อนุพันธ์ของด็อกซ์โซรูบิซินเชื่อมต่อเด็กซ์ซาเมททาโซนกระตุ้นการตายแบบอะพอพโทสิสในเอ็มซีเอฟเซเว่นเซลล์โดยไม่เข้านิวเคลียส และสามารถทำลายเซลล์ที่ถูกกระตุ้นให้ดื้อต่อด็อกซ์โซรูบิซินโดยเอ็มดีอาร์วันยีนส์

นางสาวกมลทิพย์ ไชยโกมล

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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ปีการศึกษา 2560

Doxorubicin-conjugated dexamethasone induced MCF-7 apoptosis without entering the nucleus and able to overcome MDR-1-induced resistance

Miss Kamontip Chaikomon

A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy Program in Medical Science
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Thesis Title	Doxorubicin-conjugated dexamethasone induced MCF-7 apoptosis without entering the nucleus and able to overcome MDR-1-induced resistance
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กมลทิพย์ ไชยโกมล : อนุพันธ์ของด็อกซ์โซรูบิซินเชื่อมต่อเด็กซ์ซาเมททาโซนกระตุ้นการตายแบบอะพอพโทสิสในเอ็มซีเอฟเซเว่นเซลล์โดยไม่เข้านิวเคลียส และสามารถทำลายเซลล์ที่ถูกกระตุ้นให้ดื้อต่อด็อกซ์โซรูบิซินโดยเอ็มดีอาร์วันยีนส์ (Doxorubicin-conjugated dexamethasone induced MCF-7 apoptosis without entering the nucleus and able to overcome MDR-1-induced resistance) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ. ดร. นพ.อมรพันธุ์ เสรีมาศพันธุ์, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: นพ.กฤษณพงศ์ มโนธรรม, หน้า.

ด็อกซ์โซรูบิซินเป็นยาเคมีบำบัดที่ถูกใช้อย่างแพร่หลายในการรักษาโรคมะเร็งต่าง ๆ รวมถึงมะเร็งโลหิตวิทยา กลไกการออกฤทธิ์หลักของด็อกซ์โซรูบิซิน คือ การยับยั้งเอนไซม์โทโปไอโซเมอร์เรสทู (topoisomerase II) โดยกลไกนี้เกิดขึ้นในนิวเคลียส ในปัจจุบันพบว่า เซลล์มะเร็งที่ดื้อต่อด็อกซ์โซรูบิซินนั้น เกิดจากการสะสมของด็อกซ์โซรูบิซินในเซลล์มะเร็งลดลง กลไกการดื้อต่อด็อกซ์โซรูบิซินเกิดจาก P-glycoprotein ซึ่งเป็นตัวขนส่งด็อกซ์โซรูบิซินออกจากเซลล์มะเร็ง P-glycorprotein นี้เกิดจากการควบคุมของยีนที่ชื่อว่า multidrug resistant gene มีการรายงานจากหลายการศึกษาถึงวิธีการเพื่อเอาชนะการดื้อต่อด็อกซ์โซรูบิซินของเซลล์มะเร็ง

ในการศึกษานี้ ได้ทำการดัดแปลงโมเลกุลของด็อกซ์โซรูบิซินที่ตำแหน่ง 3’amino group เพื่อเชื่อมต่อกับโมเลกุลเด็กซ์ซาเมททาโซน ถึงแม้ผลการศึกษาจะพบว่าพิษในการทำลายเอ็มซีเอฟเซเว่นเซลล์ของด็อกซ์โซรูบิซินที่เชื่อมต่อกับเด็กซ์ซาเมททาโซนหรือ DexDOX จะต่ำกว่าด็อกซ์โซรูบิซินแบบดั้งเดิม แต่การศึกษาพบว่า DexDOX สามารถกระตุ้นให้เอ็มซีเอฟเซเว่นเซลล์เกิดการตายแบบอะพอพโทสิสได้อย่างรวดเร็วโดยไม่เข้าไปในนิวเคลียส การวิเคราะห์ต่อมาแสดงให้เห็นว่า DexDOX สามารถทำให้เกิด oxidative stress ภายในส่วนไซโตพลาสซึมของเซลล์และไม่มีผลต่อวัฏจักรของเซลล์ นอกจากนี้ DexDOX ยังมีพิษต่อเซลล์ MDR1-overexpressed MCF-7 ที่ดื้อต่อ 16 เท่าของด็อกซ์โซรูบิซิน จากการศึกษานี้จึงได้สังเคราะห์โมเลกุลใหม่ของด็อกซ์โซรูบิซินหรือ DexDOX ขึ้นมาซึ่งสามารถทำลายเซลล์ที่ดื้อต่อด็อกซ์โซรูบิซินโดยการกระตุ้นของ MDR1 ได้ โดยหวังเป็นอย่างยิ่งว่าการศึกษานี้จะมีประโยชน์ต่อการรักษามะเร็งในอนาคตต่อไป

สาขาวิชา วิทยาศาสตร์การแพทย์ ลายมือชื่อนิสิต .....

ปีการศึกษา 2560 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

5774767030 : MAJOR MEDICAL SCIENCE

KEYWORDS: DOXORUBICIN, DEXAMETHASONE, DOXORUBICIN RESISTANT CANCER, MDR-1

KAMONTIP CHAIKOMON: Doxorubicin-conjugated dexamethasone induced MCF-7 apoptosis without entering the nucleus and able to overcome MDR-1-induced resistance. ADVISOR: ASST. PROF. AMORNPUN SEREEMASPUN, M.D., Ph.D., CO-ADVISOR: KRISSANAPONG MANOTHAM, M.D., MSc., pp.

Doxorubicin is a common chemotherapeutic drug that generally uses in many solid and hematologic malignancies. The main mechanism of doxorubicin is inhibition of topoisomerase II that occurs in the nucleus. Currently, doxorubicin resistant cancer cells which have decreased accumulation of intracellular doxorubicin resulting from an efflux pump, P-glycoprotein encoded by multidrug resistant gene. Several studies reported many different ways to overcome doxorubicin resistance.

In this study, we conjugated 3'amino group of doxorubicin to dexamethasone molecule. Despite of lower cytotoxic activity in MCF-7 cells, the conjugated product, DexDOX, exerted its actions in the different fashion to doxorubicin. DexDOX rapidly induced MCF-7 cells apoptosis without entering to the nucleus. Further analysis showed that DexDOX increased cytosolic oxidative stress and did not interfere with cell cycle. In addition, DexDOX retained cytotoxicity in MDR-1 over-expressed MCF-7 cells which had ≈16-folds resistance to doxorubicin. We here synthesized a new derivative of doxorubicin, DexDOX, which can overcome MDR-1 induced resistant. This molecule might be useful for future therapy.

Field of Study: Medical Science
Academic Year: 2017

Student's Signature .....
Advisor's Signature .....
Co-Advisor's Signature .....

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CHAPTER I
BACKGROUND AND RATIONAL

Doxorubicin (DOX) is an anthracycline antibiotic drug, extracted from *Streptomyces peucetius var. Caesius* [1]. In addition to an anti-bacterial effect, DOX is generally used as chemotherapeutic drug in many solid and hematologic malignancies [2, 3]. A number of studies have been proposed that DOX has a cytotoxic effect resulting from many mechanisms. DOX penetrates into the cell and binds to the cytoplasmic proteasome's 20S subunits [4]. The DOX-proteasome complex penetrates into the nucleus via the nuclear pores. In the nucleus, DOX inhibits topoisomerase II leading to DNA torsion [5, 6]. Moreover, DOX intercalates to DNA strand, preferably at cytosine-guanine nucleotide pair [7-9]. The result of both main mechanisms is cell death via apoptosis [10]. Reactive oxygen species (ROS) mediated cell death also has been proposed as the mechanism of DOX. The aglycone structure of DOX is reduced leading to generation of semiquinone radicle, a powerful ROS [11-13]. Mitochondria dysfunction, P53 and AMP-activated protein kinase activation are also involved in DOX-induced apoptosis [14-16]. Recently, DOX was proposed to cause cell death by autophagy and necrosis via poly (ADP-ribose) polymerase-1 (PARP-1) [17]

To date, DOX resistant cancers have been reported worldwide and causes a difficulty in cancer therapy. An overexpression of P-glycoprotein (P-gp) efflux pump encoded by multidrug resistant (MDR)-1 gene is an important mechanism [18-21]. P-gp

is an adenosine triphosphate (ATP) binding cassette (ABC) efflux pump that pumps DOX out of the cells resulting in decreased cytoplasmic DOX distribution. Previous study reported that P-gp also pumps DOX out of the nucleus [22]. A number of studies have shown the reversal of DOX sensitivity by P-gp blocking via pharmacological and gene targeted therapy [22, 23-28].

The studies have shown that a number of DOX derivatives have promising results in P-gp overexpression tumors [29-33]. In this study, we modified DOX structure to overcome P-gp mediated DOX resistant cancers. DOX was conjugated to dexamethasone. Dexamethasone is a glucocorticoid hormone which simply diffuses into the cells and binds to the cytoplasmic steroid receptor forming the dexamethasone-steroid receptor complex. The dexamethasone-steroid receptor complex penetrates into the nucleus [34, 35]. In addition, the molecule also escaped MDR-1 overexpression induced resistance. Therefore, it might be useful for future treatments.

Research objectives

To generate the new derivative of DOX that has an anti neoplastic effect against both DOX sensitive cancers and DOX resistant cancers.

Research questions

1. Does the new derivative of DOX have an anti neoplastic effect against both DOX sensitive cancers and DOX resistant cancers?

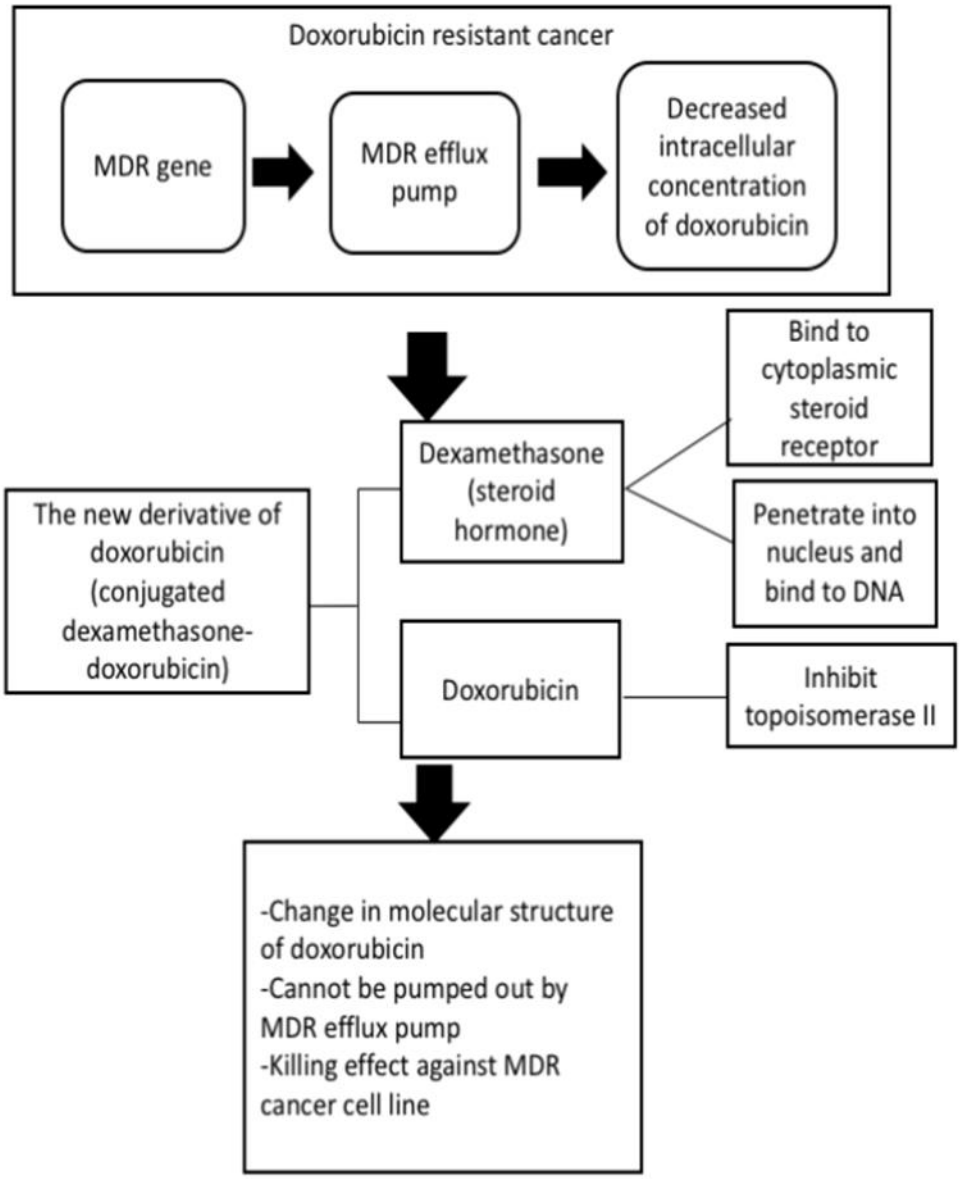
2. Does P-gp have an effect against the new derivative of doxorubicin?

