คุณสมบัติของไรนาแคนทิน-ซี ต่อการผันกลับของการดื้อยาในเซลล์มะเร็งเต้านม

นายทัศรุจน์ ชัยสิทธิ์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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MUTIDRUG RESISTANCE REVERSAL (MDR REVERSAL) OF RHINACANTHIN-C IN MCF-7 CELLS

Mr. Tassarut Chaisit

A Thesis Submitted in Partial Fulfillment of the Requirements
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Thesis Title	MUTIDRUG RESISTANCE REVERSAL (MDR REVERSAL) OF RHINACANTHIN-C IN MCF-7 CELLS
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Field of Study	Pharmacology
Thesis Advisor	Associate Professor Suree Jianmongkol, Ph.D.

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ทัศรุจน์ ชัยสิทธิ์ : คุณสมบัติของไรนาแคนทิน-ซี ต่อการผันกลับของการดื้อยาในเซลล์มะเร็งเต้านม (MUTIDRUG RESISTANCE REVERSAL (MDR REVERSAL) OF RHINACANTHIN-C IN MCF-7 CELLS) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. ดร. สุรีย์ เจียรณ์มงคล, 70 หน้า.

ไรนาแคนทิน-ซี (RN-C) เป็นสารในกลุ่ม naphthoquinone ester ที่ได้จากต้นทองพันชั่ง โดยสารนี้มีฤทธิ์ทางเภสัชวิทยาหลากหลายรวมถึงฤทธิ์ต้านมะเร็งและต้านไวรัส การศึกษานี้มีวัตถุประสงค์เพื่อตรวจวัดความสามารถของไรนาแคนทิน-ซีในการเพิ่มความเป็นพิษต่อเซลล์มะเร็งเต้านมของด๊อกโซรูบิซิน และการออกฤทธิ์ผ่านการรบกวนการทำงานตัวขนส่งสารออกนอกเซลล์ ทั้งนี้การศึกษาความเป็นพิษต่อเซลล์ทำด้วยวิธี MTT ผลการศึกษาพบว่า ไรนาแคนทิน-ซีในความเข้มข้นที่ไม่เป็นพิษต่อเซลล์ (0.1 μM) สามารถเพิ่มความเป็นพิษของด๊อกโซรูบิซินได้ในเซลล์มะเร็งเต้านม ซึ่งเห็นได้จากค่า IC50 ของด็อกโซรูบิซินลดลง 38 เท่า ที่เวลา 48 ชั่วโมง การเกิดอันตรกิริยาระหว่างไรนาแคนทิน-ซีและด๊อกโซรูบิซินมีลักษณะแบบเสริมฤทธิ์กันโดยมีค่า combination index (CI) อยู่ที่ 0.2 ทั้งนี้การเสริมฤทธิ์ดังกล่าวขึ้นกับเวลาและความเข้มข้นของสารไรนาแคนทิน-ซี นอกจากนี้ยังพบว่า ไรนาแคนทิน-ซีทำให้ระดับด๊อกโซรูบิซินใน MCF-7 เพิ่มขึ้นอีกด้วย ซึ่งเมื่อทดสอบผลการรบกวนของไรนาแคนทิน-ซี ต่อตัวขนส่งโปรตีน (MRP1 และ MRP2) โดยวัดปริมาณสับสเตรตของตัวขนส่งโปรตีนด้วยเทคนิค fluorescence spectroscopy พบว่า การให้ไรนาแคนทิน-ซีความเข้มข้น 0.1 μM แก่เซลล์ MCF-7 เป็นเวลา 12 ชั่วโมง มีผลเพิ่มการสะสมของสับเสตรทภายในเซลล์ได้ [DCDF เพิ่มขึ้น 1.20 เท่า (MRP1) และ CDCF เพิ่มขึ้น 1.90 เท่า (MRP2)] นอกจากนี้ยังพบด้วยว่า ไรนาแคนทิน-ซี สามารถเอาชนะการดื้อต่อยาด๊อกโซรูบิซินในเซลล์มะเร็งเต้านมที่ดื้อต่อยาด๊อกโซรูบิซิน ที่มีการทำงานของระดับ P-gp และ MRP2 โดยพบว่า ร้อยละของการรอดชีวิตของเซลล์ที่ลดลงอย่างมีนัยสำคัญเมื่อให้ไรนาแคนทิน-ซีร่วมกับด๊อกโซรูบิซิน ผลการวิจัยชี้ให้เห็นว่าไรนาแคนทิน-ซีสามารถที่จะเพิ่มความเป็นพิษของด๊อกโซรูบิซินและเอาชนะการดื้อต่อยาได้อย่างมีประสิทธิภาพ จากการรบกวนการทำงานของ MRP2 และ P-gp แต่อย่างไรก็ตามกลไกการเสริมฤทธิ์กันระหว่าง ไรนาแคนทิน-ซีและด๊อกโซรูบิซิน ยังต้องมีการศึกษาเพิ่มเติมต่อไป

สาขาวิชา เภสัชวิทยา ลายมือชื่อนิสิต .....

ปีการศึกษา 2558 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

5587128020 : MAJOR PHARMACOLOGY
KEYWORDS: BREAST CANCER CELL, CYTOTOXICITY, DOXORUBICIN, RHINACANTHIN-C, SYNERGY AND DRUG TRANSPORTERS

TASSARUT CHAISIT: MUTIDRUG RESISTANCE REVERSAL (MDR REVERSAL) OF RHINACANTHIN-C IN MCF-7 CELLS. ADVISOR: ASSOC. PROF. SUREE JIANMONGKOL, Ph.D., 70 pp.

Rhinacanthin-C (RN-C) is a major bioactive naphthoquinone ester found in *Rhinacanthus nasutus* Kurz (Acanthaceae). This compound has potential therapeutic value as an anticancer and antiviral agent. The purpose of this study was to determine the capability of RN-C to enhance cytotoxicity of doxorubicin (DOX) in a breast cancer cell line MCF-7 and the involvement of the ABC drug efflux transporters. The cytotoxicity was assessed by an MTT assay. RN-C at the non-cytotoxic concentration (0.1 μM) was able to significantly enhance DOX-mediated cytotoxicity in the MCF-7. The apparent IC50 of DOX at 48 hr-treatment in the presence of RN-C decreased by 38-fold. The interaction between RN-C and DOX was strong synergism with the combination index (CI) value of 0.2. The degree of synergy between RN-C and DOX was time-and concentration-dependent. In addition, intracellular DOX accumulation in the MCF-7 increased in the presence of RN-C. Furthermore, the interference of RN-C on the ABC drug transporters (MRP1 and MRP2) was evaluated by a substrate accumulation assay, using fluorescence spectroscopy technique. RN-C at 0.1 μM after 12-hr treatment could increase intracellular accumulation of transporter substrate in the MCF-7 cells [i.e., DCDF by 1.20-fold (MRP1) and CDCF by 1.90-fold (MRP2)]. Rhinacanthin-C was able overcome MDR in DOX resistant MCF-7 (MCF-7/DOX) cells, which expressed high level of P-gp and MRP2. The combination between DOX and RN-C at their non-cytotoxic concentrations when giving each compound alone significantly reduced cell viability. The findings suggested that RN-C was able to increase DOX-mediated cytotoxicity and overcome MDR effectively, possibly through interference on MRP2 and P-gp functions. The nature of interaction between RN-C and DOX was synergism. Another potential mechanism of the synergy between rhinacanthin-C and doxorubicin would be investigated further

Field of Study: Pharmacology Student's Signature .....

Academic Year: 2015 Advisor's Signature .....

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Finally, I wish to express my infinite thank to my family, especially my mother and uncle, for their endless love, care, understanding and never ending support throughout my life. Thank you for always being beside me

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LIST OF ABBREVIATIONS

ABC	= Adenosine triphosphate binding cassette
ANOVA	= Analysis of variance
ATP	= Adenosine triphosphate
BCRP	= Breast cancer resistance protein
BSA	= Bovine serum albumin
ºC	= Degree celsius
Calcein-AM	= Calcein acetoxymethyl ester
CI	= Combination index
CDCF	= 5(6)-carboxy-2',7'-dicholofluorescein
CDCFDA	= Diacetate ester of CDCF
CER	= Cytotoxicity enhancement ratio
CO_2	= Carbon dioxide
CsA	= Cyclosporine A
DOX	= Doxorubicin
DMSO	= Dimethyl sulphoxide
EDTA	= Ethylenediamine tetraacetic acid
FBS	= Fetal bovine serum
HBSS	= Hanks' Balanced Salt
IC50	= Inhibitory Concentration 50
INDO	= Indomethacin
LSD	= Least Significant Different
MCF-7	= human breast adenocarcinoma cell line
MCF-7/DOX	= human breast adenocarcinoma doxorubicin resistant cell line

MDR	= Multidrug Resistance
MTT	= 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide
mg/ml	= milligram per milliliter
ml	= milliliter
mM	= millimolar
MRP1	= Multidrug Resistance-associated Proteins 1
MRP2	= Multidrug Resistance-associated Proteins 2
NBD	= Nucleotide Binding Domain
PBS	= Phosphate Buffered Saline
RF	= Reversal ratio
P-gp	= P-glycoprotein
Phe A	= Pheophorbide A
RI	= Resistance index
RN-C	= Rhinacanthin-C
RPMI-1640	= Roswell Park Memorial Institute -1640 medium
SEM	= Standard Error of Mean
µl	= microliter
µM	= micromolar

CHAPTER I
INTRODUCTION

Breast cancer is the most common cancer in women, comprising almost one third of all malignancies in females. It is the one leading cause of cancer death worldwide. It was estimated that approximately 231,840 new cases and around 40,290 breast cancer deaths could be expected in 2015 (Society 2015). In 2012, the National Cancer Institute (NCI) of Thailand estimated that breast cancer was the most commonly new diagnosed cancer in woman at the incidence of around 39.74% of all cases (Imsamram et al., 2012). Chemotherapy has been frequently used as a standard treatment of breast cancer. Examples of cytotoxicity agents in breast cancer chemotherapy induce doxorubicin, docetaxel, paclitaxel, vinblastine and mitoxantrone (Laura Biganzolia 2004). Despite the large repertoire of therapies available a success of cancer therapy is still unlikely to achieve. A major factor contributing to the failure of chemotherapy in particular breast cancer is multidrug resistance (Schinkel and Jonker 2003; Stockler et al. 2000). For example, doxorubicin, a common chemotherapeutic drug for breast cancer, has the high resistance rate which can limit its effectiveness and use. Hence, any adjuvants or chemicals that can circumvent chemoresistance and enhance the efficacy of doxorubicin-based chemotherapy would be clinical significance.

Most likely, chemoresistance is not limited to only one specific chemical structure. Cancer cells may develop resistance toward a wide range of structurally and functionally unrelated drugs after constant exposure of a single agent (Gottesman et al. 2002; Ullah 2008). This phenomenon is known as multidrug resistance (MDR). For example, doxorubicin-induced resistant cells may be less sensitive toward other anticancer drugs such as vinblastine, cyclophosphamide and etoposide, as well

(Szakacs 2006). Consequently, cancer becomes untreatable by chemotherapy. One established mechanism by which tumors develop multidrug resistance is an overexpression of efflux transporter proteins, at the plasma membrane (Raguz and Yague 2008). Several members of the ATP-binding cassette (ABC) transporters including P-glycoprotein (P-gp), multidrug resistance associated protein 1 (MRP1), multidrug resistance associated protein 2 (MRP2) and breast cancer resistance protein (BCRP) have been linked to multidrug resistance in tumor cells, leading to chemotherapeutic failure (Cole et al. 1994; Gottesman et al. 2002).The high expression levels of these drug efflux pumps may cause less intracellular accumulation of cytotoxic drugs so that the cytotoxic concentration levels could not be reached (Choi 2005; Coley 2008). Moreover, development of multidrug resistance (MDR) in cancer cells can simultaneously reduce the effectiveness of various cytotoxic drugs. Consequently, high doses of anticancer drugs are acquired in order to overcome resistance. And achieve therapeutic success. Inevitably, the toxic effects can be anticipated.

The combination between a cytotoxic anticancer drug and a non-cytotoxic agent is one approach to enhance drug efficacy without titrating up the concentration. In this regard, a non-cytotoxic agent may be able to potentiate the cytotoxic effect of anticancer drug through several mechanisms including inhibition of drug efflux pumps (Schinkel and Jonker 2003). There are several studies on MDR reversal agents of which their mechanisms involve with direct inhibition of drug efflux pumps such as calcium channel blockers (e.g. verapamil), anti-arrhythmics (e.g. quinidine), steroids (e.g. dexamethasone) and anti-parasitics (e.g. ivermectin) (Choi 2005; Donmez et al. 2011). However, most of these MDR reversing agents have been unsuccessful clinically due to a need of their high concentrations.

In this study, the multidrug resistance (MDR) phenomenon and MDR reversal models was performed in doxorubicin-mediated cytotoxicity of human breast cancer cell line (MCF7). As known, doxorubicin (DOX) is one of the most effective cytotoxic drugs in breast cancer chemotherapy. It adverse effects include myelosuppression and cardiotoxicity (Takemura and Fujiwara 2007). Because the adverse effects are concentration-dependent, the less DOX concentrations produce the less severity of adverse events and accommodate the more patient's tolerability (Ichikawa et al. 2014). Unfortunately, DOX is one cytotoxic drug that cause high incidence of MDR.

Rhinacanthus nasutus Kurz (Acanthaceae) has been used in Thai traditional medicine for the treatment of eczema, skin diseases, tuberculosis, hepatitis, hypertension, and various parts of cancer (Siripong et al. 2006a). Rhinacanthin-C (RN-C), a major naphthoquinone ester from the leaves and roots of this plant, has been shown to possess antiviral, anti-inflammatory, antibacterial activity, anti-proliferative, cytotoxic activities, anti-tumor, immunomodulatory activity, antioxidant activity, and antiplatelet activity (Bukke et al. 2013; Gotoh et al. 2004; Punturee et al. 2004; Sendl et al. 1996; Siripong et al. 2006b). Recently, it was reported that RN-C could inhibit the function of P-gp transporter in the Caco-2 cells (Wongwanakul et al. 2013). It could be hypothesized that RN-C at the non-cytotoxic concentration was able to potentiate the cytotoxic effect of doxorubicin in MCF-7 breast cancer cell. It was possible that RN-C could be an MDR reversing agent by increasing intracellular DOX accumulation through interference on transporter efflux pump (Eid et al. 2012a; Wagner and Ulrich-Merzenich 2009).

Hypothesis

Rhinacanthin-C (RN-C) could elicit its MDR reversal effect toward DOX-resistant human breast cancer cells through inhibitions of the ABC drug efflux transporters. Consequently, RN-C could increase accumulation of DOX within the cells up to its cytotoxic levels.

