-subunit means oxygen independence and the -3α serve as a suppressor or negative regulator for HIF-1α and HIF-2α (tumor promoters due to cellular response to low oxygen). Several HIF factors are involved in the different cancer stages, whereas HIF-2α is responsible for chronic and prolonged phases of metastasis and anticancer drug resistance occurring in later stages of cancer. Meanwhile, HIF-1α is involved in the early stages of cancer that later can switch to HIF-2α through the upregulation of signaling proteins. HIF-1α and HIF-2α function can overlap during tumor development [189].

HIF-2α is a transcription factor localized in the cell nucleus, and it is expressed under hypoxic stimulation. HIF-2α activation controls the intracellular hypoxic response around the body due to its expression in endothelial, parenchyma, and interstitial cells in multiple organs. HIF-2α can modulate the expression of cytochrome c oxidase isoforms to enhance the electron transport chain. Because HIF-2α is expressed in multiple organs, it affects many different types of cancer. The cancer types affected as a consequence of the low oxygen availability in cellular and organismal levels are breast, colon, ovarian, pancreatic, prostate, renal, and hepatocellular cancers. The solid tumor cancer types, where HIF-2α is frequently detected, include; head and neck, renal, bladder, glial, breast, ovarian, prostate, and renal cancers as well as the digestive system [190].

HIF-2α's most important role is to control vascular morphogenesis, integrity, and assembly, as well as mediating p53's suppression to maintain the human embryonic stem cells. HIF-2α downregulates P53 activity under hypoxic conditions and regulates cell proliferation, angiogenesis, metabolism, metastasis, and resistance to chemotherapy as part of tumorigenesis events [190]. Overexpression of HIF-2α enhances the expression of the endothelial kinase receptor, Tie2. Tie2 helps to develop the embryonic vasculature, which persists in adulthood, and it increases cytokine protein levels and mRNA in endothelial cells, promoting angiogenesis and tumor growth [191]. HIF-2α overexpression inhibits xenobiotic sensing nuclear receptors and their gene expression, affecting the expression of MDR1 and Cytochrome P450 3A4 (which oxidizes small foreign organic molecules expression). Furthermore, HIF-2α overexpression reduces the pharmacological effects of paclitaxel, mitomycin C, imatinib, and sorafenib on gastric cancer cells [190]. Currently, the evaluation of PT2385, a HIF-2α inhibitor, in combination with nivolumab

targeted therapy to programmed death receptor-1 (PD-1) in patients with advanced clear cell renal cell carcinoma previously treated with one VEGFR targeted therapy is in clinical trial Phase I (NCT02293980). The combination of both drugs demonstrated promising anti-tumor activity in ccRCC patients [192]. EZN-2208 is a transcriptional inhibitor of HIF-1α, which in combination with All-trans retinoic acid-arsenic trioxide (ATRA-ATO) was highly effective in treating patients with acute promyelocytic leukemia (APL) who develop resistance to ATO [158].

In general, the downregulation of HIFs in tumors overexpressing this protein could be another strategy to prevent tumor drug resistance. A decrease in HIF-2α activity could be done by using drug delivery strategies to introduce small molecule inhibitors of HIF-2α, interference RNA, or by inhibiting its downstream effectors.

3.2.10 Breast cancer gene (BRCA)

There are two Breast Cancer genes (BRCA), BRCA1 and BRCA2, and each one has different tumor suppressor characteristics. Their main function is to indirectly maintain the genomic integrity collaborating with recombination repair proteins [193]. Estrogen receptor signaling is the guardian of genome stability, together with the BRCA genes and proteins that control and repair DNA damage [194]. Both (BRCA1 and BRCA2) form complexes with Rad51, a recombination protein that controls the S/G2 phase in the cell cycle process. The BRCA proteins also form complexes with each other to collaborate in the tumor suppression process [193]. The BRCA1 performs several tasks, including DNA replication, cell cycle control, apoptosis, regulation of transcription, and chromatin unfolding [195]. Concurrently, BRCA2 activity is mainly focused on DNA repair by Rad51-

mediated homologous recombination. When BRCA1 or BRCA2 genes are mutated, cancer cell lines diminish the DNA double-strand break repair ability through the process of homologous recombination (HR), promoting tumorigenesis due to genome instability [193, 196]. When patients exhibit a BRCA mutation, they usually reveal p53 mutations as well. As previously discussed, p53 gene mutations prevent further p21 expression, favoring BRCA-mutated cells to avoid apoptosis, and perpetuate developing cancer tumors [197]. Mutations in BRCA2 increased the risk in patients to develop breast, prostate, pancreas, gall-bladder/bile duct, and stomach cancer, as well as malignant melanoma [193]. Meanwhile, BRCA1 mutations increase the incidence of ovarian cancer and breast tumors [198].

BRCA1 gene upregulation is caused by estrogen-induced cell proliferation and differentiation, supporting the effect of DNA stabilization. Upregulation of the estrogen receptor expression is inhibited when BRCA genes are mutated, repressing the estrogen receptor's function. Simultaneously, BRCA gene mutations upregulate defective estrogen signaling leading to tumorigenesis [194]. CHD4 acts as a tumor suppressor gene in female cancer (i.e., ovarian cancer), promoting DNA repair like BRCA functions, reducing proliferation, and promoting sensitivity to DNA damaging agents. CHD4 modulates therapeutic responses to DNA-damaging agents in BRCA mutant cancer cells. A previous study from Guillemete et al. (2015) revealed that mRNA expression levels from CHD4 contribute to the prediction of BRCA mutation cancers. When BRCA-associated cancer exhibited CHD4 depletion, a DNA-damaging agent (e.g., cisplatin) resistance was observed [199].

Meanwhile, the downregulation of p53 transcriptional activity is related to the overexpression of BRCA2 [200]. In another study, BRCA2 inactivation decreased cell cycle progression and DNA

replication and lowered cell proliferation compared to BRCA. BRCA2 knockdown is related to innate immune response upregulation, promoting cell survival [201]. The anticancer drugs commonly used to treat breast cancer are taxanes and platinum agents. Taxane drugs include paclitaxel and docetaxel for BRCA1 gene mutations or hormone-negative cancers. The positive-hormone cancers are less sensitive to taxanes. Thus, platinum agent anticancer drugs, such as cisplatin and doxorubicin, are included as an alternative to triple-negative breast cancer (lack of estrogen and progesterone receptors and ERBB2) [197].

3.2.11 Occludin (OCLN)

Tight junctions (TJs) are structural proteins that control transportation across the cell membrane. These proteins regulate cellular permeability while maintaining cell polarity, restricting the diffusion of molecules through the membrane. Tight junctions also control cellular functions, including cellular responses to environmental stimuli, intracellular gene expression, cell differentiation, and proliferation. TJs are composed of membrane proteins that can interact with adjacent cells, functioning as a barrier [202]. An integral component of TJs, providing structure and function, is the protein occludin, encoded by the OCLN gene [203]. Occludin oxidizes NADH [204], which is essential for TJ morphology stability, barrier function, and its localization to the plasma membrane on endothelial cells [202a]. Occludin contains a transmembrane domain with four membrane-spanning regions and other protein domains such as a C-terminus coiled-coil domain to interact with other proteins [202a, 203b]. OCLN's protein expression can influence the development of several cancer types, including ovarian cancer [202a], lung adenocarcinoma [203b, 205], and breast cancer metastasis [206]. Zhang *et al.* (2018) reported OCLN overexpression increased transepithelial resistance, which indicates stronger TJs, while downregulation of OCLN resulted in a decreased

cell to cell adhesion phenotype (a common characteristic observed on tumors) [202a]. Another study reported that OCLN overexpression stimulates malignant growth of lung cancer cells promoting proliferation and blocking apoptosis [203b]. On the other side, eliminating the OCLN gene has been shown to promote tumorigenic factors and reduce susceptibility to apoptosis in squamous cell carcinoma [202b]. OCLN expression increases on A549 lung cancer cells exhibiting resistance to cisplatin, doxorubicin, and gemcitabine. As an anticancer drug resistance mechanism, there is an increased expression of OCLN in the tight junctions of lung cancer cells. The overexpression of OCLN induces drug resistance by inhibiting the flux of doxorubicin, lowering drug concentration within the cell. OCLN may not be related to cancer drug resistance acquisition directly but limits the chemosensitivity of anticancer drugs on lung cancer cells [205].

On the A549 lung cancer cell line, OCLN knockdown was not related directly to their resistance to anticancer drugs, yet it suppressed their chemosensitivity on a multicellular spheroid assay. OCLN overexpression on A549 cells decreased doxorubicin permeability due to affected signaling pathways, lowering the drug's accumulation and cytotoxicity, leading to anticancer drug resistance. Interestingly, spheroid cancer cells with an increased OCLN expression developed Cisplatin resistance, showing the importance of this gene in MDR [205].

3.3 Drug delivery systems using nanoparticles to improve the effectiveness of chemotherapeutic drugs in resistant tumors

Researchers have adopted several strategies to incorporate carriers to deliver a drug or a combination of drugs intracellularly. The development of nanoparticles has become an outstanding application of nanotechnology to medicine, where a nano-sized carrier efficiently

delivers its payload of anticancer drug moieties. Using this type of therapy, researchers and clinicians take advantage of the irregular vasculature of the tumor to selectively deliver the drug and diminish the drug's toxic side effects [31, 207]. Another important advantage supporting the use of nanoparticles as a drug delivery system in cancer therapy is that it overcomes drug resistance by deactivating or avoiding various drug efflux pumps [208]. This could be accomplished by designing a selective (targeted) uptake of an endogenously endocytosed compound and promoting an intracellular accumulation of the drug, driven by the delivery system.

A remarkable characteristic of most of the nanoparticles for drug delivery systems include a spherical shape and a large surface area-to-volume ratio. This property allows the nanocarriers to be absorbed through the cell's membrane while carrying an anticancer agent. Also, the surface of most nanocarriers provides the alternative to add modifications, improving the targetability of the nanoparticle. Chemotherapeutic nanocarriers have two major categories for both active-targeted and passive-targeted delivery systems: 1) inorganic nanocarriers (metal core) and 2) organic nanocarriers (polymers, lipids, or liposomes) [57]. Currently, all the clinically approved nanocarriers are passive-targeted delivery systems [31]. However, the clinical approval of these DDS for cancer therapy was not based on their effect against resistance, but their potential to specifically target the tumors by its irregular vasculature. Based on this, we focused our next sections on studies of organic and inorganic nanocarriers, which showed significant results against resistance.