Research hypotheses

1. The new derivative of DOX has an anti neoplastic effect against both DOX sensitive cancers and DOX resistant cancers.

2. P-gp does not affect to the new derivative of DOX.

Conceptual framework



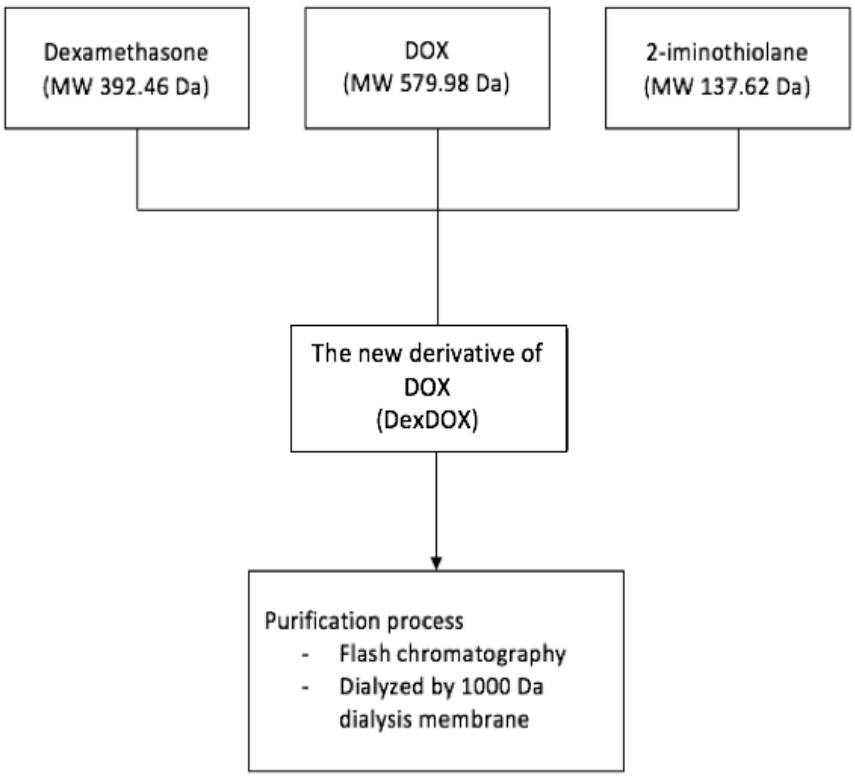
Research design

Experimental analytic study

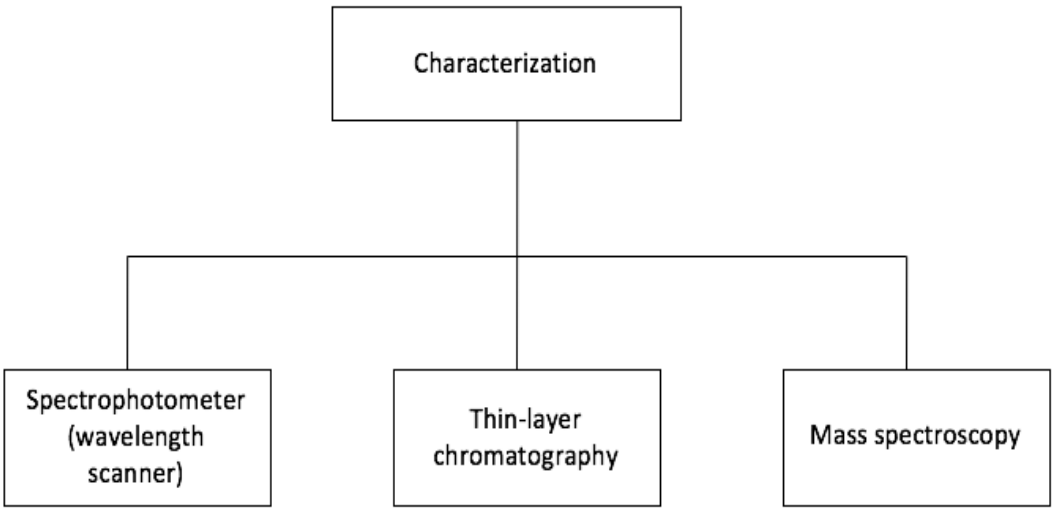
Experimental design

To synthesis and characterized DexDOX including to examine its anti neoplastic effect against DOX sensitive cancer cells and DOX resistant cancer cells.

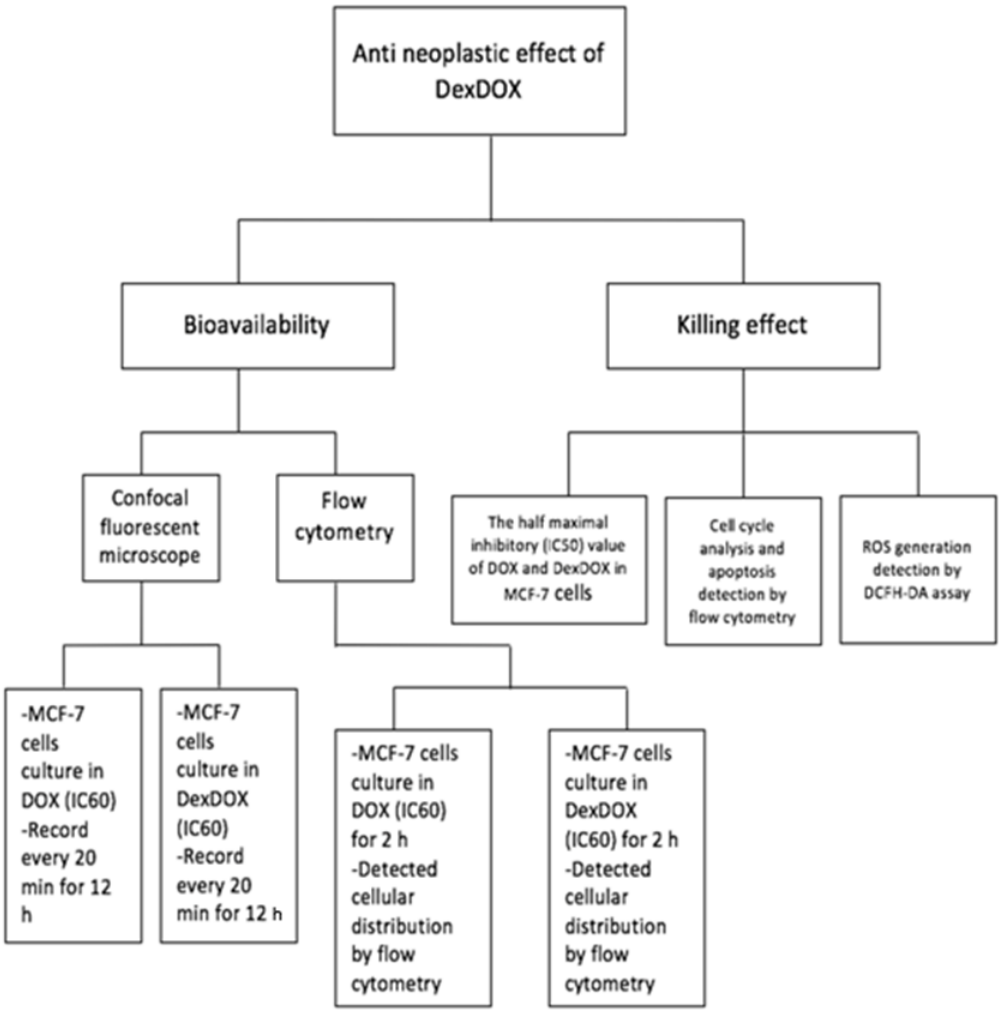
Part1: To synthesis and purify DexDOX.



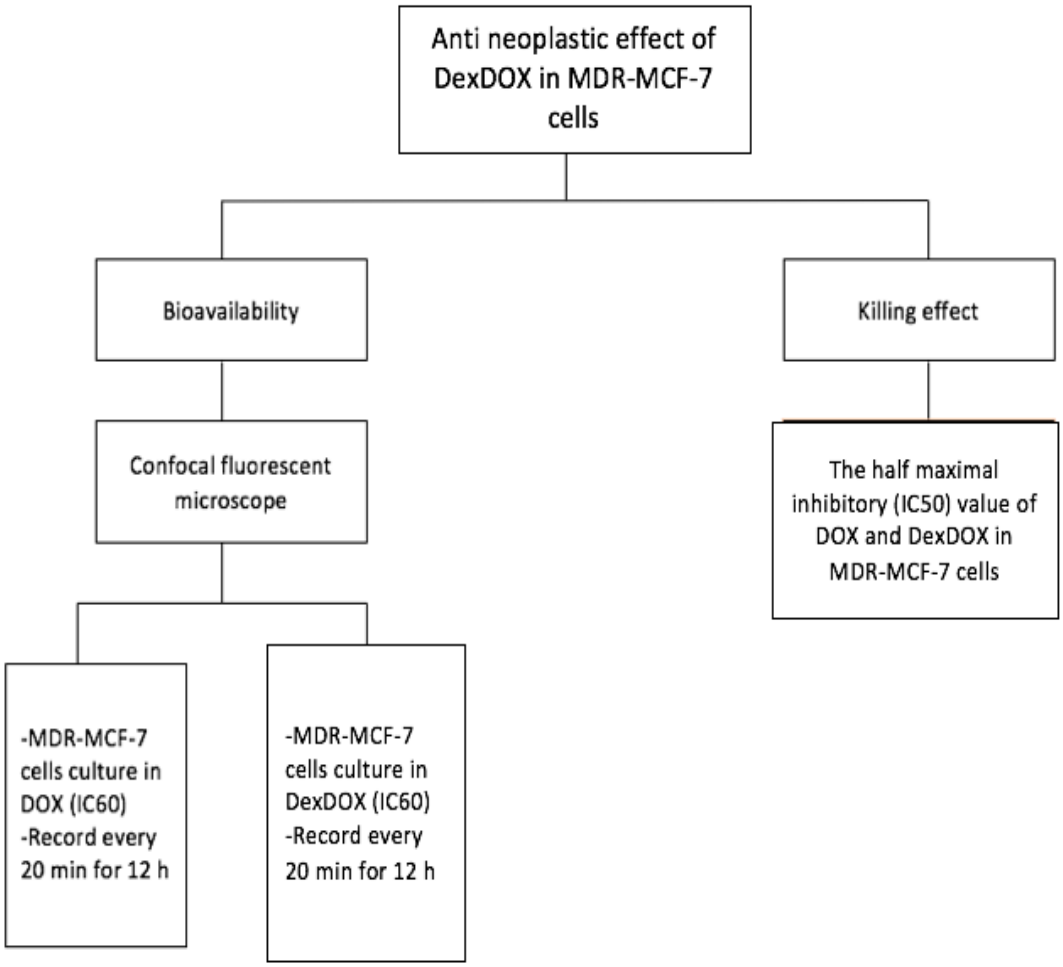
Part2: To characterize the characteristics of DexDOX and comparing to the characteristic of conventional DOX.