Objective

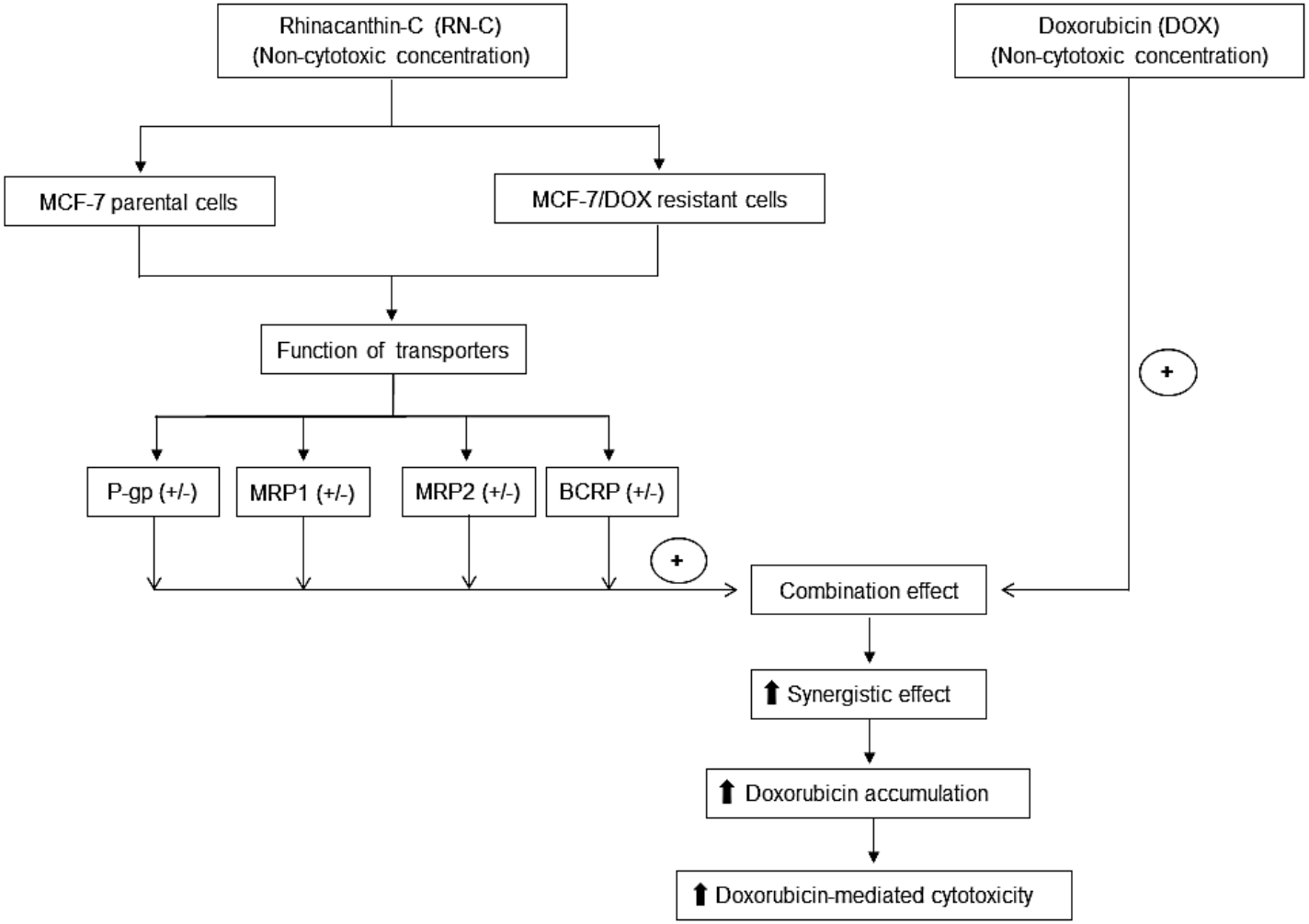
The objectives of this study were

1. To determine the cytotoxicity of rhinacanthin-C and doxorubicin, either alone or in combination, on human breast cancer cells including the parental MCF-7 cells and the doxorubicin resistant MCF-7 cells (MCF-7/DOX).
2. To examine the MDR reversal effects of rhinacanthin-C on doxorubicin-mediated resistance in human breast cancer cells, and the nature of interaction between rhinacanthin-C and doxorubicin.
3. To, investigate the mechanism of rhinacanthin-C in enhancing doxorubicin cytotoxicity in human breast cancer cells, and the involvement of the ABC drug efflux transporters.

Scope of study

The results from this study would provide preliminary data of whether rhinacanthin-C contained MDR reversal property. If so, as an MDR reversal agent, the combination effect of rhinacanthin-C and cytotoxic anticancer drugs such as doxorubicin would be helpful clinically. In addition, this information would be helpful in exploring the possibility of using rhinacanthin-C of herbal extract containing rhinacanthin-C in the standard chemotherapy as a novel treatment option to overcome MDR in cancer.

Conceptual framework



CHAPTER II
LITERATURE REVIEW

Breast cancer and Chemotherapy

Breast cancer is one of the most common cancers among women. The American Cancer Society's estimated that approximately 231,840 new cases of breast cancer will be diagnosed in American women and 40,290 women may die in year 2015 (Society, 2015). In Thailand, according to National Cancer Institute, the incidence of breast cancer has ranked first with 39.74% of all cancer cases in Thai women (Imsamran et al., 2012).

Chemotherapy is one of the common treatments for cancer with the use of cytotoxic agents that can kill cancer cells. It is sometimes the first or even the only choice to treat many cancers. However, it can be anticipated that failure of chemotherapy can occur through a number of mechanisms including multidrug resistance (MDR). Heterogeneity of cancer within a single tumor may lead to varying sensitivity to anticancer drugs among cell subpopulations, resulting in drug-resistant subpopulations. Consequently, mono-therapy with one anticancer drug may not achieve its therapeutic success. The combining multiple drugs in one regimen is one strategy to improve chemotherapeutic efficacy through having multiple hit targets and reducing cross-resistance mechanisms. Moreover, the combination drugs with multiple hit targets can provide therapeutic benefits from (1) their independent toxicity, which may allow the potential use of each compound at full dosage; and (2) their spatial cooperation, where each agent may hit different subpopulations. Consequently, the combination therapy can cause protection of normal tissues and enhance tumor response toward treatment (Swift et al., 2006).

There are various cytotoxic anticancer drugs available for different types of cancer. Anthracyclines (doxorubicin, epirubicin), taxanes (docetaxel, paclitaxel), mitoxantrone and vinorelbine are frequently used in breast cancer chemotherapy (Laura Biganzolia 2004; O'Shaughnessy, 2005). In advanced stages of breast cancers, chemotherapy are not sufficient in terms of effectiveness. Additional drugs such as bisphosphonates (BPs) are required to increase the rate of cell death and to prevent metastasis (Martin et al., 2000; O'Shaughnessy, 2005)

Multidrug Resistance (MDR) in cancer

Cancer multidrug resistance is defined as the cross-resistance or insensitivity of cancer cells to the cytostatic or cytotoxic actions of various anticancer drugs which are structurally or functionally unrelated (Gottesman et al., 2002; Saraswathy and Gong 2013). In addition, these drugs may have different molecular targets (Johnston, 2005). The resistance of the tumor cells to chemotherapeutic agents lowers the effectiveness of anticancer drugs. Consequently, patients need to take higher doses of the agents or they need to change the anticancer drugs.

Numerous mechanisms have been proposed to mediate multidrug resistance in cancer cells. Such mechanisms can be categorized as non-cellular or cellular based on the factors contributing to MDR development (Fan et al., 1994). The cellular based MDR mechanisms arise in the tumor cell as an adaptive response to cytotoxic challenge by altering gene expression (Borowski et al., 2005; Ullah, 2008). The examples of the cellular changes include an alteration of drug efflux transporters (e.g. the ABC drug efflux pumps), detoxification enzymes (e.g. Cytochrome P450 (CYP), glutathione-S-transferase (GST) and proteins in apoptosis-derived mechanisms (e.g. tumor suppressor gene p53) (Ullah, 2008). A schematic representation of mechanisms involved in multidrug resistance is illustrated in Figure 1 (Chai et al., 2010). On the other hand, the non-cellular based MDR mechanisms involve cellular adaptation

independent of gene responses. For example, lack of nutrition and hypoxia due to poor vasculature lead to lactic acid accumulation which could cause resistance against drugs that act on actively dividing cells. Moreover, accumulation of lactic aside might affect pH-dependent drug transport (Demant et al., 1990).

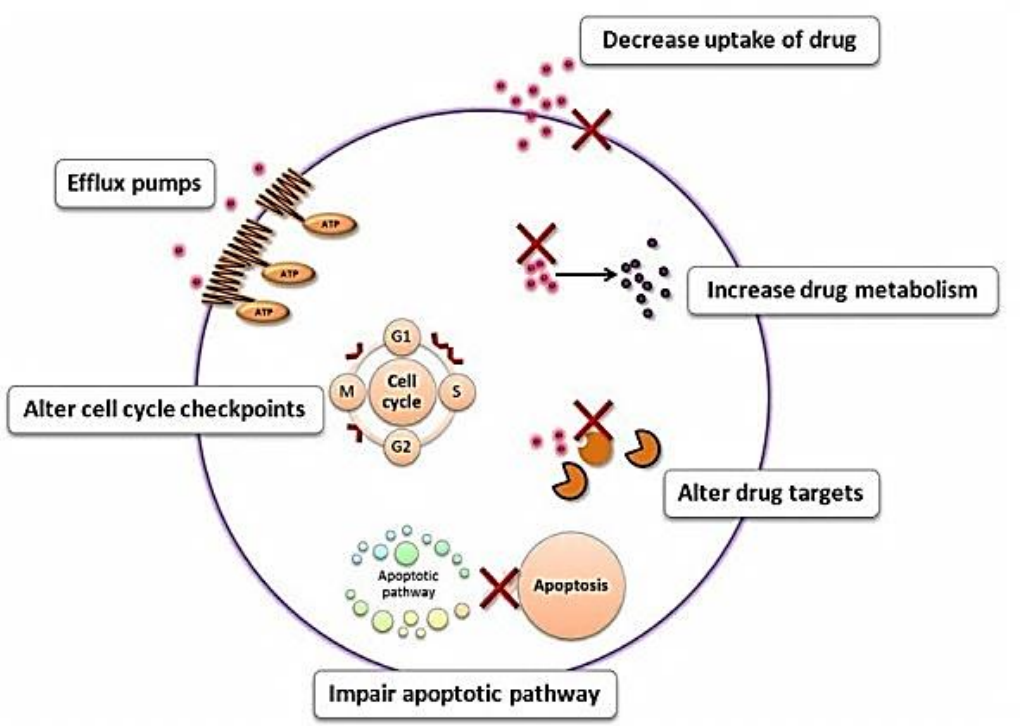


Figure 1. Proposed mechanisms involved in multidrug resistance (MDR) (Chai et al., 2010).

Multidrug Resistance (MDR) and drug transporters

Alteration of either expression or function of drug transporters can lead to MDR resistance in chemotherapy and eventually chemotherapeutic failure (Bates et al., 2001; Leslie et al., 2005; Szakacs, 2006). The transporters involved in MDR mainly belong to the family of ATP binding cassette (ABC) transporters (Figure 2). The ABC transporters actively pump cytotoxic drugs out of the tumor cells and maintain intracellular drug level below their effective concentrations (Leonessa and Clarke 2003). This transporter-based mechanism of resistance appears to be most relevant to drugs which enter cells by passive diffusion such as doxorubicin or vinblastine (Dorai and Aggarwal 2004; Gariboldi et al., 2003). The ABC drug efflux pumps that have been reported to involve with MDR in cancer include P-gp, MRP1, MRP2 and BCRP (Figure 3).

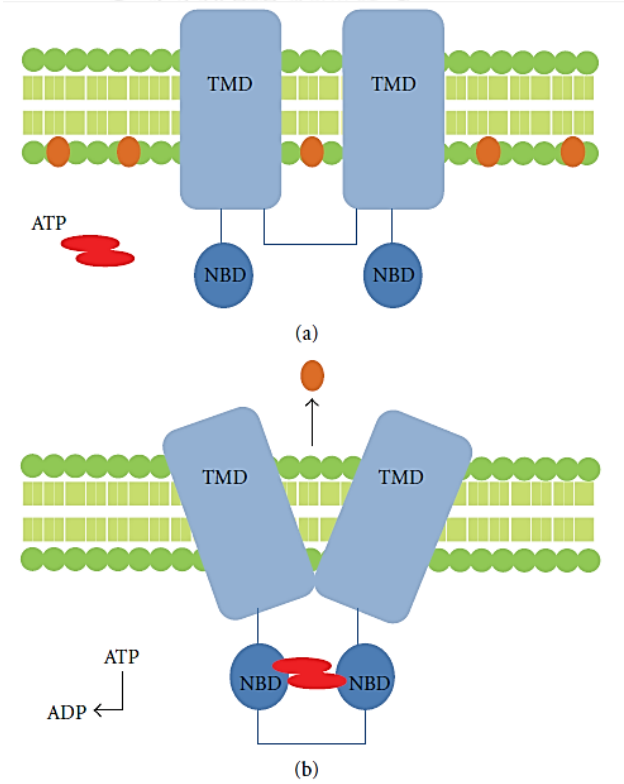


Figure 2. Structures of ABC transporters known to confer drug resistance (Bates et al., 2001)

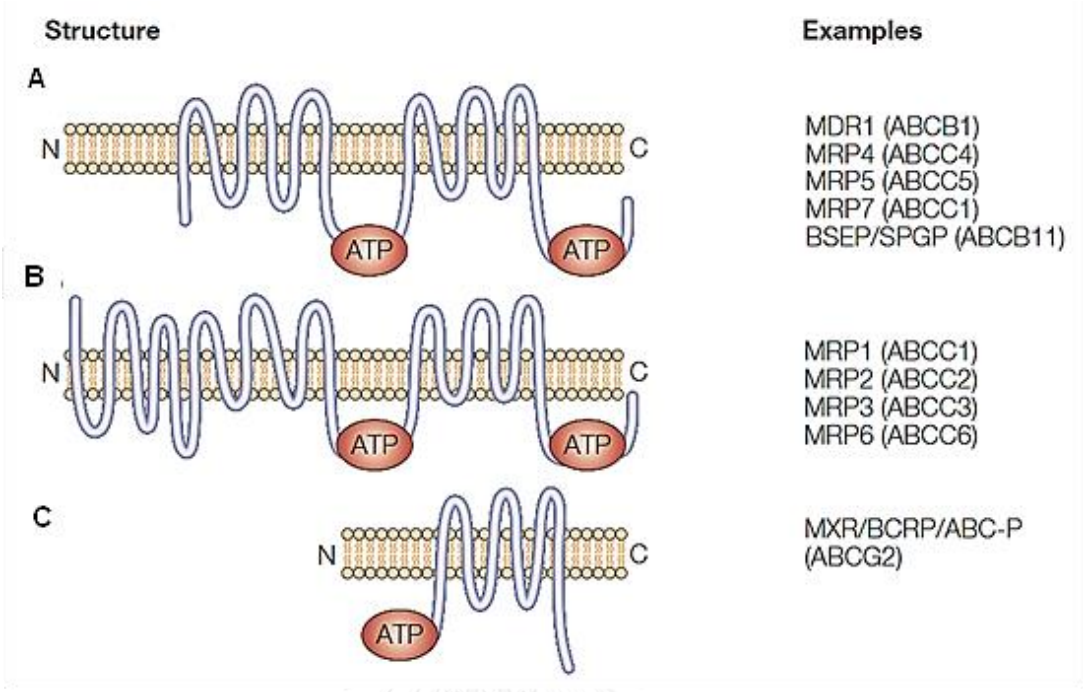


Figure 3. Structure of the ABC drug efflux pumps. (A) P-gp, (B) MRP1 and MRP 2, (C) BCRP (Gottesman et al., 2002)

The overexpression and high activity of drug efflux pumps are one of the major mechanisms responsible for MDR in cancer. Inhibition of the drug efflux pumps may overcome MDR in cancer if anticancer drugs are their substrates. The substrates of the ABC drug efflux pumps are listed in Table 1. Another approach can be suppressing MDR transcription (Borowski et al., 2005; Lee, 2004). In order to target mRNA of ABC transporters; antisense oligonucleotides, hammerhead ribozymes and RNA interference strategies have been developed. For instance Jin et al. showed a natural marine product Et743 was able to inhibit MDR1 transcription via blocking its promoter activation (Jin et al., 2000).

The failure of doxorubicin treatment has been linked to the expression and activity of multi-drug resistance (MDR) transporters, in particular the ABC drug efflux pumps including P-glycoprotein (P-gp), Multidrug resistance protein 2 (MRP2) (Gottesman et al., 2002; Walker et al., 2004). It was well known that doxorubicin is a substrate of MDR1, MRP1, MRP2 and BCRP export pump proteins (AbuHammad and Zihlif 2013). These transporters could hinder the intracellular doxorubicin accumulation up to its cytotoxic level. Thus, targeting MDR drug transporters has been a promising approach to overcome drug resistance or improve chemosensitivity without the need of higher concentration or additional chemotherapeutic drugs in the therapeutic regimen (Krishna and Mayer 2000; Leslie et al., 2005).