3.3.1 Organic nanocarriers

Nanoparticles containing an organic core are biocompatible, solid, and often biodegradables. Organic nanocarriers are accessible for synthesis as well as viable for surface modifications. Those characteristics increase the efficiency and biodistribution of the delivery system [209]. Based on our knowledge, currently, all the FDA-approved nanoparticle-based drugs are in the category of organic nanocarriers, i.e., protein-based, polymers and liposomes; also, there are various in clinical trials [31]. The following section will discuss the different organic nanocarriers and their applications in cancer resistance triggered by the genes discussed.

3.3.1.1 Polymers

In polymer nanoparticles, anticancer agents can be encapsulated through conjugation, or polymer attachments can be added to promote their release after a stimulus-response [57]. Risnayanti and his collaborators incorporated polylactic-co-glycolic acid (PLGA) and carboxylic acid-based particles to encapsulate both MDR1 and BCL2 siRNA [109]. Their design tackled drug efflux and cell death defense pathways. This dual MDR1 and BCL2 siRNA-loaded PLGA nanoparticle system was a viable strategy to overcome the chemoresistance on ovarian cancer cells (Paclitaxel-resistant cell line SKOV3-TR and Cisplatin-resistant cell line A2780-CP20) by enhancing cellular drug sensitivity [113]. Wang Z et al. (2017) developed PLGA nanoparticles to encapsulate the anticancer drug Disulfiram to protect it from degradation due to its short half-life in the bloodstream. The nanoparticles were combined with copper, resulting in an inhibition of liver cancer stem cells. In addition, the nanoparticles were combined with 5-fluorouracil, resulting in a synergistic cytotoxicity and anti-metastasis effect on a mouse model of liver cancer

[210]. Another research group's delivery system used PLGA as a water-soluble carrier. Chang et al. (2013) designed nanoparticles to encapsulate curcumin, a low water-soluble compound with anti-tumor, anti-metastasis, and anti-angiogenesis properties. The curcumin nanoparticles were used to treat cisplatin-resistant human oral cancer cells. As a result, curcumin nanoparticles induced apoptosis to the resistant cancer cells and showed low cytotoxicity to normal human oral epidermal cells. Moreover, these curcumin nanoparticles caused DNA fragmentation, upregulation of caspase-3/9, cytochrome c, and Apaf-1, while increasing the reactive oxygen species, which are known to induce apoptosis. In addition, Bcl-2 was downregulated, and the protein and mRNA expression levels of MDR1 were suppressed [211].

In other studies, Xiao et al. (2015) designed a double functionalized PLGA nanoparticle delivery system using chitosan (to enhance endocytic uptake), and as drugs, Pluronic (an MDR1 inhibitor) and Camptothecin (a topoisomerase 1 inhibitor) encapsulated into the nanoparticle to treat colon cancer in vitro and in vivo. The outcomes for this nanocarrier design were advantageous due to the downregulation of MDR1 expression and the inhibition of P-gp, which induced apoptosis, and reduced systemic toxicity [212]. Another study registered hyaluronic acid-modified Paclitaxel nanoparticles to encapsulate and deliver MDR1 siRNA inside ovarian cancer cells. This formulation resulted in the inhibitory effect of tumor growth and induction of apoptosis by decreasing P-gp and MDR1 expression. These particular nanoparticles take advantage of the cluster of differentiation 44 (CD44), targeting the hyaluronic acid receptor [144]. This active targeting strategy is an additional feature to increase drug accumulation into cells promoting nanoparticle endocytosis instead of drug internalization by the influx pumps. Finally, synthetic, and natural polymers can be used to encapsulate and deliver drugs overcoming MDR.

3.3.1.2 Liposomes

Liposomes are drug delivery nanocarriers that exhibit biodegradable and biocompatible properties, with the ability to encapsulate water-soluble agents, e.g., DNA and RNA, in their aqueous inner core and insoluble agents into their bilayer membrane [57]. Those specific characteristics make the liposome a versatile therapeutic nanocarrier. In this way, a group worked on a self-assembling nanocomplex to carry the p53 gene and targeted glioblastoma multiforme. They reported a cationic liposome composed of 1,2-dioleoyl-3-trimethylammonium propane (DOTAP) and di-oleoyl phosphatidyl ethanolamine (DOPE) as the carrier encapsulating an oligonucleotide. This nano-delivery platform successfully crosses the blood-brain barrier, targeting glioblastoma multiforme (GBM) and cancer stem cell lines: U87, T98G, and LN-18. The nanoparticles carried p53 and Temozolomide. This treatment revealed an increased antitumor efficiency sensitizing cancer stem cells and tumor cells to the drug, activating apoptosis and decreasing cancer drug resistance in human cancer [213]. In another recent study, researchers developed curcumin loaded into a cationic liposome-polyethylene-glycol–polyethyleneimine complex (LPPC), together with the drug Herceptin (trastuzumab) non-covalently intercalated on the surface of the carrier. Curcumin-LPPC-Herceptin and doxorubicin-LPPC-Herceptin complexes displayed an enhanced HER2-positive breast cancer targeting with a potent therapeutic effect on SKBR3 (HER2 positive) and Hs578T (HER2 negative) breast cancer cells [214].

3.3.1.3 Micelles

Micelles as spherical drug nanocarriers are self-assembly systems of water-soluble components in an aqueous solution that results in a hydrophobic core and a hydrophilic shell. The hydrophilic

shell can stabilize the hydrophobic core while keeping a non-water-soluble drug inside. The resulting nanoparticle is an excellent candidate to carry non-water-soluble drugs that can be incorporated in the polymeric micelle through physical, chemical, or electrostatic interactions [209]. In a study, researchers prepared micelles of mPEG modified with Transferrin (Tf) containing R547 drug as a drug delivery system. R547 is an ATP-competitive CDK inhibitor that specifically induces cell-cycle arrest and apoptosis. However, R547 is poorly soluble in an aqueous solution at physiological pH conditions, making it a candidate molecule to be integrated into a drug delivery system. These transferrin-modified micelles showed cytotoxicity against ovarian carcinoma cells, A2780, and induced tumor growth inhibition on A2780 tumor-bearing mice compared to non-drug and non-modified micelles [215]. Another group showed the synergistic effect of using the combination of Verapamil (P-gp inhibitor) and paclitaxel targeted-delivery into breast carcinoma cell lines, using a folate-conjugated deoxycholic acid micelle to overcome MDR. The cells used were MCF-7 and MCF-7/ADR (multi-drug-resistant variant). Synergistic effects of the folate receptor mediating the internalization and the effect of both drugs worked to diminish the MDR. Side effects and toxicities to healthy tissues or organs were reduced [88a]. In another study, a preparation of Herceptin conjugated to micellar nanoparticles consisting of d-α-tocopherol polyethylene glycol succinate (TPGS), was evaluated for the concomitant targeted delivery of Docetaxel drug and the Polo-like kinase 1 siRNA to MCF7 and SK-BR-3 breast cancer cells. The synergistic effects of the co-delivery of drugs into the cells with different HER2 expression levels resulted in a sustained and controlled delivery of Docetaxel, showing an increase in its therapeutic effect [216]. Finally, theranostic iron oxide-coated nanoparticles combined with cisplatin and with a tumor imaging infrared-dye- labeled HER2 antibody were presented in an

interesting study. The cells used for this study were HER2-positive chemo-resistant ovarian cancer cells (SKOV3), while the in vivo model used was female athymic nude mice. The outcomes of the in vivo studies were the inhibition of primary tumor growth and metastasis and the downregulation of HER2 in an ovarian cancer xenograft model [217].

3.3.1.4 Solid lipid nanoparticles

Solid lipid nanoparticles (SLN) are nanocarriers parallel to liposomes and lipid emulsions. SLN can incorporate drugs and perform targeted and controlled drug delivery [218]. Eskiler et al. (2018) developed solid lipid nanoparticles to treat resistant triple-negative breast cancer due to the BRCA1 mutation. These nanoparticles are composed of Poly (ADP-ribose) polymerase (PARP) inhibitors to induce DNA damage and to overcome HR-mediated resistance in HCC1937 and HCC1937R cell lines while delivering the anticancer drug Talazoparib (BMN673). PARP is a family of proteins associated with the regulation of many cellular processes such as genomic stability, DNA repair, and apoptosis [219]. The results from this study were DNA double-stranded breakage, G2/M cell cycle arrest, and PARP cleavage [220]. Differently, Choi et al. (2008) reported a cationic solid lipid nanoparticle design to deliver a non-viral vector-mediated p53 gene into H1299 lung cancer. SLN enters the cell to deliver the p53 gene through cell membrane permeabilization. After treatment, in vitro and in vivo results showed an increase and restoration of p53 function and apoptosis as well as decreased cancer cell growth. Those nanoparticles reported higher efficiency of the p53 gene delivered than wild type p53 mRNA and protein expression levels in lung cancer cells. Besides, the nanoparticles promoted lung cancer cell restoration of apoptotic pathways and reversed deficiencies on in vitro and in vivo tumor models [221].

3.3.2 Inorganic nanocarriers

Inorganic nanocarriers have shown various advantages over organic nanocarriers, for example, high stability on most organic solvents, large surface area, superior drug loading capacity, enhanced bioavailability, low toxicity, and controlled drug release [57]. Based on our knowledge, to date, there are several inorganic DDS under clinical trials for cancer therapy, i.e., gold nanoparticles, but none of them have been approved yet [31].

A study incorporated an inorganic material carrier (i.e., nanodiamond) to effectively deliver the anticancer drug Epirubicin to the hepatic cancer stem cell line LT2-MYC (murine hepatoblastoma) [222]. These nanodiamonds reduced the toxicity primarily through passive targeting to increase tumor-specific drug accumulation. This nanodiamond-Epirubicin complex exhibited high stability and adsorption, promoting a significant uptake and retention in tumor cells. Also, these nanodiamonds prevented the efflux of Epirubicin by ABC transporters, enhancing drug retention that led to overcoming resistance triggered by the CHD4 gene [222]. In contrast, Zhang et al. (2020) developed PEGylated tetrasulfide organosilica shell nanoparticles, exploring the co-delivery of cisplatin and Acriflavine drugs to suppress HIF functions inhibiting metastasis. This delivery system was able to synergistically co-deliver the drugs into A549 adenocarcinoma lung cancer cells in vitro and in vivo. The results revealed the versatility of this system to combat anticancer drug resistance [223]. These nanocarrier designs demonstrated the effective incorporation of inorganic materials as a viable method to overcome anticancer drug resistance.

