THE INVESTIGATION OF *IN VITRO* ANTICANCER EFFECTS OF THE COMBINATION OF SULFORAPHANE AND DOXORUBICIN

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ÖZET

SÜLFORAFAN VE DOKSORUBİSİN KOMBİNASYONUNUN *İN VİTRO* ANTİKANSER ETKİLERİNİN ARAŞTIRILMASI

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Kanser hastalığı dünyada önde gelen bir ölüm nedenidir ve insidansı ve mortalitesi artmaktadır. Dünya kanser verilerine (GLOBOCAN) göre, 2020'de dünya çapında yaklaşık 19,3 milyon yeni kanser vakası ve yaklaşık 10,0 milyon kanser ölümü olmuştur.

Bu çalışma, bir antrasiklin kemoterapi ilacı olan Doksorubisin ve fitokimyasal bir izotiyosiyanat olan Sülforafan ile kombinasyonunun 3T3, Caco-2, U2OS ve HepG2 hücrelerinde sinerjistik/sitotoksik etkilerini araştırmak amacıyla yapılmıştır. Bunun için Doksorubisin ve Sülforafan'nin tekli ve kombine uygulamalarının bu hücre hatlarında sitotoksik etkilerini araştırmak için MTT yöntemi kullanılmış ve buradan elde edilen sonuçlara göre de Coefficient of Drug Interaction (CDI) değerleri hesaplanmıştır. Doksorubisin ve Sülforafan'nin 3T3, Caco-2, U2OS ve HepG2 hücrelerindeki sitotoksik etkileri, konsantrasyon artışına bağlı olarak artmıştır. Hücre canlılık değerlerine göre hesaplanan CDI sonuçlarına göre de, Doksorubisin ile Sülforafan'nin kombine uygulamasının Dox 25+SFN 25 ve DOX 25+SFN 50 (CDI < 1) konsantrasyonunda U2OS hücreleri üzerinde sinerjistik bir etki gösterirken, 3T3, Caco-2 ve HepG2 hücrelerinde Sülforafan ve Doksorubisin kombine uygulamalarının ise antagonist etkiye (CDI > 1) neden olduğu belirlemiştir.

Anahtar Sözcükler: Doksorubisin, Sülforafan, MTT, CDI, Kanser hastalığı.

ABSTRACT

THE INVESTIGATION OF *IN VITRO* ANTICANCER EFFECT OF THE COMBINATION OF SULFORAPHANE AND DOXORUBICIN

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Department of Pharmacology

Anadolu University, Institution of Graduate School, January 2024

Supervisor: Prof. Dr. Miriş DİKMEN

Cancer disease is a leading cause of death in the world, its incidence and mortality are on the rise. According to the Global Cancer Observatory (GLOBOCAN), there were approximately 19.3 million new cancer cases and nearly 10.0 million cancer deaths worldwide in 2020.

This study was performed to determine the synergistic/cytotoxic effects of Doxorubicin which is an anthracycline chemotherapy drug and its combination with Sulforaphane an oily phytochemical isothiocyanate in 3T3, Caco-2, U2OS and HepG2 cells. For this, The MTT method was used to investigate the cytotoxic effects of single and combined applications of Doxorubicin and Sulforaphane in these cell lines, and CDI values were calculated in each cell line. The cytotoxic effects of Doxorubicin and Sulforaphane in 3T3, Caco-2, U2OS and HepG2 cells occur depending on concentration as shown in the MTT results. The CDI calculation results due to cell viability have determined that combined application of Doxorubicin with Sulforaphane has a synergistic effect on U2OS cells at a concentration of Dox 25+SFN 25 and DOX 25+SFN 50 (CDI < 1), while combined applications of Doxorubicin with Sulforaphane at different concentrations used in this study have antagonist effect on 3T3, Caco-2 and HepG2 (CDI > 1).

Keywords: Doxorubicin, Sulforaphane, MTT, CDI, Cancer disease.

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19/01/2024

STATEMENT OF COMPLIANCE WITH ETHICAL PRINCIPLES AND RULES

I hereby declare that this thesis is my own original work which prepared by me; that I have acted in compliance with scientific ethical rules principles during all stages and processes including preparation, data collection, analysis, report presentation and presentation of the results in my work; I have cited all of the sources of data and information obtained from this study, which are listed and included in the reference section.; And that this study has been checked for plagiarism with "scientific plagiarism detection program" used by Anadolu University, and “There is no plagiarism in it”. I declare that I consent all moral and legal consequences that will arise in the event of a problem contrary to this statement I have made regarding my work. I also declare that I agree to all moral and legal consequences that may arise if a problem arises that contradicts this statement I have made about my work.

Sondos Hasan HlALI

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LIST OF ABBREVIATIONS

AIF	: Apoptosis-inducing factor
ATP	: Adenosine triphosphate
Bax	: Bcl-2-Associated X Protein
BCL2	: B-Cell Lymphoma 2
CBR	: Carbonyl reductases
CHF	: Congestive heart failure
CPR	: Cytochrome P450 reductase
CSCs	: Cancer stem cells
CVD	: Cardiovascular disease
DMSO	: Dimethylsulfoxide
DOX	: Doxorubicin
DSB	: Double-strand break
FTP	: FluoreneTetraphenylethene-Polyethylene glycol
GSH	: Glutathione
GCL	: Glutamate-cysteine ligase
MTT	: 3-(4,5-Dimetiltiyazol-2-il)-2,5-difeniltetrazolyum
mPTPs	: Mitochondrial permeability transition pores
NCDs	: Noncommunicable diseases
NSCLC	: Non-small-cell lung cancer
OXPHOS	: Oxidative phosphorylation
Pgp	: P glycoprotein
SOD	: Superoxide dismutase
SFN	: Sulforaphane
TOPO II	: Topoisomerase II
TNF- α	: Tumor necrosis factor- α

1- INTRODUCTION

Cancer disease is a leading cause of death; it plays an important role in decreasing life expectancy in almost every country of the world. The global burden of cancer occurrence and mortality is increasing rapidly, there were approximately 19.3 million recently diagnosed cases as well as roughly ten million deaths due to cancer in the whole world in 2020 based on the Global Cancer Observatory (GLOBOCAN). Current ranks and trends indicate that by the end of this century, cancer disease may surpass cardiovascular diseases CVD as leading cause of death prematurely in most countries. As cancer is considered now the main reason e for premature mortality in 57 countries (including China), and three persons die of cancer for every ten persons who die prematurely of non-communicable diseases (NCDs) [1-3].

Many efforts and continual development of new drugs and structures are being done in the medical field in order to fight cancer, maximize benefits and minimize harm for cancer patients, especially in chemotherapy which represents the backbone of cancer treatment in the various stages of the disease. Cytotoxic chemotherapeutic drugs are used to treat both haematological and solid tumours. Patients with haematological malignancy respond well when treated with chemotherapy, especially in the first instance. At the same time, patients with metastatic solid cancers are commonly treated with cytotoxic chemotherapy which reduces tumour volume as a response [4,5].

Chemotherapeutic drugs should target only the cancer cells in the body with minimal collateral damage and harm to the normal cells. However, in the clinical use the efficacy of chemotherapy has suffered from many confounding factors that led to a limitation in the use of chemotherapy. Rapid drug metabolism, lack of specificity resulting in systemic toxicity, and both intrinsic and acquired drug resistance are common reasons for treatment failure [6].

Combined chemotherapy is routinely used in treating several types of cancer even the incurable ones, since it produces a longer control than treatment with monotherapy. In Combined chemotherapy, it is believed that the possibility of neoplastic cell resistance to therapies work differently is lower than that of a single drug, and therefore this has the potential to minimize tumor heterogeneity, leading to more long-term recovery, and possibly also cures [7].

Drugs included in the combination regimens should comply with specific guidelines that state that every single drug should have anticancer activity, and from different classes to have different mechanisms of action and, as a result, different forms of drug resistance to broaden the scope of activity against resistant cells. The combined drugs are supposed to be tolerable with little dosage adjustments. These combination regimens clinically have proven to be able to treat many hematological cancers and some solid cancers, in addition to adjuvant and neoadjuvant combination therapies which are used in cancers that are surgically removed to improve the rates of cure [7].

In the process of drugs combinations, the addition of any single agent known to have no activity against cancer or with little independent clinical effect should be avoided, even if there is a reasonable biochemical rationale for their selection because in this case additive toxicity is more likely to be produced rather than any additive anticancer effect [7].

Tumor heterogeneity and chemoresistance are main complications in the treatment of cancer. The resistance of cancer cell to antitumor drug can be intrinsic or can be acquired over time. The type of cancer being treated is the most important point in choosing the appropriate treatment technique, considering that the efficacy of the same therapies differs regarding the cancer type as they might be highly effective in one cancer and less effective in another [7,8].

Chemotherapy agents are classified into groups based on a variety of factors such as their chemical composition and function [9]. Anthracyclines (antitumor antibiotics) are one of these groups that affect enzymes involved in DNA replication leading to cytotoxicity no matter which stage of the cell cycle the cell is going through, but still, the mitotic phase is preferred [9].

Doxorubicin (DOX) is a class I anthracycline, it has a high treatment potential [10] and due to its ability in attacking rapidly dividing cells and thus slow the progression of the disease [11] is considered among the most potent anticancer drugs approved by the Food and Drug Administration (FDA) [10].

Sulforaphane (SFN) is an isothiocyanate compound found naturally in cruciferous vegetables such as broccoli. Sulforaphane is active against various types of cancer including breast, pancreatic, lung, cervical, prostate, and colorectal cancers, and it exhibit its anticancer activity through a variety of mechanisms, in addition to its ability to

improve the therapeutic activity of many classes of antitumor agents including doxorubicin [12].

Fimognari and collaborators suggested that the combination of sulforaphane with doxorubicin might enable doxorubicin to be taken at doses that are less and that could decrease its potential toxicity, and at the same time increasing the cytotoxic effects of DOX [13].

The purpose of this study is to determine the cytotoxic effects of Doxorubicin and Sulforaphane at concentrations of 50 µM, 25 µM, 12.5 µM, and 6.25 µM and their cytotoxic effects on 3T3, Caco-2, U2OS and HepG2 cells with MTT method, and to evaluate the agonist, antagonist, synergist, and additive effects of DOX and SFN combination by using Coefficient of Drug Interaction (CDI) calculations.

2. LITERATURE REVIEW

2.1. General Information about Cancer

Cancer disease is a widespread global public health issue. It involves a wide variety of malignant tumors that can affect nearly every organ and tissue in the body. It is primarily caused by genetic mutations and physiological changes within a cell, which lead to abnormal cell growth (neoplasia). In most cancer patients the invasion of tumor cells of adjacent tissues and organs that are distant is the main reason of morbidity and mortality [14,15].

Cancer disease has six cellular physiological alterations that could be a significant cause of malignant cell growth, these alternations have been identified as the hallmarks of almost every type of cancer. These hallmarks include lack of need of external growth factors to stimulate growth, absence of response to antigrowth signals, escape from apoptosis (programmed cell death), no end to their potential for replication, angiogenesis, and tissue invasion and metastasis. These changes are considered as new capabilities gained during tumor development and represent the effective breaking of an anticancer defense system present in cells and tissues [16].

Many factors were identified as a carcinogenic which stimulate the occurrence of this disease [17]. These factors were classified to include internal factors (like hereditary mutations, immune conditions, and hormones) and external (environmental and acquired) factors (like alcohol, radiation, food, obesity, and diseases that are contagious). The genetic factors contribute about 5-10% to cancer risk while its 90–95% for environmental and lifestyle factor [18].

People with cancer may experience a variety of symptoms related to the disease and its treatment. Symptoms that are poorly controlled in cancer patients are associated with decreased compliance with treatment and quality of life. Among the most common troubling symptoms experienced by cancer survivors are insomnia, fatigue, pain, and Neuropathy [19].

There are many of cancer treatment modalities that are divided into conventional and modern ones. The traditional treatment methods are chemotherapy, surgery, and radiotherapy, while modern methods include stem cell therapies, hormone therapy, immunotherapy, stem cell therapies, and dendritic cell-based immunotherapy [20].

According to the statistics, between 2018 and 2040, It is expected that the number of cancer cases worldwide would rise to twenty six million with fifteen million persons

who require treatment with chemotherapy, mainly for lung (16.4%), breast (12.7%), and colorectal cancer (11.1%) [21].

2.2. Doxorubicin (DOX)

2.2.1. General information about DOX

Doxorubicin (DOX, Adriamycin) the first identified anthracyclines with daunorubicin. Daunorubicin; (DNR) was isolated from pigment-producing Streptomyces Peucetius. In 1969, this fungus was exposed to the mutagen N-nitroso-N methyl urethane, which resulted in the variant species S. peucetis var. caesius, which produced doxorubicin. By 1974, doxorubicin had been authorized for marketing as a chemotherapy drug in the USA and still in clinical use until today as one of the most potent antitumor drugs [22, 23].

Doxorubicin is a photosensitive chemotherapeutic drug that is soluble in water and orange to red in color at neutral pH [24]. The chemical structure of doxorubicin consists of an aglycone ring which coupled with an amino sugar. The aglycone comprised of a tetracyclic ring with quinone-hydroquinone adjacent groups, a methoxy substituent and a short side chain with a carbonyl group. The sugar component (called daunosamine) is attached by a glycosidic bond to one of the rings and consists of a 3-amino-2,3,6-trideoxyL-fucosyl moiety and the side chain of DOX terminates with a primary alcohol [25].

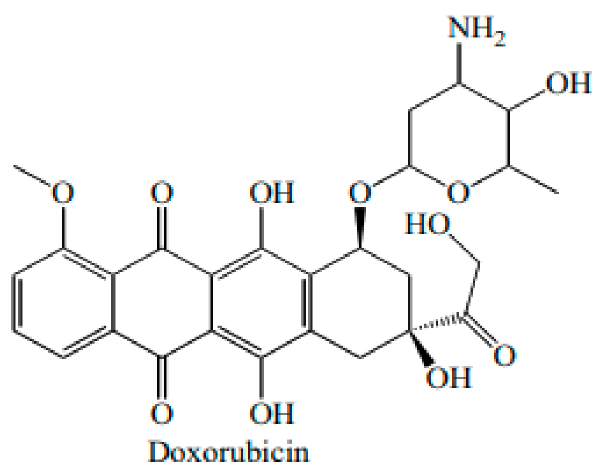


Figure 2.1. *The chemical structure of Doxorubicin [10]*

DOX is one of the most effective clinical chemotherapy medications that administered by itself or combined with other drugs, and it has the broadest range of activity compared to the rest of its class agents. In the therapeutic field, despite the production of other derivatives, DOX is still the most commonly used clinical chemotherapy drug, with low doses effective against a wide range of cancers [26]. DOX is used to treat hematological malignancies and solid tumors, which include bile ducts, uterus, prostate, breast, ovary, stomach and oesophagus, liver tumours, osteosarcomas and soft tissue sarcomas, childhood solid tumors, Kaposi's sarcoma, Wilms Tumor, acute myeloblastic and lymphoblastic leukaemia [10].

Doxorubicin, like the majority of cancer therapies, is rarely utilized alone. It's used in combination with other anticancer drugs such as taxanes, nitrogen mustard analogs, platinum drugs, vinca alkaloids, and fluoropyrimidines [27]. The most common doxorubicin-containing regimens are AC (Adriamycin, cyclophosphamide) [28],TAC (taxotere,AC),CHOP(cyclophosphamide, hydroxydaunorubicin, vincristine, prednisone) [29], ABVD (Adriamycin, bleomycin, vinblastine, dacarbazine) [30]. The combination of paclitaxel and doxorubicin has been shown to be a preferable and active regimen in the initial therapy for metastatic breast cancer[31].