Table 1. Selected substrates of P-glycoprotein and other efflux transporters (AbuHammad and Zihlif 2013)

P-gp (MDR1)	MRP1 (ABCC1)	MRP2 (ABCC2)
Anthracyclines Doxorubicin Daunorbicin Epirubicin Mitoxantrone	Anthracyclines Doxorubicin Daunorbicin Epirubicin	Anthracyclines Doxorubicin Mitoxantrone
Topoisomerase inhibitors Etoposide Teniposideazatoxin	Topoisomerase inhibitors Etoposide	Topoisomerase inhibitors Etoposide Irnotecan and SN-38
Vinca alkaloids Vincristine Vinblastine	Vinca alkaloids Vincristine Vinblastine	Vinca alkaloids Vincristine Vinblastine
Alkaloids Cepharanthine Homoharringtonine		
Taxanes Paclitaxel Docetaxel		
Antitumor antibiotics Actinomycin D Mitomycin C	Antitumor antibiotics Actinomycin D	
Antimetabolites Cytarabine	Antimetabolites Methotrexate	Antimetabolites Methotrexate

The control of function of ABC transporters in particular P-gp is one of popular strategies for overcome MDR in cancer. The pump inhibitors are sometime called MDR modulators. To date, MDR modulators through P-gp inhibition have been developed and grouped into four generations as listed in Table 2 (Modok et al., 2006; Morjani and Madoulet 2010).

Table 2. Example of MDR modulators

Generation	P-gp Inhibitor
First	Amiodarone, Cyclosporin A (CSA), Quinidine, Quinine, Verapamil, Nifedipine, Dexniguldipine
Second	PSC833 (Valspodar) VX-710 (Biricodar)
Third	GG918 (Elacridar), LY475776, LY335979 (Zosuquidar), XR-9576 (Tariquidar), V-104, R101933 (Laniquidar), S9788
Fourth	Curcumin Flavonoids

Most of the fourth generation P-gp inhibitors were originated from natural products (Coley, 2008). It was reported that fruits like orange, grapefruit and strawberry contained substances were able to inhibit P-gp function (Deferme et al., 2002). Moreover, in addition to P-gp, a number of natural products including and flavonoids were also shown to be effective against MDR through inhibition of MDR1, MRP1 and BCRP (Limtrakul, 2007; Limtrakul et al., 2007; Limtrakul et al., 2005). In previous study, naringenin were demonstrated its ability to enhance doxorubicin-mediated cytotoxicity through selective inhibition of MRP function in breast cancer cells line (Zhang et al., 2008).

Doxorubicin (DOX)

Doxorubicin (DOX) an anthracycline derivative isolated from *Streptomyces peucetius* var. *caesius*, is anticancer antibiotic with a four-membered ring system containing an anthraquinone chromophore, and an aminoglycoside (Figure 4). Doxorubicin is one of the most important anticancer drugs, with major clinical activity in carcinomas of the breast, endometrium, ovary, testicle, thyroid, stomach, bladder, liver, and lung cancer (Cutts et al., 2005; Kim et al., 2014). Doxorubicin is also generally used in combination with other anticancer agents (e.g. cyclophosphamide, cisplatin, and fluorouracil) in order to increase therapeutic success. The combination regimens demonstrated a better responses and remission duration, in comparison with single-agent therapy (Wagner and Ulrich-Merzenich 2009). Doxorubicin exerts its cytotoxic action through four major mechanisms. They are (1) inhibition of topoisomerase II; (2) DNA intercalation, with consequent blockade of the synthesis of DNA and RNA, and DNA strand scission; (3) alteration of membrane fluidity and ion transport; and (4) generation of semiquinone free radicals and oxygen free radicals through an enzyme-mediated reductive process (Cutts et al., 2005; Suzuki et al., 2005).

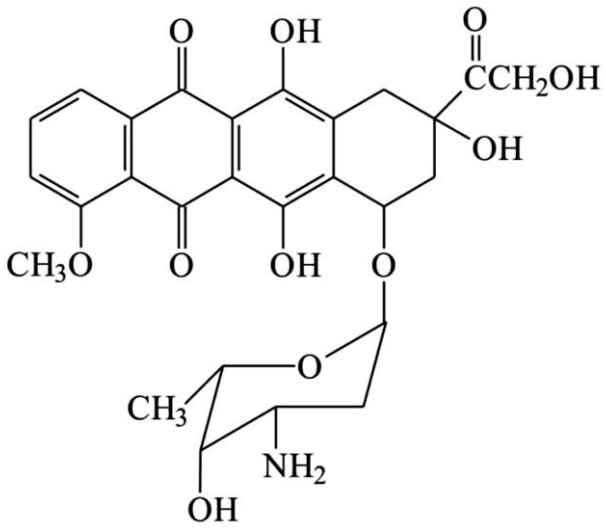


Figure 4. Chemical structure of doxorubicin (DOX) (Kim et al., 2014)

Doxorubicin has been very well known for its cardiotoxicity and severe cumulative dose-related development of drug resistance (Ichikawa et al., 2014). When doxorubicin is given as a single-agent treatment, response rates are typically 40% to 60%. Sometimes, the rate can be as high as 80% (Faneyte et al., 2002). Despite the successful treatment of primary cancer, resistance to DOX may later develop and becomes a major clinical problem of treatment failure (AbuHammad and Zihlif 2013). One of these suggested mechanisms is the overexpression of multi-drug resistance (MDR) transporters, in particular the ABC drug efflux pumps including P-glycoprotein (P-gp), Multidrug resistance protein 2 (MRP2) (Gottesman et al., 2002). It was well known that doxorubicin is a substrate of MDR1, MRP1, MRP2 and BCRP export pump proteins (Shen et al., 2008). Thus, targeting MDR drug transporters has been a promising approach to overcome drug resistance without reducing the need high dose or additional chemotherapeutic drugs in the therapeutic regimen (Kowalski et al., 2005; Leslie et al., 2005).

Most of the fourth generation P-gp inhibitors were originated from natural products (Coley, 2008). It was reported that fruits like orange, grapefruit and strawberry contained substances that were able to inhibit P-gp function (Borowski et al., 2005; Deferme et al., 2002; Kars et al., 2008). Moreover, in addition to P-gp, a number of natural products including curcumin and flavonoids were also shown to be effective against MDR through inhibition of MDR1, MRP1 and BCRP (Kars et al., 2008; Limtrakul et al., 2007; Limtrakul et al., 2005). In previous study, naringenin were demonstrated its ability to enhance doxorubicin-mediated cytotoxicity through selectively inhibition of MRP function in breast cancer cells line (Zhang et al., 2009).

Synergy research of phytomedicine in cancer therapy

Simultaneously giving two chemicals can cause interaction which may affect the bioactivity of each compound or both. The interaction can be classified into addition, synergy and antagonism. Synergy is a new key activity to improve therapeutic outcome in chemotherapy with cytotoxic agents. The synergy is can be defined as *'an effect seen by a combination of substances that is greater than would have been expected from a consideration of individual contribution'* (Heinrich, 2004).

Pharmacological experiments either in vitro or in animal models can be used for demonstration of the isoboles of a mixture of two substances. This isoboles method provides a graphic demonstration with linearly arranged x and y axes reflecting the dose rates of the single individual components (Figure 5). From isobologram, "zero interaction" or "additive interaction" suggests that the effect of two substances A and B is a pure summation effect. Correspondingly, the overall effect with antagonistic interaction is less than expected from the summation of the separate effects. And when the overall effect of two drugs A and B giving simultaneously is larger than it would be expected by the summation of the separate effects, this interaction is synergy.

Talalay induced a scientific term “combination index” (CI) in order to quantitatively depict synergism (CI < 1), additive effect (CI = 1), and antagonism (CI > 1) and developed the generation computer “CalcuSyn” software for dose-effect analysis based on the “mass-action law” (Chou, 2006; Chou, 2010; Chou and Talalay 1984).

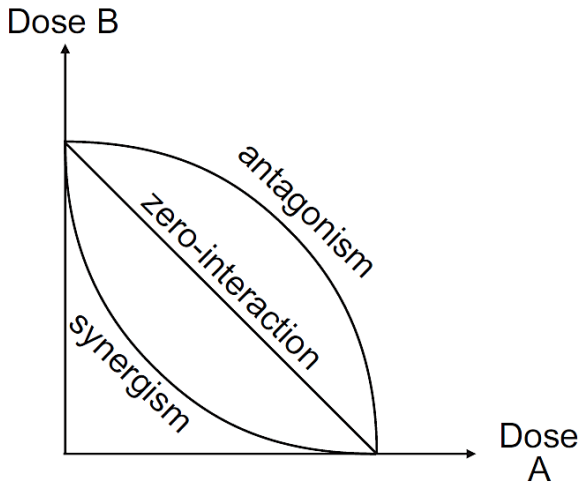


Figure 5. Isoboles for additive, synergism and antagonism

The synergistic interaction can occur from a number of circumstances including synergistic multi-target effects, pharmacokinetic effects (e.g., enhancement of bioavailability), overcome of MDR mechanisms. It has been demonstrated that several plant compound could be useful adjuvants in cancer therapy due to their ability to modulate MDR in tumor cells and increase chemosensitivity toward anticancer agents (El-Readi et al., 2010; Li et al., 2011; Ma and Wink 2008; Modok et al., 2006; Wink et al., 2012). The MDR reversal abilities of some phytochemicals, including alkaloids, phenolics and terpenoids alone or in combination with the digitonin in enhancing drug sensitivity in multi-drug resistance cancer cells toward treatment with doxorubicin were reported (Eid et al., 2012b). The combination of doxorubicin and non-toxic concentrations of individual aromadendren, harmine and sanguinarine synergistically

sensitized and enhanced the cytotoxicity of doxorubicin in cancer resistant cells line (Eid et al., 2012c). Furthermore, it was reported that certian phenolic, terpenoids and alkaloids interfered directly and/or indirectly with P-gp function, resulting reversal of MDR in cancer cells (Eid et al., 2012a). Limonin from *Citrus* species was one of the most potent P-gp reversal agents (El-Readi et al., 2010). It was reported that limonin significantly enhanced doxorubicin-mediated cytotoxicity in cancer resistance cells (El-Readi et al., 2010). Moreover, salinomycin a monocarboxylic ionophores isolated from *Streptomyces albus.,* enhanced doxorubicin-induced cytotoxicity in multidrug resistant MCF-7/MDR human breast cancer cells via decreased efflux of doxorubicin (Kim et al., 2015). In addition, SZ-685C purified from the mangrove *endophytic fungus* No. 1403 from the South China Sea, was found to reverse chemoresistance by suppressing the Akt proliferation signaling and to induce apoptosis in ADR-resistant MCF-7/ADR and MCF-7/Akt breast cancer cells (Zhu et al., 2012).

Rhinacanthus nasutus

Rhinacanthus nasutus (Linn.) Kurz (*R. nasutus*) (Figure 6) is one of the herbal medicine in the family Acanthaceae. This plant has been used in traditional medicine for the treatment in diseases such as eczema, pulmonary tuberculosis, herpes, hepatitis, diabetes, hypertension and several skin diseases (Siripong et al., 2006b).

Figure 6. Rhinacanthus nasutus (Linn.) Kurz

R. nasutus plant is well known as the sources of flavonoids, steroids, terpenoids, anthraquinones, lignans and naphthoquinones. Rhinacanthins were naphthoquinones derivatives isolated from leaves and roots of *R.nasutus* plant (Sendl et al., 1996; Siripong et al., 2006a; Siripong et al., 2006b; Wu et al., 1988). Currently, there are 15 rhinacanthin derivatives identified (rhinacanthins A, B, C, D, G, H, I, J, K, L, M, N, O, P, and Q). Rhinacanthin-C is a major naphthoquinones found in this plant (Figure 7) (Sendl et al., 1996; Siriwatanametanon et al., 2010; Wu et al., 1988). These naphthoquinone compounds have gained big attention as new potential therapeutic agents for cancer.

The pharmacological effects of *R. nasutus* and Rhinacanthin-C in particular anti-cancer and cytotoxic activities were reported in several in vitro cell culture studies. For examples, either extracts of *R. nasutus* or rhinacanthin-C were found effective as cytotoxic substances in a number of cancerous cells including human epidermoid carcinoma (KB), human laryngeal carcinoma (Hep2), human breast adenocarcinoma (MCF-7), human hepatocellular carcinoma (HepG2), human cervical carcinoma (SiHa), human amelanotic melanoma carcinoma (C-32), lewis lung carcinoma (LLC), murine colon adenocarcinoma (Colon 26), P-388 mouse lyphocytic leukemis (P-388), human cervix adenocarcinoma (HeLa), MDR1 overexpressing subline of human cervical carcinoma (Hvr100-6), human prostatic cancer cell (PC-3) and human bladder carcinoma (T24) (Gotoh et al., 2004). Rhinacanthin-C was capable of inhibiting cell proliferation and induced apoptosis of human cervical carcinoma (HeLS3) in concentration- and time-dependent manners (Siripong et al., 2006b). Moreover, it was reported that rhinacanthin-C exhibited significant cytotoxic activity against murine leukemia (P-388), human lung carcinoma (A-549), human colon adenocarcinoma (HT-29) and leukemia (HL-60) cells (Wu et al., 1988). Rhinacanthin-C was found to inhibit mosquito cytochrome P450 enzymes in synergism with cypermethrin to induce cytotoxicity in *Spodoptera frugiperda* cells (Pethuan et al., 2012). The anti-inflammatory action of *R.nasutus* and rhinacanthin-C was also reported. Extract of *R.nasutus* containing rhinacanthin derivatives were shown to inhibit iNOS and COX-2 gene expressions and to suppress release of nitric oxide (NO), PGE2 and TNF-α in LPS-activated RAW 264.7 cells (Siriwatanametanon et al., 2010).

Rhinacanthin-C was recently reported its ability to inhibit the function P-gp and MRP2 in Caco-2 cells (Wongwanakul et al., 2013). The inhibitory effect of rhinacanthin-C on transporter function was reversible. Moreover, rhinacanthin-C was a more potent inhibitor toward P-gp than MRP2 (Wongwanakul et al., 2013). These findings suggested that rhinacanthin-C might raise the problem of herb-drug interaction when co-

administered with other P-gp substrates (Pedersen et al., 2008; Sharom et al., 1999; Wongwanakul et al., 2013). This potential interaction might be beneficial in chemotherapy when giving rhinacanthin-C simultaneously with doxorubicin, a known substrate of P-gp and MRPs. It could be hypothesized that the interference of rhinacanthin-C on P-gp and MRP2 activities could in enhance accumulation doxorubicin in MDR cancer cells. Consequently, rhinacanthin-C was able to enhance the anti-cancer effect of cytotoxic doxorubicin.

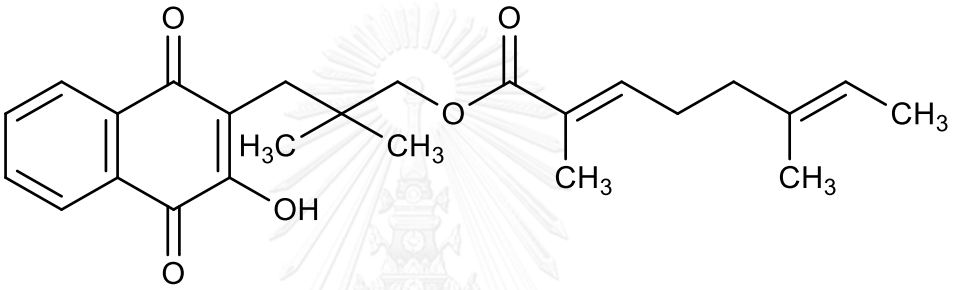


Figure 7. Chemical structure of Rhinacanhin-C (RN-C)

CHAPTER III

MATERIA AND METHODS

1. Materials

1.1 Test compound

Rhinacanthin-C (RN-C) was kindly provided by Dr. Pongpun Siripong, Natural Products Research Section, Research Division, National Cancer Institute, Bangkok, Thailand. RN-C, naphthoquinone ester was isolated from leaves and roots of *R. nasutus* Kurz., using the purification and identification processes as previously described in (Siripong et al., 2006b).