3.3.2.1 Transitional metals

Another type of inorganic nanocarriers include the transitional metals. Most nanomaterials are metallic compounds due to their inherent properties as nano-sized particles to facilitate transportation through biologicals barriers. Thus, nickel is considered as a highly abundant metallic material candidate to be developed for health-based applications. Ingestion, inhalation, and skin absorption are the best routes for nickel to enter the human body, making the lung and kidney its primary targets [224]. Researchers have studied nickel-containing nanoparticles. Bioavailability and toxicological properties of metallic Ni nanoparticles and NiO nanoparticles have been examined on lung cancer cells. These NiO nanoparticles promoted nuclear translocation of HIF-1α, leading to the upregulation of NDRG1 in H460 human lung epithelial cells. However, these metallic nanoparticles showed moderate toxicity. Both nickel nanoparticles generated the activation of apoptosis through caspase and poly (ADP-ribose) polymerase [225]. On the other hand, a study using zinc oxide nanoparticles as treatment reported the upregulation of NDRG1 expression, and other cell growth and differentiation proteins essential in specific pathways to the ovarian granulosa cells of hens [226].

Some studies have been incorporating metallic-based nanomedicine to treat cancer resistance caused as a response to hypoxia. Silver nanoparticles were incubated with the MCF7 (breast cancer cells) and HeLa (ovarian cancer cells) in hypoxic conditions. These silver nanoparticles promoted the inhibition of HIF-1 reporter and vascular endothelial growth factor (VEGF) to disrupt angiogenesis. In addition, silver nanoparticles disrupted the cellular function of HIF signaling pathway [227]. Setapathy et al. (2013) reported the implementation of silver-based nanoparticles as an alternative to treat HCT116 human colon cancer cells. These nanoparticles

contributed to the growth inhibition and increased the cytotoxicity. This research group reported an increase in BAX/BCL-XL ratio, p53, p21, caspase 3, 8, and 9 activation leading to apoptosis as well as a decrease in AKT and NF-κB levels [228]. The NF-κB and AKT levels were determined due to their importance in cell proliferation promoting survival in resistant cancer. Silver-based nanoparticles can also be considered as an anticancer strategy to treat p53-dependent cancer cell development[228].

In addition, the study of titanium dioxide nanoparticles to determine their effects on the blood-brain barrier has increased. Disdier et al. (2015) study revealed that Titanium is internalized in the liver, lungs, and spleen, persisting for up to a year. However, in brain epithelial cells, Ti circulated for a short period having no effect on blood-brain barrier integrity, although brain inflammation was reported. Interestingly, the presence of Titanium in the liver increased TJ protein concentration, including occluding, and promoted the modulation of P-gp mRNA expression[229]. Although the principal objective of this study was to analyze the effect of the nanoparticles on the blood-brain barrier, their results showed the influence of Ti on the regulation of Occludin. The incorporation of Ti nanoparticles to influence cancer resistance by the modulation of the occludin protein has excellent potential for future studies.

In parallel to all the results presented in this section, the incorporation of nanomedicine to increase the intracellular drug concentration, changing the cell uptake route of the drug (e.g., endocytosis instead of passive diffusion), and targeting cancer resistance-related genes, also showed great potential as the next steps to improve cancer therapy. Due to this, there are many

nano-sized DDS under different stages of translational research for cancer therapy. Tumors are very heterogeneous in their cell population. In this way, the physiological barriers, e.g., microenvironment that must overcome these DDS are more complex than the in vitro models. In addition, it is still unknown the inherent physicochemical impact of the DDS nanoparticles inside the human body that would completely change the fate and therapeutic effect of the drug. Thus, the uptake of drugs delivered by the nanocarriers is not significantly higher than the free chemo drug in patients. Due to this, researchers are still working on the development of more robust DDS to overcome these limitations.

Table 3.4 summarizes the different nanocarriers that have been studied to treat cancer resistance triggered by genes.

Table 3.4: Drug delivery system nanoparticles[#] and their effect on cancer resistance.

DDS carrier	Nanoparticles modification	Encapsulated drug or toxic agent	Cells or tumor treated	Genes affected	Effect over resistance	Ref.
PLGA*	Dual RNAi delivery system (MDR1 and BCL2 siRNA)	Paclitaxel* and cisplatin*.	ovarian cancer cells: SKOV3-TR and A2780-CP20	MDR1 and BCL2	Stimuli inhibition of drug efflux and cell defense pathways (enhanced drug sensitivity)	[113]
PLGA*	PLGA-encapsulated Disulfiram	Disulfiram*	Hepatocellular carcinoma (Huh7, PLC/PRF/5)	CHD4	Extended the half-life of Disulfiram	[210]

PLGA*	Pluronic and chitosan surface-functionalized PLGA nanoparticles	Camptothecin*	Colon-26 cells (Colon cancer cells)	MDR1	Downregulate the expression of MDR1 expression and enhanced tumor uptake. Induced tumor cell apoptosis, reduced systemic toxicity, and inhibited P-gp.	212
PLGA*	PLGA-curcumin nanoparticles	Curcumin*	CAL27-cisplatin-resistant human oral cancer cells	MDR1 Bcl-2	Suppress the protein and mRNA expression levels of MDR1. Downregulate the protein levels of Bcl-2. Intrinsic apoptotic pathway through regulating the function of (MDR1) and the production of (ROS)	211
PEG* and PEI	hyaluronic acid (HA) based nanoparticle	MDR1 siRNA with paclitaxel*	SKOV-3TR and OVCAR8TR Ovarian cancer cells	MDR1	Down-regulation of MDR1 and Pgp expression. Inhibitory effect on the tumor growth. Decreased Pgp expression and increased apoptosis in MDR ovarian cancer mice model	144
ModifiePEG-PE micelles	Tf-conjugated polymeric micelles	R547 (a potent and selective ATP-competitive CDK inhibitor)	A2780 ovarian carcinoma cells	P21	*In vitro* and *in vivo* studies in ovarian cancer confirmed cytotoxicity and tumor growth inhibition.	215
Deoxycholic acid micelles	Folate-conjugated	Verapamil*, a P-gp inhibitor, and Paclitaxel*	MCF-7 and MCF-7/ADR (multi-drug-resistant variant), human breast carcinoma cell lines	MDR and P-gp	Verapamil-mediated overcome MDR solid tumors by targeting the delivery of micellar Paclitaxel into tumor cells.	88a
Cationic liposome DOTA/DOPE *	systematic nanodelivery platform encapsulating human p53 or oligonucleotide	Temozolomide * and P53 therapy	Human GBM cell lines U87, T98G, and LN-18	P53	DDS crosses the blood-brain barrier and efficiently targets cancer stem cells and tumor cells, activating apoptosis.	213
Cationic liposome-PEG-PEI complex	Herceptin was non-covalently associated onto the surface of the nanocarrier	Curcumin* and doxorubicin*	SKBR3 (HER2-positive) and Hs578T (HER2-negative) breast cancer cells	HER2	Cytotoxicity improved. Anti-proliferative effect increased.	214
Micells TPGS* and siRNA	Herceptin-conjugated micelles	Docetaxel* and polo-like kinase 1 siRNA	MCF7 and SK-BR-3 cell lines Breast cancer cell	HER2	Co-delivery of drugs was sustained and controlled	216
amphiphilic polymer nanoparticle	coated magnetic iron oxide	Cisplatin* and near-infrared dye labeled HER2 antibody	SKOV3 ovarian cancer cell line. In vivo models	HER2	Inhibited the growth of the primary tumor, peritoneal, and lung metastasis in ovarian cancer. Shrinkage	217

			female athymic nude mice		of tumor and primary tumors that had low levels of HER2.	
Nanodiamond	Epirubicin* nanodiamond complex	Epirubicin*	LT2-MYC cell line from murine hepatoblastoma tumor model	CHD4	Nanodiamond-drug complex with epirubicin exhibited high stability and adsorption, promoting uptake and retention on tumor cells	[222]
Nickel oxide	Nickel-containing nanoparticles		H460 human large cell lung cancer	NDRG1 and HIF-1a	Activate a toxicity pathway characteristic of carcinogenic Ni compounds	[225]
Zinc oxide	ZnO nanoparticles		Jinghong-1 laying hen's ovarian granulosa cells	NDRG1	Upregulated the expression of NDRG1 and regulate proteins	[226]
Silver nanoparticles	Ag nanoparticles		MCF7 (breast cancer) and HeLa (cervical cancer) cells	HIF-1	HIF-1a signaling pathway disrupted and vascular endothelial growth factor to inhibit angiogenesis.	[227]
Silica matrix	microporous organosilica shell-coated cisplatin nanoparticle*	Cisplatin* and acriflavine	A549 lung cancer cells	HIF-1	Synergistic co-delivery of drugs. Inhibit metastasis and enhancing cisplatin efficiency	[223b]
Solid lipid nanoparticles	PARP inhibitor to induce toxicity	Talazoparib* (BMN 673)	HCC1937 and HCC 1937R Triple-negative breast cancer	BRCA1	DNA double-stranded breakage, G2/M cell cycle arrest and PARP (protein regulator of genomic stability) cleavage	[220]
Titanium dioxide nanoparticles	TiO_2* nanoparticles	Titanium	Brain epithelial cells (brain microvasculature endothelial cells) and Male Fisher F344 rats	OCLN	Occludin protein is regulated while crossing blood-brain barrier with not affected integrity. Upregulation of tight junction proteins, modulation of P-gp mRNA expression	[229]

Abbreviations: PLGA: poly(lactic-co-glycolic acid), RNAi: RNA interference, siRNA: Small interfering RNA, PEG: Poly(ethylene glycol), PEI: Polyetherimide, DOTA/DOPE: 1,2-dioleoyl-3-trimethylammonium propane/di-oleoyl phosphatidyl ethanolamine, TPGS: d-α-tocopherol polyethylene glycol succinate, PE: phosphatidylethanolamine, PARP: poly ADP ribose polymerase.

*Food and Drug Administration (FDA) approved drug, polymer of particle.

[#]All these DDS have been tested in vitro, in vivo or both but none of them have been FDA approved.

3.4 Conclusion

Resistance to therapy continues to be the most significant medical challenge in cancer today. The current multimodal approach of cancer treatment is not enough to cure many tumor types and

to decrease relapse. Since there are many underlying mechanisms of resistance, it is vital to understand the biological determinants. Identifying the biological drivers of drug resistance will result in new therapeutic strategies focused on targeting the internal tumor characteristics that develop malignancies. The introduction of targeted and specific drug delivery systems such as nanocarriers is a big step towards the correct path in drug design. Non-toxic and targeted new cancer treatment alternatives will help overcome therapy resistance, providing hope to patients dealing with this devastating disease.