2.2.2. Cell entering

Doxorubicin drug passes through tumor cells by using simple diffusion and then binds to the 20S proteasomal subunit of the proteasome with high affinity in the cytoplasm to form (DOX proteasome complex), which moves into the nucleus that considered the primary site of drug localization through nuclear pores aided by signals of nuclear localization. Since the affinity of DOX for DNA is higher than for the proteasome, DOX will bind to the DNA after separating itself from the proteasome as it shown in step 1,2,3 respectively in Fig.2.2 [32].

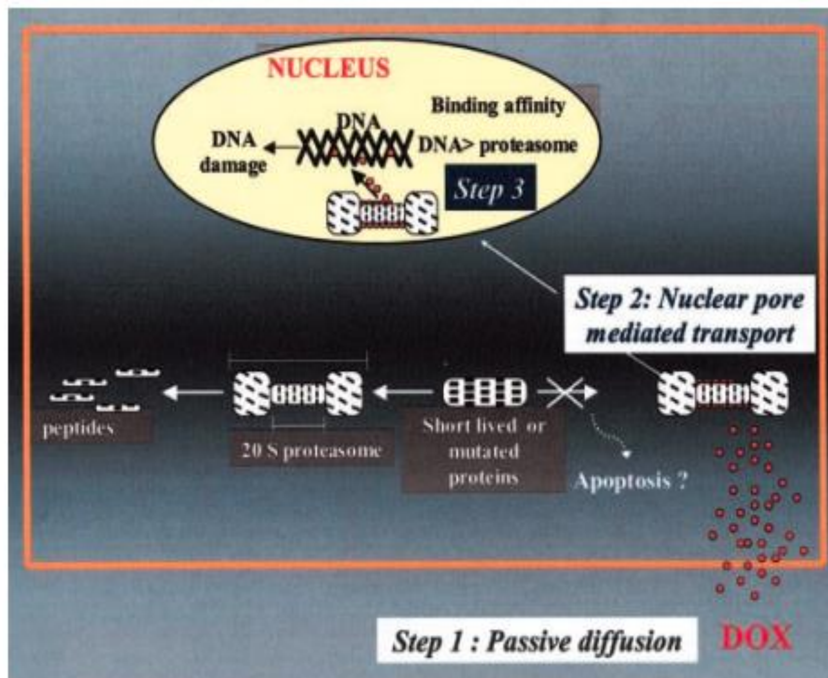


Figure 2.2. *Entry of DOX into the nucleus [25]*

2.2.3. Mechanism of action

The cytotoxic effects of DOX come from multiple mechanisms of action that are worked together or independently [8]. Dividing cells are the main target of DOX which affects them at various phases of the cell cycle: cells at M and S phases are most affected (by inhibiting of DNA synthesis), G2 phase (may has an impact on protein synthesis or structural configuration) and to a lower degree G1 phase (maybe by inhibiting RNA synthesis but it still unclear). On the other hand, cells that have not yet divided collect less drug and may die as a result of RNA synthesis inhibition [33].

The nucleus accounts for approximately 80% of the intracellular distribution of DOX [13] which means the concentration of doxorubicin in nuclear compartments is 50 times higher than in the cell cytoplasm [9]. After localizing in the nucleus, DOX is capable of intercalating into the DNA helix and binding to proteins that play important roles in DNA replication and transcription. These kinds of interactions inhibit DNA, RNA, and protein synthesis, eventually causing cell death [8, 34].

Also, it can activate wide variety of molecular signals like AMPK (AMP-activated protein kinase) inducing apoptosis and can alter the ratio of Bcl-2/Bax which in turn will affect the apoptosis pathway that will result in activation of various caspases lead to apoptosis [9].

The primary mechanism of action of DOX is topoisomerase II inhibition, which causes DNA damage. Many other mechanisms and molecular effects of DOX were studied and documented; induction of oxidative stress and formation of free radicals that induces damaging of DNA or lipid peroxidation, DNA cross-linking, affects directly to the membrane, intercalation into DNA causing the inhibition of macromolecules synthesis, DNA binding and alkylation, and the ability to interference with DNA winding or DNA strand separation and helicase activity are also included (shown in Fig 2.3) [8, 35]

2.2.3.1. ***Topoisomerase II inhibition***

- Topoisomerases Enzymes

DNA topoisomerases are divided into two types, type I and type II, based on the number of DNA strands they break and how they alter the genetic material's topological state. This family of enzymes is essential for all organisms' survival, and they alter DNA topology without changing its structure and sequence. They work by causing transient breaks in the double helix for DNA transcription and replication by cleaving one strand of a DNA duplex and passing a second duplex through this transient cleavage. The resulting intermediate is known as a "cleavable complex." [37, 38]. Topoisomerase I causes transient single-strand DNA breaks that reseal after the double helix's twisting status is changed [25].

Topoisomerase II enzyme contains multiple polypeptide chains and has tyrosyl residues in the active site that initiate DNA cleavage. It works by attacking nucleophilic on the phosphate of the nucleic acid backbone and it needs ATP to function. When adenosine triphosphate (ATP) is present or deoxyadenosine triphosphate (dATP), this DNA TOPO II is capable of acting as a catalyst for a variety of topological isomerization reactions. Like for instance; catenation and decatenation, knotting and unknotting, and by temporarily breaking the double strands of DNA, it relaxes superhelical twists and then resealing the breaks [39, 40].

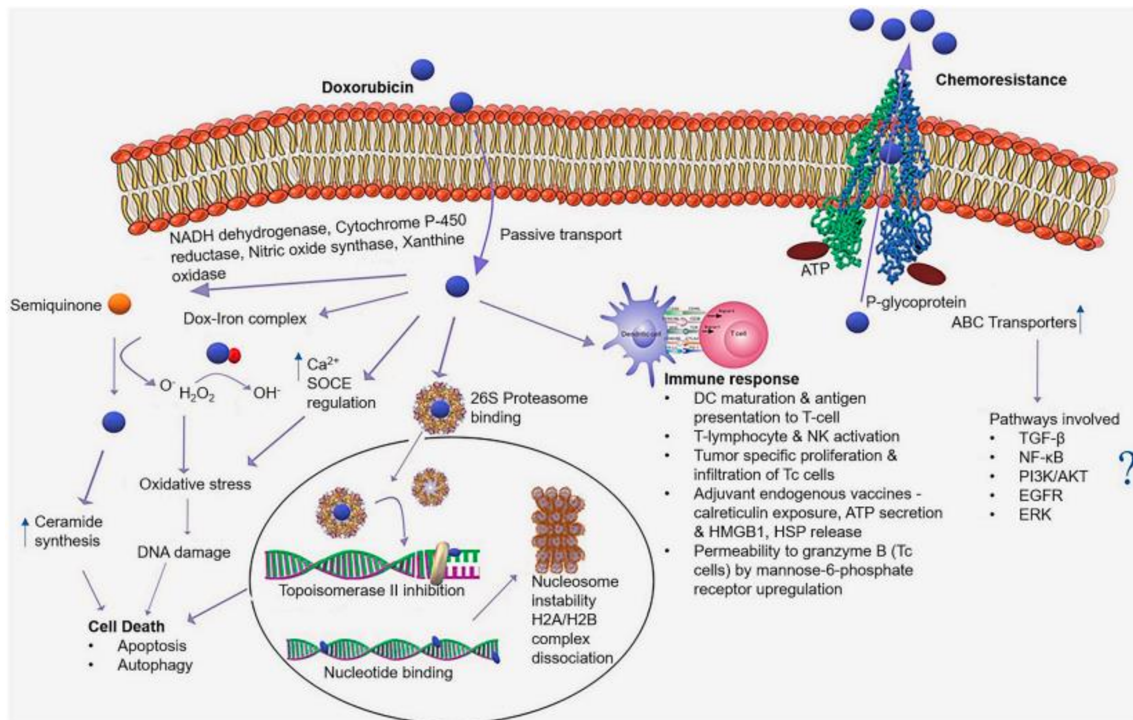


Figure 2.3. *DOX mechanism of action and all molecular targets of doxorubicin [36].*

- Inhibition of topoisomerase II **and** I

Topoisomerases are targets for doxorubicin, it takes advantage of topoisomerases' fatal nature and corrupts them by raising steady-state levels of DNA cleavage complexes, Such an action transforms topoisomerases into powerful physiological toxins capable of inducing mutagenic and lethal events [41].

DOX works on the cleavable complex which is formed as an intermediate during the process of cleavage of DNA. It links and places itself in the protein-DNA interface of the active site of topoisomerase II, forming a DOX-DNA-topoisomerase II ternary complex, which leads to impeding DNA resealing and DNA double-strand break (DSB). This damage in DNA which is mediated by topoisomerase II, is accompanied by a G1 or G2 growth arrest and a programmed death of cells. DOX has also the ability to inhibit DNA topoisomerase I enzyme [17-20, 42-45].

The mechanism of action of DOX in the formation of this ternary complex and confirming its stability is dependent on well-defined structural determinants. The planar ring system is significant for intercalation into DNA as rings B and C overlap with adjacent base pairs and ring D passes through the intercalation site. The sugar residue and the cyclohexane ring A appear to be important. More specifically, the sugar moiety that

exist in the minor groove is crucial to the function of DOX as poison to topoisomerase II. Increasing the drug activity can be occur by removal of the 4-methoxy and 3'-amino substituents [44, 45].

There are many candidates of pharmacogenes that modulate this DOX antitumor pathway. These include the enzymes that take part in the DNA repair mechanisms and in the controlling of cell cycle, and these are; *MLH1, TOP2A, MSH2, TP53,* and *ERCC2* genes, shown in Fig 2.3, [27].

2.2.3.2. *Generation of free radicals*

Formation of free radical due to redox-cycling:

Generating free radicals is one of the important antitumor mechanisms that DOX uses to cause oxidative damage in cancer cells [25].

The chemical structure of DOX contains quinone moiety in ring C, which acts as an electron acceptor. One-electron addition to the quinone from reduced nucleotides and by cellular flavoproteins result in formation of an unstable metabolite semiquinone. The reaction is mediated by a variety of cellular oxidoreductive enzymes such as nicotinamide adenine dinucleotide NADH dehydrogenase, endothelial nitric oxide synthase, NADPH cytochrome P450 reductase (CPR), and xanthine oxidase. Semiquinone quickly reverted to DOX in the presence of oxygen by reducing molecular oxygen to reactive oxygen species (ROS) such as superoxide (O2 -) and hydrogen peroxide (H2O2), Fig 2.4.

Generating these free radicals result in cleavage / degradation of DNA, oxidative stress, lipid peroxidation, membrane damage and apoptosis. In addition, the semiquinone in the absence of oxygen can undergo a reaction and van be oxidized with the bond between ring A and daunosamine, leading to reductive deglycosidation (loses its sugar moiety) and the formation of 7-deoxyaglycone, which is a potent DNA alkylating species. Aglycones have high lipid solubility, which enables them to intercalate into biologic membranes and generate reactive oxygen species near to sensitive targets [46, 47].

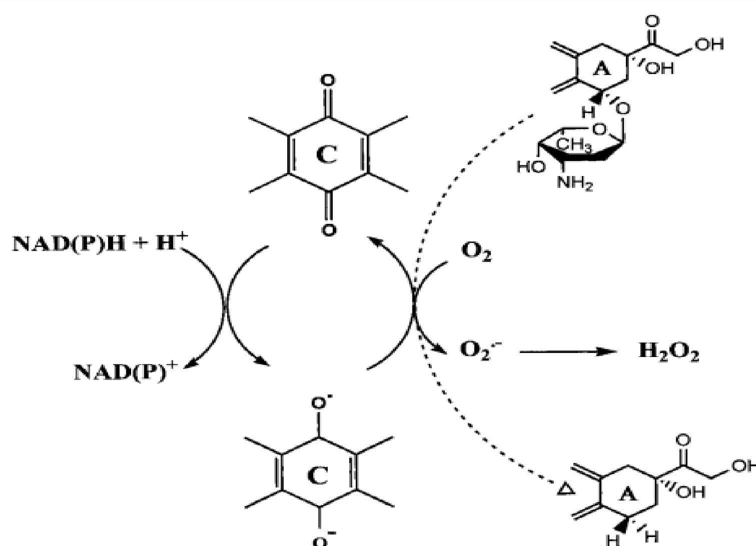


Figure 2.4. *One-electron redox cycling of DOX [25].*

Formation of free radical due to Doxorubicin-iron complex:

DOX is an iron chelator; it can form complexes with iron. Free radical production can be induced by the doxorubicin-iron II complex in two distinct ways., the first one needs a reducing system while the second mechanism depends on the complex itself without a reducing system [48]. When a reducing system is present, this DOX-Fe 2+ complex can react with oxygen (O2) which causes superoxide (O2 -) production as a result, which subsequently undergo dismutation to create hydrogen peroxide (H202). H202 also can interact with the complex to form hydroxyl (OH) radicals, and the complex is transformed into doxorubicin-iron III (DOX-Fe3+) [49, 50].

Enzymatic reduction of DOX-Fe3+ occurs by NADH cytochrome P-450 reductase or by an interaction with reduced thiols such as glutathione (GSH) or cysteine to form back DOX Fe2+. This DOX Fe2+ complex can, intern, react again with 02 or H202 as shown in the upper part of Fig 2.5. In this mechanism there is no formation of any metabolites and production of these radicals can continue without stopping. [48].

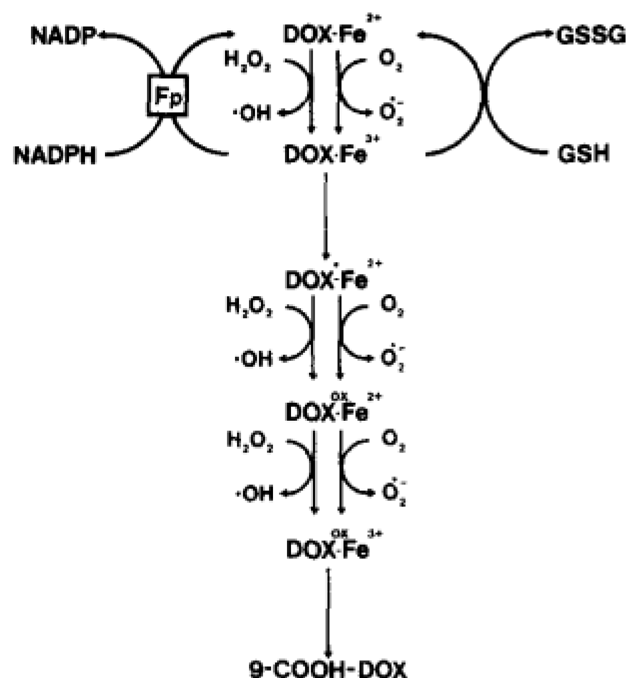


Figure 2.5. *Production of free radical from doxorubicin-iron complex [48].*

In the second mechanism where is no reducing system, an intramolecular redox reaction allows the DOX-Fe 3+ to reduce its iron through either oxidizing its hydroquinone moiety at ring B or by oxidizing the side chain on C9. Oxidizing the hydroquinone moiety at ring B produces an oxidized form of the doxorubicin semiquinone radical [51, 52]. DNA is most likely will catalyze this intramolecular iron reduction. Then, when oxygen is present, this complex can be oxidized, which in turn can leads to O2 – radicals production , but at the same time it can react with H202 to produce OH radicals. [48].

Electrons from the doxorubicin C9 chain can keep the complex's iron reduced, promoting free radical production until the side chain has been completely oxidized, which will ultimately result in the formation of the oxidized metabolite of 9-COOH-doxorubicin (doxorubicin 9-dehydroxyacetyl-9-carboxyl doxorubicin) [52]. These doxorubicin-iron complex-driven hydroxyl radical are probably involved in damaging of DNA, lipid, and protein [48].