RN-C was kept at -20 ºC until used. Throughout the study, RN-C compound was dissolved in dimethyl sulfoxide (DMSO) 99.9%. The final concentration of DMSO in each experiment was less than 0.1% (v/v). At this concentration, DMSO had no cytotoxic effect on either the parental MCF-7 or the doxorubicin resistant (MCF-7/DOX) resistance cells.

1.2 Chemicals

Chemicals in this study including cyclosporine A (CsA), Indomethacin (INDO), doxorubicin (DOX), KO143, pheophorbide A (Phe A), bovine serum albumin (BSA), Bradford reagent, ethylenediamine tetraacetic acid (EDTA), penicillin G sodium, triton X-100, streptomycin sulfate, trypsin, Hanks' balanced solution (HBSS), 0.04% tryptan blue, 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide (MTT reagent) were purchased from Sigma Chemical Company (St Louis, MO, USA). (6)-carboxy-2',7'-dichlorofluorescein diacetate (CDCFDA), calcein acetoxymethyl (calcein-AM), carboxy-dichlorofluorescein (CDCF) were purchased from Fluka (Buchs, Switzerland). RMI-1640 and fetal bovine serum (FBS) were from Gibco Life Technologies (Grand Island, NY, USA).

1.3 Experimental instruments

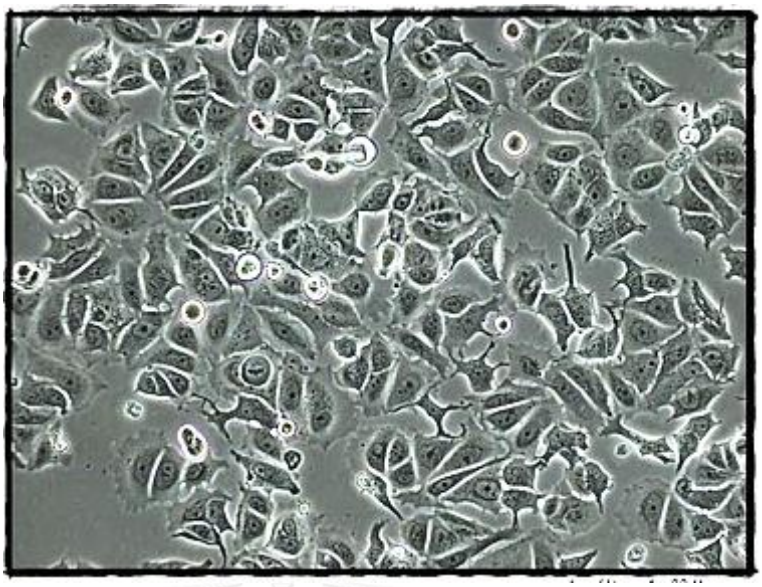
1. Autoclave: Hirayama, Saitama, Japan
2. Hot air oven: MEMMERT, Buchenbach, Germany
3. Humidified carbon dioxide incubator: Thermo Scientific Forma Series II, New York, USA
4. Inverted microscope: Meiji Techno Microscopes TC5000, Saitama, Japan
5. Laminar air flow hood: BossTech, Bangkok, Thailand
6. Microplate reader: Wallac 1420 Perkin-Elmer Victor 3, Perkin Elmer lnc., Massachusetts, USA
7. Microplate reader: Spectra MAX M5, Molecular Devices, USA
8. Multiwell plates: Coring, New York, USA
9. Orbital shaker: OS-20, Biosan, Riga, Latvia
10. pH meter: CG 842, Schott, Hofheim, Germany
11. Refrigerated centrifuge: Z 383K, Hermle Labortechink,Burladingen, Germany
12. Tissue culture flasks: Coring, New York, USA
13. Vortex mixer: mode K550-GE. Scientific Industries, New York, USA
14. Water bath: WB22, Memmert, Germany

1.4 Cell culture

Two types of cells culture including MCF-7 (ATCC® HTB-22™) and doxorubicin-resistant subline MCF-7/DOX were used in this study (Figure 8). For routine passage, the cultures were spilt at 1:3 when they reached 80-90% confluence, using 0.25% trypsin solution containing 1 mM EDTA.

- The MCF-7 was human breast adenocarcinoma cell line purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA). The cells were maintained in RPMI-1640 supplemented with 10% fetal bovine serum (FBS), 1% penicillin–streptomycin mixture at 37 °C in a humidified atmosphere of 5% CO_2.
- The doxorubicin-resistant subline MCF-7/DOX cells were in-house developed from the MCF-7 cells by culturing the cells (passage 146 to 286) in RPMI-1640 medium containing DOX. The resistant cells were selected in stepwise selection method upon increasing the concentrations of DOX (AbuHammad and Zihlif, 2013; Mealey et al., 2002). Initially, the parental MCF-7 cells were seeded at a density of 5.5 $\times 10^4$ cell/cm^2 and grown in RPMI-1640 medium containing 0.01 μM DOX. After the cells were capable of growing and reaching confluence, the cells were passaged and grown in medium containing DOX at a higher concentration. Usually, the DOX concentration increased in a stepwise manner by approximately 1.5 to 2-fold until reaching the maximum concentration of 1.5 μM. The MCF-7/DOX cells were maintained in RPMI-1640 complete medium containing 1.5 μM DOX in order to keep their resistance toward DOX.

A.



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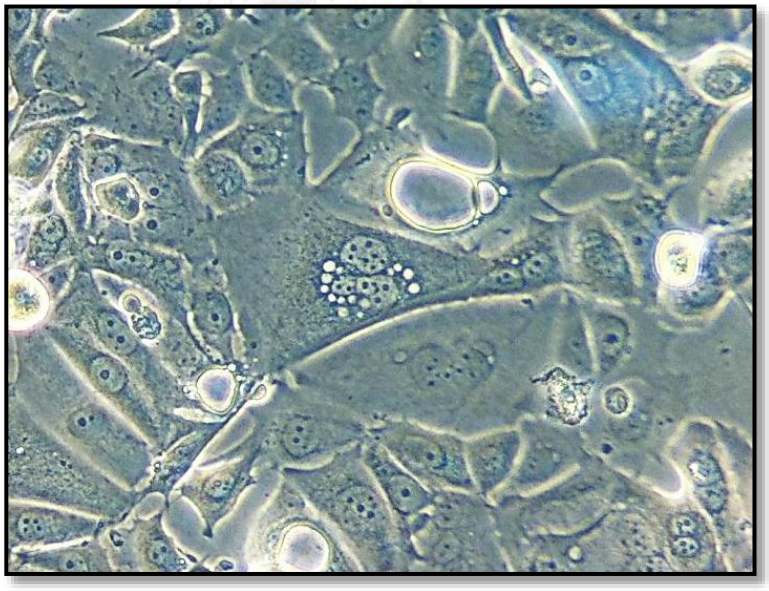


Figure 8. The morphology of human breast adenocarcinoma cell line. (A) MCF-7 parental cells line. (B) MCF-7/DOX resistant cell line.

2. Methods

2.1 Cell viability

Cell viability was determined by an MTT assay (Carmichael et al., 1987). MTT is a yellow water soluble tetrazolium dye that can be reduced by mitochondria reductase enzyme into a purple insoluble formazan (Figure 9), which can be measured spectrophotometrically with a microplate reader at 570 nm. The cells (either MCF-7 or MCF-7/DOX) were seeded onto 96 well-plates at a density 5 $\times 10^3$ cell per well. After 24 hours, the cells were treated as follows:

1. Treating the parental MCF-7 cells with various concentrations of either RN-C (0-10 μM) or DOX (0-2 μM) for 24 and 48 hours at 37 ºC in a humidified atmosphere of 5% CO_2.
2. Treating the resistant MCF-7/DOX cells with various concentrations of DOX (0, 0.1, 1 and 2 μM) for 48 hours at 37 ºC in a humidified atmosphere of 5% CO_2.
3. Treating the parental MCF-7 cells with DOX (0-2 μM) in the presence of RN-C at non-cytotoxic concentration for 24 and 48 hours at 37 ºC in a humidified atmosphere of 5% CO_2.
4. Treating the resistant MCF-7/DOX cells with DOX (0, 0.1, 1 and 2 μM) in the presence of RN-C at non-cytotoxic concentration for 48 hours at 37 ºC in a humidified atmosphere of 5% CO_2.

At the end of the treatment period, the cells were incubated with MTT solution (0.83 mg/ml in serum-free RPMI-1640) for another 4 hours at 37 ºC. The intracellular formazan crystals in each well were dissolved with 100 μl DMSO. The absorbance was read in a microplate reader at the wavelength of 570 nm. Cell viability was calculated as the percentage of the control.

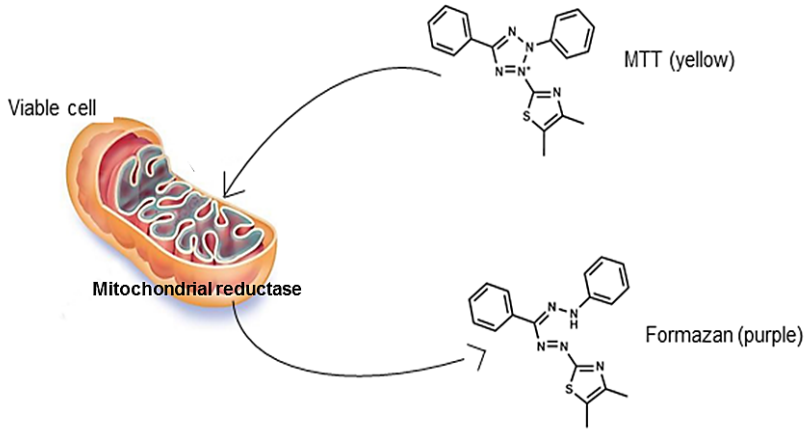


Figure 9. Principle of MTT assay.

In addition, the degree of resistance toward DOX (or resistance index; RI) of MCF-7/DOX cells was estimated in the condition that both parental MCF-7 and resistant MCF-7/DOX cells were treated with DOX for 48 hrs. The RI was calculated using following equation:

$$\text{Resistance index (RI)} = \frac{\%\ \text{cell viability (MCF}-7/\text{DOX) cells}}{\%\ \text{cell viability (MCF}-7)\ \text{cells}}$$

2.2 Determination of transporter functions

Activities of the ABC drug efflux transporters including P-gp, MRP1, MRP2 and BCRP were determined by a substrate accumulation assay as described previously (Sukhaphirom et al., 2013). The cells were seeded onto 24 well plate at density of 2.2×10^5 cells per well. On day 3 after seeding, the cells were washed and treated with serum-free RPMI 1640 medium containing either RN-C or a known pump inhibitor for 30 min at 37 ºC. Then, a specific substrate of each transporter was added and further incubated for another 30 min. In this study, the accumulation of specific substrate in the presence and absence of specific known inhibitors were listed in Table 3.

Table 3. Specific substrates and inhibitors of efflux transporters.

Efflux transporters	Specific substrate	Inhibitors	Reference
P-glycoprotein	Calcein-AM (0.4 μM)	Cyclosporine A (50 μM)	Sukhaphirom et al., 2013
MRP1	DCDF (5.2 μM)	Indomethacin (500 μM)	Kars et al., 2006
MRP2	CDCFDA (5 μM)	Indomethacin (500 μM)	Sukhaphirom et al., 2013
BCRP	Pheophorbide A (10 μM)	KO143 (10 μM)	Fardel et al., 2015

In addition, the effect of RN-C on substrate accumulation was also determined at the incubation periods up to 12 hrs. At the end of incubation period, the cells were washed with ice-cold PBS and lysed with 0.1% Triton X-100. Fluorescence intensity in the cell lysates was detected at excitation/emission wavelengths of 485/535 nm for calcein, DCDF and CDCF and at 635/ 670 nm for Phe A, using a microplate reader The fluorescent intensity was calculated and reported as % of control (i.e., substrate alone group). The amount of proteins in each sample were determined with Bradford reagent® at 595 nm.

2.3 Determination of intracellular DOX accumulation

The cell were seeded onto 12-well plate at the density of 2.2×10^5 cell per well. On day 3 after seeding, the cells were treated with DOX (1 or 2 μM), either alone or in combination with RN-C at non-cytotoxic concentration at 37 ºC for up to 12 hrs. At certain time point, the culture media was removed and the cells were washed twice with phosphate buffered saline (PBS). Subsequently, the cells were fixed in 4% paraformaldehyde for 15 min at room temperature. After washing 2 time with PBS, intracellular fluorescent signal of DOX were visualized under the fluorescence microscope at excitation/emission wavelengths of 488/550 nm.

Moreover, the intracellular DOX accumulation was quantified after 6 hr-treatment by measuring the fluorescence intensity in cell lysate with a microplate reader at excitation/emission wavelengths of 485/595 nm. DOX accumulation ratio between the cells treated with DOX alone and the cells treated with DOX in combination of RN-C was also calculated.

2.4 Data analysis

Data were expressed as mean ± SEM from 3-4 separated experiments. The nature of the interaction (synergism, additive, or antagonism) between RN-C and DOX was assessed through combination index analysis. The combination index (CI) was calculated from the following equation:

$$CI = C50\ (DOX)/C50\ (RN\text{-}C) + IC50\ (DOX)/IC50\ (RN\text{-}C)$$

Where C50 (DOX) and C50 (RN-C) were the concentration in a combination regimen that generated 50% cytotoxicity. IC50 (DOX) and IC50 (RN-C) were the concentrations that produce 50% cytotoxicity when the compound was used alone. The CI values suggested as following; < 0.1 very strong synergism, 0.1–0.3 strong synergism, 0.3–0.7 synergism, 0.7–0.85 moderate synergism, 0.85–0.90 slight synergism, 0.90–1.10 nearly additive, 1.10–1.20 slight antagonism, 1.20–1.45 moderate antagonism, 1.45–3.3 antagonism, 3.3–10 strong antagonism, > 10 very strong antagonism according to (Chou, 2010; Reynolds and Maurer 2005).

Moreover, a cytotoxicity enhancement ratio (CER) (or reversal ratio; RR) was calculated from the ratio between the IC50 values of DOX in the absence and presence of RN-C. The CER value could be a measure of an increase in cell sensitivity toward DOX treatment in the presence of RN-C (Chou, 2006).

Statistical analysis for multiple comparisons was performed by one-way ANOVA, followed by post-hoc Least Significant Different (LSD) test. Comparisons between two groups were performed by Student's t-test. Statistical significance was considered at $P < 0.05$

CHAPTER IV
RESULTS

4.1 Effect of Rhinacanthin-C (RN-C) and doxorubicin (DOX) on cell viability

The effects of RN-C and DOX on cell viability were determined by an MTT assay. The cell were treated with various concentrations of either RN-C (0.01-10 μM) or DOX (0.01-2 μM) for 24 to 48 hrs. As show in Figure 10, both RN-C and DOX caused concentration-dependent cytotoxicity on the parental MCF-7 cells after 24 and 48-hr treatment. In addition, the cytotoxicity of both compounds was affected by the treatment period. As show in table 1, the IC50 values of DOX and RN-C increased by 1.76 and 15.03 folds respectively, when the treatment period increased from 24 to 48 hrs. RN-C at the concentration up to 1.5 μM was not toxic to the cells after 24-hr exposure. Its maximum non-cytotoxic concentration decreased by approximately 150-folds when the treatment period increased from 24 to 48 hrs.