Chapter 4

Synergic lung cancer therapy by a protein-based drug delivery system loaded with doxorubicin in combination with a pentacyclic triterpene.

4.1 Background

For decades, chemotherapy has been the treatment of choice for many cancer patients. However, there is no gold-standard therapeutic approach to eradicate cancer. This prompts the development of new strategies for more specific treatments design depending on the diagnosed cancer type. [20] Considering that chemotherapy affects all growing cells, lack of tumor specificity is one of the biggest drawbacks in cancer treatments due to severe toxic side effects**.** Furthermore, the acquired chemoresistance produced more aggressive cancer cells in patients under treatment. To tackle these problems, development of nanosized DDS has been an emerging strategy to treat cancer as well as other diseases that need more target-specific treatments.[230] [230b] [231]

Doxorubicin (Dox), a natural anthracycline, is a chemotherapeutic drug recommended for several types of cancer, e.g., small cell lung carcinoma (SCLC).[230a] However, Dox is not curative to non-small cell lung carcinoma (NSCLC) [230b] and also induces resistance, diminishing its therapeutic effects. [231] The mechanism of action of Dox promotes inhibition of the DNA topoisomerase in RNA/DNA synthesis and an increase in reactive oxygen species.[232] In addition, Dox also induces

chemoresistance by the activation of epidermal growth factor receptor (EGFR).[233] Due to the lack of selectivity and chemoresistance, patients undergoing Dox treatment may experience cardiac toxicity and cancer recurrence. [232 234]

Betulinic acid (BeA), the other compound investigated herein, is a lupine-type pentacyclic triterpene mainly isolated from birch trees, is a phytochemical compound with a great potential against several diseases. Some multifunctional aspects include antimicrobial, antiviral, anti-inflammatory and anticancer activities.[49-50] The anticancer properties of BeA have shown potent cytotoxic activity against various types of cancer *in vitro* and *in vivo.* [51] In addition, researchers have reported BeA antitumor mechanism through mitochondrial oxidative stress induction, regulation of SP transcription factor Sp1 (specificity protein 1, a protein encoded by SP1 gene), and inhibiting proliferative factors mediating tumor cell death.[51] However, an essential disadvantage of BeA, which limits its biomedical use to a greater extent, is its poor water solubility of only 0.02 µg/ml at room temperature.[50] To improve this, researchers have studied different encapsulation methods, including self-assembling properties, to enhance BeA bioavailability under physiological conditions.[235] Thus, several benefits arise from drug combinations working in synergy including toxicity decrease and fewer side effects compared with higher doses of single drugs.[236] Hence, combining BeA with other drugs could work as an excellent alternative anticancer treatment.

The most abundant plasma protein is serum albumin (SA), a highly water-soluble and stable protein over a wide pH range (from pH 4 to 9). SA possesses many interaction sites (e.g., pockets) and functional groups (e.g., amines and carboxyls) ideal for covalent modification, and molecule-(polar and non-polar) and ion-loading.[237] Also, the flexibility of the SA structure, facilitates the

binding of different compounds.[237] Interestingly, SA has a primary and three-dimensional structure highly conserved in mammals, e.g., the bovine SA has 76% amino acid similarity with human SA.[238] HSA and BSA-based DDS have shown many advantages over the "naked" drugs, including a better NP size, homogeneity, storage and physiological stability, solubility and dispersion in aqueous solution, biocompatibility, biodegradability, and the possibility of surface modification leading to more specific therapy.[61] Moreover, cancer cells overexpress various albumin receptors (i.e., gp60 and SPARC) which can contribute to enhanced albumin-based nanoparticle uptake.[239] Specifically, Abraxane (albumin-based nanoparticles containing paclitaxel) shows improved drug efficiency over Taxol (paclitaxel).[240]

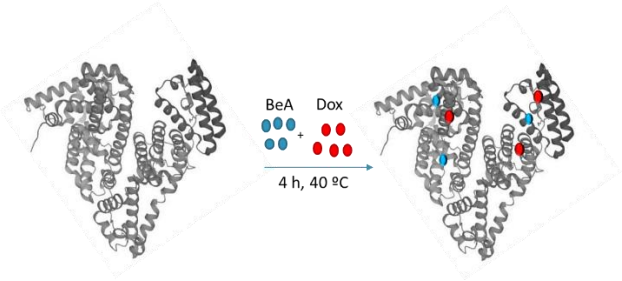
In this work, we encapsulated a drug combination of BeA and Dox in a BSA-based DDS using a cost-effective oil-in-water-like emulsion method. The resulting nanosized DDS was characterized and the drug-release in tumor-like and in normal physiological environments determined. The effect of the BEA and Dox loaded DDS and the isolated drugs on NSCLC A549 cell viability was measured and analyzed for synergistic interactions. Additionally, the drug effect on the metabolic activity of the treated A549 cells were investigated.

4.2 Results

4.2.1 Preparation and characterization of BSA-(BeA+ Dox) DDS

The preparation of the protein-based DDS NPs was achieved using an oil-in-water-like emulsion system where the drugs were dissolved in the organic solvent DMF and added to an aqueous BSA solution. The hydrophobicity of BeA contributed to the self-assembly formation of the DDS. Many conditions were varied, such as protein-drug ratios, incubation times and whether to incorporate a lipid coating to improve nanoparticle stability. Also, our DDS was also prepared

and characterized with just one drug (Dox or BeA) to compare with the DDS loaded with two drugs.



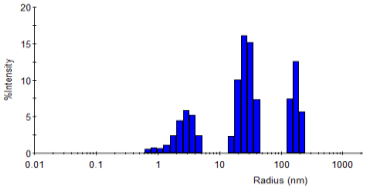
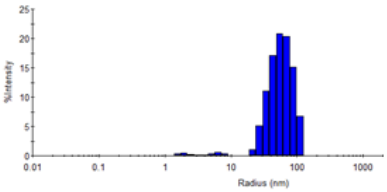
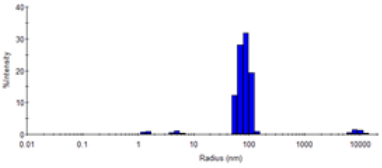
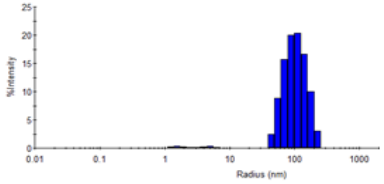
Scheme 4.1. Illustration of the preparation of BSA(BeA+Dox)

4.2.1.1 Size and charge of the DDS

The properties of the DDS were characterized to identify the best conditions in terms of size and polydispersity. These properties are important for the therapeutic success and tumor accumulation of any DDS directed towards solid tumors.[241] The diameter size, surface charge (z-potential) and polydispersity index of the DDS particle were determined by DLS (**Table 4.1**). All developed DDS showed particle sizes in the nanometer range. The BSA-BeA DDS showed the smallest diameter (size: 97 ± 1 nm, polydispersity: 27 ± 2%), followed by BSA-Dox (size: 138 ± 13 nm, polydispersity: 51 ± 14 %), and BSA(Dox+BeA) (size: 181 ± 2 nm, polydispersity: 23.2 ± 0.4 %).

The surface charge is measured using the Zeta-potential of the NP and is relevant to dispersion stability and cellular internalization of the DDS.[242] The Zeta-potential value can be related to particle stability due to the representation of the electrostatic force around the particle and the tendency to aggregation.[243] Even when positive charged NPs are favored for higher internalization in the literature, there are many studies using neutral and negative NPs that have shown high internalization[244] The Zeta-potential for all the BSA DDS with one drug (BSA-BeA: -4.6 ± 0.4, BSA-Dox: -3 ± 1) and with two drugs (BSA(BeA+Dox): -2.7 ± 0.7) were quite similar.

Table 4.1: Characterization of the protein-based drug delivery system by DLS.

DDS	Z-Potential (mV)	%PDI	Size (hydrodynamic radius, nm)	DLS graph
BSA	-16 ± 5	57 ± 0	71 ± 14	
BSA - BeA	-4.6 ± 0.4	27 ± 2	97 ± 1	
BSA - Dox	-3 ± 1	51 ± 14	138 ± 13	
BSA(BeA+Dox)	-2.1 ± 0.7	23.2 ± 0.4	181 ± 2	

4.2.1.2 Encapsulation efficiency (EE) of BeA and Dox

A vanillin-sulfuric acid method was implemented to quantify the total amount of the triterpene BeA in the DDS. The concentration of BSA and Dox in the DDS was determined using Bradford assay and Dox intrinsic absorbance at 485 nm, respectively (**Table 4.2**). Those values were used

to calculate the amount of each component in the DDS and, subsequently, the % EE for each drug and carrier yield (**Table 4.3**). The BSA-BeA DDS contained 18 ± 6 µM of BeA and 77 ± 11 µM of BSA. BSA-Dox DDS had 43 ± 6 µM of Dox and 131 ± 24 µM of BSA. Remarkably, BSA(Dox+BeA) showed the highest concentration for both drugs (61 ± 6 µM Dox and 27 ± 14 µM BeA) in 110 ± 3 µM of BSA in comparison to one-drug component DDS. Furthermore, these quantifications were used to obtain the drug EE in the NPs. The tendency observed in the drug concentrations in each DDS was very similar for the EE and the carrier yield, where BSA(Dox+BeA) showed the highest %EE (18 ± 4 % for BeA and 77 ± 15 % for Dox) and carrier yield (80 ± 12 %).