Superoxide (O_2^-) production as a result, which subsequently undergo dismutation to create hydrogen peroxide (H202). H202 also can interact with the complex to form hydroxyl (OH) radicals [48].

2.2.3.3. *Formation of DNA adducts*

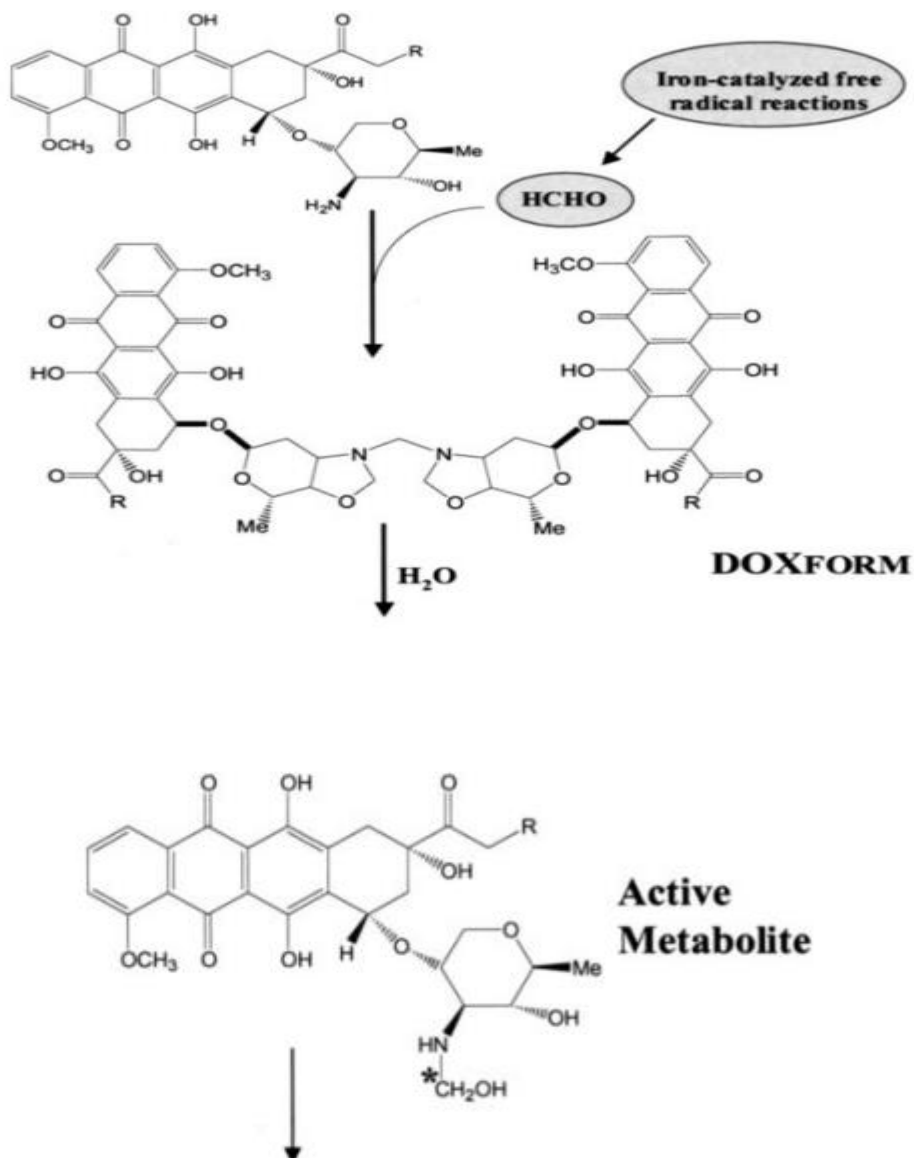
DOX can cause apoptosis through a mechanism that is not dependent on topoisomerase II, this mechanism is known as DNA adduct formation. In topoisomerase II-deficient cells, adduct formation, and apoptosis can be effectively induced, and can be mediated by formaldehyde (HCHO, FORM) [38]. DOX can produce formaldehyde in cells from carbon cellular sources such as spermine and lipids via iron-mediated free radical reactions [53]. Then, DOX and formaldehyde react to yield a compound called (DOXFORM) which is made up of two drug molecules that are joined by three methylene groups. Two of the drug molecules form oxazolidine rings, and one of the drug molecules binds the oxazolidines at their 3-amino nitrogens [54]. This preactivated form of doxorubicin is more readily absorbed by cells, and increased potential of nuclear targeting, stays in the nucleus longer and accumulates in DNA, and a decreased cellular release, which makes DOXFORM significantly more cytotoxic than doxorubicin to cancer cells that are resistant to anthracycline[38, 55].

After that, DOXFORM hydrolyzes to form an active monomeric metabolite [54]. DOXFORM has a unique ability to make intercalation into DNA by forming a covalent bonding with the 2-amino group of a G-base in the minor groove of DNA. It occurs at 5′ GpC-rich sequences via a covalent aminal (N-C-N) linkage between the doxorubicin 3′ NH2 group and the guanine N-2 amino group [36]. This unique combination of intercalation, covalent bonding, and hydrogen bonding is known as anthracycline virtual cross-linking of DNA (Figure 2.6) [53].

The adduct formation mechanism is very useful and can improve DOX cytotoxicity in sensitive cells as well as cells that had formed resistance. Resistant cells might have resistance to DOX because of P glycoprotein (Pgp) overexpression [56]. Reduced or increased expression of enzymes mediating DOX redox activation might lead to resistance also, for example, reduced expression of NADPH cytochrome P450 reductase or increased ROS detoxifying enzyme expression [e.g., superoxide dismutase (SOD), GSH peroxidase, catalase][57].

In tumors with Pgp-overexpressing, this conjugate decrease DOX affinity for Pgp and in competition with Pgp, DOX-FORM conjugates bind to DNA quickly [9]. While in cells with higher amount of ROS scavengers or lower amount of redoxactivating enzymes, preconjugation of DOX with formaldehyde would make DOX redox cycling unneeded and subsequent generation of HCHO from cellular carbon sources [53].

Additional proof of the enhanced activity of anthracycline-FORM conjugates was provided by experiments in which doxorubicin was given alongside medications that released formaldehyde in the cell [54].



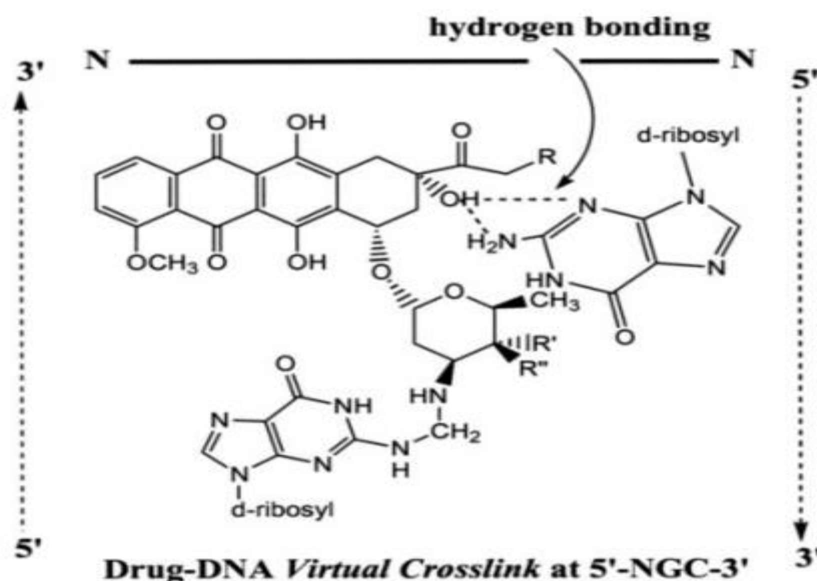


Figure 2.6. *The Formation of DOXFORM, hydrolysis to active metabolite, and mechanisms of drug-DNA virtual cross-link [25].*

2.2.4. DOX-induced cardiotoxicity and possible interventions

Some of frequent side effects of its use include acute nausea, vomiting, gastrointestinal disturbances [58], stomatitis [10], fever [26], and elevated cardiovascular toxicity rates like arrhythmias, low blood pressure, rapid heartbeat, and congestive heart failure which considered as the most serious and dose-limiting adverse feature of DOX [59, 60]. Regarding to DOX cardiotoxicity, mitochondrial toxicity was emphasized as a key factor in the causing of a recognizable cardiomyopathy through treatment with DOX [26].

Apoptosis induced by DOX in tumor cells is thought to be the same mechanism through which toxicity may occur in healthy cells, although by different routes [10]. DOX has a mitochondrial specificity and inhibits the major functions of mitochondria [61]. This anticancer drug has a high affinity to specific 4-acyl chain phospholipid that exists in the inner mitochondrial membrane called cardiolipin [62]. Cardiolipin acts as an anchor for cytochrome c and is required for the adenine nucleotide translocator to work to the maximum [63, 64]. DOX associated with cardiolipin to form a complex that prevents cytochrome c from being anchored by cardiolipin, blocks the binding of creatine kinase to the inner mitochondrial membrane, decreases the activity of many other critical cardiolipin- dependent mitochondrial enzymes, and also, cardiolipin that has been

oxidized can contribute to activate cell death initiated by mitochondria (Figure 2.7) [65-67].

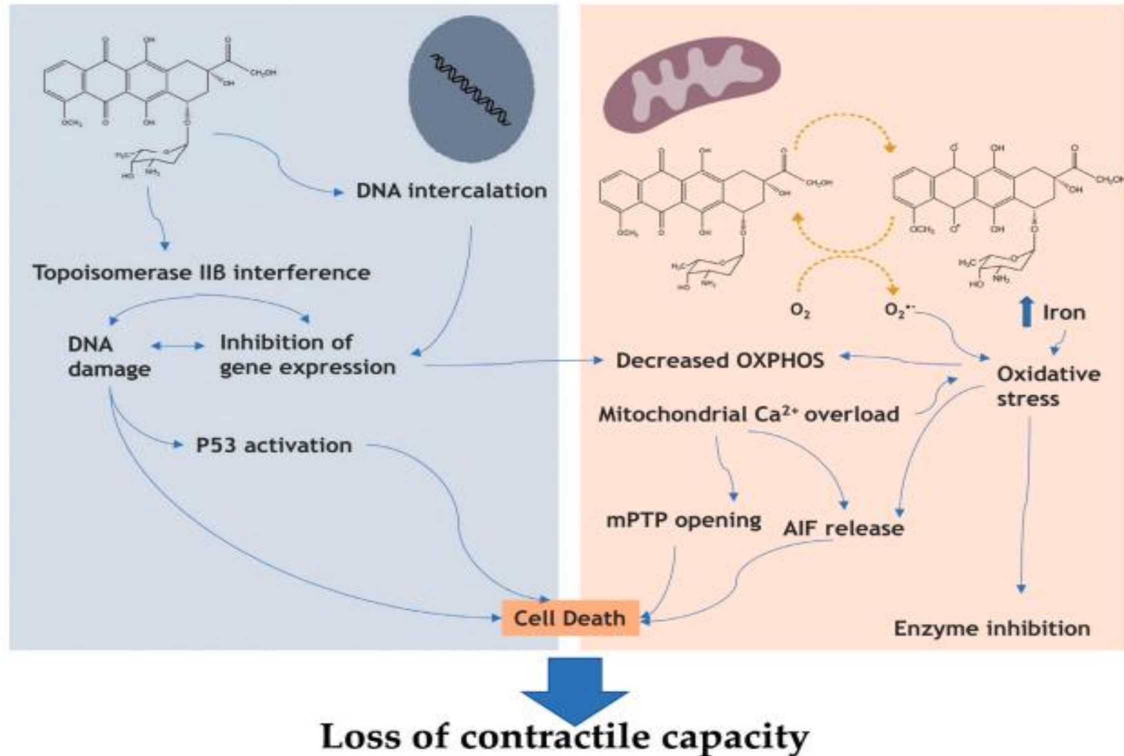


Figure 2.7. *Nuclear and mitochondrial effects of doxorubicin anticancer drug in cardiomyocytes [26].*

Davies and Doroshow demonstrated that DOX undergo a redox cycling by complex I (NADH dehydrogenase enzyme) of the mitochondrial respiratory chain [68, 69]. This happens at the expense of NADH or by the cytochrome P450 system using reducing equivalents from NADPH since NAD(P) H serves as an electron donor needed for doxorubicin reduction. The drug goes into mitochondria and reacts with complex I leading to produce of semiquinone radical intermediates, which can in turn re-oxidize in the existence of molecular oxygen (O2) generating reactive oxygen species (ROS), which can then react with the biomolecules of mitochondria in the vicinity include nuclei acids ,proteins, and lipids [10, 26, 70].

In addition to that, an excess of iron in the mitochondria can cause increasing mitochondrial oxidative stress fig 2.7. Ichikawa et al measured an increase in iron accumulation in mitochondria from isolated cardiomyocytes and hearts from patients - specifically suffering from DOX-induced cardiomyopathy [71].

Doxorubicin drug also induces mitochondria to lose the ability they have to maintain and develop electrochemical gradients across the inner membrane, and this effect is depending on the dose. This induction of the mitochondrial permeability transition result from the opening of mitochondrial permeability transition pores (mPTPs) [72]. The opening of this channel causes a non-selective permeabilization of the inner mitochondrial membrane [73] and this can lead to cell death [26]. The mPTP is a high conductance, non-selective channel that connects the inner and outer mitochondrial membranes [74, 75], being regulated by many physiological factors [76, 77], for example, both the oxidation of specific thiol residues in mitochondrial proteins and the accumulation of excessive calcium in the matrix are considered critical regulators and inducers of permeability transition pore (PTP) induction [78].

Oliveira and collaborators [79], asserts that DOX treatment induces an increase in the quantity of oxidized thiol residues in proteins of the PTP complex. Also, it increases the permeability of mitochondria leads to release of the AIF (apoptosis-inducing factor) which is involved in caspase-independent cell death [26]. The authors also noticed a drop in the amount of two key antioxidant defenses: gluthathione (GSH) and vitamin E, which result in a decreased protection against ROS produced during DOX redox cycling [79, 80].

In addition to that, Inside the mitochondria DOX has the ability to form adducts with the mitochondrial DNA (mtDNA) that disrupt mitochondrial function, protein expression, and lipid oxidation [81].

On the other hand, DOX has effect on the nuclei of cardiac cells, it intercalates into DNA after entering and accumulating in the nuclei and interferes with topoisomerase 2β. Zhang et al demonstrated that Inhibition of topoisomerase IIβ in cardiomyocytes by DOX is a primary cause of the cardiotoxic cascade which inhibits mitochondrial biogenesis and gene expression, thereby inhibiting secondary oxidative phosphorylation (OXPHOS). DOX-induced DNA damage can also result in p53 overexpression, which can lead to the increase of expression of downstream proapoptotic targets, activating cell death as shown in Figure 2.7 [82].

The heart is a particularly vulnerable organ to the toxicity caused by DOX for a variety of reasons. As previously stated, DOX forms complexes with cardiolipin, an important part of the mitochondrial inner membrane, and heart cells have a greater mitochondrial density per unit volume compared to the majority of other types of tissue.

[83]. Also, the existence of a heart-specific isoform of the mitochondrial complex I (NADH dehydrogenase enzyme) which capable of initiating DOX redox cycling and, as a thus, promoting ROS formation [84], combined with the heart's low levels of antioxidant defenses when compared to other tissues together contribute to the heart's high susceptibility to DOX-induced toxicity [85].

Possible pharmacological interventions include MitoQ, Carvedilol, and Berberine which are among the compounds that prevent mitochondrial oxidative stress (including by chelating excess iron, as in the case of carvedilol), with secondary protection against DNA damage. Dexrazoxane is the only FDA-approved agent to reduce anthracycline toxicity, and it works through two different mechanisms: iron chelation and topoisomerase 2-inhibition fig 2.8 [86]. Furthermore, weekly administration and continuous infusion schedules of DOX have been reported to be associated with reduced of plasma peak, cardiotoxicity, CHF, and higher cumulative drug dosage [87].