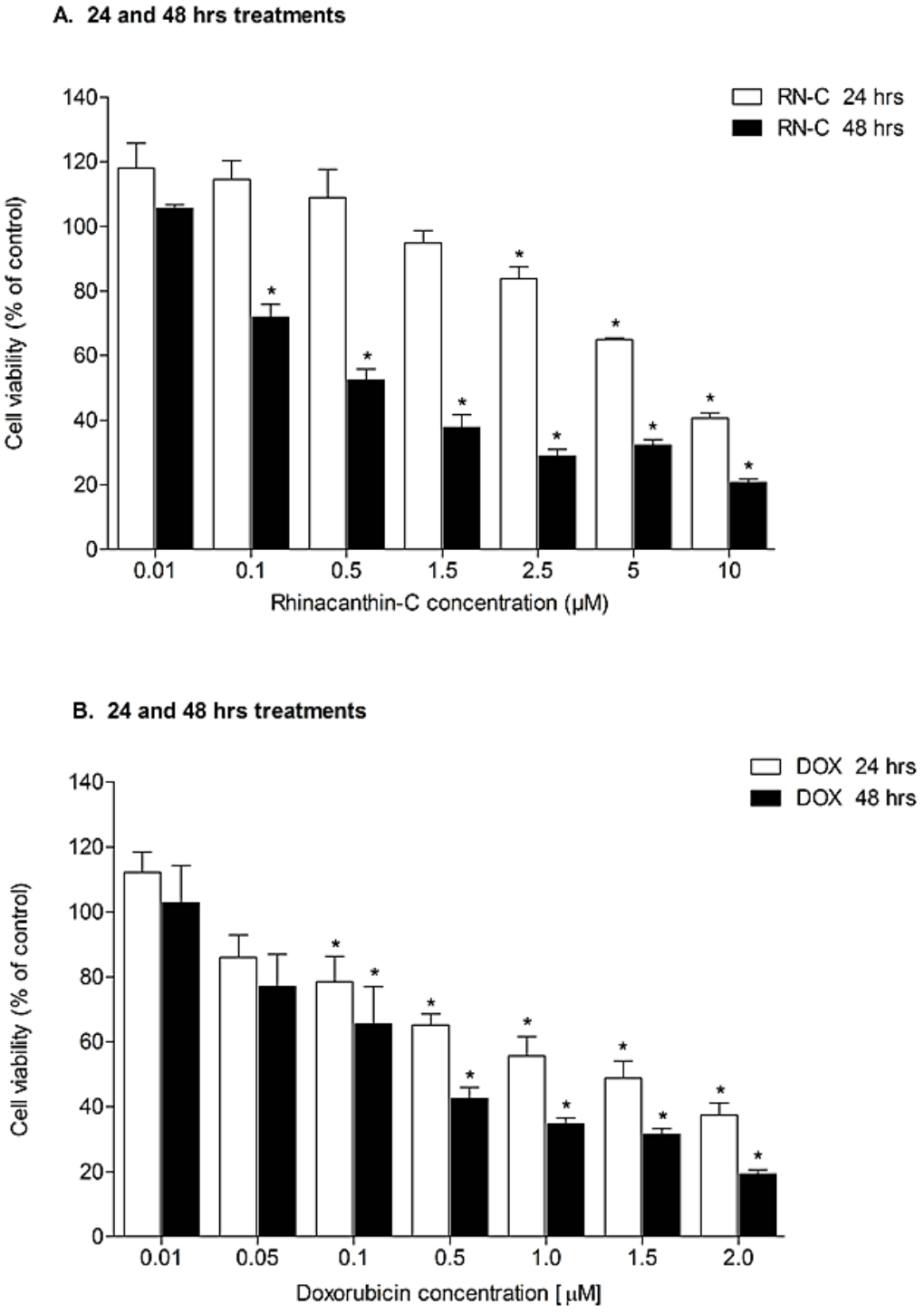


Figure 10. Effects of rhinacanthin-C (RN-C) and doxorubicin (DOX) on cell viability of parental MCF-7 cells. (A) Treatment with RN-C for 24 and 48-hrs. (B) Treatment with DOX for 24 and 48 hrs. Each value represented the mean ± SEM. (n=4). *P< 0.05 vs untreated group.

As shown in Figure 11, RN-C at the non-cytotoxic concentration (either 0.04 or 0.1 μM) was able to enhance the cytotoxicity of DOX in the parental MCF-7 cells. The enhancement effect of RN-C on DOX-mediated cytotoxicity depended on the concentration of RN-C and the treatment periods. Upon addition of RN-C 0.1 μM for 24 hrs, the IC50 of DOX decreased by 1.42-fold. But 0.04 μM RN-C had no effect on DOX-mediated cytotoxicity. An increase of treatment period from 24 hrs to 48 hrs markedly increased the enhancement effects of RN-C. As shown in Figure 12, the enhancement effects of RN-C 0.04 μM on DOX-mediated cytotoxicity increased from 1.04 to 9.63 folds when the treatment period increased from 24 hrs to 48 hrs. The enhancement effect of RN-C at the concentration of 0.1 μM was more pronounced at the treatment periods of 48 hrs. Upon increasing the treatment period from 24 hrs to 48 hrs, the enhancement effects of 0.1 μM RN-C on DOX-mediated cytotoxicity increased from 1.42 to 38.50-fold. The IC50 and CI values of each treatment were shown in Table 4.

According to the CI analysis, the nature of interaction between RN-C and DOX in the parental MCF-7 was synergism at 0.1 μM RN-C. The effect of RN-C 0.04 μM on DOX-mediated cytotoxicity at 24 hrs treatment was nearly additive (CI = 0.97). Moreover, the degree of synergism largely depended on the treatment periods. Upon increasing the treatment period from 24 hrs to 48 hrs, the effects of RN-C (either 0.04 or 0.1 μM) on DOX-mediated cytotoxicity shifted into strong synergism.

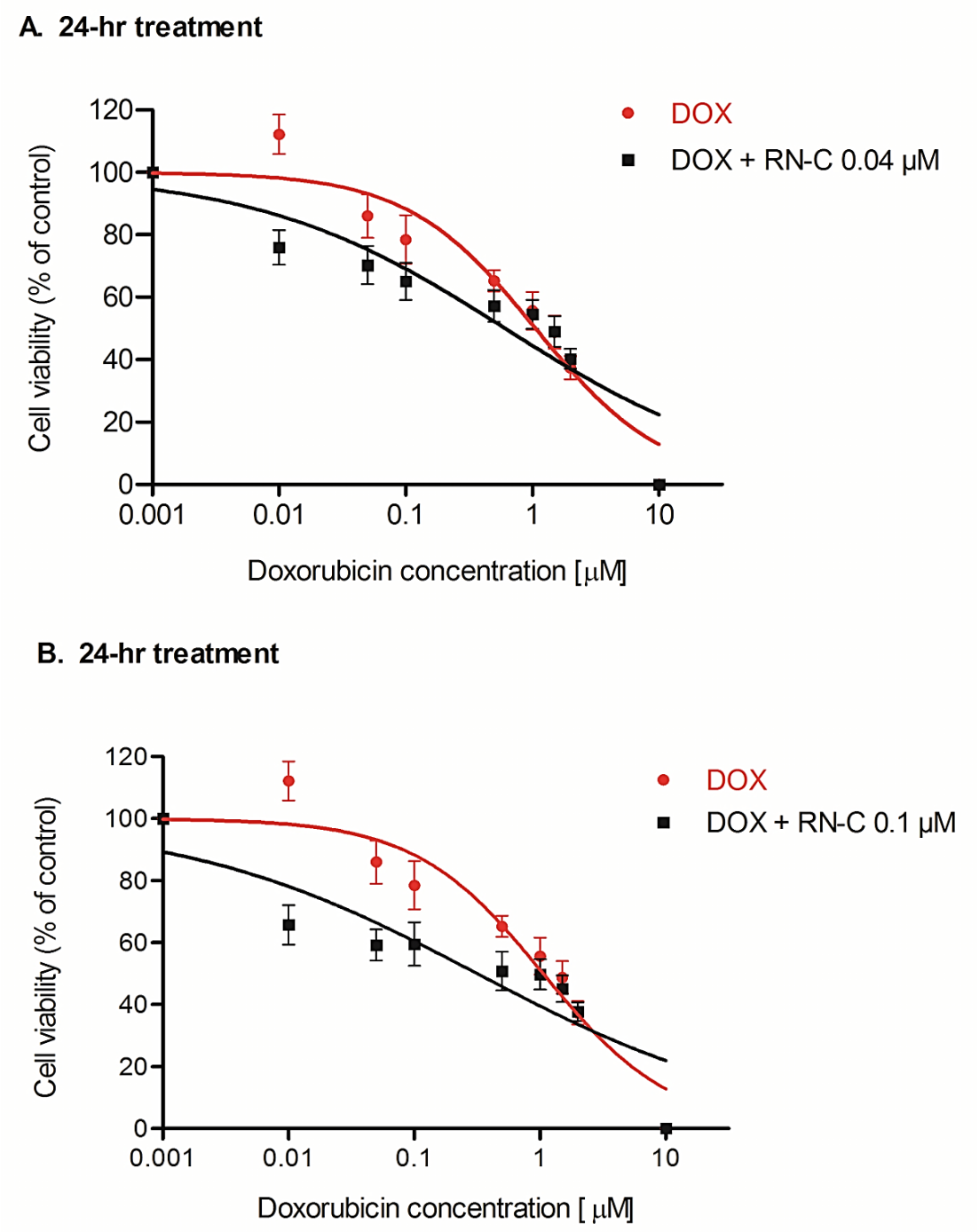


Figure 11. Cell viability after treatment with doxorubicin (DOX) in the absence and presence of 0.04 and 0.1 μM rhinacanthin-C (RN-C) for 24 hrs in parental MCF-7 cells. MCF-7 cells were incubated with DOX at various concentrations (ranging from 0.01 to 2 μM), either alone or in combination with RN-C for 24 hrs. Result are expressed as mean ± SEM (n = 4).

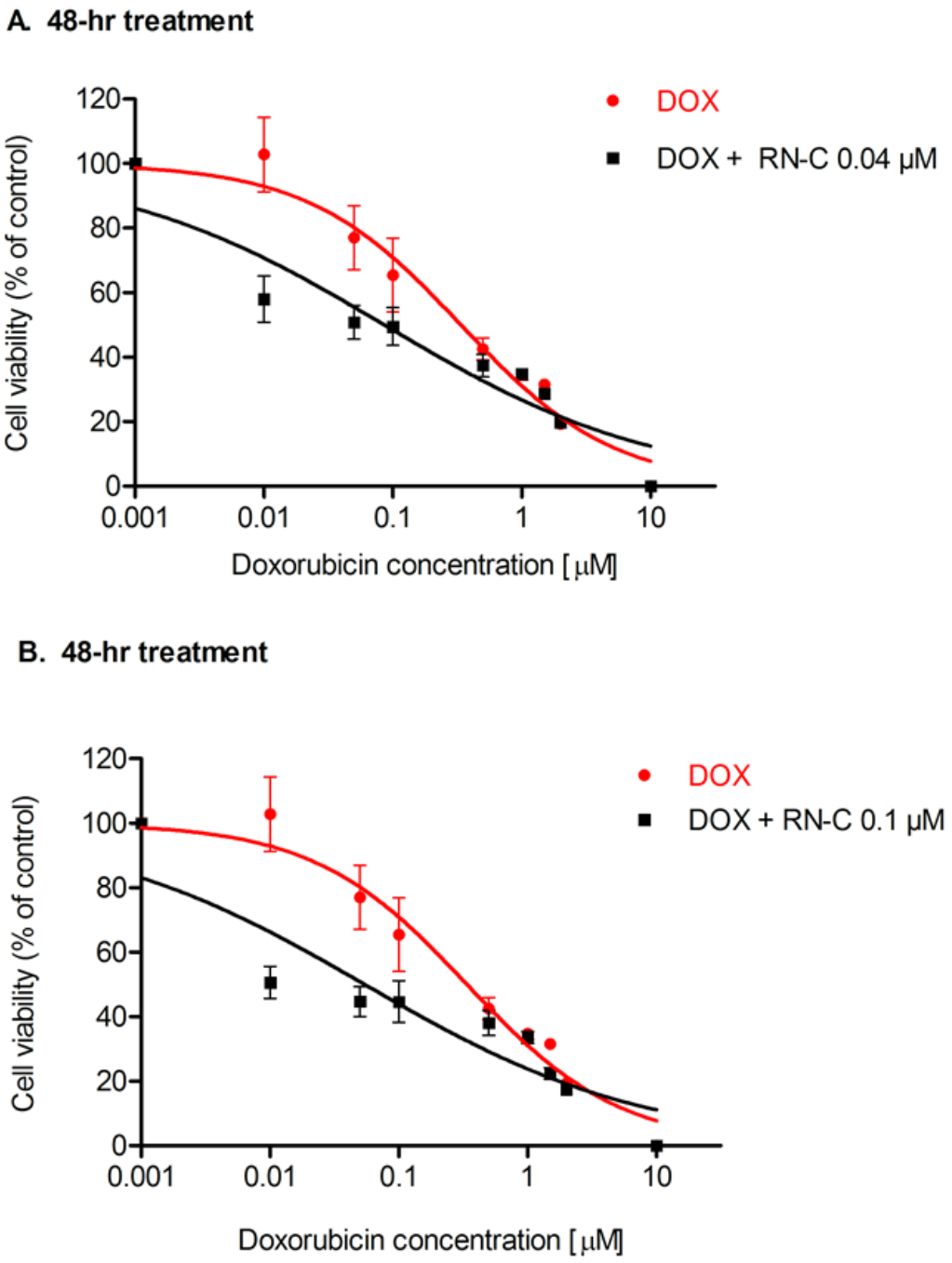


Figure 12. Cell viability after treatment with doxorubicin (DOX) in the absence and presence of 0.04 and 0.1 μM rhinacanthin-C (RN-C) for 48 hrs in parental MCF-7 cells. MCF-7 cells were incubated with DOX at various concentrations (ranging from 0.01 to 2 μM), either alone or in combination with of RN-C for 48-hrs. Results are expressed as mean ± SEM (n = 4).

Table 4. Summary of the IC50 values of rhinacanthin-C (RN-C) and doxorubicin (DOX), (either alone or in combination), and combination analysis in the parental MCF-7 cells.

	24 h				48 h			
Compounds	**IC50**	**IC50 of DOX**	**CER**	**CI**	**IC50**	**IC50 of DOX**	**CER**	**CI**
Doxorubicin	1.36 ± 0.12				0.77 ± 0.06			
Rhinacanthin-C	8.57 ± 0.15				0.57 ± 0.19			
Rhinacanthin-C 0.04 µM		1.31 ± 0.18	1.04	0.97		0.08 ± 0.12	9.63	0.17
Rhinacanthin-C 0.1 µM		0.96 ± 0.33	1.42	0.71		0.02 ± 0.09	38.50	0.20

[1] CI values were interpreted as follows: < 0.1 very strong synergism, 0.1–0.3 strong synergism, 0.3–0.7 synergism, 0.7–0.85 moderate synergism, 0.85–0.90 slight synergism, 0.90–1.10 nearly additive, 1.10–1.20 slight antagonism, 1.20–1.45 moderate antagonism, 1.45–3.3 antagonism, 3.3–10 strong antagonism, >10 very strong antagonism according to (Reynolds and Maurer, 2005; Chou, 2006).

[2] CER values indicated the fold of dose reduction of doxorubicin by a given combination.

4.2 Effect of rhinacanthin-C (RN-C) on intracellular accumulation of doxorubicin (DOX)

The effect of RN-C on DOX accumulation within the parental MCF-7 cells was determined for up to 12 hrs, using a fluorescence microscope. As known, DOX is a fluorochrome, the amount of intracellular DOX would be indirect proportion to its fluorescence. As shown in Figure 13, the amount of intracellular DOX increased in time-dependent manner. Co-treatment the cells with 1 μM DOX and 0.1 μM RN-C caused a significant increase of DOX fluorescent signals within the cells, in comparison with those treated with DOX alone. The treatment with 1 μM DOX, either alone or in combination with 0.1 μM RN-C had no effect on cell viability in this experiment condition.