Part3: To evaluate the efficacy of DexDOX in MCF-7 cells.



Part4: To evaluate the efficacy of DexDOX in MDR-MCF-7 cells.



CHAPTER II
REVIEW OF THE RELATED LITERATURES

DOX

DOX is a part of anthracycline antibiotic drug, derived from *Streotomyces peucetius var. Caesius* [1]. It is composed of the aglycone, a tetracyclic ring with hydroxylquinone-semiquinone group and sugar structures [11-13]. It rapidly diffuses into the cells and binds to cytoplasmic proteasome's 20S subunits. The DOX-proteasome complex penetrates the nuclear pores into the nucleus and localizes into the nucleus [4]. To date, a number of DOX mechanisms have been proposed. Topoisomerase II is well established the main target of DOX action. It has an important role in DNA topological stage and is a key enzyme in prevention of DNA torsion. There are two types of topoisomerase enzymes, topoisomerase I and topoisomerase II [36]. Topoisomerase I causes breaking and generating DNA nick of a single strand DNA [37]. While topoisomerase II has two isoforms, alpha and beta, these two isoforms have different functions. Alpha topoisomerase II has been proposed as the main enzyme in many cellular activities. This enzyme binds to DNA resulting in separation of double strand DNA and recoiling of DNA super helix. Therefore, DNA torsion is prevented by these processes [36, 38]. Many chemotherapeutic drugs are targeted to topoisomerase II such as etoposide and DOX [6].

DOX causes topoisomerase II poisoning by DOX intercalating to DNA strand, preferably at cytosine-guanine nucleotide pair leading to separation of double strand DNA (figure 1) [9]. The separation of double strand DNA resulting in changing of number of turns in DNA or a linking number and DNA torsion. Topoisomerase II enzyme is inhibited by DOX and cannot perform its normal activity [5-9]. Moreover, DOX also forms a covalent bond with guanine occurring in a concentration of chemotherapeutic effect [39]. ROS generation that resulting from intracellular formaldehyde generation has been reported in DOX-induced covalent bond formation with DNA [11-13]. This mechanism is one of mechanisms that is independent on topoisomerase II.

Topoisomerase II-independent mechanisms of DOX also have been proposed. DOX mainly consists of quinone and it is oxidized to semiquinone. Semiquinone is a potent ROS that mediates apoptotic cell death [11-13]. In addition, DOX-induced ceramide production also has been reported causing apoptotic cell death [40-42]. Mitochondrial dysfunction, P53, AMP-activated protein kinase activation and Bcl-2/Bax pathway are also involved in DOX-induce apoptotic cell death [14-16]. Recently, DOX has been proposed to cause cell death by autophagy and necrosis via poly (ADP-ribose) polymerase-1 and independence of P53 [17].

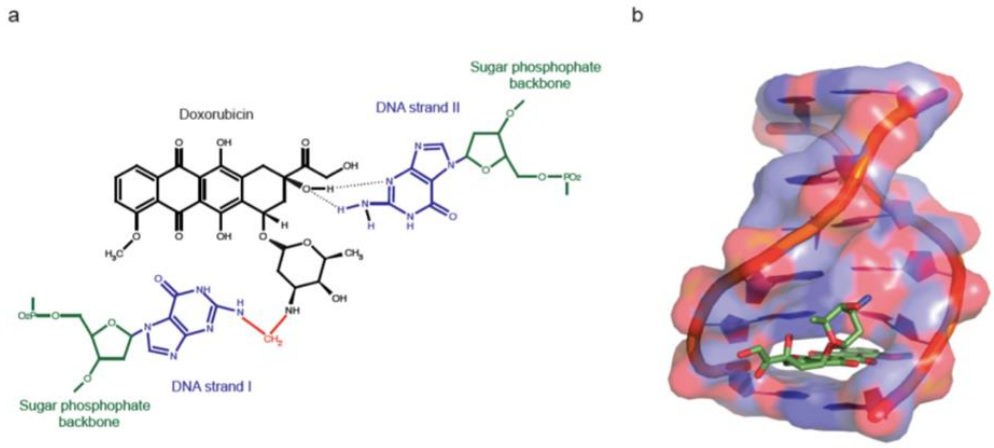


Figure 1: Intercalation of Dox and DNA strands, preferably at cytosine-guanine nucleotide pair resulting in separation of double strand DNA (Yang F, et al., 2014).

DOX has an effectiveness in tissue distribution and freely diffuses into cells after an injection. DOX consists of the ketone, sugar and aglycone structures that are mainly metabolized in liver and mostly excreted in the bile [43, 44]. The ketone structure is metabolized by aldo-ketoreductase forming doxorubicinol, the main metabolite of DOX. The sugar structure is metabolized to form doxorubicinone and doxorubicinolone. The aglycone structure is also metabolized and results in 7-deoxydoxorubicinone and 7-deoxydoxorubicinolone [45]. Moreover, the metabolism of DOX generates semiquinone, a potent ROS produced by the reductive activity of the aglycone structure of DOX [11-13].

In addition to an anti neoplastic effect, DOX also has many side effects to several organs such as bone marrow toxicity, gastrointestinal toxicity, neurological toxicity and cardiotoxicity, a well known and serious toxicity [46]. Cardiotoxicity is irreversible and depends on accumulated dose of DOX, reported over 500 mg/m2 of

DOX mediated cardiomyopathy in 4% of patients and 18% of patients when over 550 mg/ m2 of DOX [47]. DOX-related cardiotoxicity can occur in few days after DOX administration [48]. Many mechanisms of DOX-related cardiotoxicity have been reported. ROS-mediated cell death is mainly associated with DOX-related cardiotoxicity (figure 2) [49]. DOX also disrupts the mitochondrial electron transport chain by binding to cardiolipin. DOX-cardiolipin complex leads to ROS formation and mitochondrial dysfunction [50]. Mitochondrial ultrastructure also can be injured by DOX-mediated ROS formation [51]. Cardiac muscles are unable to use energy from mitochondria, the major source of energy, leading to cell death. Moreover, DOX has been proposed to cause mitochondrial dysfunction independent of ROS formation [52-54].

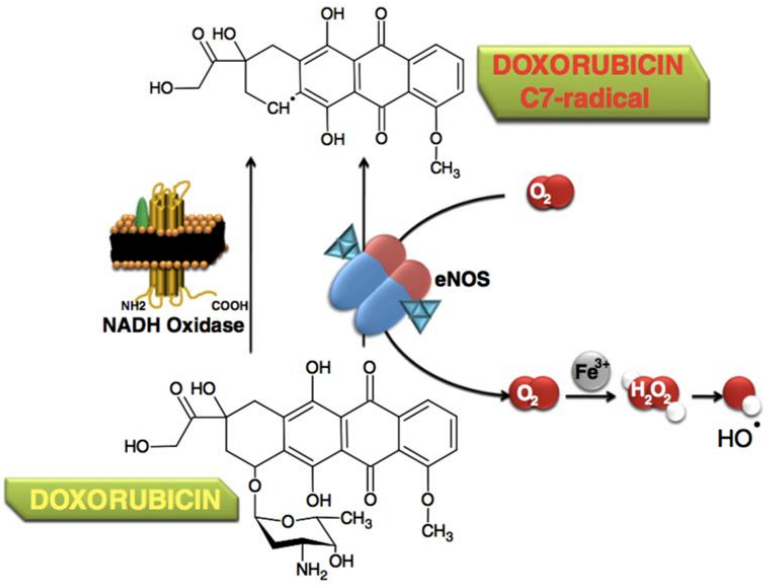


Figure 2: Cellular toxicity induced by Dox-mediated ROS formation (Octavia Y, et al., 2012).

NAD(P)H complex can be generated in DOX-related cardiotoxicity leading to ROS formation [55, 56]. Single nucleotide polymorphism (SNP) has been proposed as an important role in DOX-related cardiotoxicity from NAD(P)H complex generation. DOX also forms complex with eNOS reductase resulting in ROS formation [56]. But the role of other isoforms, inducible nitric oxide synthase and neuronal nitric oxide synthase, need further studies.

Cardiomyocytes contain with iron. DOX can be reduced by iron and forms semiquinone, a potent ROS [52, 53]. A number of studies have been suggested that ROS formation does not only depend on the interaction between DOX and intracellular iron. An increase of intracellular iron via the interaction between doxorubicinol and Fe-S group also induces ROS formation from DOX reduction by intracellular iron [57]. The animal model study has been proposed that iron storage is also involved in DOX-related cardiotoxicity [58]. In addition to intracellular iron, intra mitochondrial iron is also associated with DOX-related cardiotoxicity [54]. Many studies have shown the results of iron chelators, especially dexraxozane that has a protective effect against DOX-related cardiotoxicity in some studies [59, 60]. ABCB8, a protein transporter, involves in mitochondrial iron transport [54, 61]. This transporter also has a protective effect in DOX-related cardiotoxicity.

In addition to ROS formation, DOX-related cardiotoxicity also results from the other mechanisms. Apoptosis associates with DOX-related cardiotoxicity (figure 3) [49].

Caspase-3 activation, toll-like receptors have been proposed to be involved in apoptosis-induced cardiotoxicity [62-64]. Heat shock proteins are inducers and protective proteins that are also involved in apoptosis-induced cardiotoxicity [65, 66]. Moreover, DOX can cause abnormal repair of cardiomyocytes via increasing level of metalloprotinases-2 and metalloprotinases-9 [67]. Dysregulation of intracellular calcium, ceramide production, neuregulin signaling and COX-2 inhibitors have been shown to induce DOX-related cardiotoxicity [68-71].