In addition, nonpharmacological interventions involve both restricted calorie consumption and exercise fig 2. 8. They function by triggering metabolic modulators like AMPK (AMP activated protein kinase) and many others. In the case of exercise, it increases cell defenses like heat shock proteins and antioxidant networks. Physical activity has also been shown to reduce the quantity of DOX collected by cardiomyocytes, and indirectly lowering cardiac risk [26].

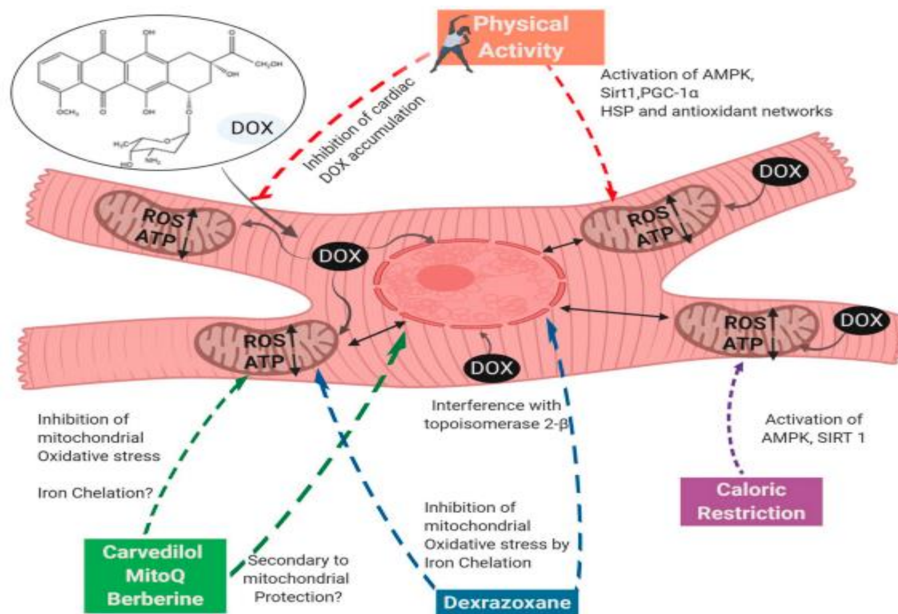


Figure 2.8. *Pharmacological and non-pharmacological interventions aimed to decrease DOX cardiotoxicity [26].*

2.2.5. Administration of DOX

Doxorubicin is typically administered intravenously at 21-day intervals. While DOX can be given quickly (over 15 to 20 minutes), slowing down the administration of the liposomal formulation is recommended to minimize the possibility of infusion reaction [88].

Many of DOX nanoformulations have been approved for therapeutic use. For example, the DOX loaded FluoreneTetraphenylethene-Polyethylene glycol (FTP) nanoparticles. FTP nanoparticles in the presence of acidic pH will release DOX and had a similar anti-neoplastic effect as free DOX [89].

Also, for photothermal and chemo combination therapy, DOX- encapsulated gold nanocages coated with thermosensitive liposomes were investigated [90]. Cholesterol-based redox-sensitive DOX-loaded nanoparticles have been synthesized, which when cleaved by intracellular glutathione (GSH) result in rapid drug release. *In vitro*, These nanoparticles were discovered to be more effective at nuclear internalization and cytotoxicity [91]. A platelet membrane-based biomimetic nanocarrier loaded with DOX has high tumor affinity and a long circulation half-life [92].

2.2.6. Doxorubicin metabolism and clearance

Doxorubicin has a rapid distribution into body tissue with a distribution half-life of 3–5mins, which indicates a rapid absorption of DOX by cells [9]. At the same time, the elimination half-life of Doxorubicin is up to 48 hours [36], suggesting that doxorubicin takes more time to be removed from the tissue than its uptake [93]. This anticancer drug accumulates mostly in the liver, because of its role in metabolism [9]. In the liver and kidney, DOX undergoes metabolism and converted into its main metabolite doxorubicinol via NADH-dependent two-electron reduction of the carbonyl side chain into a secondary alcohol, by cytosolic enzymes like aldo-keto reductases or carbonyl reductases (CBR1 and CBR3) [94, 95].

Semiquinone is the second metabolite which produced by cytoplasmic oxidoreductase enzymes. This metabolite is unstable and reconverted to DOX with generating of free radicals [96]. Enzymes like NADPH-dependent hydrolase and reductase type glycosidases, as well as xanthine dehydrogenase, cleave reductively the glycosidic bond and carbonyl side-chain groups to form the aglycones (7-deoxyaglycones and hydroxyaglycones (XDH)) which are the minor third metabolites of DOX [96]. The

7-deoxyaglycones are non-cytotoxic and formed by the microsomal or mitochondrial oxidoreductases while the hydroxyaglycones are formed by the cytosolic enzymes [47, 97]. These aglycones have the ability to enter the mitochondrial membrane and make intercalation because of their higher lipophilicity, compete with coenzyme Q10, and produce superoxide radical [98].

The hepatobiliary pathway is responsible for doxorubicin clearance. After 24 hours, about 10-20% of DOX is eliminated in the feces, and 50% after 150 hours. The percentage of the drug that is eliminated via urine out of the 5-12% as well as roughly 40% of the given dose is excreted in the bile and is eliminated within five days [9].

In addition to the intracellular mechanisms of DOX, it also stimulates immune cells to clear the tumor [36], since chemotherapy-treated tumor cells can show surface changes or release substances that assist the immune system to recognizes the tumor and eliminate it [44, 99].

2.3. Sulforaphane (SFN)

2.3.1. General information about SFN

Sulforaphane is an oily phytochemical isothiocyanate that contains sulfur. It has been derived from cruciferous vegetables such as broccoli sprouts, Brussels, cauliflower, and cabbage [100]. Sulforaphane is presents in plants in the form of glucosinolates. The myrosinase-catalyzed process converts glucosinolates to sulforaphane during glucosinolate degradation (Figure 2.9) [101].

In 1992, SFN has received attention for its own activities [108], And now it is considered as one of the promising natural chemopreventive and therapeutic agents [13]. Over time it was shown to have acted as an anti-inflammatory and antioxidative agent [102, 103] ,and can act against many number of chronic diseases, such as type 2 diabetes [104], Helicobacter pylori-inducing intestinal inflammation [105], and autism spectrum disorder [106, 107].

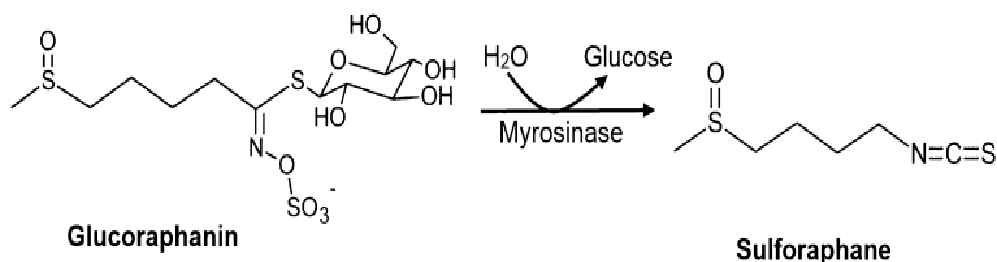


Figure 2.9. *The transformation process of glucoraphanin to sulforaphane [12].*

Besides, SFN is a potent, green anti-tumor agent used in treatment of many types of cancer [108] including breast [109], cervical [110], non-small-cell lung cancer (NSCLC) [111], bladder cancer [112], renal cell carcinoma (RCC) [113], and colon and prostate cancers [114] pancreatic, melanoma, and ovarian cancers [12].

Several clinical trials were conducted to assess the safety and tolerability of SFN at the doses used. Bioactive SFN is widely recognized as a promising chemopreventive agent with effects against many types of cancers due to its effectiveness, safety, nontoxic nature, without any side effects, and its inexpensive price [115].

2.3.2. Mechanism of anticancer activity of SFN

SFN has many pathways to exerts its antitumor action which include the following (Figure 2.10).

2.3.2.1. *Modulating phase I and II metabolic enzymes*

Modulating metabolic enzymes of both Phase I and II SFN can interfere with initiation stage of cancer. Phase I metabolic enzymes can convert procarcinogens to carcinogens and initiate carcinogenesis. To modulate phase I metabolism, SFN can directly interfere with P450 enzymes and inhibits CYP1A1 and CYP3A4 and reduces the activity of CYP3A4 [116-118]. Phase II enzymes, such as NAD(P)H: quinone oxidoreductase-1 (NQO1), catalase, glutamate-cysteine ligase (GCL) and more are included in cytoprotective genes. They can be upregulated by SFN by activating nuclear factor erythroid-2- (NF-E2-) related factor 2 (Nrf2). Nrf2 is a transcription factor that plays an important role in the antioxidant stress response [115], that mediated these phase II enzymes [119].

2.3.2.2. *Cell cycle arrest*

SFN can cause cell cycle arrest by blocking primarily cell cycle in G2/M phase [12] which can lead to increased protein levels of p21WAF1/CIP1 (an inhibitor of cyclin-dependent kinases) [120]. It also inhibits tumor proliferation which was associated with downregulation of cyclin B1 [110] and cyclin D1 genes [120]. also, blocking activity in G1/S was reported [121, 122].

2.3.2.3. *Apoptotic pathways*

Apoptosis can also be modulated by SFN. It can activate the intrinsic and extrinsic pathways of apoptosis. The activation of intrinsic or mitochondrial pathway leads to release of cytochrome C from the mitochondria, which in turn binds to apoptosis protease activation factor-1 (Apaf-1) and eventually activates caspase-9 that lead to inactivation of poly (ADP-ribose) polymerase (PARP) - a DNA repair enzyme Fig 2.10. This Caspase is a family of cysteine proteases dependent pathway of apoptosis [121].

Extrinsic or death receptor pathway includes activation of death receptors by ligands like tumor necrosis factor-α (TNF- α) which lead to induction of effector caspases-9 and caspase-8 [12, 121, 123, 124].

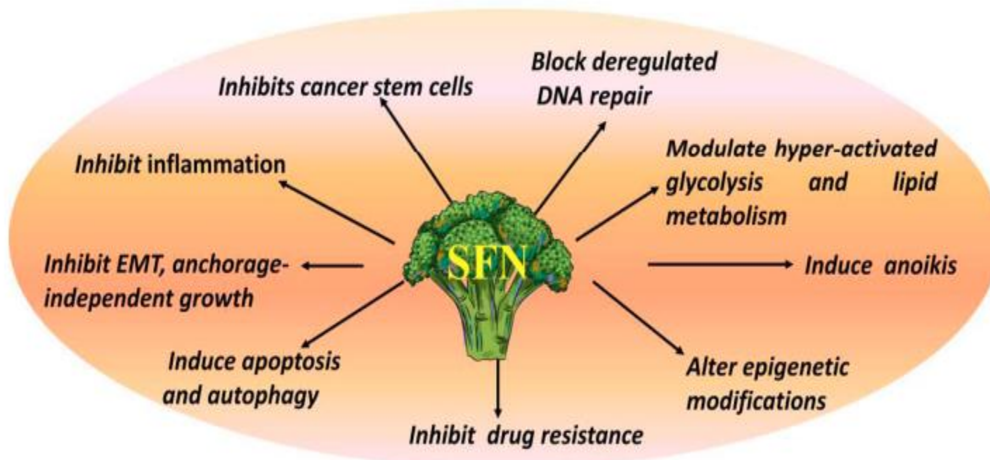


Figure 2.10. *Mechanisms contributing to anti-cancer effects of sulforaphane (SFN) [108].*

SFN-induced apoptosis can also occur by generation of reactive oxygen species (ROS)which is one of the most important mechanisms [125]. Induction of the proapoptotic Bcl-2 family members, and mitogen-activated protein kinases (MAPK) signal transduction [12, 126].

2.3.2.4. *SFN as HDAC inhibitor*

SFN is a natural histone deacetylase (HDAC) inhibitor [115]. Many types of cancer are affected by this mechanism when treated with SFN like breast, colon [127], prostate [128], and lung cancer [129].

HDAC enzymes remove acetyl groups added by Histone acetyltransferase (HAT) to lysine residues in the N-terminal tail, which leads to repression of the transcription and eventually cell growth inhibition with an increase in local or global histone acetylation [108, 115].

In breast cancer treatment, SFN inhibits the development of the tumor by inhibiting histone deacetylases 5 (HDAC5) through suppressing of upstream transcription factor 1, which is responsible for controlling transcription of HDAC5 [130]. Also, in hepatoma carcinoma cells, SFN acts by inhibiting of HDAC 5 and 11 which accompanied by increasing of methylation level of many genes related to cell proliferation, cell cycle, and antiapoptosis, which partly accounts for the apoptosis, cell arrest, and destruction of DNA induced by SFN [131].

2.3.2.5. *SFN inhibits cancer stem cells*

Cancer stem cells (CSCs) are a small group of cancer cells within malignant tumors. They are responsible for initiation and recurrence of malignant tumors after therapy and they possess special characteristics like potent metastatic and invasive ability, high resistance to chemotherapy, and capabilities of self-renewal [132, 133]. The chemopreventive effect of SFN can inhibits these cells through various mechanisms, such as activating apoptosis and autophagy pathways, targeting self-renewal signaling, and altering miRNAs [108].

There are numerous other mechanisms by which SFN can induce anti-cancer activity. SFN inhibits inflammatory signaling, modulates glycolysis and lipid metabolism [108], and is a potential DNA methylation modulator in cancer development and progression [115].

2.3.3. Enhancement of anticancer activity in combination therapy

A lot of studies demonstrated that SFN enhances the activity and the effectiveness of drugs and perform a synergistic effect when taken with them simultaneously via several mechanisms (Table 2.1) [12].

Table 2.1. *Synergistic anticancer effect of sulforaphane with therapeutic molecules in different cancer types[12].*

Cancer Types	Molecules showing synergism with Sulforaphane	Cell lines tested	Mechanism
Pancreatic Cancer	Gemcitabine, doxorubicin, 5-flurouracil, 17-allylamino 17-demethoxygeldanamycin, ibuprofen, aspirin, and curcumin	Mia-Paca-2 and Panc-1	Enhancement of cytotoxicity Enhancement of apoptosis Attenuation of cancer resistance Inhibition of self-renewal capacity/spheroid formation Inhibition of migration potential and Invasion Inhibiting clonogenic potential Inhibition of tumor growth
Breast Cancer	Paclitaxel, docetaxel, gemcitabine, clofarabine, afimoxifene, and lapatinib	SUM149, SUM159, MCF-7, T47D, SKBR-3, and BT-474	
Colorectal Cancer	Oxaliplatin, diindolylmethane, and (-) epigallocatechin-3-gallate	Caco-2 cells, 40-16, and HT-29	
Prostate Cancer	Taxol, cisplatin, and tumor necrosis factor (TNF)-related apoptosis ligand (TRAIL)	DU145 and PC3	
Cervical Cancer	Eugenol	HeLa	
Glioma	Resveratrol and temozolomide	U251 and LN229	
Multiple Myeloma	Arsenic trioxide	PCNY-1, MM1.S, KMS-11, and ARP-1	
Adenoid Cystic Carcinoma	5-Flurouracil	ACC-M and ACC-2	
Melanoma	Quercetin	B16F10	

2.3.4. Metabolism and absorption of SFN

Sulforaphane has –N=C-S group with an electrophilic carbon atom [134].The metabolism of Sulforaphane usually occurs by mercapturic acid pathway which is activated by the spontaneous in vivo reaction between glutathione and electrophilic –N¼C-S group [135]. The main SFN metabolite is SFN-N-acetylcysteine (SFN–NAC) (SFN-NAC) [136]. After 1-3 hours of SFN administration, the drug and metabolites reach the highest levels and within 24 hours they are cleared in human plasma to be excreted in the urine [137, 138]. The absorption and bioavailability of Sulforaphane is high. Once it goes into cells, SFN quickly interacts with GSH to form SFN–GSH, leading to its intracellular accumulation. This property favors SFN accumulation in cancer cells due to their high GSH content, promoting the anti-cancer effect of SFN [108].