Table 4.2: DDS component quantification

DDS	**[BSA] (µM)**	**[Dox] (µM)**	**[BeA] (µM)**
BSA - BeA	77 ± 11	-	18 ± 6
BSA - Dox	131 ± 24	43 ± 6	-
BSA(BeA+Dox)	110 ± 3	61 ± 6	28 ± 12

Table 4.3. DDS encapsulation efficiency and carrier yield

DDS	**EE (%)**		**Carrier yield (%)**
	BeA	Dox	BSA
BSA-BeA	10 ± 2	/	58 ± 2
BSA-Dox	/	53 ± 7	37 ± 5
BSA(BeA+Dox)	18 ± 4	77 ± 15	80 ± 12

4.2.1.3 Circular dichroism (CD) analysis

To determine the perturbation on the structure of BSA in the formation of the DDS NPs, its secondary structure was observed through circular dichroism (CD). Also this analysis can be used to confirm the drugs (BeA and Dox) loading into the BSA cavities throughout secondary structure changes. The structural patterns of native BSA and after drug (BeA and Dox) loading were determined and shown in **Figure 4.1**. All the samples were analyzed using the same concentration (0.5 mg/mL). For the secondary structure, we can appreciate a similar tendency pattern but with less intensity. This decrease is more notorious for the DDS loading two drugs. The change in secondary structure spectra (190-250 nm) increases when BeA and Dox are bound to BSA. At 200 nm, the absorbance decreases, which corresponds to the protein backbone at the absorption of a cyclic ring. The decrease was considered because of configurational changes due to the increase of α-helix content.[245]

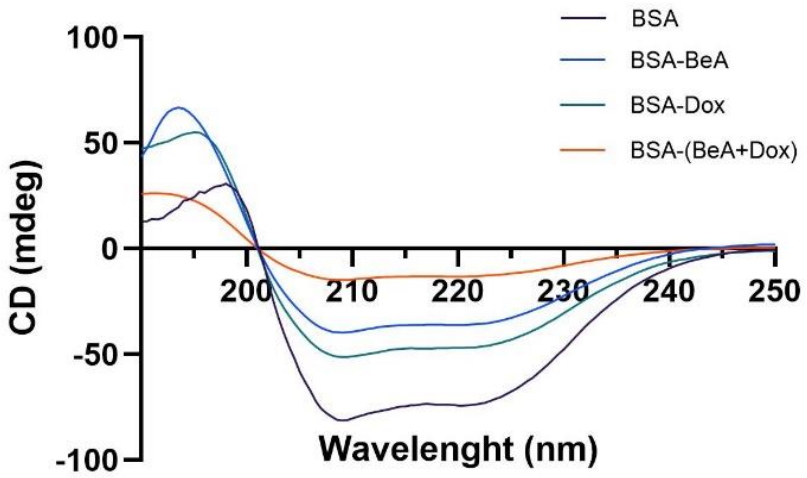


Figure 4.1. CD analysis of BSA-based DDS NPs. The secondary structure spectrum of BSA, BSA-BeA, BSA-Dox, and BSA-(BeA+Dox) are presented.

4.2.1.4 Cumulative drugs release profile

In a DDS, the drug release responding to an external stimulus is a key property to increase the drug specificity and decrease toxic side effects.[246] In this way, the cumulative Dox and BeA release profile was acquired by incubating our DDS loading the two drugs at 37 ºC in the most abundant physiological buffer, sodium bicarbonate (25 mM) at normal plasma pH 7.4 and at a more acidic pH 6.8 to mimic the tumor micro environment. Results of the cumulative release are shown in **Figure 4.2**. Our system (BSA(Dox+BeA)) at pH 7.4 showed a low initial burst (~16% for both drugs) in the first hour (**Figure 4.2 A & B zoom inset pH 7.4**). The initial burst at pH 6.8 was drastically increased for BeA, while it remained quite similar for Dox (**Figure 4.2 A & B zoom inset pH 6.8)**. At pH 6.8, 52.4 ± 0.7 % of Dox and 62 ± 1 % of BeA was released within 24 h. A release in which the DDS can accumulate in the tumor followed by fast drug release is desired, At pH 7.4, the release was significantly slower and within 24 h 28.9 ± 0.7 % of Dox was released and 38.8 ± 0.4 % of BeA. At pH 6.8, both agents were fully released from the DDS after 72 h. In contrast, at pH 7.4, BeA and Dox were fully released at 96 h and 144 h, respectively. A faster release in the tumor micro environment is a desired property.

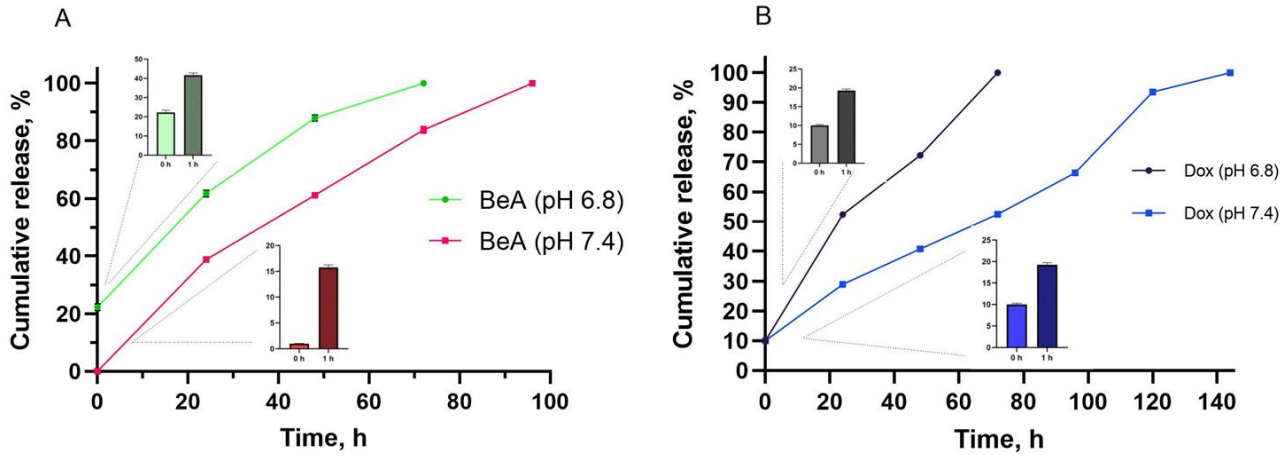


Figure 4.2: Cumulative in vitro release of the encapsulated drugs BeA (A) and Dox (B) from the DDS. The insets represent the initial burst release of each drug at 0 and 1 h insets. DDS was incubated in sodium bicarbonate buffer (pH 6.8 and 7.4) at 37 ºC and sink conditions were maintained. All data are represented as mean ± SD.

4.2.2 In vitro assays

The following in vitro assays were performed with A549 cells. To allow for comparison with normal non-cancerous cells, MRC5 fibroblast cells were used. In the mechanistic assays, only A549 cell were used. The incubation time was 24 h and the drug concentrations employed were the initially determined IC_{50} values. Native fatty acid-free BSA was used as a negative control for all the in vitro assays.

4.2.2.1 Cytotoxicity of BeA and Dox in A549 cells

A dose-response curve was created for A549 cells to confirm each drug's ability to induce cell death. Each drug was incubated at several µM concentrations for 24 h in a confluency of 1 x 10^4 cells/well to obtain the IC_{50} using MTS viability assay. The IC_{50} values obtained were 98 ± 18 µM for Dox and 42 ± 2 µM for BeA (**Figure 4.3**). As expected, cell viability decreases at increasing drug concentration. Then, A549 cells were co-incubated with both drugs, a slightly decrease (25 ± 2 µM BeA and 86 ± 1 µM Dox) in the IC_{50} concentration of each drug from this Dox + BeA combination (**Figure 4.4**).

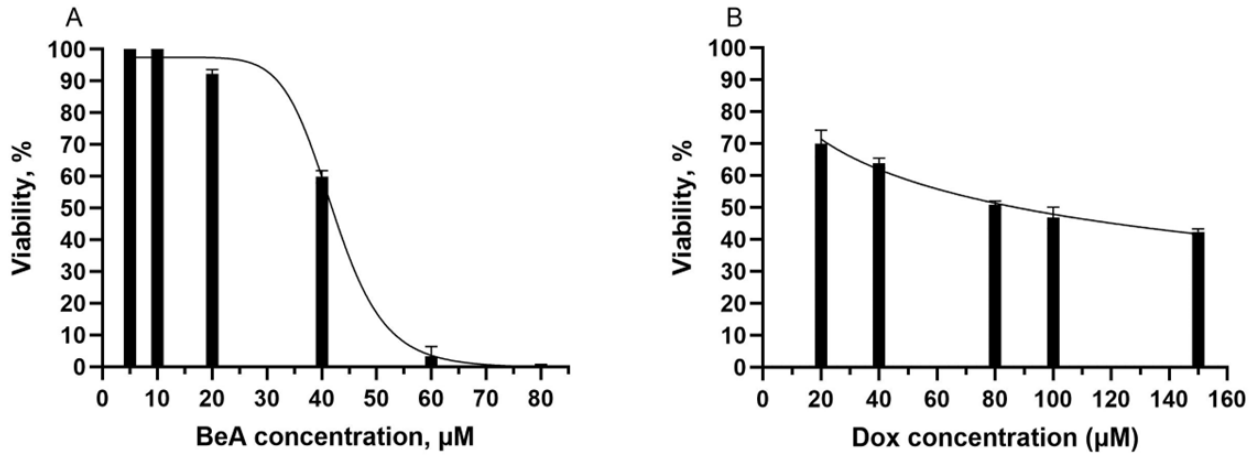


Figure 4.3: Viability of A549 cells incubated with different concentrations of BeA (A) and Dox (B) determined by MTS assay. All data are represented as mean ± SD

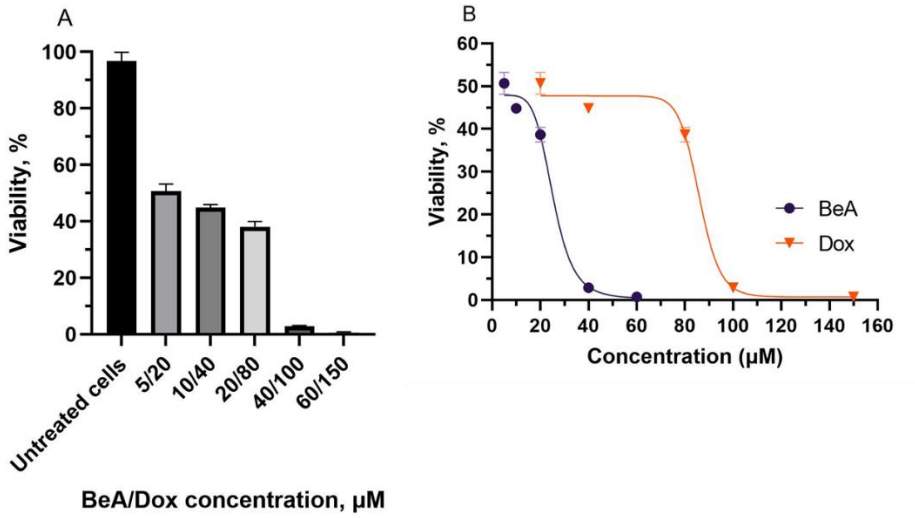


Figure 4.4: Cell viability of BeA and Dox co-incubation. (A) Viability of A549 cells simultaneously incubated with a range of concentrations of BeA and Dox determined by MTS assay. (B) Dose response curve of BeA and Dox in the drug combination. All data are represented as mean ± SD.