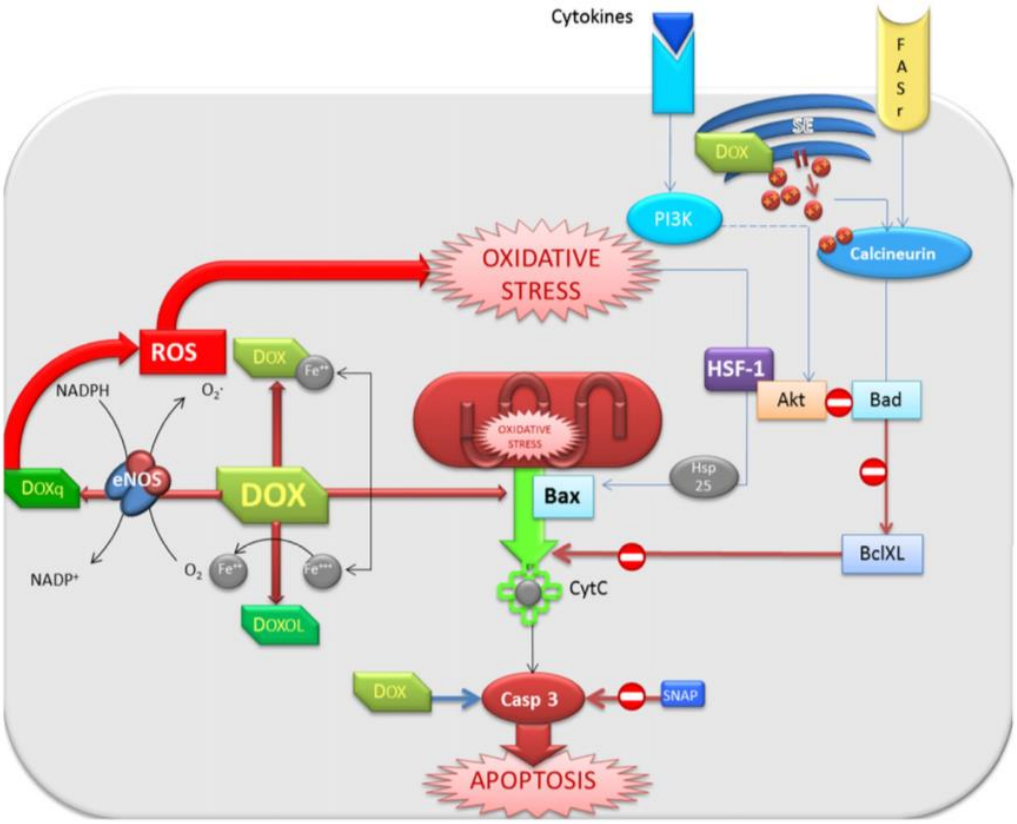


Figure 3: Mechanism of apoptotic cell death in Dox-induced cardiotoxicity (Octavia Y, et al., 2012).

Cardiomyopathy from DOX toxicity causes many changes of histologies and morphologies. A large area of fibrosis, inflammatory cells and fibroblast proliferations

are usually shown in cardiomyocytes. Vacuolated cardiomyocytes and loss of myofibrils are the specific characteristic of DOX-induced cardiomyopathy. Four chambers of heart are dilated resulting in dilated cardiomyopathy. Reduced cardiac contractility also leads to clinical significance in patients [48].

The ABC transporters and P-gp

The ABC transporters are well-established as a drug transporter and have an important role in many drug-resistant cancers. ATP hydrolysis energizes these transporters to transport substrates throughout cell membrane. Moreover, ABC transporters protect many cells and maintain normal physiology of cells [72, 73]. These transporters consist of two parts of six transmembrane proteins and two nucleotide-binding sites. Walker A and B motifs are found in ATP-binding sites within intracytoplasmic nucleotide-binding sites. A signature (C) motif is only found within nucleotide-binding sites of ABC transporters. Multiple substrates transport between two parts of transmembrane proteins (figure 4) [74]. Several transportation mechanisms of ABC transporters have been demonstrated (figure 5) [73, 75]. Conformational change of transmembrane proteins, inward and outward was the first model of transportation mechanism [76].

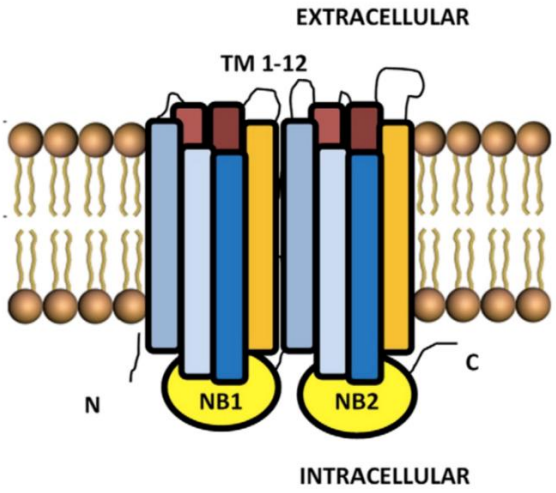


Figure 4: Structure of ABC transporter containing with two parts of six transmembrane proteins and two nucleotides-binding sites (García-Carrasco M, et al, 2015).

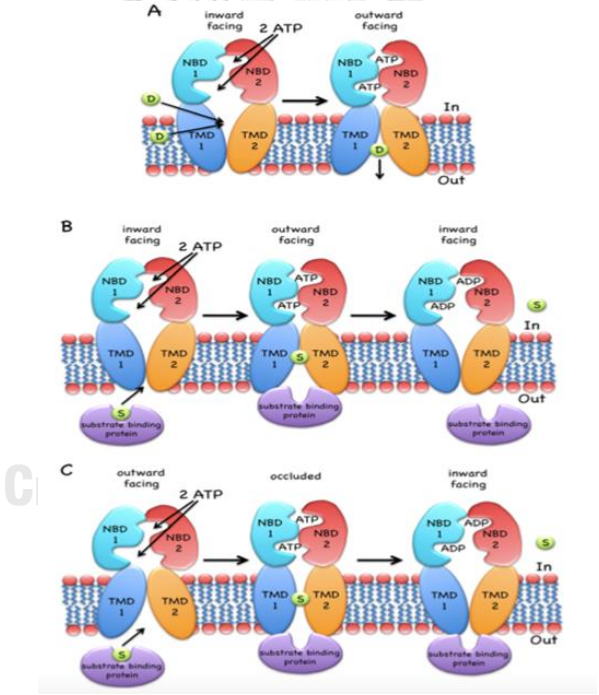


Figure 5: Patterns of transportation mechanisms of P-gp (Wilkens S, 2015).

(A) The inward-facing exporter binds to substrate leading to conformational change to the outward-facing exporter and transport substrate outward, (B) The inward-facing type I transporter, (C) The outward-facing type II importer.

There are forty-nine superfamilies of the human ABC genes with seven subfamilies, A to G. ABCB or MDR and ABCC encoded multidrug resistance-associated protein (MRP) are associated with multidrug resistances [77-79]. ABCB1 is usually found overexpression in drug-resistant cells. Many drugs such as doxorubicin, vincristine and colchicine are affected. T3587G, SNP was found in nonfunctional ABCB1 [80]. The other ABCB defects can cause many diseases in humans depending on functions of ABCBs. For example, ABCB4 and ABCB11 are involved in cholestasis liver diseases [81, 82]. ABCC1 weighs 190 kDa and locates on chromosome 16p13.1 containing with two parts of six transmembrane proteins, one part of five transmembrane proteins and two intracytoplasmic nucleotide-binding proteins [83, 84]. ABCC1 is also the gene of a chemotherapeutic drug transporter, MRP1. An overexpression or mutation of ABCC1 also causes chemotherapeutic drug-resistances such as doxorubicin and vincristine. The study has been reported drug-sensitive ovarian cancer in G2168A variant of ABCC1 [85]. Moreover, ABCC1 variants are also associated with treatment complications. ABCC1 with IVS16 and A1695T variant have shown to develop peripheral neuropathy from vincristine [86]. A recent study has demonstrated ABCC1 prevents doxorubicin-induced cardiomyopathy. In addition, patients with doxorubicin-induced cardiomyopathy have been reported having G2012T variant. The mechanism of cardiomyopathy in patients with G2012T has been proposed that is independent on transportation function of ABCC1 [56]. Moreover, the defects of ABCC1 can cause many diseases in humans. For example, cystic fibrosis, it is an inherited, autosomal recessive

disease with high prevalence in Caucasians. The mutation of ABCC7 that encodes cystic fibrosis transmembrane conductance regulator (CFTR), a chloride channel leading to exocrine gland dysfunctions and mucociliary clearance defects especially in respiratory tract [87].

The other subfamilies of ABC transporters also function in normal physiology and can cause many diseases. Subfamiliy A has an important function in lipid transport. High-density lipoprotein deficiency, Tangier disease and retinitis pigmentosa develop in ABCA mutations [88]. ABCE and ABCF are members of ABC transporters that lack of transmembrane proteins and do not relate to any diseases [78, 89]. ABCG2 has been proposed to be involved in steroids resistance. Mutations of ABCG5 and ABCG8 result in hypercholesterolemia [72].

P-gp was firstly reported in 1970s [90]. A number of studies have demonstrated P-gp characterization by using P-gp-specific monoclonal antibodies, cDNA sequence analysis and P-gp transfection [91, 92]. A detection of intracellular rhodamine 123 has been used to indicate the activity of P-gp [93, 94]. P-gp is also a member of 49 superfamilies of ABC transporters encoded by MDR-1 or ABCB1 located on chromosome 7q21.12. The molecular weight of P-gp is 170 kDa consisted of 1280 amino acids [72-74]. Transmembrane protein domains 1, 5, 6, 11 and 12 are specific binding-sites for various substrates [95, 96]. When substrates bind to specific binding sites, two parts of six transmembrane proteins and nucleotide-binding sites form a

transmembrane pore. Substrates are transported via this transmembrane pore. Of interest, an expression of P-gp is controlled by many mechanisms. A number of studies have suggested that posttranscriptional modifications are involved in P-gp expression. Inhibitions of mitogen-activated protein kinase (MAPK) and heat shock protein 90 can reduce P-gp expression in colorectal tumor cells. In addition, an overexpression of MAPK members has been reported resulting in increasing P-gp expression. Degradation of P-gp by autophagy and ubiquitin-proteasome pathway are also involved in regulation of P-gp expression [96]. P-gp expression are also depend on transcription of MDR1. Many transcriptional factors such as AP-1 and NF-κB have been reported to be involved P-gp expression through binding to MDR-1 promoter and activation of MDR1 transcription [97, 98]. Moreover, many microRNAs have been proposed to downregulate and upregulate MDR-1 transcription [74].

There are three types of P-gp that two types, type I and II, have been reported to cause drug resistances. Moreover, type I and III are found in many organs and control normal homeostasis [99]. Normally, P-gp localizes in many organelles and transported-epithelium of many organs such as kidney, liver, intestines, blood brain barrier and placenta. MDR1a knock out mice have shown an important role of P-gp in blood brain barrier which has a selective permeability to drugs and toxins. Moreover, the study has been reported that MDR1a knock out mice have shown higher level of neurotoxin than wild type mice [100]. Recently, many reports have shown an overexpression of P-gp in

epileptic drug-resistant patients by decreasing drug concentration. The roles of antiepileptic drugs as a substrate or an inhibitor for P-gp also have been proposed as one of mechanism in antiepileptic drug resistance. Because P-gp presents in gastrointestinal tract, drug absorption and drug interaction are also influenced by P-gp [101].

Several drugs are transported by P-gp. Therefore, drug interactions resulting from P-gp effects need to be considered for drugs co-administration. Neurotoxicities of antidiarrheal drug, loperamide have been reported in patients receiving this drug with P-gp inhibitors. The mechanism has been proposed that P-gp in gastrointestinal tract is inhibited resulting in increase of drug absorption to central nervous system. Therefore, P-gp has an important role in the pharmacokinetic which need to be considered in clinical drug uses [102].

In human immunodeficiency virus (HIV) infected patients, there have had the studies that report an increase in P-gp expression on T-cells whereas P-gp activity does not increase [103, 104]. Moreover, the study has shown an increase in P-gp expression T-cells in HIV patients that are associated with a number of HIV replications [105]. Some antiretroviral drugs are well-known as substrate for P-gp especially protease inhibitor drugs. In contrast, ritonavir is an exceptional protease inhibitor, it is a well known P-gp inhibitor that is used for combination with other protease inhibitors for HIV therapy [106]. P-gp-knockout mice have shown an increase in ratio of atazanavir, protease

inhibitor concentration in brain to blood comparing to wild type mice [107]. Zidovudine is one of antiretroviral drug that has less efficacy in P-gp expressed and HIV infected cells [108]. Moreover, nevirapine, delavirdine and efavirine can induced P-gp expression [109]. Because of the effects of P-gp, they cause many problems in HIV treatment especially in an effectiveness of viral suppression.