SFN is an unstable compound where appropriate formulations of it are needed so it can be used in clinical settings to achieve higher bioavailability and better bioactive effects. Usually, formulations rich in glucoraphanin, which is the stable precursor of SFN,

are used clinically and the bioavailability of these formulations is also being investigated [138, 139].

Besides, formulations contain glucoraphanin with myrosinase enzyme in active form exhibit higher SFN bioavailability (3–4 times) than formula doesn’t compose of active myrosinase. Moreover, Fahey and colleagues demonstrates that Omeprazole (a proton pump inhibitor drug) or enteric coating myrosinase can increase the bioavailability of SFN of formulations contain broccoli sprout extract and active myrosinase. This might because of the myrosinase's protection from the effect of the stomach acidity [140].

2.4. Combination of Doxorubicin and Sulforaphane

The co-administration of natural agents with anticancer drugs is a promising approach to enhance the pharmacological and toxicological profile of cancer chemotherapy [141,142]. The phytochemicals in tumor tissues may affect and interact with many molecular targets and may increase the activity of traditional anticancer drugs. At the same time, they might decrease the adverse consequences caused by chemotherapeutic agents on normal cells [125].

The combination of DOX and SFN has improved the activity of DOX. SFN increased DOX proapoptotic activity in different cell lines and reversing doxorubicin resistance in p53-mutated cells and inducing apoptosis [13, 143]. Moreover, SFN also increases the ability of DOX to damage RNA [144] and can induce the autophagy through epigenetic pathways that lead to improve the sensitivity to DOX [145].

In fact, according to Bose and co-workers [146],the co-administration of DOX and SFN together could reduce the effective dose of DOX by 50% and thus a reduction of its adverse effects will occur, and SFN has shown the cardioprotective effects when given before DOX or administrated with Dox at the same time [125, 146].

3. MATERIALS

3.1. Chemicals and Cell Types

Doxorubicin Hydrochloride (DOX: Sigma-Aldrich, Germany), Sulforaphane (SFN: Bio vision), Minimum Essential Medium Eagle (DEMEM) (Sigma-Aldrich, Germany), Fetal Bovine Serum (FBS) (Sigma-Aldrich, Germany), Dimethylsulfoxide (DMSO) (Sigma-Aldrich, Germany), MTT (3-[4,5-dimethylthiazol2-yl]-2,5 diphenyl tetrazolium bromide)(Sigma-Aldrich, Germany), Caco-2 human colorectal cancer cell line (ATCC® HTB-37TM), NIH/3T3 mouse embryonic fibroblast cell line (ATCC® CRL-1658), U2OS human osteosarcoma cell line (ATCC® HTB-96) , HepG2 human hepatocellular carcinoma cell line (DSMZ ACC 180).

3.2. Instruments and Tools

Cedex smart Slide (Roche, Germany),Cedex (Innovatis), innovates Cedex XS Image Based Cell Analyzer AG (Roche, Germany), Desktop refrigerated centrifuge (Eppendorf), Cytation 3 Cell Imaging Multi-Mode Reader (Biotek),LEICA DFC495 microscope (Leica DM 300 Inverted microscope), Laminar Flow cabin (Heal Force), Tripsin-EDTA 10X (Pan, Biotch), Deep freezer refrigerator(Altus), Precision balance (Ohaus), Eppendorf AG centrifuge 5810 R, Water bath (Nuve), 12-channel automatic pipette (Axygen, UK) 10, 100, 1000 and 5000 µl automatic pipettor (Eppendorf, Canada), 15 and 50 ml centrifuge tubes (Isolab, Almanya), 96 well cell culture plate (TPP, Switzerland).

4. METHOD

To ensure sterilization, glass and metal materials were wrapped in aluminum foils and in a sterilizer with dry heat at 180ºC for 2 hours; liquid solutions were sterilized in an autoclave at 121ºC, 1.5 atm/Hg for 20 minutes.

4.1. Preparation of DOX and SFN Concentrations:

Master stocks of 100 mM concentrations were prepared by dissolving Doxorubicin and Sulforaphane in powder form in certain volumes of Dimethylsulfoxide (DMSO; Sigma) with known molecular weights. A certain number of aliquots were made and stored at 20ºC. During the experiments, it was diluted in fresh medium and used by preparing different concentrations. It was applied to the control group by adding 0.1% DMSO (solvent) in the medium.

4.2. Cell Culture

Cell culture is frequently preferred in cancer research, vaccine studies, *in vitro* cytotoxicity studies and in drug development. In cell culturing, we control conditions such as temperature, nutrients, and humidity to ensure the survival of cells of multicellular organisms and keep them away from contamination [147].

3T3, Caco-2, U2OS and HepG2 cells used in the study were used from the cell culture stocks of Anadolu University Faculty of Pharmacy Pharmacology Laboratory.

Caco-2 cells is used in RPMI-1640 medium containing 10% fetal bovine serum, 1% penicillin-streptomycin. NIH/3T3, U2OS and HepG2 cell lines are also used in DMEM medium containing 1% L-glutamine, 1% sodium pyruvate and containing 10% fetal bovine serum. All cells were incubated in an incubator with 5% CO2, 95% relative grown in culture medium at 37°C at humidity.

When the culture flask had a cell density of 80%, it was divided into subcultures and grown and used in the experiments.

4.3. Cell Counts

During the studies, the media in the flasks were changed every 2-3 days, and when almost the entire surface of the flask was covered with cells, the cells were passaged using the trypsinogen method. First of all, the medium on the adhered cells was removed with the help of a pipette. Cells were washed with 5 ml of PBS. After adding 1 ml of Trypsin-

EDTA (for 75 cm²) on the adherent cells and in contact with the entire surface, they were kept in an incubator for 3-5 minutes. Mechanical force was applied by lightly tapping to lift the cells completely. 5-7 ml of medium was added directly on it and the cells taken into the falcon tube with the help of a pipette were centrifuged for 5 minutes/1200 rpm. The liquid part accumulated in the falcon was poured in the opposite direction of the horizontal stance in the centrifuge.

It was ensured that only the cells remained in the falcon. 1 ml of medium was added to the cells and mixed well with the help of a pipette.

10 µl of Trypan Blue and 10 µl of cell suspension were added on a coverslip and subjected to pipetting. After, 10µl of the prepared mixture was added to the wells of disposable Cedex Smart Slides and placed in the Cedex XS (Innovatis) 27 automatic cell counter device. Thus, the number of cells in 1 ml was determined.

4.4. Determination of Cytotoxicity by MTT Method

By acquiring electrons, MTT (3-[4,5-dimethylthiazol2-yl]-2,5 diphenyl tetrazolium bromide), one of the tetrazolium salts in heterocyclic organic structure, is reduced to a structure called formazan. MTT is yellow, while the formazan produced is purple. The color produced by reduction is calorimetrically measured. Because formazan does not dissolve in water and crystallizes, it has been demonstrated that the use of dimethyl sulfoxide (DMSO) for solubilization is appropriate in the tests. The color reaction can only be carried out by living cells since the tetrazolium ring can only be broken by functioning mitochondria. For this reason, the amount of formazan produced gives the number of living cells. The dead cells lose the ability to reduce tetrazolium salts into colored formazan products. Viable cells with active metabolism convert MTT into a purple-colored formazan product [147, 148].

3T3, Caco-2, U2OS and HepG2 cells were cultured in growth medium at 37°C in a 5% CO_2 incubator. In order to evaluate whether the cells proliferated adequately and cell viability, counting was done and 5×10^3 cells in each well were seeded into the medium in 96-well plates. Cells were allowed to incubate for 24 hours to adhere to the plate. From 100 µM stock solutions prepared by dissolving Doxorubicin and Sulforaphane in DMSO; Dilutions of 50 µM, 25 µM, 12.5 µM and 6.125 µM were made. The prepared concentrations were applied to the cells individually and in combinations, and the cells

were again allowed to incubate for 24 hours. As a control, 0.1% DMSO (solvent) in the medium was used.

At the end of the incubation period, the medium in the plate was discarded and 100 µL of 1/10 MTT (5 mg/mLmedium) solution was added to the cells in the well, and the plates were kept in the incubator for 3 hours. At the end of the incubation, the medium containing MTT was discarded and 100 µL of DMSO was added to each well. The plates were read in the Cytation 3 Cell Imaging MultiMode Reader at a wavelength of 540 nm with 8 replicates (8 wells) for each group. The average absorbance values of the control wells were accepted as 100% and the cell viability values of the groups in which the concentrations of the substances were applied were calculated.

4.5. Evaluation of the Synergistic cytotoxic effects of combined concentrations

4.5.1. Coefficient of drug interaction (CDI) calculation

The CDI (Coefficient of Drug Interaction) is using to assess drug combinations' effects. The equation that is used to calculate CDI is: CDI = AB/ (A × B). According to the absorbance of each group, the ratio of AB combination groups to the control group is the ratio of the A or B single agent group to the control group. Thus, the CDI <1, $= 1$ or > 1 demonstrates that drugs have synergistic, additive, or antagonistic effects, respectively CDI <0.7 indicates that the drug is significantly synergistic [149].

4.6. Statistics

Graphs and CDI tables prepared according to cell viability% obtained as a result of MTT analysis were analyzed by applying one-way analysis of variance ANOVA and Tukey test as post-hoc in GraphPad Prism 8. Data are expressed as mean± standard error of the mean (SEM). Significance values; $p>0.05$ no difference, *$p<0.05$ difference, **$p<0.01$ significant difference, ***$p<0.001$ very significant difference, **** $p<0.0001$ most significant difference.

5. RESULTS AND DISCUSSION

5.1. Evaluation of the 24-Hour Cytotoxic Effects of Doxorubicin and Sulforaphane Single and in Combinations on 3T3 Cells

According to MTT results, cytotoxic effects of single applications of 50, 25, 12.5 and 6.125 µM concentrations of Doxorubicin and Sulforaphane on 3T3 cells and the combined applications of these two drugs on cell viability % were given in Figure 5.1-Figure 5.3.

A cell viability assay is performed based on the ratio of live and dead cells. As seen in Figure 5.1, it is seen that the viability of the cells decreased depending on increased concentration in the groups treated with 6.125, 12.5, 25 and 50 µM Doxorubicin compared to the control. The viability of 3T3 cells decreased due to the cytotoxic effect in the groups caused by DOX.

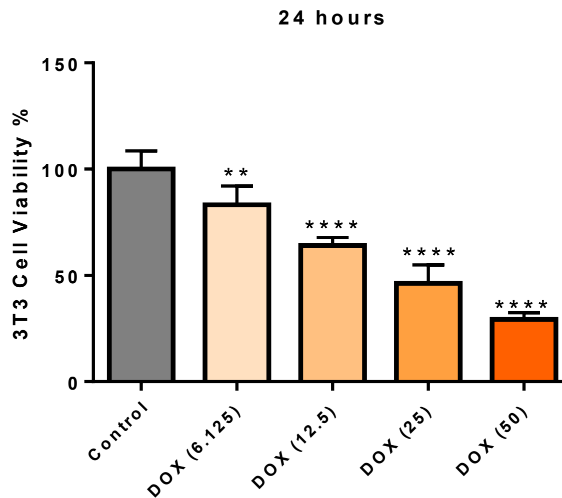


Figure 5.1. *In 3T3 cells, cell viability % rates of 6.25, 12.5,25, and 50 µM of DOX, according to MTT test results at 24 hours (Mean±SEM, n=8, **p<0.01, ****p <0.0001) (Control: 0.1% DMSO)*

According to our MTT results, the cell viability % rates in the groups treated with 6.125, 12.5, 25 and 50 µM concentrations of Doxorubicin in 3T3 cells were determined as 83,24%, 64,07%, 46,36% and 29,28% respectively, compared to the control. Statistical significance among the single groups in which 6.125, 12.5, 25, and 50 µM concentrations were applied was found to be **p<0.01 for 6.125 µM and ****p<0.0001 for all other concentrations when compared to the control group (Figure 5.1).

As shown in Figure 5.2 the viability of the cells decreased in the groups treated with 6.125, 12.5, 25 and 50 µM Sulforaphane compared to the control. The viability % of 3T3 cells was reduced in the groups due to the cytotoxic effect of increased SFN concentrations.

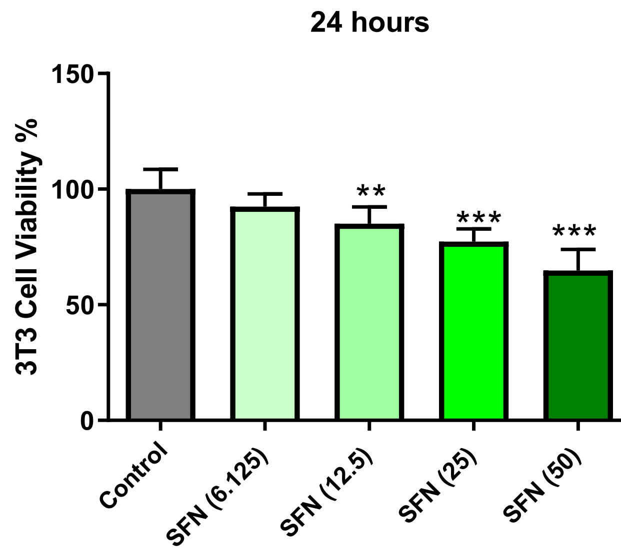


Figure 5.2. *In 3T3 cells, cell viability % rates of 6.125, 12.5, 25 and 50 µM of SFN, according to MTT test results at 24 hours (Mean±SEM, n=8, **p<0.01, ***p<0.001) (Control: 0.1% DMSO).*

According to our MTT results, the cell viability % rates in the groups treated with 6.125, 12.5, 25 and 50 µM concentrations of Sulforaphane in 3T3 cells were determined as 92,42%, 85.03%, 77.33% and 64,83% respectively, compared to the control.

Statistical significance among the single groups in which 6.125, 12.5, 25, and 50 µM were applied was found to be **p<0.01 for 12.5 µM, and ***p<0.001 for 25 and 50 µM when compared to the control group (Figure 5.2).

As seen in Figures 5.1 and 5.2, certain concentrations were selected for combined applications according to the MTT results of Dox and SFN. Concentrations of 6.125, 12.5, 25 µM for Dox and 12.5, 25, 50 µM for SFN were determined for combined applications.

As illustrated in Figure 5.3, it is seen that the viability % of the cells decreased in in the groups treated with 6.125, 12.5 and 25 µM of Dox and also with 12.5, 25, and 50 µM of SFN compared to the control. Also, the viability % of 3T3 cells decreased due to the cytotoxic effect in the groups treated with DOX and SFN combine groups, (DOX

6,125+SFN12,5), (Dox12.5+SFN25), (Dox12.5+SFN50), (Dox25+SFN25) and (DOX25+SFN50).