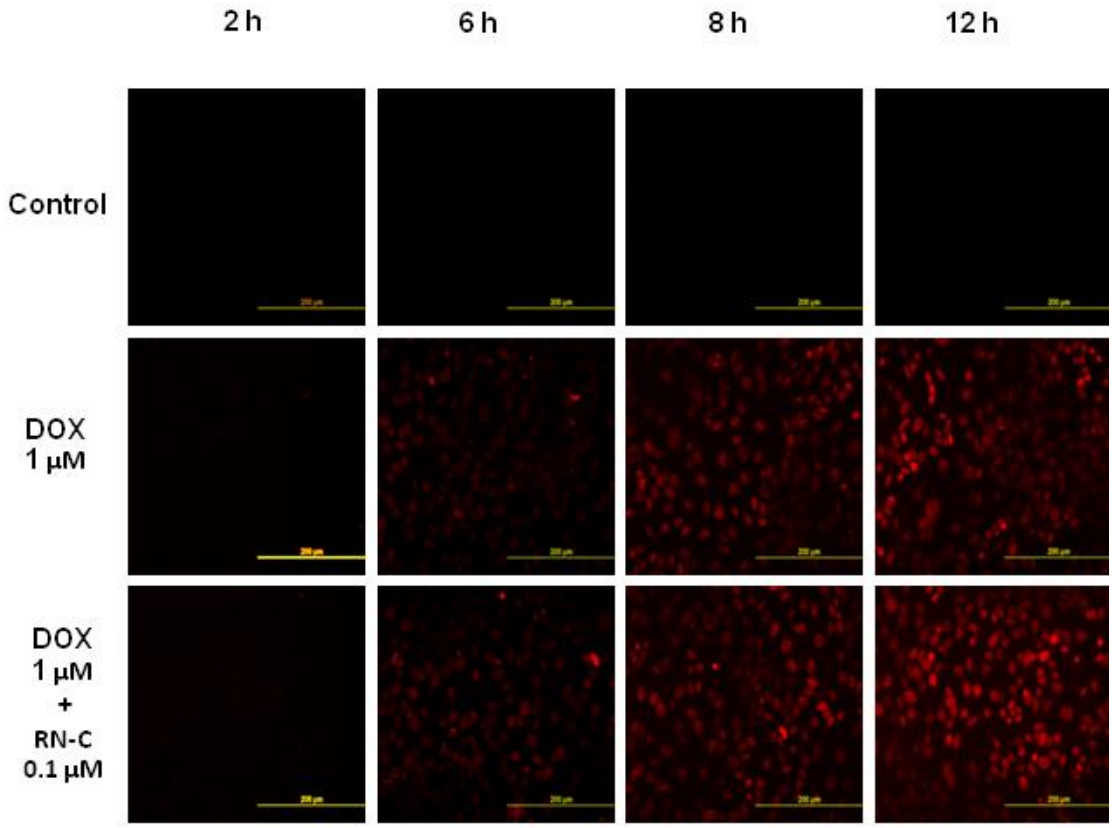


Figure 13. RN-C increased the intracellular accumulation of doxorubicin in parental MCF-7 cells. Cells were treated with 1 μM DOX alone or in combination with 0.1 μM RN-C for up to 12 hrs. Representative fluorescence image of parental MCF-7 cells, comparing the distribution of red fluorescence for DOX treatment alone or in combination with RN-C at various time points for up to 12 hrs. Treatment with RN-C enhanced the intracellular accumulation of DOX in parental MCF-7 cell line. DOX appeared to be exclusively located within the parental MCF-7 cells. The original magnification of all images was 200 μm (X20), and the images were captured under the same microscope settings. All data were representatives of at least three independent experiments.

Furthermore, the amount of DOX accumulated within the cells after treatment period of 6 hrs was also quantified with spectrofluoroscopy technique, as described in Methods. As shown in Figure 14 and Figure 15, the intracellular DOX accumulation ratio in both parental MCF-7 and resistant MCF-7/DOX cells increased significantly in the presence of 0.1 μM RN-C (1.17-fold increase in parental MCF-7 and 1.18-fold in resistant MCF-7/DOX cells). Moreover, the effect of RN-C on intracellular DOX accumulation depended on its concentration. At the co-treatment period of 6 hrs, 0.04 μM RN-C had no significant effect on DOX accumulation (1.04- fold increase in parental MCF-7 and 1.03-fold in resistant MCF-7/DOX cells).

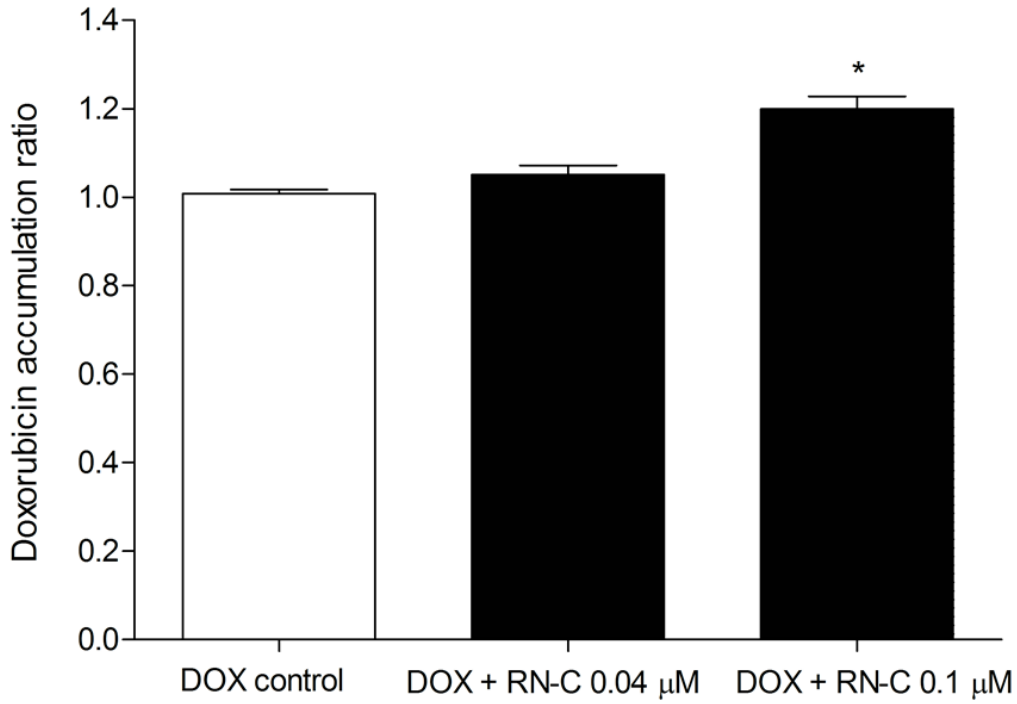


Figure 14. Effect of rhinacanthin-C (RN-C) on the accumulation of doxorubicin (DOX) in parental MCF-7 cells. Data represented mean ± SEM from 3 independent experiments. *$P < 0.05$ compared with control group (DOX alone).

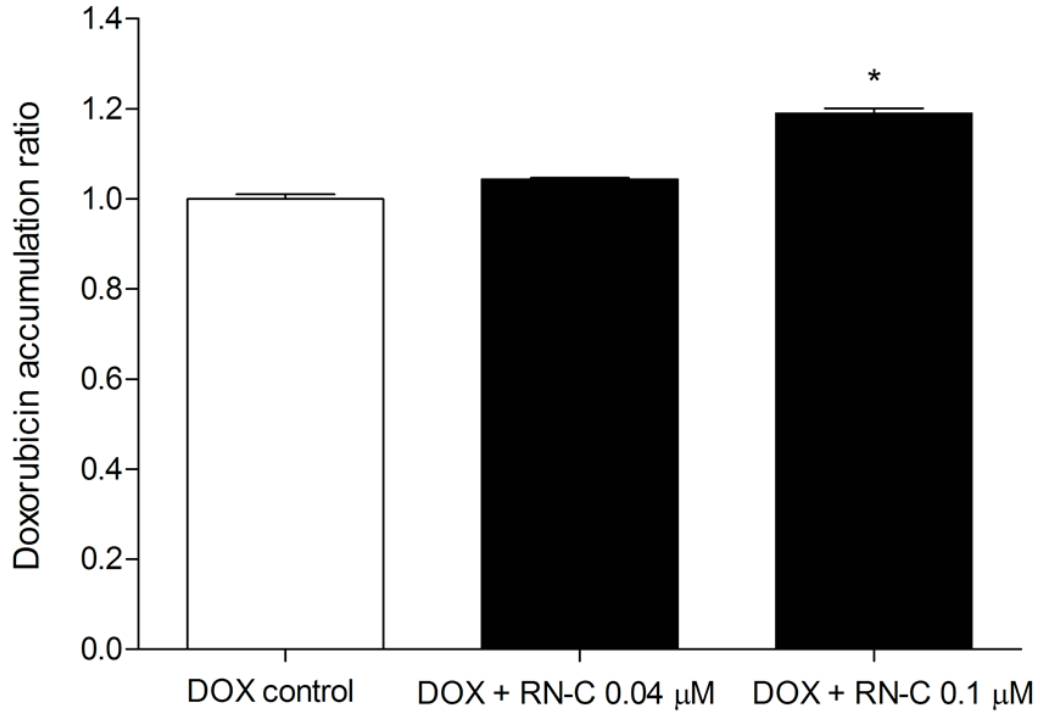


Figure 15. Effect of rhinacanthin-C (RN-C) on the accumulation of doxorubicin (DOX) in MCF-7/DOX resistant cells. Data represented mean ± SEM from 3 independent experiments. **P*< 0.05 compared with control group (DOX alone).

4.3 Activities of BCRP, MRP1, MRP2 and P-gp transporters in parental MCF-7 and resistant MCF-7/DOX cells

In this study, the specific substrate and inhibitor of BCRP, MRP1, MRP2 and P-gp transporters were used to demonstrate the existence of these transporters in the parental MCF-7 cells and resistant MCF-7/DOX cells. As shown in Figure 16, indomethacin (500 μM), which was a known inhibitor of MRP1 and MRP2, increased intracellular accumulation of DCDF and CDCF by approximately 2.90- and 3.50-fold. In addition, cyclosporine A (50 μM) and KO143 (10 μM), which were known inhibitors of P-gp and BCRP, had no effect on calcein and pheophorbide A accumulation in the parental MCF-7 cells. The result suggested that the parental MCF-7 cells expressed appreciable level of MRP1 and MRP2 activities, but the cells had no BCRP and P-gp functions.

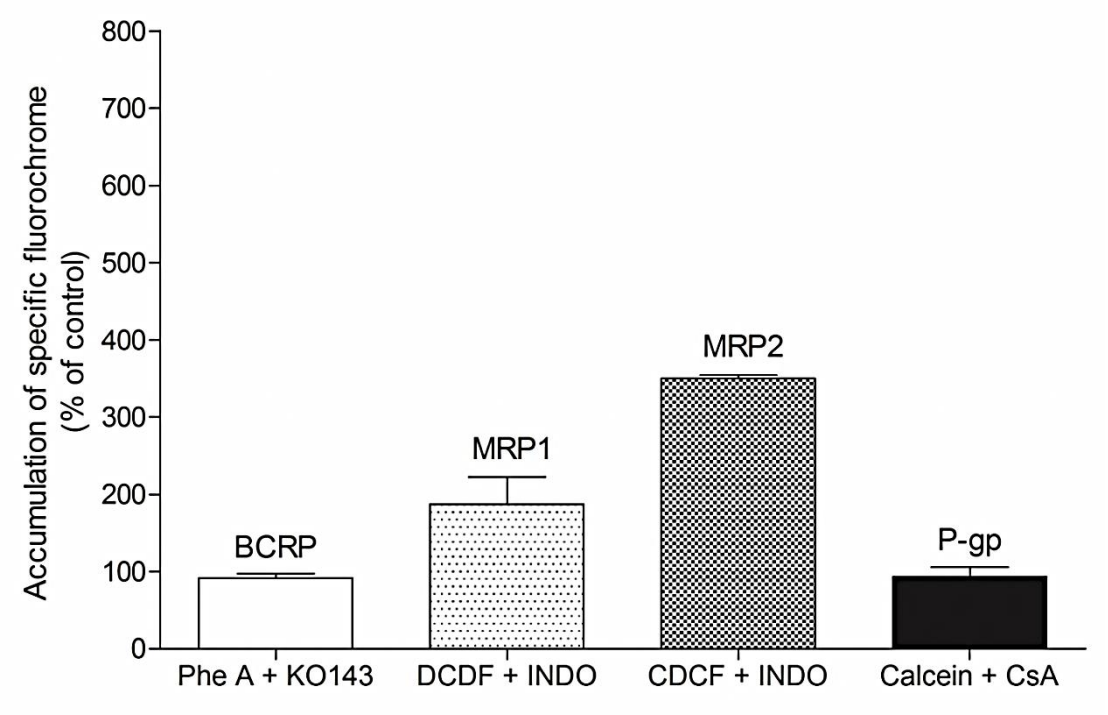


Figure 16. Activities of the ABC drug efflux transporters (BCRP, MRP1, MRP2 and P-gp) in the parental MCF-7 cells. Intracellular accumulation of Phe A, DCDF, CDCF and calcein were determined in the presence and absence of known inhibitors KO143 (KO143; 10 μM), indomethacin (INDO; 500 μM) and cyclosporine A (CsA; 50 μM). Data were calculated and expressed as the percentage of the untreated group.

Furthermore, functions of MRP2 and P-gp transporters were observed in the MCF-7/DOX resistance cells. As shown in Figure 17, cyclosporine A (50 μM) and indomethacin (500 μM), increased intracellular accumulation of calcein and CDCF by approximately 6.52-fold and 3.18-fold. On the contrary, indomethacin (500 μM) and KO143 (10 μM), which were known inhibitors of MRP1 and BCRP, had no effect on DCDF and pheophorbide A accumulation in the MCF-7/DOX resistant cells. The result suggested that the MCF-7/DOX resistant cells expressed appreciable levels of P-gp and MRP2 activities, but the cells had no MRP1 and BCRP functions.

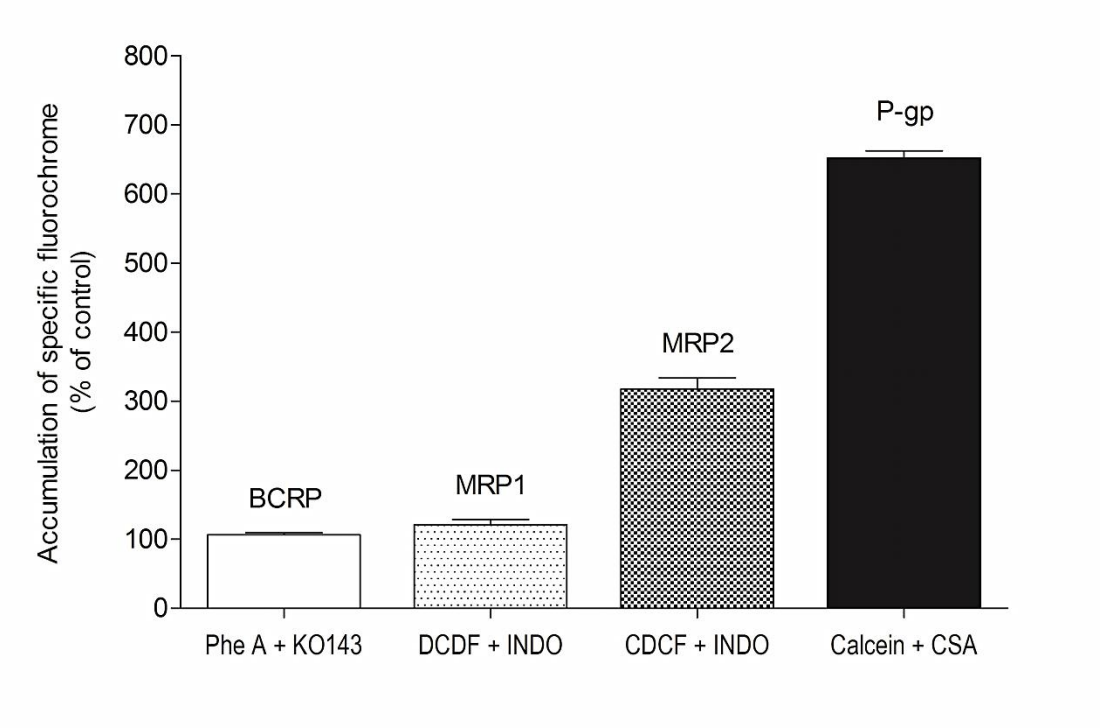


Figure 17. Activities of the ABC drug efflux transporters (BCRP, MRP1, MRP2 and P-gp) in the MCF-7/DOX resistant cells. Intracellular accumulation of Phe A, DCDF, CDCF and calcein were determined in the presence and absence of known inhibitors KO143 (KO143; 10 μM), indomethacin (INDO; 500 μM) and cyclosporine A (CsA; 50 μM). Data were calculated and expressed as the percentage of the untreated group.