Moreover, many reports have demonstrated that P-gp is involved in various diseases such as neurological diseases and autoimmune diseases. The study has shown the variants of P-gp, G2677T and C3435T are associated with inflammatory bowel diseases [110]. P-gp expressed lymphocytes have been reported to be associated with severity and treatment responsiveness in systemic lupus erythematosus and rheumatoid patients [74]. A number of studies have shown overexpression of P-gp in many chemotherapeutic drug resistances such as vinca alkaloids, taxanes, especially in anthracyclines, doxorubicin and daunorubicin. Cross resistances between chemotherapeutic drugs also have been reported. Of interest, chemotherapeutic drug resistances can occur without prior exposure to chemotherapeutic drugs [111].

DOX-resistant cancer

DOX resistant cancer is an important problem that limits chemotherapeutic treatments and causes difficulty in cancer treatments. Many cancers develop chemotherapeutic drug resistance such as breast cancer, lung cancer and hematologic malignancies. Cross resistance can develop independently to relations of structural

and functional group, known as pleiotropic drug resistance. But there was no report of cross resistance of DOX to antimetabolites and alkylating drugs [111].

Many resistant mechanisms of cancers have been investigated and have been proposed. P-gp-related resistance is a well-known mechanism including other MDR proteins such as MRP and breast cancer resistance protein (BCRP). Expression of P-gp has been detected to correlate with NCI-60 tumor resistant cell lines. Overexpression of P-gp in cancer cells results in drug resistance [112]. Decrease in intracellular drug accumulation is mediated by P-gp efflux pump effect [22]. MRP also causes resistances to chemotherapeutic drugs same as P-gp such as anthracyclines and vincristine [84]. Alteration of topoisomerase II expression is one of the resistance mechanism that has been reported in etoposide resistant cancer cells that has a cross resistant activity to doxorubicin [113]. Moreover, DOX-induced ceramide production contributes to DOX resistance. Ceramide upregulates overexpression of glucosylceremide synthase (GCS) that induces ceramide glycosylation. By this process, generation of positive feedback through Sp1 transcription factor causes anti apoptotic process resulting in DOX resistance [114]. In addition, the study has reported that high mobility group box 1 (HMGB1)-mediated autophagy is associated with doxorubicin-resistant osteocarcinoma cell lines [115]. In melanomas, doxorubicin resistance has reported contributing to ABCB8 by protecting mitochondria from ROS [116].

Recently, many studies have been demonstrated several therapeutic modifications to overcome the overexpression of P-gp in resistant tumors. Initially, P-gp inhibitors have been developed. Verapamil, a calcium channel blocker and cyclosporine A, a calcineurin inhibitor are the first generation of P-gp inhibitor [117, 118]. These two drugs block P-gp activity resulting in increased intracellular accumulation of substrates or drugs. In clinical uses, higher doses of verapamil and cyclosporine A are required and lead to undesirable side effects especially cardiovascular and immunosuppressive side effects [118, 119]. Therefore, the new generation of P-gp inhibitors are undergoing development. For example, dexverapamil, the second generation of P-gp inhibitor, can block P-gp but also causes toxicity in animals. Biricodar, the third generation of P-gp inhibitor blocks both P-gp and MRP [120, 121]. Moreover, the other new generation of P-gp inhibitors such as tariquidar block P-gp and BCRP [121]. Of interest, the other P-gp inhibitors have been synthesized from natural derivatives such as coumarins, flavonoids and terpenoids [122].

To overcome P-gp-mediated drug resistances, modulations of drug bioavailability and transportation are important ways to increase drug efficacy. Polyethylene glycol (PEG) based surfactants is hydrophilic and also uses in drug formulations to increase the ability of drug dissolution resulting in improvement of bloodstream transportation. In addition, there have been many studies about nanoparticles that have been used as drug carrier [123, 124]. Doxorubicin and

curcumin, the p-gp inhibitor-loaded nanoparticle with PEG and biotin outer layers has been reported to has an antineoplastic effect against MDR breast cancer cell lines [125]. The outer layers have been proposed to protect the nanoparticle from degradation by immune system and improve drug transportation.

Glucocorticoids and dexamethasone

Dexamethasone is one of synthetic glucocorticoids, a steroid hormone. Glucocorticoids are cholesterol-derived hormones. The zona fasciculata, the cortex of adrenal glands synthesizes and secrets glucocorticoids under hypothalamic-pituitary-adrenal (HPA) axis regulation. The serum level of glucocorticoids is influenced by circadian rhythm that peak concentration is found in the morning. Many physiologic responses are controlled by glucocorticoids such as stress responses, cell metabolisms and cell growth [126]. Glucocorticoids action by binding to glucocorticoid receptor (GR) and mineralocorticoid receptor (MR). NR3C1 gene locates on chromosome 5 q31-q32. This gene is responsible for cytoplasmic GR encoding, while MR is encoded by NR3C2. GR is a member of nuclear receptor superfamily. N-terminal transactivation domain (NTD, activation functional domain-1, AF-1), DNA-binding domain (DBD), C-terminal ligand-binding and heat shock protein-binding domain (LBD, activation functional domain-2) and hinge region are consisted of GR (figure 6) [127]. When ligands absence, GR binds to heat shock proteins, immunophilins and several kinases at LBD.

Dysfunction of NTD which containing AF-1 reduces GR gene expression. Moreover, activation of AF-1 and AF-2 activate transcriptional activity [128, 129].

Two isoforms of GR, GRα and GRβ isoform have different actions. The GRα isoform binds to glucocorticoids and nucleus leading to activation of transcriptional activity. On the other hand, the GRβ isoform actions as a negative control of the GRα isoform and does not bind to glucocorticoids [128].

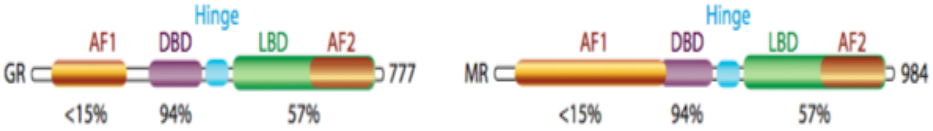


Figure 6: Structures of GR and MR containing with DBD, LBD, AF-1 and AF-2 (Sacta MA, et al., 2016).

GR normally locates in the cytoplasm when glucocorticoids bind to GR resulting in glucocorticoid-GR complex conformational change and translocation into the nucleus. The intranuclear glucocorticoid-GR complex binds to DNA at glucocorticoid response elements (GREs) locating on DBD that results in activation of transcriptional activity, called transactivation. Many side effects glucocorticoids such as osteoporosis, exogenous cushing and diabetes are mediated by transactivation effect. Productions of many anti-inflammatory proteins are activated. In contrast to transactivation, glucocorticoids can suppression transcription factors by directly binding to transcription factors or competition with transcription factors to bind to coactivation factors. This suppression mechanism is called transrepression. The useful ant-inflammatory effects

of glucocorticoids are believed to cause by transrepression mechanism [128, 130]. Moreover, GR can action independently to transcriptional activation that results in non-genomic effects. The study has been reported that the membrane-bound GR can activate mitogen-activated protein kinase pathway resulting in earlier onset and duration of glucocorticoid effects comparing to activation via binding to DNA (figure 7) [130]. There are two types of glucocorticoid membrane receptors. The α membrane receptor activation results in activation of intracellular signals. The G-coupled protein receptor is believed to be the other type of glucocorticoid membrane receptor. Because of non-genomic effect of glucocorticoids, the high-dose and very high-dose glucocorticoid treatment are used to treat an acute and active diseases such as active systemic lupus erythematosus and systemic vasculitis. The high-dose and very high-dose glucocorticoid can saturate intracytoplasmic GR and the unbound glucocorticoid bind to membrane receptors leading to non-genomic effects. The rapid and high efficacy of non-genomic effects action as anti-inflammation and immunosuppression to an active and life-threatening diseases [128, 130].

The anti inflammatory property of glucocorticoids was firstly established in patient with high blood cortisol who had rheumatoid arthritis [131]. The mechanisms of anti inflammatory effect relate to inhibition of inflammatory cytokines such as interleukins and prostaglandins. Moreover, glucocorticoids inhibit vascular changes following inflammatory process. Cell death via apoptosis also occurs in glucocorticoid

treatment. Because of these mechanisms, glucocorticoids have been used in many hematologic and solid cancer treatments [132]. The study has been reported that dexamethasone mediates apoptotic cell death via inducing the expression of apoptotic-mediated gene in lymphoma cells [133]. In solid cancers, glucocorticoids have shown benefits in estrogen receptor positive breast cancer and androgen receptor positive prostate cancer [134, 135]. Moreover, the inhibitions of MMP2/9 and interleukin-6 and inhibition of angiogenesis have been proposed as the mechanisms of glucocorticoids in metastatic cancer treatment [136]. Glucocorticoids also used for relieving pain and complications of cancer treatments such as nausea. For example, Dexamethasone is generally used in cancer treatments for antiemetic effect, edema reduction in brain tumors and chemotherapeutic regimens of hematologic malignancies [137].

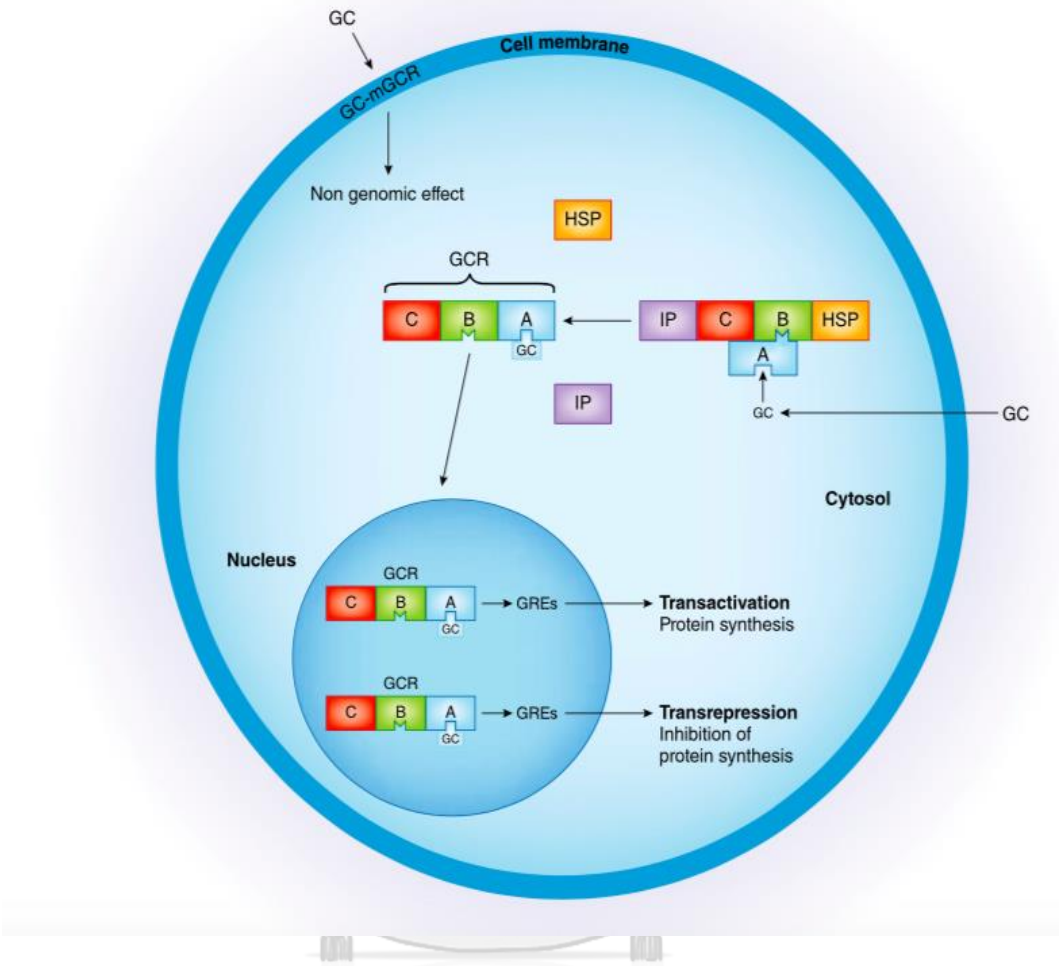


Figure 7: Mechanisms of glucocorticoids-mediated genomic (transactivation and transrepression) and non-genomic effects via binding to intracytoplasmic GR and membrane-bound GR (Ponticelli C, et al., 2018).