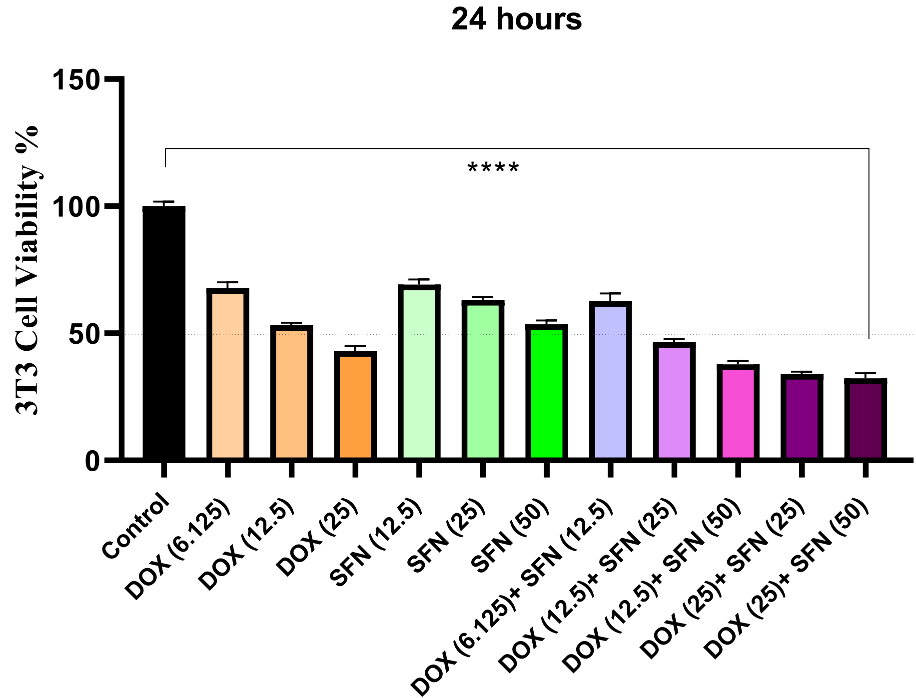


Figure 5.3. *In 3T3 cells, cell viability % rates of different concentrations of DOX and SFN single and in combine treatment, according to MTT test results at 24 hours (Mean±SEM, n=8, P values found in GraphPad Prism 8; ****p<0.0001) (Control: 0.1% DMSO).*

Our MTT results indicate that the cell viability % rates in the groups treated with 6.125,12.5 and 25 µM concentrations of Dox in 3T3 cells were determined as 67,85%, 53,18% and 43,09% respectively, compared to the control. Cell viability rates were found to be 69,17%, 63.16% and 53.58%, respectively, in the groups treated with 12.5, 25, and 50 µM concentrations of SFN. Whereas, in the groups treated with DOX and combined concentrations of SFN (DOX6,125+SFN12,5), (DOX12.5+SFN25), (DOX12.5+SFN50), (DOX25+SFN25) and (DOX25+SFN50) the cell viability % rates were determined as 62,72%, 46,62%, 37,87%, 34,08% and 32,25% respectively.

Statistical significance among all groups was found to be ****p<0.0001 when compared to the control group (Figure 5.3).

5.2. Evaluation of the 24-Hour Cytotoxic Effects of Doxorubicin and Sulforaphane Single and in Combinations on CaCo2 Cells

The cytotoxic effects of single applications of 6.125, 12.5, 25 and 50 µM concentrations of Doxorubicin and Sulforaphane on CaCo-2 cells and the combined applications of these two drugs on cell viability% were given in Figure 5.4 -Figure 5.6.

As seen in Figure 5.4, it is seen that the viability of the cells decreased depending on increased concentration in the groups treated with 6.125, 12.5, 25 and 50 µM Doxorubicin compared to the control group.

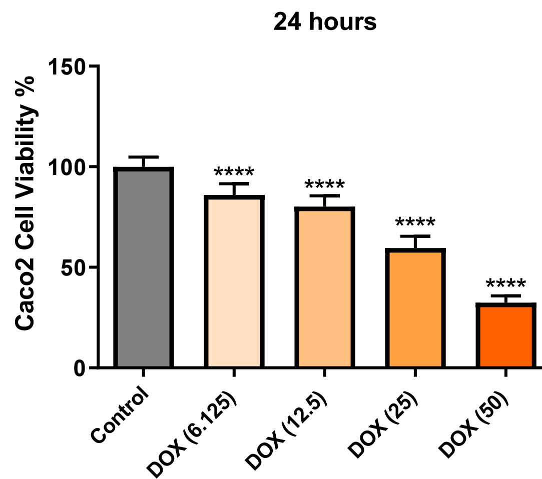


Figure 5.4. *In CaCo-2 cells, cell viability % rates of 6.125, 12.5, 25 and 50 µM of DOX, according to MTT test results at 24 hours (Mean±SEM, n=8, ****p <0.0001) (Control: 0.1% DMSO)*

According to our MTT results, the cell viability % rates in the groups treated with 6.125, 12.5, 25 and 50 µM concentrations of Doxorubicin in CaCo2 cells were determined as 85.88%, 80.12%, 59.50% and 32.43% respectively, compared to the control. Statistical significance among the single groups in which 6.125, 12.5, 25 and 50 µM DOX. concentrations were applied was found to be ****p<0.0001 when compared to the control group (Figure 5.4).

As shown in Figure 5.5 the viability of the cells decreased depending on increased concentration in the groups treated with 6.125, 12.5, 25and 50 µM Sulforaphane compared to the control. The viability of Caco-2 cells was reduced in the groups due to the cytotoxic effect of SFN.

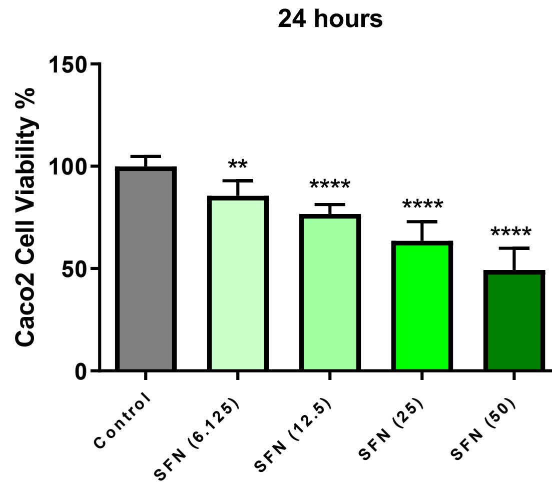


Figure 5.5. *In CaCo-2 cells, cell viability % rates of 6.125, 12.5, 25 and 50 µM of SFN, according to MTT test results at 24 hours (Mean±SEM, n=8,**p<0.01, ****p <0.0001) (Control: 0.1% DMSO).*

According to our MTT results, the cell viability % rates in the groups treated with 6.125, 12.5, 25 and 50 µM concentrations of Sulforaphane in CaCo-2 cells were determined as 85,51%, 76,65%, 63,56% and 49,31% respectively, compared to the control. Statistical significance among the single groups in which 6.125, 12.5, 25, and 50 µM SFN were applied was found to be **p<0.01 for 6.125 µM and ****p<0.0001 for 12.5, 25 and 50 µM when compared to the control group (Figure 5.5).

As illustrated in Figure 5.6, it is seen that the viability of the cells decreased depending on increased concentration in the groups treated with 6.125, 12.5 and 25 µM of Dox and also with 12.5, 25, and 50 µM of SFN compared to the control group. Moreover, the viability of CaCo-2 cells decreased due to the cytotoxic effect in the groups in which combined concentrations of DOX and SFN (DOX6,125+SFN12,5), (Dox12.5+SFN25), (Dox12.5+SFN50), (Dox25+SFN25) and (DOX25+SFN50) were applied.

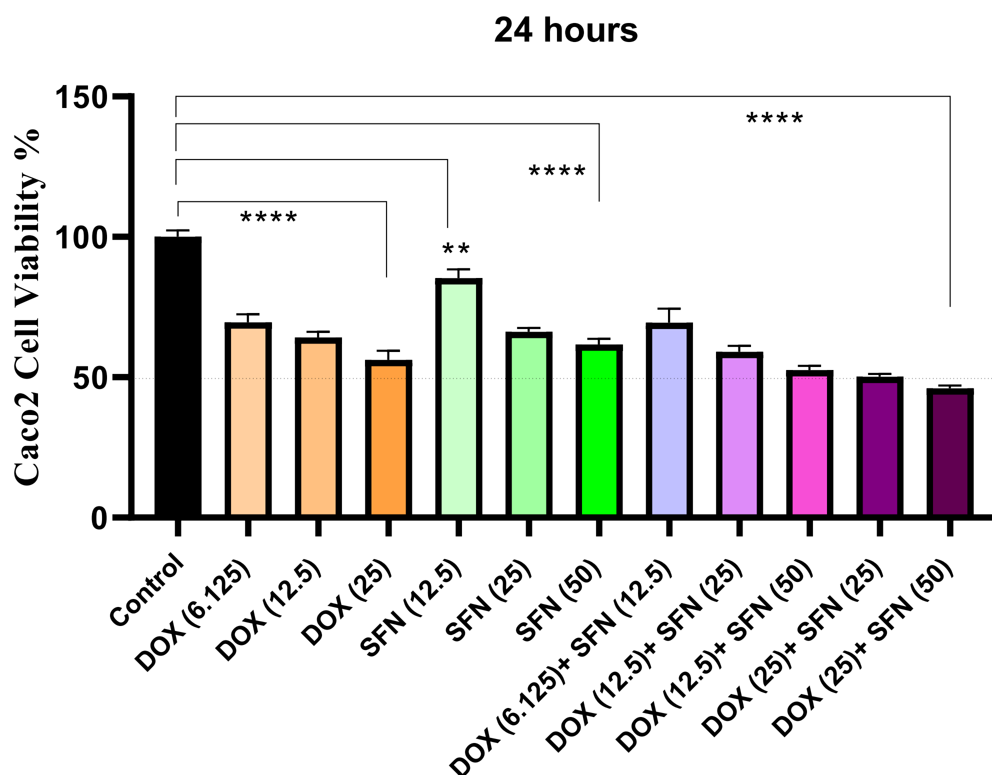


Figure 5.6. *In CaCo-2 cells, cell viability % rates of different concentrations of DOX and SFN single and in combined applications, according to MTT test results at 24 hours (Mean±SEM, n=8, P values found in GraphPad Prism 8; **p<0.01, ****p<0.0001) (Control: 0.1% DMSO).*

Our MTT results indicate that the cell viability % rates in the groups treated with 6.125, 12.5 and 25 µM concentrations of DOX in CaCo-2 cells were determined as 69,52%, 64,20% and 56,23% respectively, compared to the control. Cell viability rates were found to be 85,27%, 66.17% and 61,73%, respectively, in the groups treated with 12.5, 25, and 50 µM concentrations of SFN.

In the groups treated with combinations of DOX and SFN (DOX 6.125+SFN 12.5), (DOX12.5+SFN25), (DOX12.5+SFN50), (DOX25+SFN25) and (DOX25+SFN50) the cell viability % rates were determined as 69.46%, 59.08%, 52.59%, 50.14% and 46.04.% respectively.

Statistical significance was found to be **p<0.01 for SFN 12.5 µM and ****p<0.0001 for all other groups when compared to the control group (Figure 5.6).

5.3. Evaluation of the 24-Hour Cytotoxic Effects of Doxorubicin and Sulforaphane Single and in Combinations on U2OS Cells

The cytotoxic effects of single applications of 6.125, 12.5, 25 and 50 µM concentrations of Doxorubicin and Sulforaphane on U2OS cells and the combined applications of these two drugs on cell viability % is given in Figure 5.7 - Figure 5.9.

As seen in Figure 5.7, it is seen that the viability of the cells depending on increased concentration in the groups treated with 6.125, 12.5, 25 and 50 µM Doxorubicin compared to the control group. The viability of U2OS cells decreased due to the cytotoxic effect in the groups caused by DOX.

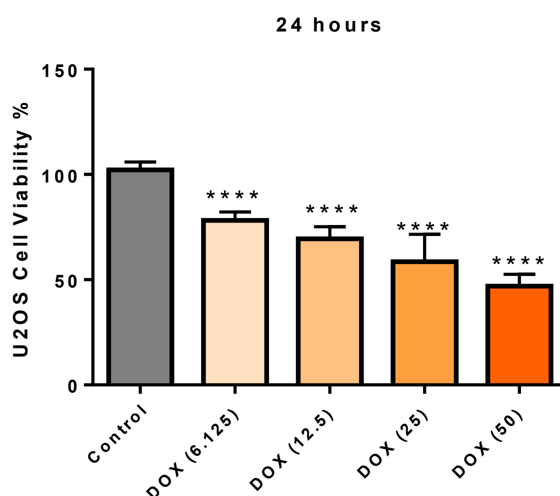


Figure 5.7. *In U2OS cells, cell viability % rates of 6.125, 12.5, 25 and 50 µM of DOX, according to MTT test results at 24 hours (Mean±SEM, n=8, ****p <0.0001)(Control: 0.1% DMSO).*

According to our MTT results, the cell viability % rates in the groups treated with 6.125, 12.5, 25 and 50 µM concentrations of Doxorubicin in U2OS cells were determined as 78.23%, 69.44%, 58.60% and 46.90% respectively, compared to the control. Statistical significance among the single groups in which 6.125, 12.5, 25, and 50 µM concentrations were applied was found to be ****p<0.0001 when compared to the control group (Figure 5.7).

As shown in Figure 5.8 the viability of the cells decreased depending on increased concentration in the groups treated with 6.125, 12.5, 25 and 50 µM Sulforaphane

compared to the control. The viability of U2OS cells was reduced in the groups due to the cytotoxic effect of SFN.

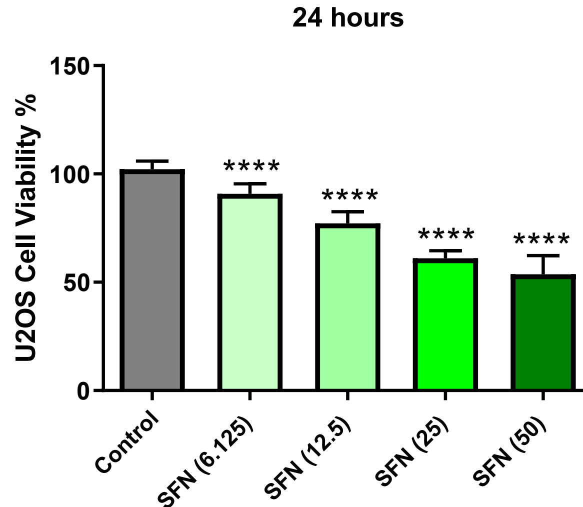


Figure 5.8. *In U2OS cells, cell viability % rates of 6.125, 12.5, 25 and 50 µM of SFN, according to MTT test results at 24 hours (Mean±SEM, n=8, ****p <0.0001) (Control: 0.1% DMSO).*

According to our MTT results, the cell viability % rates in the groups treated with 6.125, 12.5, 25 and 50 µM concentrations of Sulforaphane in U2OS cells were determined as 90.84%, 77.15%, 61.08% and 53.67% respectively, compared to the control. Statistical significance among the single groups in which 6.125, 12.5, 25, and 50 µM were applied was found to be ****p<0.0001 when compared to the control group (Figure 5.8).

As illustrated in Figure 5.9, it is seen that the viability of the cells decreased depending on increased concentration in the groups treated with 6.125,12.5 and 25 µM of DOX and also with 12.5, 25, and 50 µM of SFN compared to the control. The viability of U2OS cells decreased due to the cytotoxic effect in the groups in which DOX and combined concentrations of SFN (DOX 6,125+SFN 12,5), (Dox12.5+SFN25), (Dox12.5+SFN50), (Dox25+SFN25) and (DOX25+SFN50) were applied.