4.4 Effect of RN-C on MRP1 and MRP2 function

The non-cytotoxic concentrations of RN-C at 0.04 and 0.1 μM were chosen to assess its inhibitory effects on MRP1 and MRP2 activities.

4.4.1 MRP1 function

RN-C at the concentration 0.1 μM was unable to significantly increase intracellular accumulation of DCDF in parental MCF-7 cells after 12-hr treatment (Figure 18). However, the effect of RN-C on DCDF retention within the cells was comparable to that of 0.1 μM indomethacin. It was worth noting that the concentration of indomethacin, a known MRP1 and MRP2 inhibitor, in this experiment was 5000-folds less than its standard effective concentration of 500 μM. The result suggested that 0.1 μM of RN-C could not interfere with MRP1 activity in the parental MCF-7 cells.

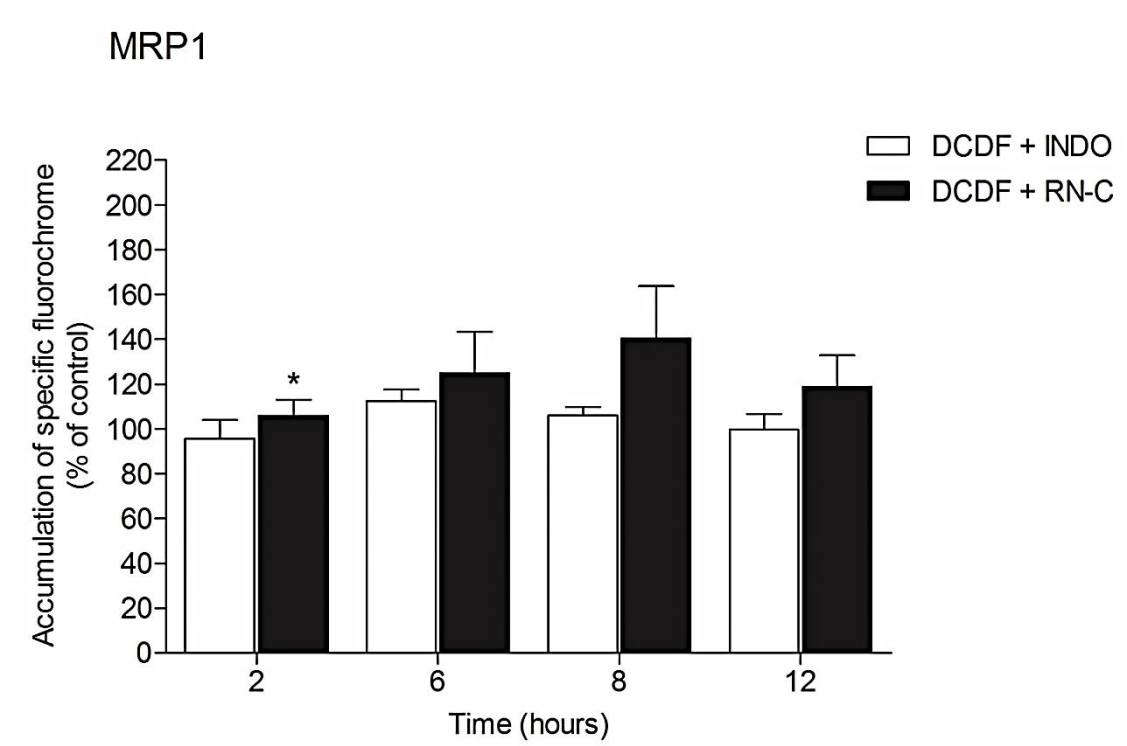


Figure 18. Effect of rhinacanthin-C (RN-C) and indomethacin (INDO) at the eqimolar concentration of 0.1 μM on intracellular accumulation of DCDF in the MCF-7 cells after 2, 6, 8 and 12-hrs treatments. Data were calculated and expressed as the percentage of the untreated group.

4.4.2 MRP2 function

RN-C at the concentration 0.1 μM was able to significantly increase intracellular accumulation of CDCF in parental MCF-7 cells after 12-hr treatment (Figure 19). Moreover, RN-C caused more CDCF retention within the cells than a known MRP2 inhibitor indomethacin at the equimolar concentration of 0.1 μM did. These data suggested that RN-C at the very low concentration was able to suppress MRP2 activity. Moreover, the effect of RN-C on CDCF retention was time-dependent. As the treatment period with RN-C was longer, the amount of CDCF retained within the cells increased by 1.07-fold (for 2-hr), 1.65-fold (for 6-hr), 1.73- fold (for 8-hr) and 1.90-fold (for 12-hr). The data suggested that RN-C at the non-cytotoxic concentration was able to interfere with MRP2 activities, resulting in an increase of MRP2 substrate accumulation in a time-dependent manner.

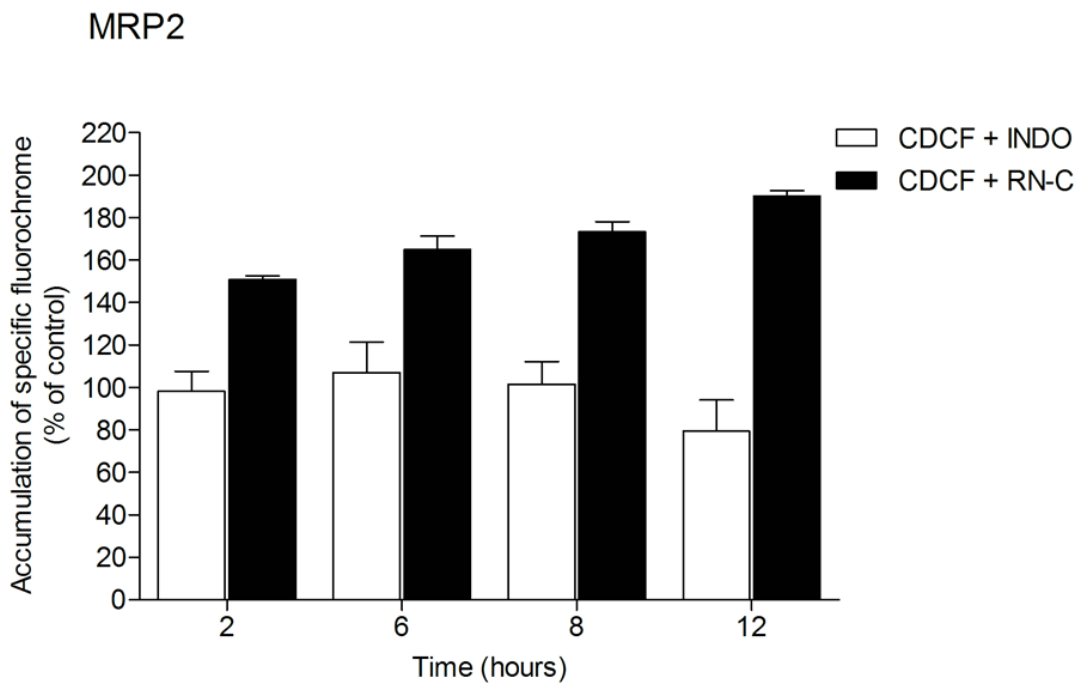


Figure 19. Effect of rhinacanthin-C (RN-C) and indomethacin (INDO) at the eqimolar concentration of 0.1 μM on intracellular accumulation of CDCF in the parental MCF-7 cells after 2-, 6-, 8- and 12-hr treatments. Data were calculated and expressed as the percentage of the untreated group.

4.5 MDR reversal effect of RN-C on doxorubicin resistant MCF-7 cells

As shown in Figure 20, the doxorubicin resistant MCF-7 cells (MCF-7/DOX) were less sensitive toward doxorubicin-mediated cytotoxicity than their parental MCF-7 cells. The relative resistance of MCF-7/DOX cells, as compared to the MCF-7 parental cells was demonstrated in Table 5. The reversal index (RI) apparently increased when the concentration of DOX increased. The results demonstrated that the resistance of MCF- 7/DOX cells toward DOX treatment increased from 1.70-fold to 4.73-fold, when the concentration of DOX increased from 0.1 to 2 μM.

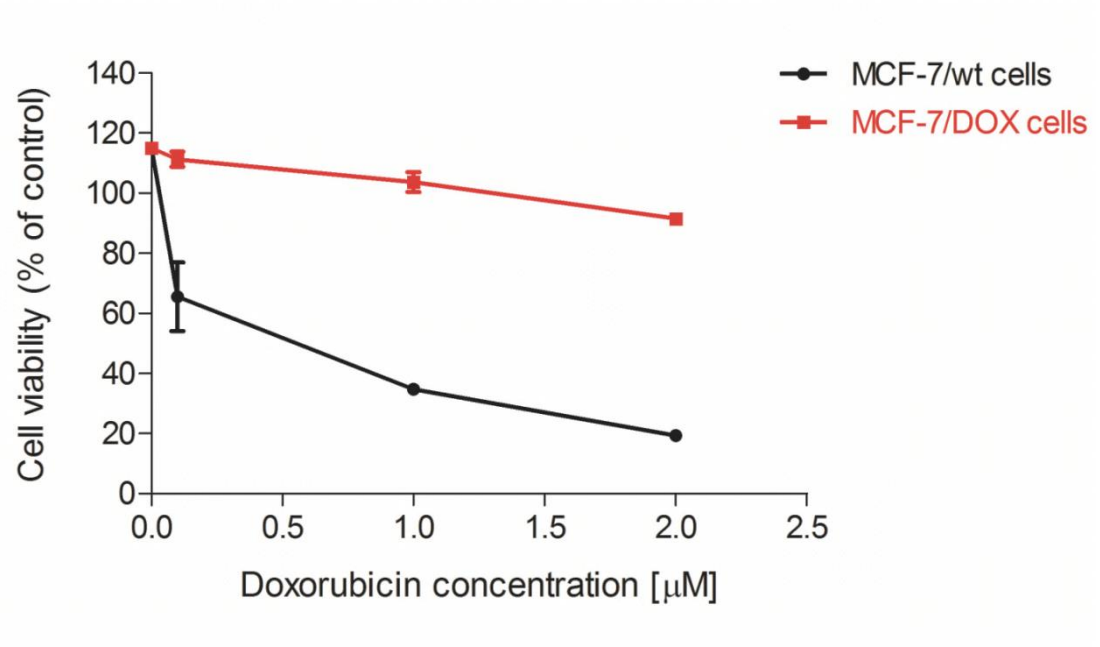


Figure 20. Doxorubicin mediated cytotoxicity in the resistance MCF-7/DOX and parental MCF-7 cells.

Table 5. The relative resistance of MCF-7/DOX resistance cells, as compared to the MCF-7 parental cells. Cells were treated with various concentrations of DOX for 48 hrs. The percentage of cell viability of both cell types in the presence of DOX were calculated and used for RI estimation.

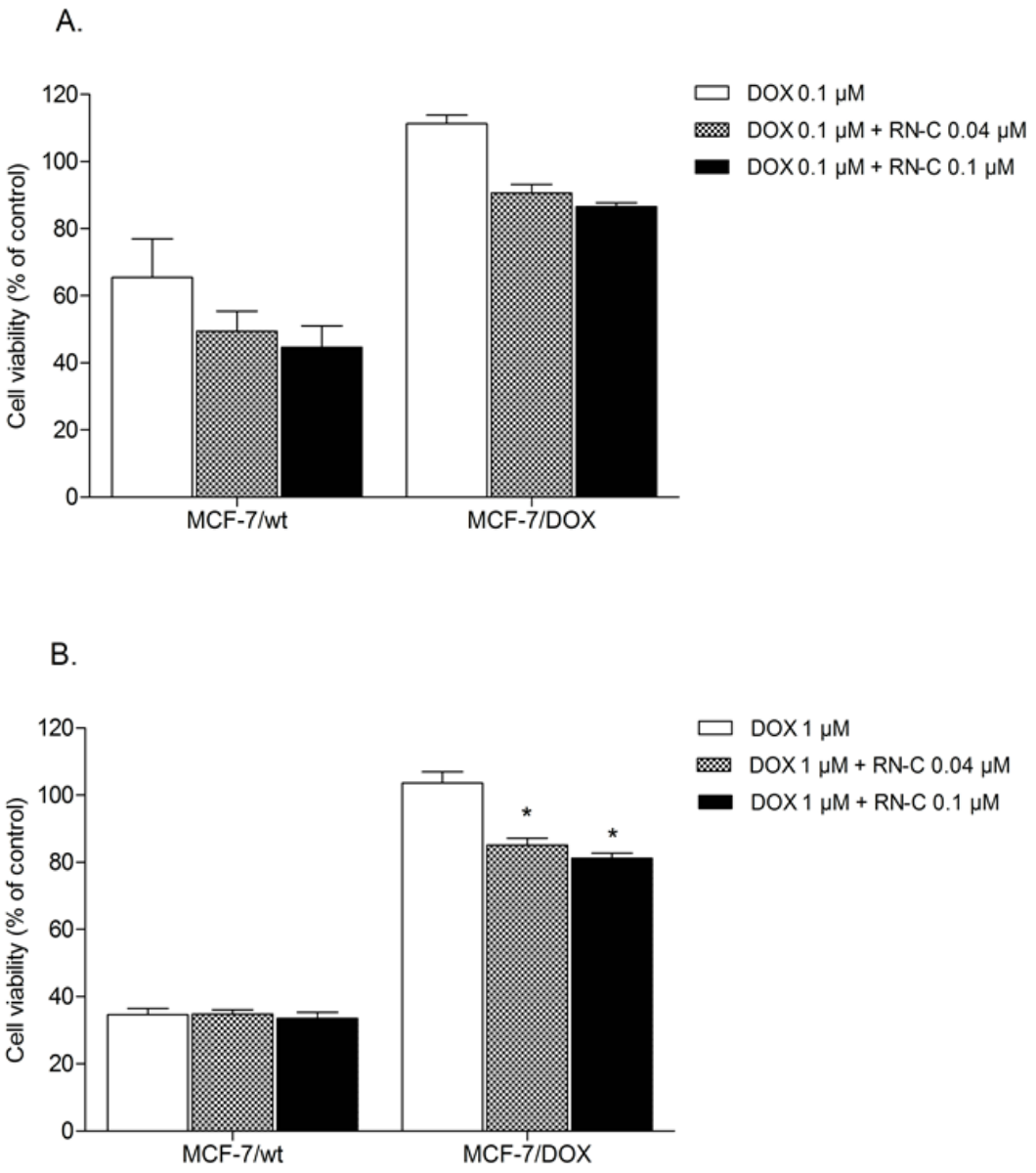
Compounds	MCF-7 parental cells	MCF-7/DOX resistant cells	RI
DOX 0.1 μM	65.46 ± 2.86	111.29 ± 0.83	1.70
DOX 1 μM	34.71 ± 0.44	103.66 ± 1.07	2.99
DOX 2 μM	19.30 ± 0.33	91.47 ± 0.46	4.73

[1] Determination of % cell viability of various concentration doxorubicin (DOX). The % cell viability of DOX was determined in MCF-7 parental and MCF-7/DOX resistant cells.

[2] The degree resistance was calculated by resistance index (RI), which obtained from the data = % cell viability of MCF-7/DOX cell vs % cell viability of MCF-7 cells.

[3] Data were expressed as means ± SEM of three independent experiments.