CHAPTER III
MATERIALS AND METHODS

Cell lines and Cell culture

The human breast cancer cell line, MCF7, was obtained from American Type Cell Collection (ATCC; Manassas, VA, USA). The cells were cultured to 70-80% confluence in Dulbecco's modified Eagle medium (DMEM; Gibco®, Thermo Fisher Scientific, Waltham, MA, USA) with 10% fetal bovine serum (FBS) (Gibco®) at 37ºC and 5% CO_2.

MDR-1 overexpression

To stimulate drug-resistance in MCF-7, we transfected MCF-7 cells with an ABCB1 pCMV GFP-tagged plasmid (OriGene Technologies Inc., Rockville, MD, USA). One day prior to transfection, the cells were plated at 10,000 cells per cm^2 in a 25 ml flask. Transfection was performed by mixing 2 µg of ABCB1 pCMV GFP-tagged plasmid with Xfect transfection reagent (Clontech, Takara Bio USA Inc., Mountain View, CA, USA) to a final volume of 100 µl. Next, 2.5 µl of Xfect polymer was added into the tube containing the plasmid. The tube was then vortexed and incubated for 10 min at room temperature (RT). The contents of the tube were then added to the cells. Our preliminary analysis showed that approximately 85% of the cells were expressing GFP at day 5 following transfection.

One-pot, two-steps conjugation of dexamethasone to DOX

One pot, two-step reactions were performed as follows. In the first step of the reaction, equal moles of DOX (Selleck Chemicals, Houston, TX, USA) and 2-iminothiolane (Sigma-Aldrich Co., St. Louis, MO, USA) were mixed together in DMSO (Sigma-Aldrich Co.) for 48 h at RT. Equal moles of dexamethasone (Selleck Chemicals) comparing to the moles of DOX in DMSO was then added drop-wise to the first reaction, and then mixed, and incubated for 96h at RT. The products were further purified by flash chromatography using a pre-cast silica column (StarFlash™ Column, Starlab Scientific Co Ltd., Xi an City, Shaanxi Province, People's Replublic of China). In some experiments, the products were dialyzed in deionized water with a molecular weight cut-off of 1000 Dalton (Pur-A-Lyzer™ Mega 1000 Dialysis Kit, Sigma-Aldrich, St Louis, MO, USA) before carrying out the flash chromatography. The chromatography fractions were dried via negative pressure evaporation. The product was kept dry by placing it into a desiccator.

Thin-layer chromatography (TLC)

TLC was performed at each step of the reaction and purification process. Samples were dropped onto and dried in pre-cast silica gel (Miles Scientific, Newark, DE, USA). TLC was run using a 15: 1.5: 5 ratio of chloroform: acetic acid: methanol (V/V). The gels were directly visualized or visualized under UV light.

Mass spectroscopy

Molecular weight of DexDOX was determined by atmospheric pressure chemical ionization (APCI) microTOF mass spectroscopy (Bruker Daltonics, Billerica, MA, USA). The sample was dissolved in 0.05% formic acid in methanol (v/v). The acquisition parameters of operating were positive ion polarity, 5000 V capillary, 1.6 Bar nebulizer, 200 °C dry heater and 8.0 l/min dry gas. The spectrum was scanned from 100 m/z to 1800 m/z.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay

MCF-7 cells and MDR-1-transfected MCF-7 cells were plated overnight in a 96-well plate (4,000 cells per well). Cells were treated with either DOX at a final concentration of 0-12.5 μg/ml, or DexDOX at a final concentration 0-120 μg/ml, in triplicate for 24 h. Next, 10 μl/well of WST-1/ECS solution from the Quick Cell Proliferation Colorimetric Assay Kit (BioVision, Inc., Milptas, CA, USA) was added, and the solution incubated for 2 h at 37°C and 5% CO_2. Absorbance was measured at 480 nm with the Biochrom Anthos 2010 microplate reader (Biochrom Ltd., Cambourne, Cambridge, UK). IC50s were calculated from linear regression from the absorbance OD of each triplicate concentration [34].

Video confocal fluorescent microscopy

Confocal microscopy was performed to allow analysis of the intracellular distribution and cytopathic effects. Microscopy was performed using an LSM 800 confocal microscope (Carl Zeiss, Jena, Germany). Low numbers of MCF-7 cells were seeded into 24-well plate (3,000 cells/well) overnight. On the day of the experiment, culture media was replaced with 10% FBS DMEM added as a supplement, together with either 100 μg/ml DexDOX, or 3 μg/ml DOX (approximately IC60). Videos were recorded using ZEN software version 2.1, every 20 min for a total duration of 12 h.

Flow cytometry for cell cycle analysis and apoptosis detection

MCF-7 cells were treated with either 100 μg/ml of DexDOX or 3 μg/ml of DOX for 48 h. Both adherent and floating cells were collected and washed twice with cold PBS. The cell pellet was resuspended in 200 μl of PBS and then 10 μl of PI staining mixture was added to each of the samples. The samples were then incubated for 30 min at RT. Analysis was carried out using FACs Calibur (BD Biosciences, San Jose, CA, USA). For analysis of apoptosis, cells were stained with FITC Annexin V Apoptosis kit and PI (BioLegend®, San Diego, CA, USA) according to the manufacturer's protocol.

Fluorescent microscopy of dichloro-dihydro-fluorescein diacetate (DCFH-DA) assay and 2'-[4-ethoxyphenyl]-5-[4-methyl-1-piperazinyl]-2,5'-bi-1H-benzimidazole trihydrochloride trihydrate (Hoechst 33342) fluorescent stain

MCF-7 cells were seeded overnight into 8-well chamber slides at 4,000 cells per well. Cells were washed with HBSS buffer. Pre-warmed DCFH-DA solution was then added to a final concentration 100 µM. Next, the cells were incubated for 30 min at 37°C, 5% CO_2. The DCFH-DA solution was then removed. Fresh media containing either 3 µg/ml of DOX or 100 µg/ml of DexDOX was then added. The expression of dichlorofluorescin (DCF) in treated cells was observed at 0,20, 40, and 60 min. Hoechst 33342 (Thermo Scientific™, Rockford, IL, USA) was diluted in PBS to final concentration, 1 mg/ml. Later, 0.1 µg/ml Hoechst 33342 was prepared by 1:10,000 dilution of 1 mg/ml Hoechst 33342 in Assay Buffer (0.1 M NaCl, 10 mM EDTA, 10 mM Tris, pH 7.0.). Hoechst 33342 was added to the treated MCF-7 cells before the expression of DCF was observed by fluorescent microscopy (Olympus BX51, Tokyo, Japan) and images taken using the Olympus DP71 system [35].

Statistical analysis

Numerical data are expressed as mean ± SD. Comparison between two groups was assessed by pair t-tests (SPSS version 13). A $P<0.05$ was considered to be statistically significant.

CHAPTER IV
RESULTS

Purified and characterization of DexDOX product

The conjugation process was carried out in DMSO at RT. The product was purified by flash chromatography. The fractions collected from the chromatography were then subjected to vacuum drying, resulting in a dried purple material. This material could not be dissolve in water, methanol, or ethanol, suggesting that the molecule is strongly non-polar (Figure 8a). TLC analysis showed the presence of a single, different band that had different retardation factor (Rf) to the original molecule and to un-purified product (Figure 8b). The solution of purified product in DMSO showed a purple color and had a peak absorbance wavelength at 505 nm as compared to 484 nm for DOX (Figure 8c). Electrospray ionization (ESI) mass spectroscopy revealed that the molecule had a mass of 1037 Dalton (Figure 8d).

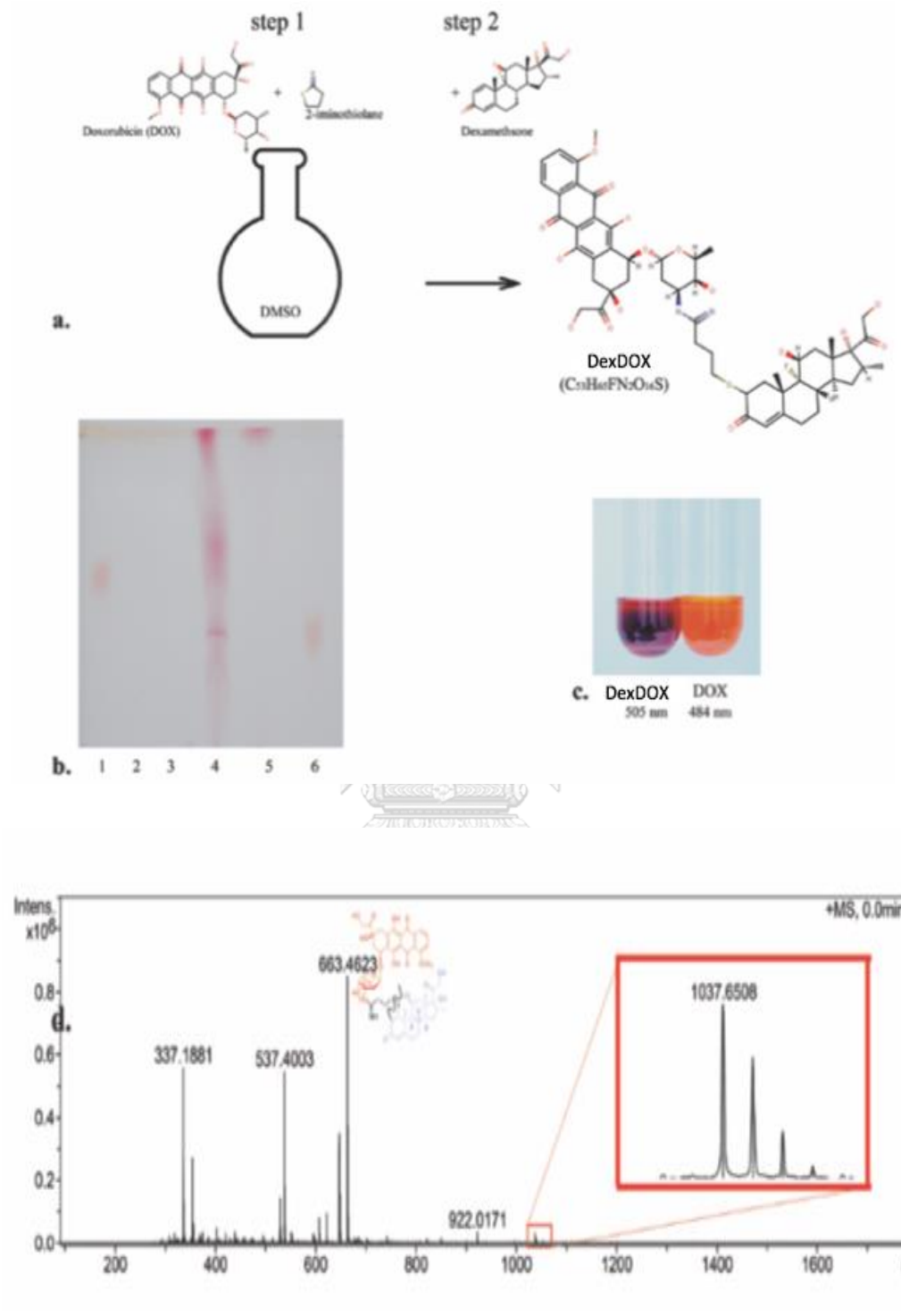


Figure 8: Conjugation scheme and characterization of DexDOX.

a) The diagram shows the 1-pot, 2-step conjugation process, 1st step: a reaction of doxorubicin and 2-iminothiolane at 1:1 mole, 2nd step: equal molarity of

dexamethasone was added. The reactions were performed in DMSO at RT. b) Representative image of TLC showing lane 1: doxorubicin, lane 2: dexamethasone, lane 3: 2-iminothiolane; lane 4: reactions products; fraction A, targeted products, fraction B, lane 5: DexDOX (purified fraction a), lane 6: doxorubicin + dexamethasone c) Representative color of DexDOX and doxorubicin in DMSO solution and D) APCI mass-spectroscopy analysis of DexDOX.