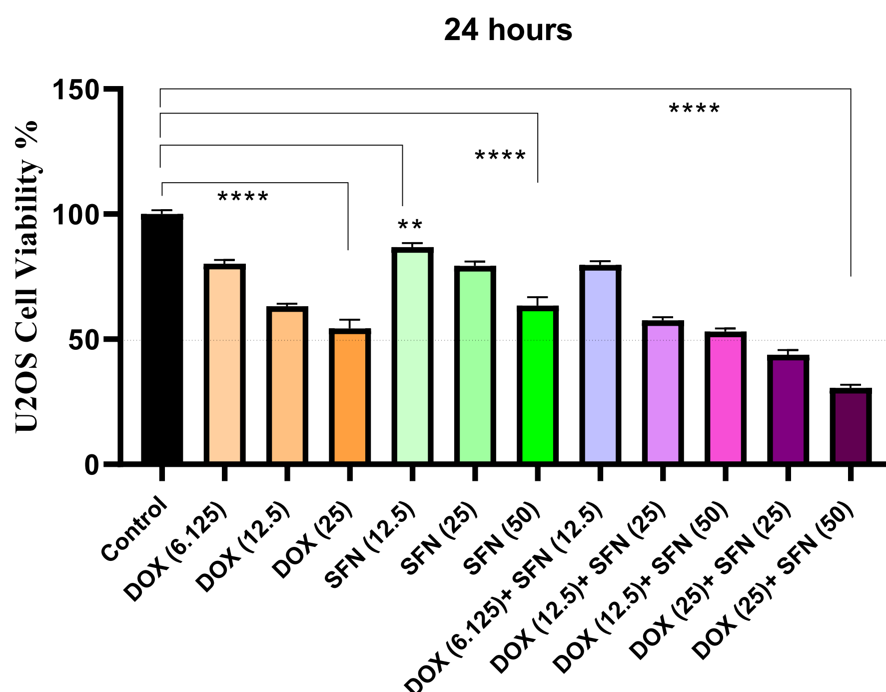


Figure 5.9. *In U2OS cells, cell viability % rates of different concentrations of DOX and SFN single and in combined applications, according to MTT test results at 24 hours (Mean±SEM, n=8, values found in GraphPad Prism 8; **p<0.01,****p<0.0001) (Control: 0.1% DMSO).*

Our MTT results indicate that the cell viability % rates in the groups treated with 6.125,12.5 and 25 µM concentrations of DOX in U2OS cells were determined as 80.14%, 63.14% and 54.31% respectively, compared to the control. Cell viability rates were found to be 86.79%, 79.29% and 63.40%, respectively, in the groups treated with 12.5, 25, and 50 µM concentrations of SFN.

In the groups treated combinations of DOX and SFN concentrations (DOX 6.125+SFN12.5), (DOX12.5+SFN25), (DOX12.5+SFN50), (DOX25+SFN25) and (DOX25+SFN50) the cell viability % rates were determined as 79.70%, 57.51%, 53.09%, 4.87% and 30.53% respectively. Statistical significance was found to be **p<0.01 for SFN 12.5 µM and ****p<0.0001 for all other groups when compared to the control group (Figure 5.9).

5.4. Evaluation of the 24-Hour Cytotoxic Effects of Doxorubicin and Sulforaphane Single and in Combinations on HepG2 Cells

The cytotoxic effects of single applications of 6.125, 12.5, 25 and 50 μM concentrations of Doxorubicin and Sulforaphane on HepG2 cells and the combined applications of these two drugs on cell viability% is given in Figure 5.10-Figure 5.12.

As seen in Figure 5.10, it is seen that the viability of the cells decreased depending on increased concentration in the groups treated with 6.125, 12.5, 25 and 50 μM Doxorubicin compared to the control group. The viability of HepG2 cells decreased due to the cytotoxic effect in the groups caused by DOX.

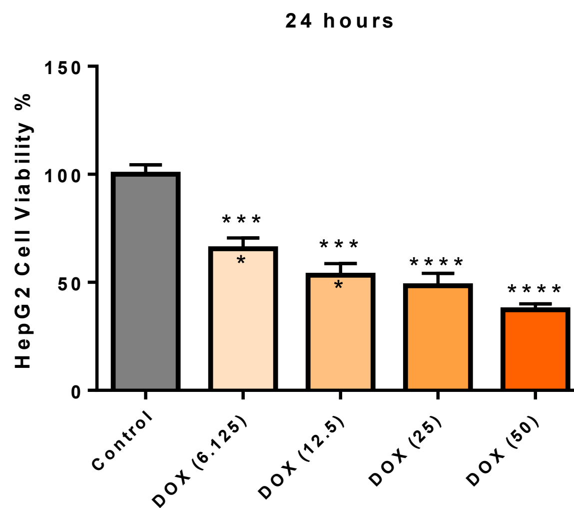


Figure 5.10. *In HepG2 cells, cell viability % rates of 6.125, 12.5, 25 and 50 μM of DOX, according to MTT test results at 24 hours (Mean±SEM, n=8, ***p<0.001, ****p <0.0001) (Control: 0.1% DMSO).*

According to our MTT results, the cell viability % rates in the groups treated with 6.125, 12.5, 25 and 50 μM concentrations of Doxorubicin in HepG2 cells were determined as 56.59%, 53.33%, 48.44% and 37.34% respectively, compared to the control. Statistical significance among the single groups in which 6.125, 12.5, 25, and 50 μM DOX were applied was found to be ***p<0.001 for 6.125 and 12.5 μM DOX, while ****p<0.0001 for 25 and 50 μM when compared to the control group (Figure 5.10).

As shown in Figure 5.11 the viability of the cells decreased depending on increased concentration in the groups treated with 6.125, 12.5, 25and 50 μM Sulforaphane

compared to the control. The viability of HepG2 cells was reduced in the groups due to the cytotoxic effect of SFN.

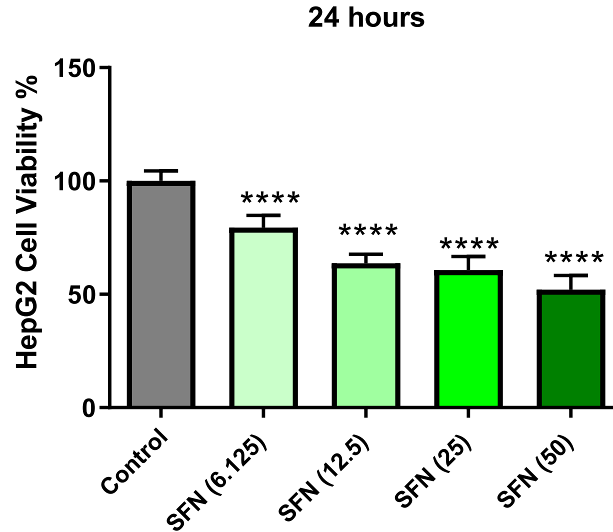


Figure 5.11. *In HepG2 cells, cell viability % rates of 6.125, 12.5, 25 and 50 µM of SFN, according to MTT test results at 24 hours (Mean±SEM, n=8, ****p <0.0001) (Control: 0.1% DMSO).*

According to our MTT results, the cell viability % rates in the groups treated with 6.125, 12.5, 25 and 50 µM concentrations of Sulforaphane in HepG2 cells were determined as 79.40%, 63.73%, 60.63% and 52.06% respectively, compared to the control. Statistical significance among the single groups in which 6.125, 12.5, 25, and 50 µM SFN were applied was found to be ****p<0.0001 when compared to the control group (Figure 5.11).

As illustrated in Figure 5.12, it is seen that the viability of the cells decreased depending on increased concentration in the groups treated with 6.125,12.5 and 25 µM of Dox and also with 12.5, 25, and 50 µM of SFN compared to the control group. Moreover, the viability of HepG2 cells decreased due to the cytotoxic effect in the groups in which combined concentrations of DOX and SFN (DOX 6,125+SFN 12,5), (Dox12.5+SFN25), (Dox12.5+SFN50), (Dox25+SFN25) and (DOX25+SFN50) were applied.

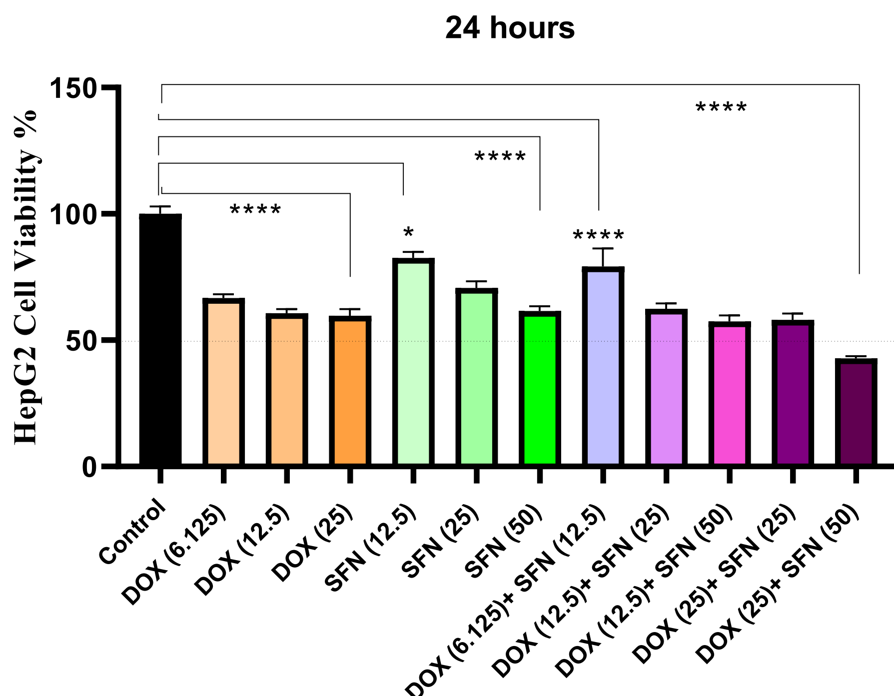


Figure 5.12. *In HepG2 cells, cell viability % rates of different concentrations of DOX and SFN single and in combined applications, according to MTT test results at 24 hours (Mean±SEM, n=8, P values found in GraphPad Prism 8; *p<0.05, ****p<0.0001) (Control: 0.1% DMSO).*

Our MTT results indicate that the cell viability % rates in the groups treated with 6.125, 12.5 and 25 µM concentrations of Dox in HepG2 cells were determined as 66.72%, 60.66% and 59.66% respectively, compared to the control. Also, cell viability rates were found to be 82.51%, 70.68% and 61.54%, respectively, in the groups treated with 12.5, 25, and 50 µM concentrations of SFN.

In the groups treated with combined concentrations of DOX and SFN (DOX 6.125+SFN12.5), (DOX12.5+SFN25), (DOX12.5+SFN50), (DOX25+SFN25) and (DOX25+SFN50), the cell viability % rates were determined as 79.16%, 62.43%, 57.40%, 58.02% and 42.80% respectively. Statistical significance was found to be *p<0.05 for SFN 12.5 µM and ****p<0.0001 for all other groups when compared to the control group (Figure 5.12).

5.5. Evaluation of Synergistic/Cytotoxic Effects of Doxorubicin with Sulforaphane Combinations in Different Concentrations on 3T3, Caco-2, U2OS and HepG2 Cells

The antagonist/synergist interactions of Doxorubicin in combination with Sulforaphane at different concentrations (DOX6.5+SFN12.5), (DOX12.5+SFN25), (DOX12.5+SFN50), (DOX25+SFN25) and (DOX25+SFN50) on 3T3, Caco-2, U2OS and HepG2 Cells are determined by the following coefficient of drug interaction (CDI) formula. CDI was calculated and evaluated by converting absorbance results to cell viability % values. According to this;

$$CDI = \frac{\text{Cell Viability \% of the combined group (A+B)}}{\text{Cell Viability \% of A treated group} \times \text{Cell Viability \% of B treated group.}} \times 100$$

The control group was accepted as 100% in the calculation made with this formula. The calculated coefficients of drug interaction are as follows: CDI < 1 synergistic effect, CDI < 0.7 strong synergistic effect, CDI = 1 additive effect, and CDI > 1 as antagonist effect [150].

5.5.1. Antagonist/synergist interactions of combined concentrations of Doxorubicin with Sulforaphane on 3T3 cells

According to the cell viability % values calculated from MTT results, as a result of combined applications of DOX and SFN in 3T3 cells, CDI > 1 (antagonist effect) for all combined concentrations (Table 5.1).

Table 5.1. *Cell viability % rates (Mean ± SEM) and CDI values of DOX and SFN combined applications at 24 hours in 3T3 cells. Control group was accepted as 100%. CDI: Coefficient of Drug Interaction.*

Concentrations (µM)	Mean ± SEM (3T3 cell viability%)	CDI value (Coefficient of Drug interaction)
DOX (6.125)	67,85 ± 2,259	-
DOX(12.5)	53,17 ± 1,033	-
DOX (25)	43,09 ± 1,877	-
SFN (12.5)	69,17 ± 2,052	-
SFN (25)	63,16 ± 1.09	-
SFN (50)	53,58 ± 1.54	-
DOX (6.125)+ SFN (12.5)	62,72 ± 2,939	1.3
DOX (12.5)+ SFN (25)	46,62 ± 1,249	1.3
DOX (12.5)+ SFN (50)	37,87 ± 1,361	1.3
DOX (25)+ SFN (25)	34,08 ± 0,889	1.2
DOX (25)+ SFN (50)	32,25 ± 2,066	1.4

5.5.2. Antagonist/synergist interactions of combined concentrations of Doxorubicin with Sulforaphane on Caco-2 cells

Cell viability % values obtained from MTT results as a result of DOX and SFN combined applications in Caco-2 cells, indicate that CDI > 1 (antagonist effect) for all combined concentrations (Table 5.2).

Table 5.2. *Cell viability % rates (Mean ± SSEM) and CDI values of DOX and SFN combined applications at 24 hours in Caco2 cells. Control group was accepted as 100%. CDI: Coefficient of Drug Interaction.*

Concentrations (µM)	Mean ± SEM (Caco-2 cell viability)	CDI value (Coefficient of drug interaction)
DOX (6.125)	69,52 ± 2,925	-
DOX (12.5)	64,20 ± 1,979	-
DOX (25)	56,23 ± 3,153	-
SFN (12.5)	85,27 ± 3,162	-
SFN (25)	66.17 ± 1.385	-
SFN (50)	61,72 ± 2,014	-
DOX (6.125)+ SFN (12.5)	69,46 ± 4.986	1.1
DOX (12.5)+ SFN (25)	59,08 ± 2,057	1.3
DOX (12.5)+ SFN (50)	52,59 ± 1.464	1.3
DOX (25)+ SFN (25)	50,14 ± 0,995	1.3
DOX (25)+ SFN (50)	46,03 ± 1,049	1.3

5.5.3. Antagonist/synergist interactions of combined concentrations of Doxorubicin with Sulforaphane on U2OS cells

According to the cell viability % values obtained from MTT results, as a result of combined applications of DOX and SFN in U2OS cells, CDI > 1 (antagonist effect) for

(DOX 6.5+SFN 12.5), (DOX12.5+SFN25) and (DOX12.5+SFN50); CDI < 1 (synergistic effect) for (DOX25+SFN25) and (DOX25+SFN50). (Table 5.3).

Table 5.3. *Cell viability % rates (Mean ± SEM) and CDI values of DOX and SFN combined applications at 24 hours in U2OS cells. Control group was accepted as 100%. CDI: Coefficient of Drug Interaction.*

Concentrations (µM)	Mean ± SEM (U2OS cell viability%)	CDI value (Coefficient of Drug interaction)
DOX (6.125)	80,15 ± 1,579	-
DOX (12.5)	63,14 ± 1,066	-
DOX (25)	54,31 ± 3,550	-
SFN (12.5)	86,79 ± 1,646	-
SFN (25)	79,29 ± 1,791	-
SFN (50)	63,39 ± 3,407	-
DOX (6.125)+ SFN (12.5)	79,70 ± 1,499	1.1
DOX (12.5)+ SFN (25)	57,51 ± 1,273	1.1
DOX (12.5)+ SFN (50)	53,09 ± 1,247	1.3
DOX (25)+ SFN (25)	43,87 ± 1,790	**0.8**
DOX (25)+ SFN (50)	30,53 ± 1,299	**0.8**

5.5.4. Antagonist/synergist interactions of combined concentrations of Doxorubicin with Sulforaphane on HepG2 cells

According to the cell viability % values obtained from MTT results, as a result of combined applications of DOX and SFN in HepG2 cells, CDI > 1 (antagonist effect) for all combined concentrations (Table 5.4).