4.2.2.2 Molecular interaction effect of the BeA and Dox combination

Because we determined a decrease in the IC_{50} of the drugs BeA and Dox combined, We decided to use the CompuSyn software to quantitatively analyze the molecular interaction effect of BeA and Dox and calculate the combination index (CI) values using the Chou and Talalay equation.[67] The mean CI value obtained was 0.73 ± 0.2, indicating a synergistic effect (< 1) of the drug combination against A549 cells. We also used Synergy Finder 2.0, a web application for the

analysis of drug combination screening,[68] to calculate the synergy score of the drug combination to confirm the results from the CompuSyn. The synergy score was 19.06 (where >10 indicates synergistic effect) (**Figure 4.5**). This score can be interpreted as the average excess response due to drug interactions (i.e., synergy score of 19.06 corresponds to 19.05% of response beyond expectation), revealing that the combination of BeA and Dox exhibits a synergistic effect on the viability of A549 cells. The more synergistic area score is 34.46, shown in the gray highlighted square of **Figure 4.5A**, when the concentrations are 40 µM and 100 µM for BeA and Dox, respectively.

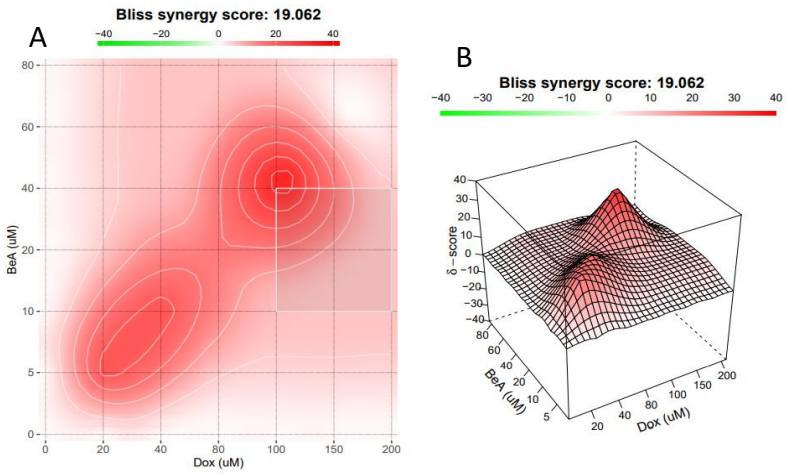


Figure 4.5: Synergy distribution (A) and 2D surface plot (B) of dose-response analysis using the Bliss method in A549 cells treated with BeA and Dox simultaneously. Synergy Finder 2.0 software was used for this analysis.

4.2.2.3 Cytotoxicity of the BSA(Bea+Dox) DDS

After we confirmed that the combination of BeA and Dox worked synergistically together, we tested our BSA-(BeA+Dox) DDS to determine its effect against NSCLC A549 cells. Once the DDS were prepared and characterized, A549 cells were treated with a range of concentrations for 24 h and 48 h (**Figure 4.6**). The highest concentration of drugs loaded in the DDS (25.5 µM Dox and 13.0 µM BeA), reduced the viability to 42 ± 2 % after 24 h and to 5.9 ± 0.8 % after 48 h of

treatment. Normal lung fibroblast MRC5 cells were also treated with the DDS. Cell viability was 58.0 ± 0.4 % after 24 h and 0.5 ± 0.4 % after 48 h. This means that, when employing isolated cells, any growing cell is impacted by the drugs, as expected. One has to keep in mind, however, that passive accumulation of the nanosized DDS under in vivo conditions should reduce the impact on healthy cells in the real treatment. This in general is the idea of nanosized systems loaded with cytotoxic drugs.

Then, the IC_{50} values of each drug in the DDS was calculated. When the DDS contains 13 ± 3 µM of BeA and 27 ± 6 µM of Dox, the cell viability of A549 cell reached 50%. This implies that the IC_{50} of BeA and Dox are significantly lower in the BSA(BeA+Dox) DDS than the naked drugs individually and also in combination. In addition, when MRC5 cells were treated with BSA(Bea+Dox) DDS, we observed a slightly less cytotoxic effect in comparison to A549 cancer cells after 24h incubation, but an even larger effect after 48 h (Figure 4.6).

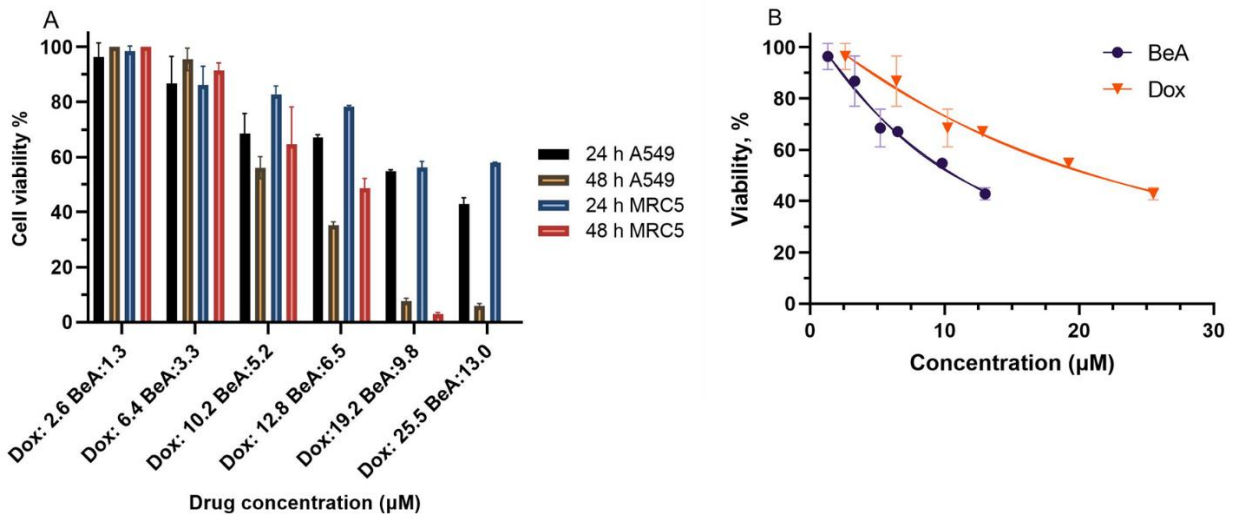


Figure 4.6: Cell viability of BSA-(BeA+Dox). (A) A549 and normal lung fibroblast MRC5 cells were incubated with a range of concentrations of the BSA(BeA+Dox) DDS. (B) Dose-response curves to determine the IC_{50} of BeA and Dox in the DDS against A549 cells. BSA (carrier) concentration was from 5 to 54 µM, showing no cytotoxicity.

4.2.2.4 Cellular internalization of the BSA(Dox+BeA) DDS

A549 cells were grown on chambered slides and incubated with the FITC-labeled DDS for 24 h to trace the specific cellular internalization of the system. DAPI dye (blue fluorescence) was used to stain cell nuclei, Dox intrinsic fluorescence was observed as red fluorescence, and Vybrant Dio dye (green fluorescence) was used to stain the cellular membrane. After the A549 cells were treated with the samples, considerable amounts of BSA(Dox+BeA) were internalized and observed in the membrane areas and the cell nucleus, respectively (**Figure 4.7 B**). In addition, as a DNA intercalator, Dox was colocalized with cell nuclei [232] (**Figure 4.7 B and D).**

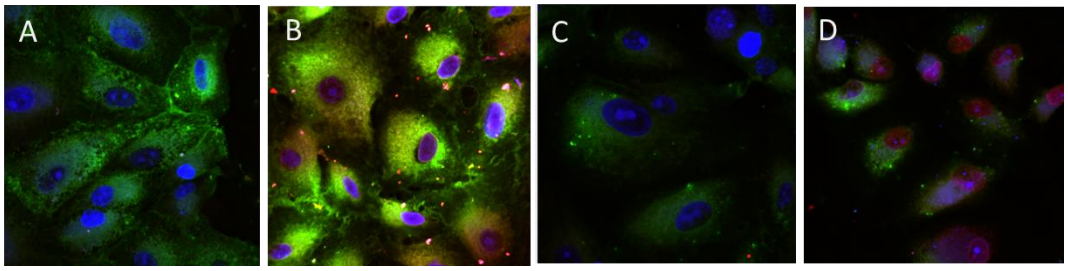


Figure 4.7: Confocal microscopy images after incubation of A459 cells with the FTIC labeled DDS. From A-B, samples were modified with FITC (green fluorescence) to visualize cellular uptake in A549 cells, DAPI (blue fluorescence) was used to stain cell nucleus and the red signal is from the Dox fluorescence. Cells were incubated with (A) 10 µM free FITC as control, (B) FITC-labeled BSA(Dox+BeA) DDS. In C and D cells were stained with Vibrant Dio dye (green fluorescence) to visualize the cell membrane, with DAPI to stain the nucleus and the red signal is from Dox fluorescence. Cells were incubated with (C) just Vibrant Dio and DAPI, (D) the former and BSA(Dox+BeA) for 24 h.

In images 4.7 B and D, where the cells were treated with BSA(Dox+BeA), quenching of the blue and red dyes is common due to Dox and DAPI binding to similar areas of the DNA in the nucleus.[247]

4.2.3 Flow cytometry analysis

Using the IC_{50} of the drugs delivered via DDS as reference, we selected to use the preparation that contains 5.2 µM of BeA and 10.2 µM of Dox (~IC_{50}/2) at 24 h of incubation in A549 cells for the remainder of the mechanistic experiments. As negative control, we used 50 µM fatty acid depleted-BSA. As positive controls, we used the free drugs at 20 µM of BeA and 20 µM of Dox.