Cytotoxicity of DexDOX in MCF7 cells

We evaluated the anti-proliferative effect of DexDOX by MTT assay. Following a 24 h exposure to varying concentrations of DOX and DexDOX, the number of viable MCF7 cells was found to decrease. An average of calculated IC50s for DexDOX from four, independent experiments was 88.25 ± 6.9 μg/ml. In contrast, the average IC50s for DOX under the same conditions, was 2.8 ± 0.9 μg/ml. This result clearly indicates that DexDOX has less cytotoxicity to MCF-7 cells than DOX.

Difference of cellular distribution between DexDOX and doxorubicin

Cellular entry of DexDOX was evaluated by flow cytometry analysis. MCF-7 cells were treated with DexDOX or DOX for 2 h prior to flow cytometry analysis. The results indicate that >70% of DexDOX-treated MCF cells and almost of the DOX-treated cells emitted a fluorescence signal at the time of flow cytometry analysis (Figure 9).

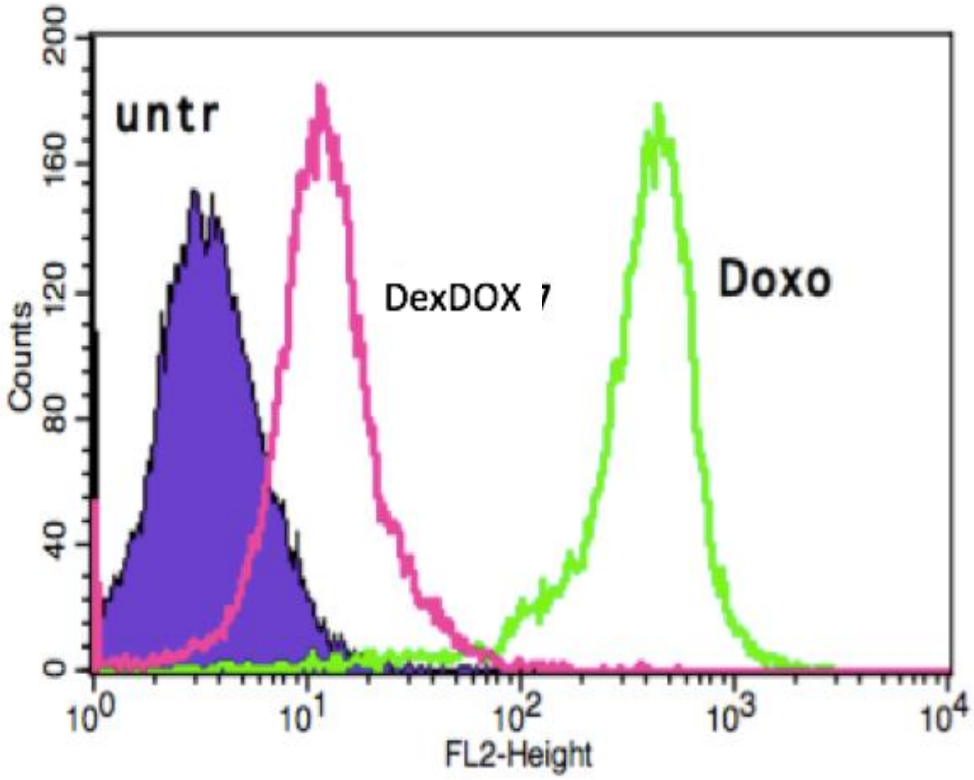


Figure 9 Cellular penetration and retention of DexDOX.
Flow cytometry of MCF-7 cells 2h following incubation with DexDOX and Dox.

As both DOX and DexDOX emitted florescent signals, we employed live cell confocal video microscopy to study the intracellular distribution of both molecules. The fluorescence signals of DOX were found to increase gradually over time during the observation period. The signals were observed in both the cytoplasm and nucleus, but predominately inside the nucleus (Figure10). Interestingly, the number of pyknotic cells (arrow), which indicate the presence of apoptotic cells, gradually increased with increased intra-nuclear accumulation of DOX. The intracellular distribution and the accumulation of DexDOX observed were different from that of DOX. Fluorescence

signals of DexDOX peaked at 3 min after exposure, suggesting that this molecule was more efficient at entering MCF-7 cells than DOX. The DexDOX fluorescence signal rapidly decreased. Notably, apoptotic cells were observed at the early phase of the exposure to DexDOX and their numbers increased further as the fluorescence signal decreased. Interestingly, fluorescence signals from DexDOX were observed only in the cytoplasm, suggesting that this molecule could not enter the nucleus. Taken together, these differences in the patterns of cellular diffusion and distribution suggest that the mechanisms of cytotoxicity employed by DexDOX and DOX are not identical.

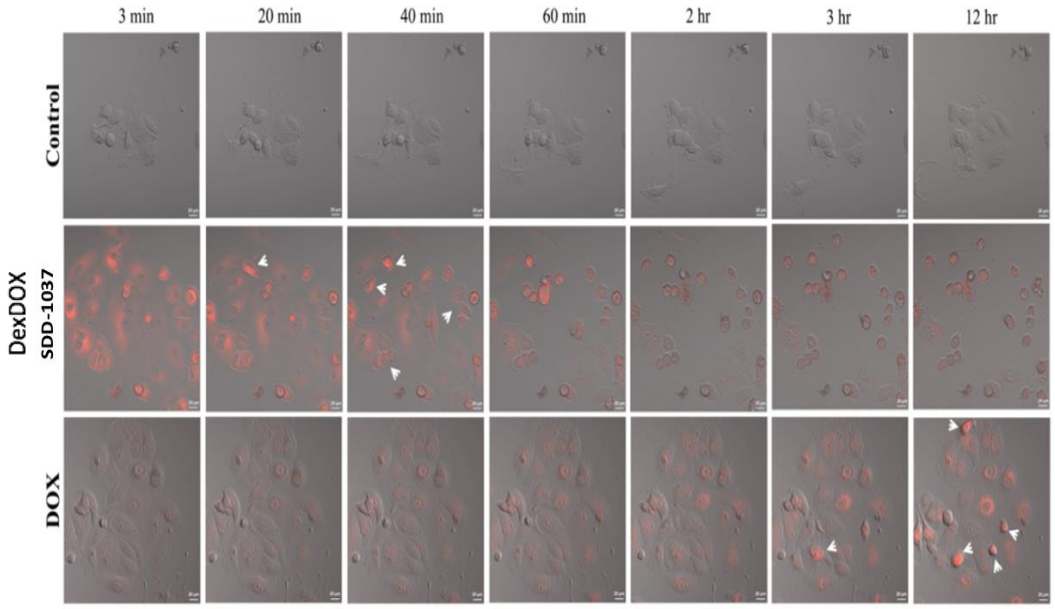


Figure 10: Cellular penetration and subcellular distribution of DexDOX.
Representative images from video-assisted confocal microscopy showed that DexDOX entered MCF-7 more rapidly than doxorubicin. The fluorescence signal was rapidly lost

without entering the nucleus. Interestingly, pyknotic cells which are indicative of apoptosis, were observed (arrow) after the fluorescence signal was lost. In contrast, doxorubicin gradually diffused into cells and accumulated in the nucleus. The apoptotic cells (arrow) were observed in the later phase.

DexDOX-induced cell death via ROS generation, independent of the cell cycle interruption

Flow cytometry analysis of the cell cycle showed that under the conditions used, the majority of the MCF-7 treated with DOX, 55.0%±1.3% of the cells were in G0/G1 phase and approximately 27.4%±0.7% of the cells were in G2/M phase. In contrast, 48 h treatment of the cells with DexDOX did not affect cell cycle progression, about 75.4%±2.2% of the cells were in G0/G1 and 11.5%±1.4% were in G2/M phase as compared to the untreated cells in which 77.4%±2.4% and 10.4%±1.8% of the cells were in G0/G1 and G2/M, respectively (Figure 11a).

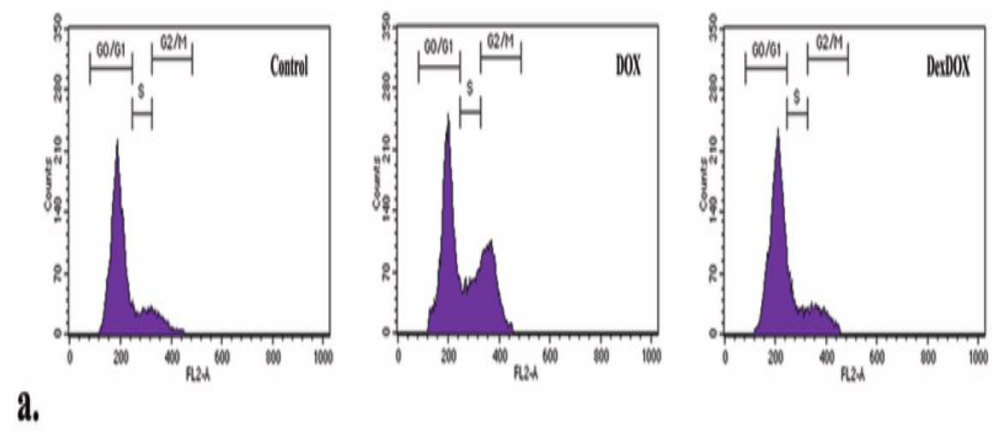


Figure 11a: Cell cycle analysis demonstrated that treatment with DexDOX did not induce cell cycle arrest, whereas doxorubicin induced cycle arrest in the G2/M phase.

Flow cytometry of Annexin V showed that following a 48 h exposure to DOX, approximately 70% of the cells were Annexin+ PI-, indicating that those cells were in an early apoptotic phase/state. In contrast, exposure of DexDOX to IC60 showed the presence of double-positive Annexin V and PI, indicating that the cells were in late apoptosis (Figure 11b). These findings supported the results of video confocal microscopy which showed that an early apoptosis occurred following exposure to DexDOX. To further understand the mechanisms leading to cell death, we indirectly evaluated intracellular ROS generation in DexDOX-treated cells. The expression of intracellular dichlorofluorescin (DCF), an oxidized product of DCFH-DA, was subsequently analyzed/assessed/evaluated. Our results demonstrate that DexDOX increased DCF expression during the early phase of the cell cycle (20-60 min) (Figure 11c). The increase in DCF signals during the first 60 min suggest that cellular ROS increased during this period.