The doxorubicin-mediated cytotoxicity in both resistance (MCF-7/DOX) and parental (MCF-7) cells were tested at 48 hrs treatment. In this study, DOX at the concentrations of 0.1, 1 and 2 μM could produce the cytotoxicity in the parental MCF-7 cells, but not in the resistant MCF-7/DOX cells. As shown in Figure 21, RN-C at the concentration of 0.04 and 0.1 μM giving in the combination with 1 and 2 μM doxorubicin were able to enhance doxorubicin-mediated cytotoxicity in the resistant MCF-7/DOX cells. The cytotoxic effects of 2 μM doxorubicin in combination with RN-C at 0.04 and 0.1 μM were more pronounced than those generated from RN-C in combination with 1 μM DOX. The presence of RN-C either 0.04 or 0.1 μM slightly decreased the viability of the parental MCF-7 treated with 0.1 μM DOX for 48 hrs. At the concentration of 0.1 μM, DOX even in the combination with RN-C 0.1 μM could not significantly produce its cytotoxic effect in the resistant MCF-7/DOX cells. Furthermore, the non- cytotoxic concentration of DOX when giving in combination of RN-C could generate cytotoxicity in concentration dependent manner.


A.
Cell viability (% of control)
120
100
80
60
40
20
0
MCF-7/wt
MCF-7/DOX
DOX 0.1 μM
DOX 0.1 μM + RN-C 0.04 μM
DOX 0.1 μM + RN-C 0.1 μM
B.
Cell viability (% of control)
120
100
80
60
40
20
0
*
*
MCF-7/wt
MCF-7/DOX
DOX 1 μM
DOX 1 μM + RN-C 0.04 μM
DOX 1 μM + RN-C 0.1 μM

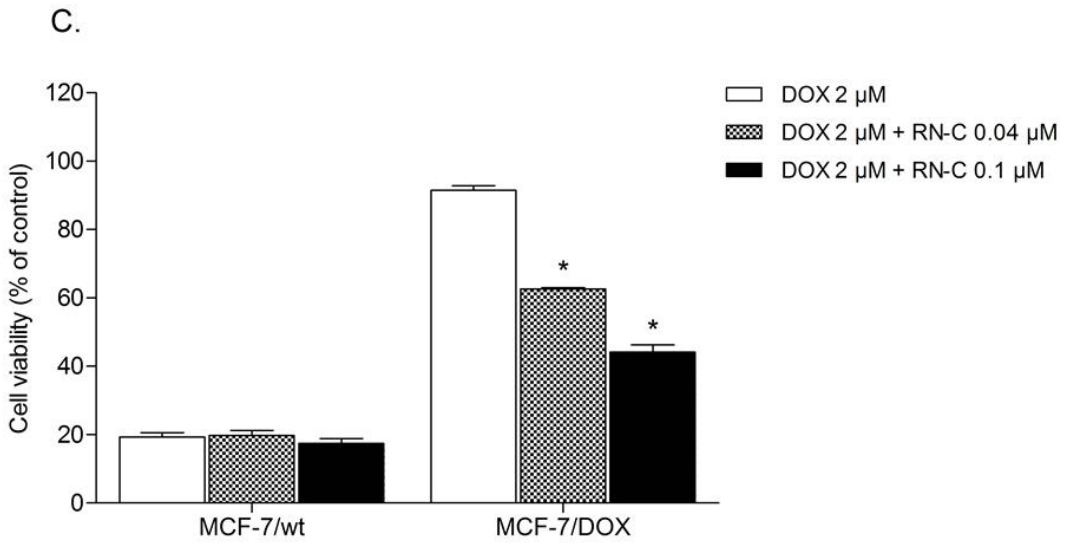


Figure 21. Rhinacanthin-C (RN-C) enhanced chemosensitivity of doxorubicin in MCF-7 parental cells and MCF-7/DOX cells. Cell were treated with doxorubicin (DOX) at 0.1 μM (A) or 1 μM (B) or 2 μM (C) in the presence and absence of RN-C 0.04 μM and 0.1 μM for 48 hrs. Cell viability were expressed as a percentage of those in the untreated control cells. Data represents the mean ± SEM of 3 independent experiments. *P<0.05 indicated statistically significant difference from DOX alone group.

The effectiveness of RN-C as a MDR reversal agent could be calculated and expressed as the reversal fold, which was the % cell viability in the presence or absence of RN-C. As shown in Table 6, RN-C (at 0.04 μM and 0.1 μM) gave reversal of resistance to DOX in MCF-7/DOX cells in the range of 1.22 to 2.07-fold. The maximum reversal effect of 2.07 fold was observed when giving RN-C 0.1 μM in combination with DOX 2 μM.

Table 6. Effect of RN-C on the sensitivity of MCF-7 parental and MCF-7/DOX resistant cells toward DOX treatment.

Compounds	MCF-7 parental cells	MCF-7/DOX resistant cells	RF (MCF-7/DOX cells)
DOX 0.1 μM	65.46 ± 2.86	111.29 ± 0.83	
DOX 1 μM	34.71 ± 0.44	103.66 ± 1.07	
DOX 2 μM	19.30 ± 0.33	91.47 ± 0.46	
DOX 0.1 μM + RN-C 0.04 μM	49.49 ± 1.47	90.70 ± 0.83	1.23
DOX 1 μM + RN-C 0.04 μM	34.87 ± 0.31	85.15 ± 0.70	1.22
DOX 2 μM + RN-C 0.04 μM	19.80 ± 0.35	62.61 ± 0.14	1.46
DOX 0.1 μM + RN-C 0.1 μM	44.66 ± 1.61	86.57 ± 0.38	1.28
DOX 1 μM + RN-C 0.1 μM	33.56 ± 0.46	81.20 ± 0.51	1.28
DOX 2 μM + RN-C 0.1 μM	17.46 ± 0.33	44.11 ± 0.73	2.07

[1] The cells were treated with various concentrations of DOX in the presence of RN-C (0.04 and 0.1 μM) for 48 hrs.

[2] The reversal fold (RF) was calculated from % cell viability for DOX alone vs the % cell viability for DOX in the presence of RN-C. Data were means ± SEM from three independent experiments.

[3] Data were expressed as means ± SEM of three independent experiments.

CHAPTER V
DISCUSSIONS

In this study, rhinacanthin-C (RN-C), a napthoquinone ester isolated form *R. nasutus*, was able to enhance chemosensitivity of human breast cancer cells toward doxorubicin treatment. As known, doxorubicin is an anthracycline cytotoxic agent commonly used for the treatment of breast cancer in patients with either endocrine resistant or with metastasis conditions (Gariboldi et al., 2003; Swift et al., 2006). Doxorubicin incorporates into DNA of cancer cells, leading to DNA damage. In addition, doxorubicin also prevents cell replication by inhibiting protein synthesis (Wang et al., 2014). The uses of doxorubicin can be limited due to its serious adverse effects and development of multidrug resistance later on. Moreover, a number of chemotherapeutic drugs even with different chemical structures or mechanisms often fail to overcome cancers with doxorubicin resistance. Therefore, increasing the sensitivity of cancer to doxorubicin would be an attractive goal for improving the clinical management of breast cancers.

In order to increase a success of cancer chemotherapy, several approaches have been applied such as searching of novel potent cytotoxic compounds or MDR reversal agents. In this study, rhinacanthin-C was investigated its potential sensitizing effect on cancer cells treated with doxorubicin. Previous studies demonstrated that rhinacanthin-C exerted its cytotoxic action, inhibited cell proliferation and caused apoptosis in several cancer cells (Siripong et al., 2006a; Siripong et al., 2006b; Wu et al., 1988). In this study, rhinacanthin-C was apparently less potent than doxorubicin in both MCF-7 parental cancer and MCF-7/DOX resistant cells. Moreover, the effect of rhinacanthin-C was further investigated beyond its cytotoxicity. The results demonstrated that rhinacanthin-C at the non-cytotoxic concentrations was able to increase the cytotoxic effect of doxorubicin in both MCF-7 parental cancer cell and

MCF-7/DOX resistant cells. The concentrations of rhinacanthin-C and doxorubicin in the combinatorial synergistic study did not produce cytotoxicity when giving alone. However, when giving both compounds at the non-cytotoxic concentrations simultaneously, cell death increased significantly. The synergy was also time-and concentration-dependent. This combinatorial effect was observed in both parental and resistant MCF-7 cells.

The nature of interactions between rhinacanthin-C and doxorubicin was characterized through analysis of combination index (CI) (Chou, 2010). At 24 hr-treatment, the synergistic interaction was observed when the concentration of rhinacanthin-C was at 0.1 μM, but not at the concentration of 0.04 μM. The IC50 value of doxorubicin remained almost unchanged in the presence of RN-C 0.04 μM for 24 hr-treatment period. The degrees of synergy were leveled up into strong interaction when the co-treatment periods, even with 0.04 μM rhinacanthin-C, increased from 24 hrs to 48 hrs. Hence, rhinacanthin-C and doxorubicin at their non-cytotoxic concentrations would produce the cytotoxicity in MCF-7 breast cancer cells when giving both compounds together. The nature of interaction between rhinacanthin-C and doxorubicin was synergy which could be at strong level in 48 hr-treatment. Consequently, this combination markedly reduced the cytotoxic concentration of doxorubicin by 38 folds (with 0.1 μM RN-C), as seen by the increased CER.

The mechanism of synergy between rhinacanthin-C and doxorubicin was investigated in this study. It was likely that rhinacanthin-C could increase intracellular doxorubicin accumulation in MCF-7 cancer cells. This hypothesis was based upon the report about the intrinsic property of rhinacanthin-C in inhibition of P-gp and MRPs activities (Wongwanakul et al., 2013). Data in this study indicated that the presence of rhinacanthin-C at the non-cytotoxic concentration caused an increased amount of doxorubicin within the parental MCF-7 cells in time-dependent manner. Co-treatment the cells with 1 μM doxorubicin and 0.1 μM rhinacanthin-C caused a significant increase

of doxorubicin fluorescent signals within the cells, in comparison with those treated with doxorubicin alone. As known, doxorubicin is a well-known P-gp and MRP2 substrate. It was likely that rhinacanthin-C 0.1 μM significantly decreased the efflux of doxorubicin through inhibition of P-gp and MRP2, resulting in an increase of intracellular doxorubicin accumulation up to its cytotoxic level. Eventually, doxorubicin mediated cell death could be observed in the combination treatment.

Mechanisms of MDR in cancer include high expression of the ATP binding cassette (ABC) transporters increase, DNA repair, alteration of the cytochrome P450 oxidases, down-regulation of drug targets, alteration of cell-cycle regulation (Desoize and Jardillier 2000; Perez-Tomas, 2006). The roles of membrane transporters including P-gp and MRPs were well established in MDR of cancers, which could lead to chemotherapeutic failure. Suppression of P-gp and MPR2 activities would help to circumvent MDR and enhanced chemosensitivity of the resistant cells toward their cytotoxic drug substrates. Hence, several MDR reversal agents were known P-gp and MRP2 inhibitors. It was reported that verapamil or cyclosporine A and indomethacin significantly reduced P-gp and MRP2 expression and transporter activities in MCF-7/DOX cells (Akiyama et al., 1988; Borowski et al., 2005; Lee, 2004; Zhang et al., 2009).

In this study, effects of rinacanthin-C on activities of the ABC efflux transporters including P-gp, MRP1, MRP2 and BCRP were determined in both the parental and resistant MCF-7 cells, using the specific substrates and inhibitors of each transporter. Calcein-AM, DCDF, CDCFDA and pheophorbide A (Phe A) were used as a specific substrate for P-gp, MRP1, MRP2 and BCRP, respectively. Calcein-AM and CDCFDA would be converted into fluorescent substances after the cleavage by intracellular esterase. In addition, DCDF and pheophorbide A (Phe A) were autofluorescent substrates. The positive inhibitors in this study included cyclosporine A (a known P-gp inhibitor), indomethacin (a known MRP1 and MRP2 inhibitor) and pheophorbide A (Phe A) (a known BCRP inhibitor). In this study, the results demonstrated that the MCF-7 parental

cells expressed MRP1 and MRP2 activities at appreciable level, but not P-gp, and BCRP activities. The MCF-7/DOX resistant cells contained both P-gp and MRP2 activities. In addition, the MRP2 activity in the MCF-7/DOX resistant cells was higher than that in MCF-7 parental cells by 4.73-fold (Coley 2008; Donmez et al., 2011).

Inhibition of drug efflux pump is a known mechanism for improving cell sensitivity to cytotoxic drugs in multidrug resistant (MDR) expression of the ABC transporters in particular P-gp, MRP1 and MRP2 in cancer cells (Gottesman et al. 2002; Li et al. 2011). The inhibition of these efflux transporters can increase intracellular accumulation of its cytotoxic drug substrate up to the level that can cause cell death (Kitagawa et al., 2004; Krishna and Mayer 2000). In the parental MCF-7 cells, rhinacanthin-C even at the low concentration (0.1 μM) was able to increase intracellular levels of specific substrate CDCF, suggesting its interference on MRP2 function. Moreover, the inhibitory effect of rhinacanthin-C on MRP2 function was higher than that of indomethacin, a known MRP2 inhibitor, at the equimolar concentration. Hence, it was likely that rhinacanthin-C was more potent MRP2 inhibitor than indomethacin. Even at the low concentration, rhinacanthin-C was still capable of inhibiting MRP2 function, leading to an increase of MRP2 substrate accumulation within the cells. Thus, rhinacanthin-C could increase doxorubicin mediated cytotoxicity via its inhibition of MRP2 activity in the parental MCF-7 cells.

Doxorubicin resistant (MCF-7/DOX) breast cancer cells were in-housed developed by stepwise selection of MCF-7 cells at increasing concentrations of doxorubicin. The final cells were found to be resistant to 1.5 μM doxorubicin. In order to determine the fold-resistance of MCF-7/DOX cell line, the resistance index (R) of doxorubicin was calculated. The results of cell viability study suggested that the MCF-7/DOX subline cells were approximately 4.73-fold resistant to doxorubicin, in comparison with the parental MCF-7 cells. Moreover, the resistant cells apparently contained both P-gp and MRP2 activities.

Effects of rhinacanthin-C on MDR reversal in the MCF-7/DOX cells were demonstrated by a decrease of cell viability in the treatment containing both rhinacanthin-C and doxorubicin. The doxorubicin-mediated cytotoxicity in resistance MCF-7/DOX cells were tested at 48 hr-treatment. Rhinacanthin-C at the concentration of 0.04 and 0.1 μM giving in the combination with doxorubicin (1 and 2 μM) were able to enhance doxorubicin-mediated cytotoxicity in the resistant MCF-7/DOX cells. Although the accumulation level of doxorubicin in MCF-7/DOX cells was not measured in the resistant MCF-7/DOX cells, it was very likely that rhinacanthin-C could inhibit P-gp and MRP2 expressed in these cells, resulting in an increased intracellular doxorubicin accumulation up to its cytotoxic level.

Combinations of drug with different modes of action would be benefit in improving therapeutic efficacy. In addition, the concentrations of cytotoxic anticancer agents could be reduced while maintaining the similar effectiveness with less toxicity (Chou, 2006). Previously, rhinacanthin-C in combination with cypermethrin synergistic increased effect cytotoxicity in *Spodoptera frugiperda* cells (Pethuan et al., 2012). The combination was beneficial for resistance management strategies in mosquito vector control (Pethuan et al., 2012). In the aspect of cancer treatment, most of the anti-cancer cytotoxic agents caused untolerated high toxicity that subsequently limited their application. Searching of non-toxic chemosensitizers would be useful in clinical cancer management. This study demonstrated that rhinacanthin-C would be a good candidate as an MDR reversal agent. By targeting at the ABC drug efflux pumps, rhinacanthin-C was able to increase doxorubicin mediated cytotoxicity and overcome MDR effectively. Due to the synergism interaction, the concentrations of both rhinacanrhin-C and doxorubicin in the combination were significant lesser than those giving alone.

In conclusion, rhinacanthin-C enhanced the doxorubicin sensitivities in both MCF-7 and MCF-7/DOX cells through its interference on the functions of P-gp and MRP2. Consequently, rhinacanthin-C was able to increase doxorubicin-mediated cytotoxicity. Another potential mechanism of the synergy between rhinacanthin-C and doxorubicin would be investigated further.

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Poster Presentation

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