Table 5.4. *Cell viability % rates (Mean ± SEM) and CDI values of DOX and SFN combined applications at 24 hours in HepG2 cells. Control group was accepted as 100%. CDI: Coefficient of Drug Interaction.*

Concentrations (µM)	Mean ± SEM (HepG2 cell viability%)	CDI value (Coefficient of drug interaction)
DOX (6.125)	66,72 ± 1,432	-
DOX (12.5)	60,66 ± 1,707	-
DOX (25)	59,66 ± 2,615	-
SFN (12.5)	82,51 ±2,377	-
SFN (25)	70,68 ±2,690	-
SFN (50)	61,54 ±1,933	-
DOX (6.125)+ SFN (12.5)	79,16 ±7,205	1.4
DOX (12.5)+ SFN (25)	62,44 ±2,142	1.5
DOX (12.5)+ SFN (50)	57,39 ±2,452	1.5
DOX (25)+ SFN (25)	58,02 ±2,514	1.3
DOX (25)+ SFN (50)	42,80 ±0,9007	1.1

5.6. Determination of Real Time Cell Proliferation of U2OS Cells

The effect of single applications of 6.125, 12.5, 25 and 50 µM concentrations of DOX and SFN on U2OS cells and the combined applications of these two drugs on cell viability % was evaluated by using the MTT method. According to the MTT results, the viability of the U2OS cells decreased with increasing concentrations at 24 hours of incubation.

In the groups of combination concentration (DOX25+SFN25) and (DOX25+SFN50) , CDI value was calculated 0.8 (CDI < 1) for two groups. This result was evaluated that it was shown synergistic effect treated with (DOX25+SFN25) and (DOX25+SFN50) on U2OS cells. Therefore, the antiproliferative effect of these concentrations on U2OS cells was evaluated also by using real time cell proliferation analyses system. U2OS cells were treated for 24h with 25 and 50 µM of SFN, and 25 µM of DOX as a single application, and the combined applications of DOX and SFN were DOX (25)+ SFN (25) and DOX (25)+ SFN (50) µM. According to the result, the substances showed decreasing in U2OS proliferation in dose-time dependent manner (figure 5.13) . This means the DOX and SFN induce significant anti proliferation effects on U2OS cells and the combined concentration DOX (25) + SFN (50) had the most antiproliferation effect.

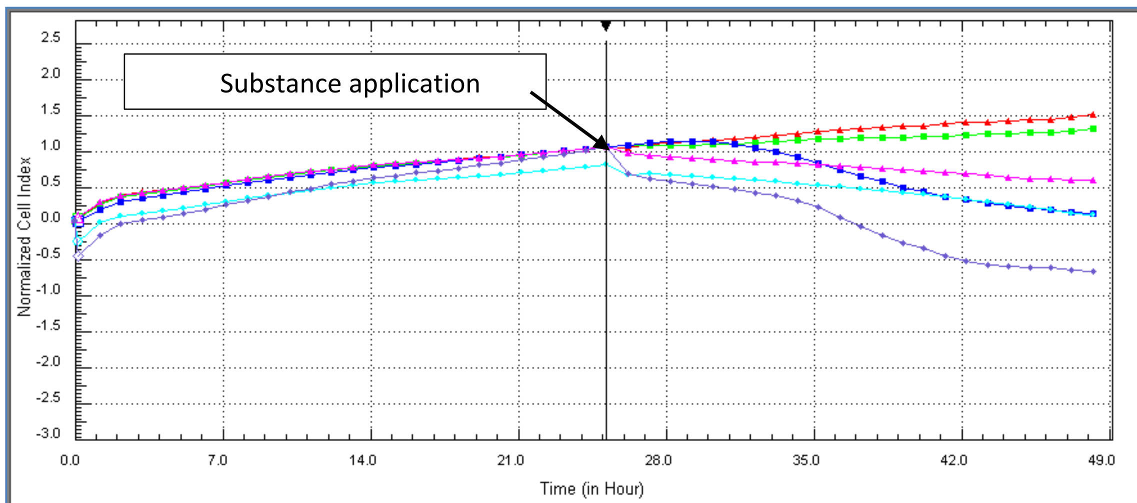


Fig 5.13. *The antiproliferative effect of DOX and SFN drugs on U2OS cells during 24h incubation.*

Kontrol grubu. SFN 25. SFN 50. DOX 25. DOX 25+ SFN 25. DOX 25+ SFN 50.

In this thesis, the cytotoxic effects of Doxorubicin and Sulforaphane alone and in combination with each other on 3T3, Caco2, U2OS and HepG2 cells were investigated, and their anticancer activities were evaluated. For this purpose, the cytotoxic effects of these two drugs alone and in combination at different concentrations on 3T3 healthy fibroblast cells, Caco-2 colon cancer cells, U2OS osteosarcoma cells and HepG2 hepatocellular cancer cells were investigated by MTT test; The relationship synergist/antagonist activities of DOX and SFN was also determined by calculating CDI (Coefficient of Drug Interaction) values.

Doxorubicin is an anticancer agent that used in clinical trials for various cancers [151], and in many settings, it is often used in combination with many other chemotherapy drugs as an essential chemotherapeutic regimen [152] and showing potent anti-tumor activity against metastatic breast cancer [151]. The cytotoxic effects of Doxorubicin on metastatic breast cancer cells were evaluated in combination with Paclitaxel [153]. It was concluded that it has a potential value for the treatment of metastatic breast cancer with high safety from cardiac toxicity by taking a bolus of DOX followed 30 min later by a 3-h infusion of Paclitaxel [153].

According to Sadeghi-Aliabadi and his colleagues in a study performed with Doxorubicin on HeLa (Human cervix carcinoma) cells, the cell survival was decreased in a dose dependent manner after 72 hours incubation with doxorubicin alone between 40 to 83% at 0.1, 1 and 2 μM concentrations and it was a cell growth inhibitor at all tested concentrations [154].

In our study, it was determined that Doxorubicin has a dose-dependent cytotoxic effect on 3T3, Caco2, U2OS and HepG2 cancer cells after 24 hours of incubation. It has been shown that the viability of 3T3 cancer cells was decreased between 17 to 71% at 6.125, 12.5, 25 and 50 μM concentrations, and the viability of Caco-2 cancer cells was also decreased between 15 to 68 % according to the MTT results in the concentration-dependent results of cell viability comparing to the control group cells. In addition, a decrease in cell viability was determined on U2OS between 22 to 54% and between 44 to 63% for HepG2 cancer cells at 6.125, 12.5, 25 and 50 μM concentrations comparing to the control group cells. According to our MTT results, Doxorubicin had the most effect on 3T3 cells and at a concentration of 50 μM, because 3T3 cells were healthy cells, so more cytotoxic effects were seen.

Among the most effective and promising chemopreventive agents is sulforaphane. In various tumor cell lines, sulforaphane has proven to be able to inhibit cell proliferation and enhance apoptosis. SFN plays an effective role in reversing chemoresistance by its proapoptotic potential when taking alone or in conjunction with other techniques for therapy [13].

In a study done by Kallifatidis and his partners MIA-PaCa2 (CSChigh) a human pancreatic cancer cells were exposed to sulforaphane (5 µM), cisplatin (CIS), gemcitabine (GEM), doxorubicin (DOX), and 5-furouracil (5-FU) either alone or combined with each other. After 72 hours of incubation, MTT assay and morphological inspection were used to analyze the cell viability and it has been found that Sulforaphane increased the *in vitro* cytotoxic effect of these anticancer drugs [155].

It was found that by targeting eighty percent of the MIA-PaCa2 (CSChigh) cells, the combination of sulforaphane and 5-FU was the most effective among other combinations. Apoptosis assay also revealed that GEM alone enhanced therty percent apoptosis in MIA-PaCa2 cells, while GEM in combination with sulforaphane induced fourty percent apoptosis. In a similar way, combining sulforaphane and cisplatin treatment significantly increased apoptosis when compared with either agent alone [155].

In our study, it was determined that Sulforaphane has a dose-dependent cytotoxic effect on 3T3, Caco2, U2OS and HepG2 cancer cells after 24 hours of incubation. It has been shown that the viability of 3T3 cancer cells was decreased between 8 to 36 % at 6.125, 12.5, 25 and 50 µM concentrations, and the viability of Caco2 cancer cells was also decreased between 15 to 51 % according to the MTT results in the concentration-dependent results of cell viability comparing to the control group cells. In addition, a decrease in cell viability was determined on U2OS between 10 to 47% and between 21 to 48% for HepG2 cancer cells at 6.125, 12.5, 25 and 50 µM concentrations comparing to the control group cells. According to our MTT results, Sulforaphane had the most effect on Caco-2 cells and at a concentration of 50 µM.

Doxorubicin which is a highly effective anticancer drug has a cardiotoxicity side effect caused by production of free radical, and reactive oxygen species (ROS) resulting in oxidative stress [12].

Singh et al. demonstrated that treatment of cardiomyoblast H9c2 cells with sulforaphane in a safe dose of 2.5 µM before giving Doxorubicin protects against *in vitro* toxicity caused by DOX treatment. The cell viability assay of H9c2 cells was 45% when

treated with 5 µg/ml DOX alone which was greatly enhanced to ~ 76% when treated with SFN (2.5 µM) + DOX (5 µg/ml) together [156].

Also, *in vivo* study done with wild type 129/sv mice showed that treatment with combination of DOX and SFN give protection against cardiotoxicity induced by DOX through many ways like reducing the formation of 4-hydroxynonenal (4-HNE) protein adducts, improving the activities of mitochondrial respiratory complex, activating the Nrf2 in hearts of treated mice, and preventing the suppression of antioxidant and antielectrophile enzymes GSTA4-4, SOD2, NQO1, and heme oxygenase 1 (HO-1). It was reported by Li et al. that cardioprotective effect of SFN against DOX-induced cardiotoxicity is mediated by the activation of the Keap1/Nrf2/ARE pathway, which as a result induce HO-1 [12, 157].

In our study, it was determined that the combined applications of Doxorubicin and Sulforaphane has a dose-dependent cytotoxic effect on 3T3, Caco-2, U2OS and HepG2 cancer cells after 24 hours of incubation and had the most effect on U2OS cells and at a concentration of DOX25+SFN50 µM, reducing cell viability in concentration-dependent results of cell viability according to MTT results.

According to CDI calculation results based on cell viability on 3T3 cells, an antagonist effect was found at all different combined concentrations of DOX and SFN (DOX 6,125+SFN 12,5), (DOX12.5+SFN25), (DOX12.5+SFN50), (DOX25+SFN25) and (DOX25+SFN50) since CDI > 1.

According to CDI calculation results based on cell viability on Caco-2 cells, an antagonist effect was found at all different combined concentrations of DOX and SFN (DOX 6,125+SFN 12,5), (DOX12.5+SFN25), (DOX12.5+SFN50), (DOX25+SFN25) and (DOX25+SFN50) since CDI > 1.

According to CDI calculation results based on cell viability on U2OS cells, an antagonist effect was found at the first three combined concentrations of DOX and SFN (DOX 6,125+SFN 12,5), (DOX12.5+SFN25) and (DOX12.5+SFN50) since CDI > 1 and a synergistic effect (CDI < 1) at (DOX25+SFN25) and (DOX25+SFN50).

According to CDI calculation results based on cell viability on HepG2 cells, an antagonist effect was found at all different combined concentrations of DOX and SFN (DOX 6,125+SFN 12,5), (DOX12.5+SFN25), (DOX12.5+SFN50), (DOX25+SFN25) and (DOX25+SFN50) since CDI > 1.

6. CONCLUSION AND RECOMMENDATIONS

In this thesis study, determining the anticancer effects of Doxorubicin and Sulforaphane, and the antiproliferative effects of agents at different concentrations and in combination on 3T3, Caco-2, U2OS and HepG2 cells. In this study, it is aimed to evaluate the synergist, antagonist, and additive effects on cells at varying concentrations by CDI calculations.

In summary, in our study, according to the MTT results, in the concentration-dependent results of cell viability, the combined applications of DOX with SFN at different concentrations (DOX6,125+SFN12,5),(DOX12.5+SFN25), (Dox12.5+SFN50), (Dox25+SFN25) and (DOX25+SFN50) on 3T3, Caco-2, U2OS and HepG2 cells has been determined.; A significant decrease in cell viability was determined on 3T3 at DOX25+SFN25 and DOX25+SFN50, and U2OS cells at DOX25+SFN50 concentrations.

The cytotoxic effects of combinations of DOX with SFN in 3T3, Caco-2, U2OS and HepG2 cells occur depending on the concentration. According to the CDI calculation results based on cell viability; Combined application of DOX and SFN has a synergistic effect on U2OS cells at a concentration of Dox25+SFN25 and DOX25+SFN50 (CDI < 1), while combined applications of DOX with SFN at different concentrations have antagonist effect on 3T3, Caco-2 and HepG2 (CDI > 1).

In our study, although the highest effect of DOX was on 3T3 cells- healthy fibroblast cells- there was an antagonistic effect of DOX+SFN drugs combination according to our CDI results on 3T3 cells. This may be due to the fact that the SFN drug in normal healthy cells makes DOX stay outside the nucleus (the primary site of DOX localization and action) which in turn reduces DOX-cytotoxic effects and induces a decrease of mitochondrial ROS level elevated by DOX in normal cells[158] and protects cells against the translocation of Bax to the mitochondria by DOX and therefore prevents cytochrome c releasing into the cytosol[159]. Also, SFN is a powerful stimulant of Nrf2(Nuclear Factor-E2-related factor 2), which controls how cells react to oxidative and electrophilic stress [160]. The presence of SFN leads to the translocation of Nrf2 into the nucleus, which in turn leads to increased gene expression of ROS-eliminating enzymes and induces heme oxygenase 1 (HO-1) mRNA and protein expression [158].

The antagonistic effects of DOX+SFN on cancer cells may be coming from the effect of SFN on lowering the intracellular ROS level caused by DOX in cancer cells, and as in normal cells, SFN stimulates the translocation of Nrf2 from the cytosol into the nucleus which enhances gene expression of enzymes that responsible of ROS eliminating [158].

The synergic impact of DOX+SFN on cancer cells comes from the SFN induction of two effects which include increasing the accumulation of DOX in the nucleus, which enhances DNA and chromatin damage. The second effect is inducing the inhibition of mitosis. Together, impede the growth of tumor [158].

Also, the synergy of DOX and SFN action can enhance the regression of tumor by decreasing the number of Myeloid-derived suppressor cells (MDSCs). These cells are recognized for their ability to inhibit anti-tumor immunity. Decreasing the accumulation of these cells occurs by inhibiting the COX-2/PGE2 pathway in tumor cells by DOX and SFN. Prostaglandin PGE2 in cancer cells stimulates the accumulation of MDSCs in the tumor sites which decreases the anti-tumor immunity. While DOX increases the production of prostaglandin, adding SFN treatment dramatically works on decreasing the levels of COX-2 mRNA. As a result, SFN reduces COX-2 expression, which in turn decreases the production of PGE2 thus improving the therapeutic effects of DOX [159].

The results obtained in terms of both the cytotoxicity tests performed with the MTT method and the evaluations made with the coefficient of drug interaction (CDI) are important in terms of providing data and shedding light on the studies carried out in cancer treatments of drug combinations. Also, further studies are needed to support these results.

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