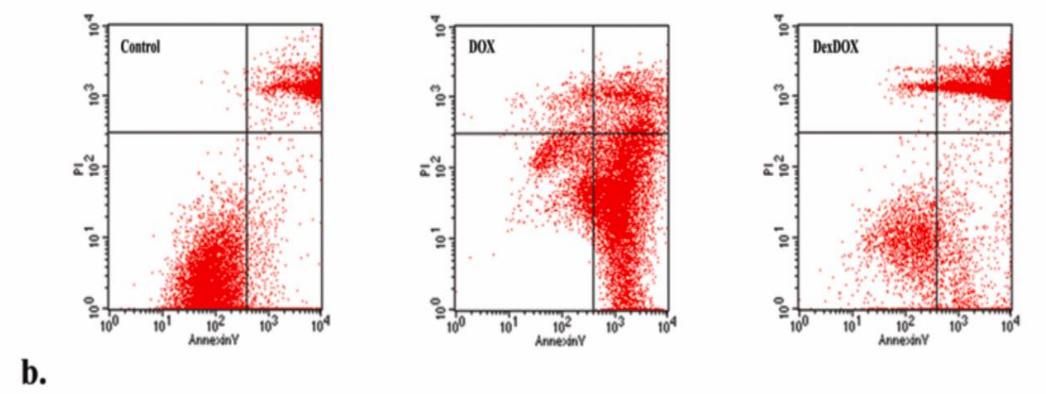


Figure 11b: Representative analysis of Anenxin V and PI showed that the majority of the MCF-7 cells were double-positive for Anexin V and PI following 48 h of treatment with DexDOX. In contrast, MCF-7 induced MCF-7 expressed Anexin V.

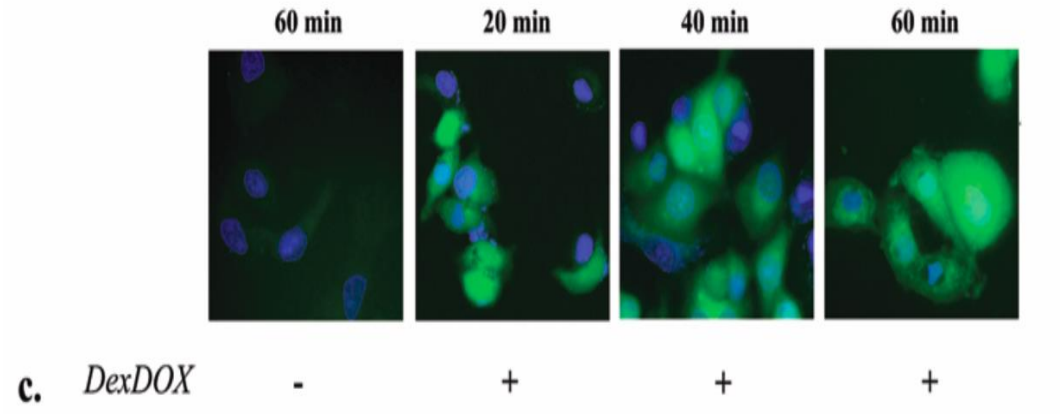


Figure 11c: Representative images of DCFH-DA-treated cells and Hoechst 33342 nuclear-stained cells showing sequential intracytoplasmic expression of DCF (green fluorescence) were generated from oxidaization of DCFH-DA ROS following exposure to DexDOX.

DexDOX can overcome MDR-1 induced resistance in MCF-7 cells

We tested the effect of DexDOX in MDR-1-overexpressing cells. Five days following transfection, approximately 87% of MCF-7 cells displayed a green fluorescent signal. The MTT assay showed that MDR-1-transfected cells could be defined as being resistant to DOX. The IC50s of DOX in MDR-1-transfected cells were 45.15 ± 7.1 μg/ml, or approximately 16-fold of the IC50s of DOX in MCF-7 cells. In contrast, the IC50s of DexDOX in MDR-1-overexpressing cells were 101.82 ± 14.0 μg/ml, which did not differ to those of the control, MCF-7, indicating that overexpression of MDR1 did not lead to a decrease in the cytotoxicity of DexDOX (Figure 12).

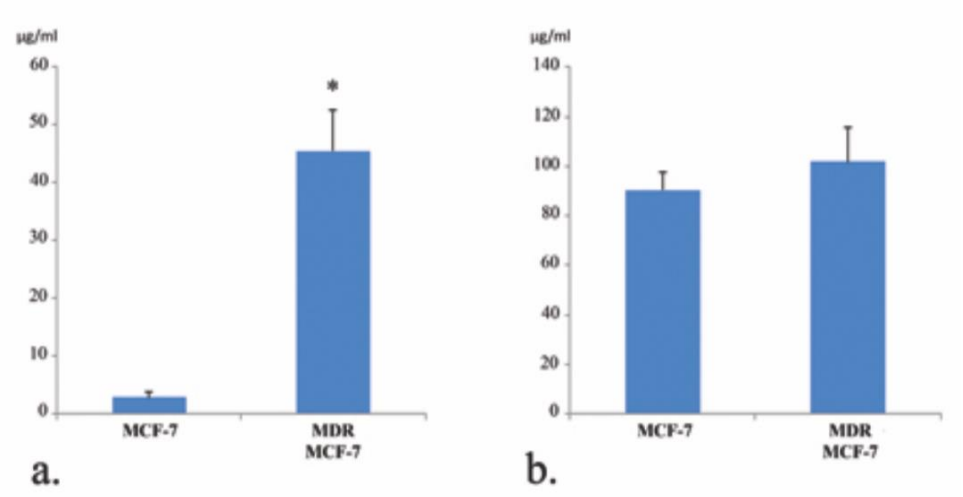


Figure 12: IC50 of DexDOX and doxorubicin in MDR1-overexpressing cells.
Bar graph comparing IC50s of doxorubicin (a) and DexDOX (b) in control MCF-7 and MDR-1-overexpressing MCF-7 cells.

CHAPTER V
DISCUSSION

Bio-conjugation, a process used to attach a bioactive molecule to another molecule via a covalent bond, leads to the formation of a novel chemical structure with may have enhanced properties compared to those of the original molecule. In this study, we synthesized a novel derivative of DOX by simple conjugation with dexamethasone. The chemical reaction is simple and does not require any catalyst or exogenous energy. We have demonstrated that the new derivative has considerable cytotoxicity but acts via a different mechanism to that of DOX.

Dexamethasone is a potent glucocorticoid analog, synthetic, lipophilic, hormone-based drug that is widely used to treat a variety of medical conditions and is included in many standard chemotherapeutic regimens. The 3' amino group in the sugar moiety of DOX which was the conjugation site is known to be a crucial site allowing the DOX molecule to intercalate to the cytosine molecule in the DNA strand. Therefore, it is hypothesized that modification at this residue is likely to interfere with DNA intercalation and topoisomerase inhibition, both of which are known to be key cytotoxic mechanisms employed by DOX. Our video-assisted microscopy study clearly demonstrated that DexDOX did not enter the nucleus showing an obvious difference with DOX in this respect. Interestingly, transferrin-conjugated DOX that was modified at 3' amino group, was also found to be unable to enter the nucleus [33]. Taken

together with our results, the data suggest that this amino group of the sugar moiety may be crucial for giving these molecules the ability to enter the nucleus.

Despite a loss of ability to enter the nucleus, DexDOX elicited potent and rapid cytotoxicity. This molecule showed a greater efficiency in cellular penetration than DOX. This may be due to the lipophilic property of dexamethasone. Peak cytoplasmic fluorescence emission occurred within the first 3 minutes of exposure to DexDOx and was followed by a rapid loss of fluorescence signal. Interestingly, initiation of apoptosis was found to coincide with loss of DexDOX´s fluorescence signal. It is known that the fluorescence signal of DOX and other anthracyclines originates from a free electron traveling along the aglycone structure. However, fluorescence signal loss in our experiment is too rapid to be the result of energy consumption efflux pump function. Therefore, we speculated that a break-down of the aglycone structure of DexDOX had occurred in the cytoplasm. Redox-cycling of aglycone breaks the aglycone structure down, forming semiquinone radicals which are a potent ROS and this is the core process of DOX-mediated oxidative injuries [12]. Our results demonstrate evidence of increased ROS in MCF-7 cells treated with DexDOX in parallel with the observation of a reduction in the cytoplasmic signal of DexDOX. This suggests that the loss of DexDOX fluorescence may contribute to cellular ROS generation. The presence of ROS was detected within 15 min, relatively early stage, following exposure to DOX [138]. It was previously reported that potentiating oxidative stress of DOX can overcome the effect

of efflux pump-induced resistance [13]. DexDOX-induced cellular cell cycle independent apoptosis after entering cells. We speculated that the rapid action of DexDOX in the generation of ROS may be crucial for overcoming the P-gp efflux pump, which needs sufficient time to extrude the drug from cells. Therefore, the over-expression of P-gp had little effect on DexDOX cytotoxicity.

DOX is best known for its cardiotoxicity. Again, the molecular mechanisms of DOX induced myocardial damage remains unclear. A line of evidence suggests that ROS generation contribute to myocardial injury [3]. However, antioxidant treatments failed to improve myocardial damage. Of interest, a recent study showed that mice lacking of topoisomerase 2β in cardiomyocyte were resisted to DOX induced myocardial damage [139]. Since DexDOX generated oxidative stress without entering to the nucleus, this molecule should be useful for further understanding the mechanism of DOX induced cardiotoxicity.

In summary, we have synthesized a new derivative of DOX by conjugating the dexamethasone molecule to DOX. This molecule showed potent cytotoxicity to MCF-7 cells in a manner that is different to that of DOX. Furthermore, we have shown that this molecule has the ability to escape the effect of P-gp overexpression on drug resistance and that therefore, it may be useful for the treatment of drug-resistant tumor cells.

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APPENDIX

LIST OF ABBREVIATIONS

ABC = Adenosine triphosphate binding cassette

AF-1 = Activation functional domain-1

ATP = Adenosine triphosphate

BCRP = Breast cancer resistance protein

CFTR = Cystic fibrosis transmembrane conduction regulator

DBD = DNA-binding domain

DOX = Doxorubicin

GCS = Glucosylceremide synthase

GR = Glucocorticoid receptor

HIV = Human immunodeficiency virus

HMGB1= High mobility group box 1

LBD = Ligand-binding domain

MDR = Multidrug resistance

MR = Mineralocorticoid receptor

MRP = Multidrug resistance-associated protein

NTD = N-terminal transactivation domain

P-gp = P-glycoprotein

ROS = Reactive oxygen species

BUFFER AND REAGENTS

1. Xfect transfection reagent

ABCB1 pCMV GFP-tagged plasmid	2	µg
Adjust volume to 100 µl with Xfect transfection reagent.		

2. Mobile phase of TLC

Chloroform	15	ml
Acetic acid	1.5	ml
Methanol	5	ml

3. Hank's balanced salt solution (HBSS) buffer

NaCl	8	g
KCl	0.4	g
$CaCl_2$	0.14	g
$MgSO_4$-$7H_2O$	0.1	g
$MgCl_2$-$6H_2O$	0.1	g
Na_2HPO_4	0.06	g
KH_2PO_4	0.06	g
Glucose	1	g
$NaHCO_3$	0.35	g
Distilled water	1,000	ml

4. Phosphate buffer saline (PBS)

NaCl	8	g
KCl	0.2	g
Na2HPO4	1.44	g
KH2PO4	0.24	g
Dissolve in distilled water and adjust pH to 7.4 with HCl.		
Distilled water	1,000	ml

5. 0.1 M NaCl

NaCl	0.84	g

	Distilled water	1,000	ml

6. 10 mM EDTA

EDTA	2.96	g
Distilled water	1,000	ml

7. 10 mM Tris-HCl pH 7.0

For 1 M Tris-HCl pH 7.0:

Tris	12.11	g

Dissolve in distilled water and adjust pH to 7.0 with HCl.

Distilled water	100	ml

To obtain 10 mM Tris-HCl 7.0, dilute 1 ml of 1 mM Tris-HCl pH 7.0 in 99 ml distilled water.

VITA

Miss Kamontip Chaikomon was born on December 7, 1987 in Bangkok. She graduated Doctor of Medicine Program from Srinakharinwirot University. She is third year residency of internal medicine, Lerdsin General Hospital.