4.2.3.1 Effect of BSA(Bea+Dox) DDS on the cell cycle

Cell cycle is an ordered sequence of events to prepare for cell growth and division. In G0, a cell is in a resting or quiescent stage and then, it can enter to the cell cycle phases, where the cell has a size increase (G1 phase), synthesize DNA (S-phase), synthetize proteins for cell division (G2-phase) and finally the cell divides by mitosis (M-phase).[248] **Figure 4.8** summarizes the results of cell cycle analysis by flow cytometry. Treatment with 20 µM of free BeA did not cause significant changes in the cell cycle phases compared to untreated cells, where the highest population was in the G0/G1 phase. This could mean that BeA induced cell cycle arrest at G0/G1 phase, as demonstrated in the literature.[249] Meanwhile, Dox showed 51.4% G0/G1, 44.0% S, and 4.3 % G2/M, showing a marked increase in cells in the S-phase. Exposure of the cells to the BSA(BeA+Dox) DDS caused an even more remarkable increase of cells in S-phase (50.9%). From the literature, Dox revealed cell proliferation inhibition through cell cycle arrest at the S- and G2/M- phases in a kidney cell line.[250]

4.2.3.2 Effect of BSA(Bea+Dox) on caspase activity

Caspases are cysteine proteases that executes apoptosis (intrinsic and extrinsic programmed cell death). Dox can induce apoptosis through caspases activation.[251] Untreated A549 cells registered a total caspase activation of 57.2%. Treating the cells with the free drugs caused 68.3% activation

in case of BeA, and 97.4% in case of Dox 97.4%, respectively. An even higher level of caspase activation (99.3%) was caused by exposure to the BSA(BeA+Dox) DDS. Comparison of the DDS with the free anticancer drugs (BeA and Dox) revealed a slight advantage of the DDS in caspase activation and cell death (**Figure 4.9**).

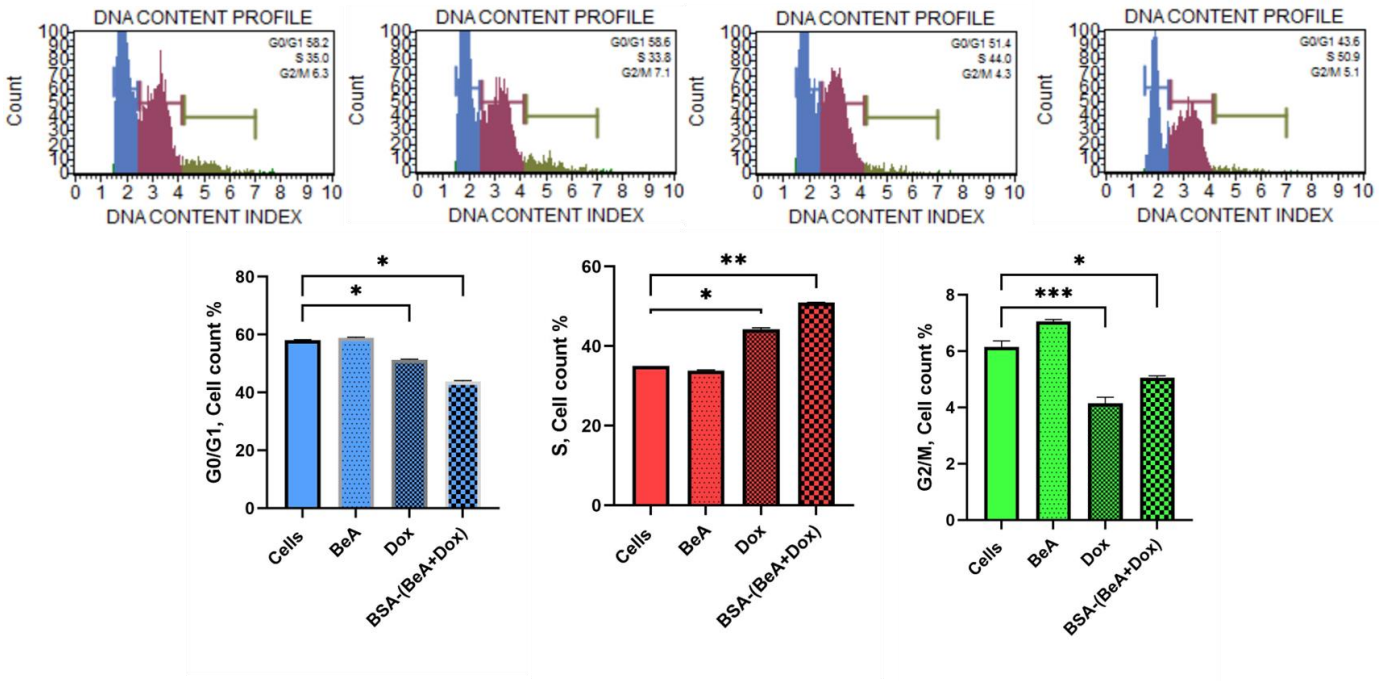


Figure 4.8. Cell cycle index induced by treatment of A549 cells with BSA(BeA+Dox). The cell cycle distribution of untreated A549 cells (A) and cells treated with BeA (B), Dox (C), and BSA(Bea + Dox) (D) is presented. Comparison of cell populations in G0/G1 (E), S (F), and G2/M (G) phases of A549 cells for each sample are shown in (A)-(D). Data were shown as mean ± SD and statistical significance is indicated.

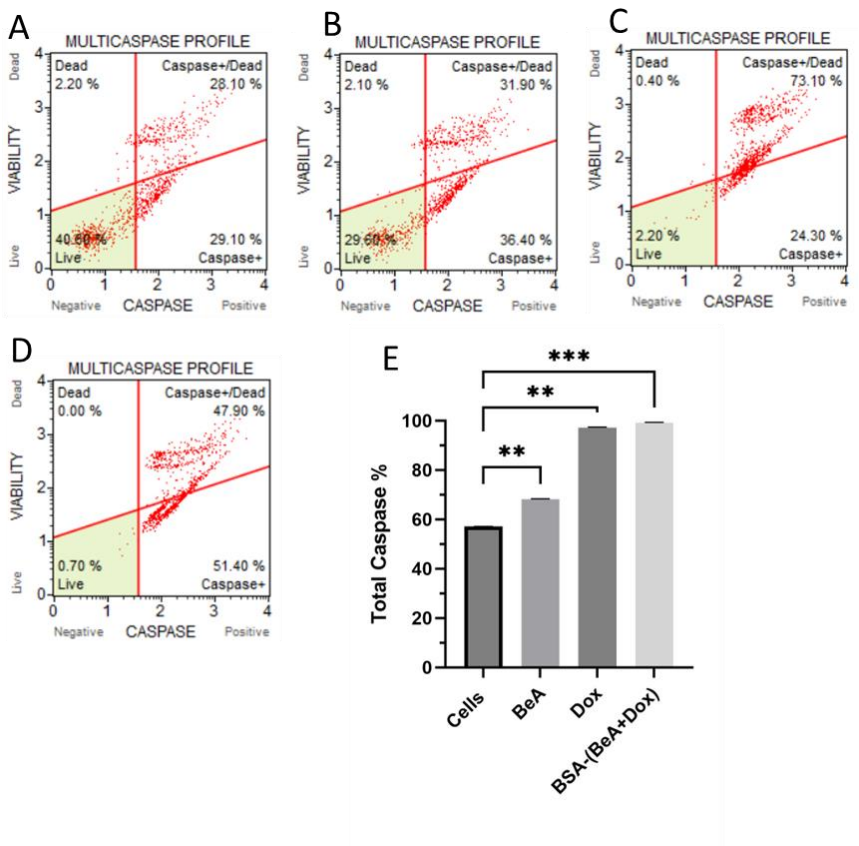


Figure 4.9. Activation of caspases by BSA(Bea+Dox). The percentage and distribution of cells exhibiting caspase activity; (A) untreated A549 cells, and cells treated with BeA (B), Dox (C), and BSA(Bea+Dox) (D). Data are presented as the means ± SD and statistical significance for all treated cells versus the control is indicated.

4.2.3.3 Effect of BSA(Bea+Dox) on the DNA processing machinery

DNA damage frequently affects the function of genes encoded.[252] DNA repair mechanisms are activated in the affected cell to correct the problem. Nevertheless, after a double-strand break when damage might be too severe for repair, phosphorylation of the histone variant X (H2A.X) and ataxia telangiectasia mutated (ATM) kinase is promoted leading to cell death.[253] The assay to assess the magnitude of this pathway indirectly measures double stand breaks by measuring the amount of phosphorylated H2A.X and ATM. We found that Dox induced an increase in the total DNA damage (97.04% vs 20.85% in untreated cells), while the BSA(Bea+Dox) DDS induced

92.48% (**Figure 4.10**). This result confirms the presence and functionality of Dox in the DDS. BeA did not show any increase in DNA damage.

4.2.3.4 Effect of BSA(Bea+Dox) on oxidative stress production

Perturbation in redox homeostasis can increase the concentration of reactive oxygen species (ROS) which subsequently can cause cancer development. However, drastic increments in ROS could also promote cell death.[254] Cellular ROS production was measured by intracellular detection of hydroxyl (HO*) and superoxide (O_2*) free radicals in A549 cells (**Figure 4.11**). BeA and Dox treated cells exhibited ROS production of 27.4% and 49.20%, respectively, compared with untreated cells. In addition, BSA-(BeA+Dox) promoted a high ROS production of 49.60%.

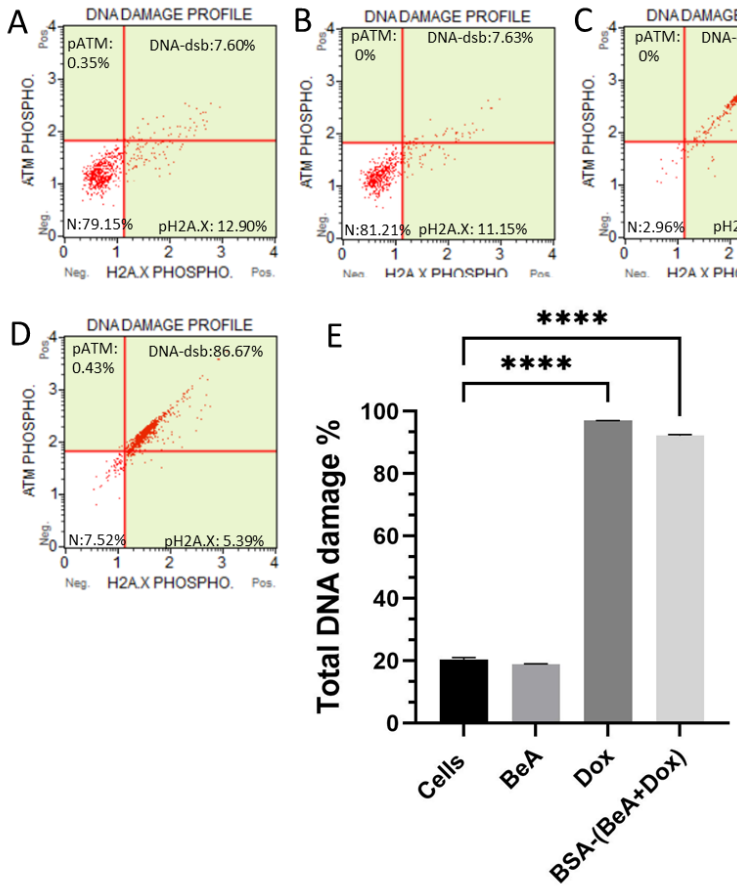


Figure 4.10. DNA damage determination induced by the BSA(Bea+Dox) DDS in A459 cells. The distribution of DNA damage in untreated cells (A) and cells treated with BeA (B), Dox (C), and BSA(Bea+Dox) (D) is presented. Data are presented as the means ± SD.

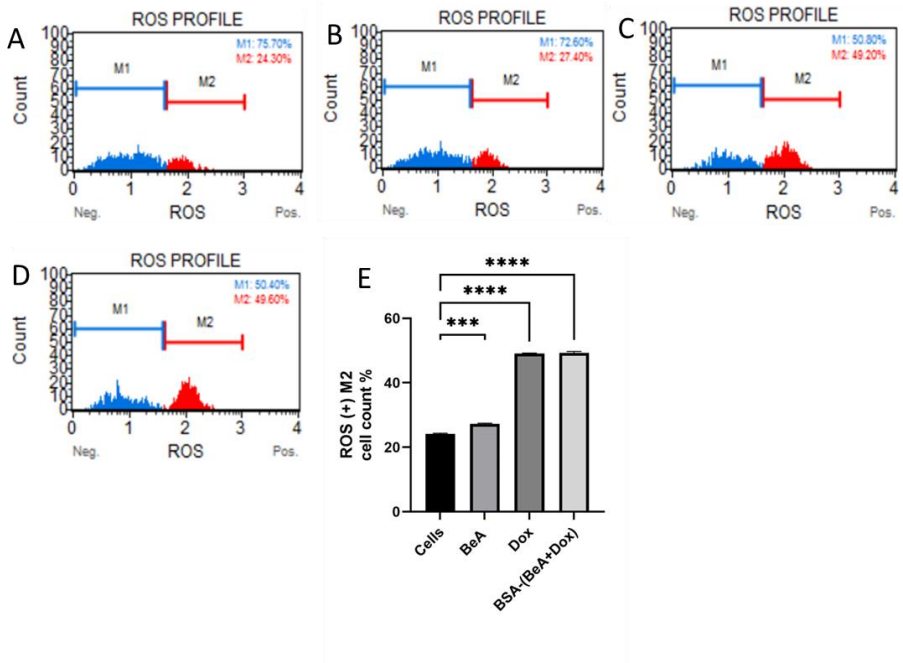


Figure 4.11: Histograms obtained from oxidative stress measurements of untreated and treated A459 cells. The distribution of A459 cells not producing ROS (M1) and producing ROS (M2) is shown for untreated cells (A) and cells treated with BeA (B), Dox (C), and BSA-(Bea+Dox) (D). Data are presented as the means ± SD. All treated cells were significantly different from non-treated cells.

4.2.3.5 Effect of BSA(Bea+ Dox) on Epidermal Growth Factor Receptor (EGFR) expression

EGFR is a transmembrane glycoprotein that is overexpressed in several cancers, especially NSCLC and it promotes cell proliferation, invasion, and chemoresistance.[255] In this study, we identified the EGFR expression by flow cytometry and show the non-expressing and expressing cells (inactivated and activated via phosphorylation). The untreated cells produced 24.0% of EGFR, whereas BeA and Dox treated cells produced 22.2% and 80.8%, respectively. Treatment of the A459 cells with the BSA(BeA+Dox) DDS produced 41.7% of EGFR. We conclude that EGFR expression was significantly decreased when BeA was combined with Dox in the BSA(BeA+Dox) DDS (**Figure 4.12**)

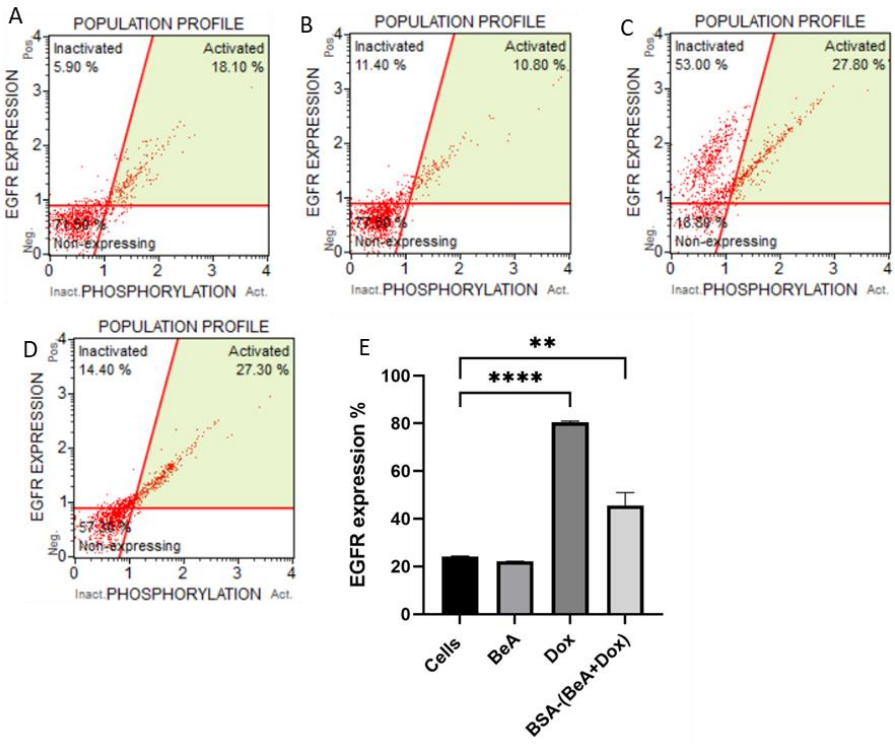


Figure 4.12. Determination of EGFR expression produced by treatment of A459 cells. Distribution of EGFR expression in untreated cells (A) and cells treated with BeA (B), Dox (C), and BSA(Bea+Dox) (D). Data are presented as the means ± SD.

4.3 Discussion

Many albumin-based NPs have been developed into DDS.[256] Our system, BSA(BeA+Dox) possesses properties (size and charge) that would allow for passive delivery and accumulation in tumors through the enhanced permeation and retention (EPR) effect caused by irregular tumor vasculature [20]. Furthermore, our DDS could be considered as a self-assembling complex between BSA and two molecularly different drugs (BeA and Dox). The triterpene BeA and the anthracycline Dox exposed strong interactions with the BSA structure pockets. Furthermore, the encapsulation efficiency (EE) was better when both drugs were loaded into the protein than just with one drug alone.

Previous studies of BSA using molecular dynamic simulation methods revealed how BeA[257] and Dox[258] are bound to BSA. BSA possesses large hydrophobic binding pockets on its surface. BeA binds to these large hydrophobic cavities of drug binding site I of subdomain IIA and IIB through hydrophobic and hydrogen bonding interactions.[257] The other drug, Dox, strongly binds to BSA by hydrophobic, hydrophilic, and hydrogen bonding interactions for stabilization. Those binding interactions alter the protein's secondary structure causing partial protein destabilization [257] [258] which were registered in the CD analysis.

The term combination therapy is used when two or more drugs positively affect the drugs' pharmacodynamics interaction (i.e., additive and synergistic). The primary purpose of combination therapy is to lower drug doses so that the patients experience less side effects while the efficiency of the treatment is enhanced.[259] In our experiments a synergistic effect is revealed in the drug combination of Dox and BeA increasing the cytotoxic effect. This synergy was demonstrated by co-incubating A459 cells with free Dox and BeA and this effect was increased by the assistance of the nanosized BSA carrier

Considering that the extracellular pH in healthy tissue is 7.4, the pH in cancer tissues is more acidic (between 6.3 and 7.0) due to the dysregulation of the acid-base homeostasis in tumors.[260] BSA(BeA+Dox) DDS released the drugs faster in an acidic environment (pH 6.8) than in physiological conditions environments (pH 7.4), revealed by in vitro cumulative drug release studies adding some selectivity by this stimulus to the DDS. Under acidic conditions, the full release took 72 h, and after 24 h, the drug release was slightly higher than 50%. This tendency is also observed in cell viability studies on A549 cells at 24 h and 48 h. Confocal microscopy validated that the BSA(BeA+Dox) DDS was internalized and Dox was localized in the nucleus.

Normal cells, MRC5, were also treated with BSA(BeA+Dox) DDS. The BSA(BeA+Dox) DDS was more aggressive on cancer cells (A549) than normal cells (MRC5) after 24 h of treatment. However, when the treatment was extended for up to 48 h, the normal lung MRC5 cells also exhibited high mortality in the highest drug concentration. An important consideration about MRC5 fibroblast cells, is that MRC5 are tumor associated cells which enhance invasive migration of cancer cells promoting the Warburg effect (intensification of aerobic usage of glucose and/or lactate promoting cancer reinforcement) and contributing to chemoresistance.[261] Thus, cytotoxicity to MRC5 after 48 h of treatment might provide an additional support to reduce chemoresistance.

The results from the metabolic cellular studies revealed that BSA(BeA+Dox) produce S-phase cell cycle arrest which was confirmed by the increase in DNA damage. The increase in oxidative stress through ROS could induce DNA disruption and/or mitochondrial membrane permeabilization. As a result, we found an increase in caspase activation confirming the induction of apoptotic pathways. Interestingly, the EGFR expression significantly decreased after DDS treatment, contrary to Dox alone. The BSA(BeA+Dox) DDS might provide an alternative to diminish and evade multidrug chemo-resistance in lung cancer patients.

4.4 Conclusions

A BSA-based DDS NPs was developed with the capacity to incorporate lipophilic (i.e., BeA) and hydrophilic moieties (i.e., Dox). The BSA-(BeA+Dox) has the characteristics of size and charge suitable for delivery in lung tumors and to be internalized by A549 cancer cells. The DDS NP surface charge results were slightly negative, and this property has shown prolonged circulating

half-live or better accumulation in tumors. Besides the synergistic effect of BeA and Dox combination, the cytotoxicity of the BSA-(BeA+Dox) against A549 cells was studied to demonstrate the efficiency of drug combination on in vitro cancer therapy as well as lowering the anticancer drugs resistance tendency that Dox caused to develop in cancer tumors. Based on the results, the BeA and Dox combination encapsulated into BSA-based NPs was very effective against A549 cells demonstrating that DDS NPs have the potential to increase the bioavailability of Dox and BeA during intravenous administration. However, further studies with drug combination DDS should be continued to explore anticancer efficacy for in vivo studies.

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