ผลของไรนาแคนทินซีต่อการทำงานและการแสดงออกของตัวขับยาออกนอกเซลล์ในเซลล์คาโค-2

นางสาวรัตน์จิกา วงศ์วนากุล

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EFFECTS OF RHINACANTHIN-C ON FUNCTION AND EXPRESSION OF EFFLUX DRUG TRANSPORTERS IN CACO-2 CELLS

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รัตน์จิกา วงศ์วนากุล : ผลของไรนาแคนทินซีต่อการทำงานและการแสดงออกของตัวขับยาออกนอกเซลล์ในเซลล์คาโค-2. (EFFECTS OF RHINACANTHIN-C ON FUNCTION AND EXPRESSION OF EFFLUX DRUG TRANSPORTERS IN CACO-2 CELLS) อ.ที่ปรึกษาวิทยานิพนธ์หลัก : ผศ.ดร. สุรีย์ เจียรณ์มงคล,อ. ที่ปรึกษาวิทยานิพนธ์ร่วม : ผศ.ดร. นนทิมา วรรธนะภูติ, 86 หน้า.

ไรนาแคนทินซีเป็นสารในกลุ่มแนพโทควินโนนที่มีฤทธิ์ทางเภสัชวิทยาหลากหลาย เช่น ฤทธิ์ในการต้านมะเร็ง ฤทธิ์ต้านการอักเสบ งานวิจัยนี้มีวัตถุประสงค์ที่จะศึกษาผลของไรนาแคนทินซีต่อการทำงานและการแสดงออกของตัวขับยาออกนอกเซลล์ในกลุ่มของ ABC transporters โดยเฉพาะ พี-ไกลโคโปรตีน (P-gp) และ มัลติดรักรีซิสแทนซ์โปรตีน 2 (MRP2) โดยใช้เซลล์คาโค-2 เป็นแบบจำลองในการศึกษา การทำงานของตัวขับยาออกนอกเซลล์ทั้งสองชนิดศึกษาโดยวัดค่าการเรืองแสงของ calcein และ CDCF ที่สะสมในเซลล์ ส่วนการแสดงออกของโปรตีน P-gp ศึกษาโดยใช้ immunofluorescent probe ที่จับกับ P-gp ที่ผิวเซลล์ ผลการศึกษาพบว่าสารไรนาแคนทินซีสามารถยับยั้งการทำงานของ P-gp และ MRP2 ได้ โดยยับยั้งการทำงานของ P-gp ได้มากกว่า MRP2 ทั้งนี้การยับยั้งการทำงานของ P-gp มีลักษณะผันกลับได้ นอกจากนี้ การให้สารไรนาแคนทินซีที่ความเข้มข้นสูง (100 μM) กับเซลล์คาโค-2 เป็นระยะเวลา 1 วัน มีผลเพิ่มการแสดงออกของ P-gp ที่ผิวเซลล์ โดยไม่มีผลต่อการทำงานของ P-gp และเมื่อให้สารดังกล่าวที่ความเข้มข้นสูงสุดที่ไม่ทำให้เกิดความเป็นพิษกับเซลล์ (0.625 μM) เป็นเวลา 7 วัน ไม่พบว่าสารมีผลต่อการแสดงออกและการทำงานของ P-gp แต่อย่างใด ผลการศึกษาชี้แนะว่าควรต้องศึกษาเพิ่มเติมถึงประสิทธิภาพและความปลอดภัยในการนำสารไรนาแคนทินซีไปใช้ร่วมกับสารที่เป็น substrates ของ P-gp ต่อไป

ภาควิชา เภสัชวิทยาและสรีรวิทยา	ลายมือชื่อนิสิต
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RATJIKA WONGWANAKUL : EFFECTS OF RHINACANTHIN-C ON FUNCTION AND EXPRESSION OF EFFLUX DRUG TRANSPORTERS IN CACO-2 CELLS. ADVISOR : ASST.PROF.SUREE JIANMONGKOL, Ph.D., CO-ADVISOR : ASST.PROF.NONTIMA VARDHANABHUTI, Ph.D., 86 pp.

Rhinacanthin-C is a bioactive naphthoquinone compound with several pharmacological actions such as anticancer and anti-inflammatory activities. The purposes of this study were to determine the effects of this compound on the function and expression of the ABC efflux transporters, in particular P-gp and MRP2, using the *in vitro* model of Caco-2 cells. The activities of P-gp and MRP2 were determined by following the intracellular accumulation of calcein and CDCF in the uptake assays. The expression of P-gp was monitored with the use of immunofluorescent probe against P-gp expressed on the cell surface. The results showed that rhinacanthin-C was able to inhibit the activities of both P-gp and MRP2. The inhibitory effect of this compound was more potent toward the function of P-gp than that of MRP2. The inhibition against P-gp activity was reversible. Treatment of the cells with rhinacanthin-C at the high concentration of 100 μM for 1 day was able to increase P-gp expression without any appreciable change in its function. At the highest non-cytotoxic concentration (0.625 μM) with 7 day treatment, rhinacanthin-C had no effect on both the expression and the function of P-gp. These findings suggested that concomitant uses of rhinacanthin-C and other P-gp drug substrates should be investigated further to evaluate the efficacy and safety.

Department: Pharmacology and Physiology	Student's Signature
Field of Study: Pharmacology	Advisor's Signature
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LIST OF ABBREVIATIONS

ABC	=	ATP-binding cassette
ABCB1	=	ATP-binding cassette, subfamily B
ABCC2	=	ATP-binding cassette, subfamily C
ABCG2	=	ATP-binding cassette, subfamily G
ATP	=	adenosine triphosphate
BCRP	=	Breast cancer resistance protein
°C	=	Degree Celsius
Caco-2	=	human colon adenocarcinoma
Calcein-AM	=	Calcein Acetoxymethyl Ester
CDCF	=	5(6)-carboxy-2',7'-dichlorofluorescein
CDCFDA	=	diacetate ester of CDCF
DMEM	=	Dulbecco's Modified Eagle's Medium
DMSO	=	Dimethyl sulphoxide
e.g.	=	exempli gratia
et al.	=	et alibi
EDTA	=	ethylenediaminetetraacetic acid
FBS	=	fetal bovine serum
Fig	=	figure
HBSS	=	Hank s' balanced salt solution
HIV	=	Human immunodeficiency virus
IR	=	Infrared
MDR	=	Multidrug resistance
ml	=	millilitre

MRP2	=	Multidrug resistance-associated protein
NEAA	=	non-essential amino acid
PBS	=	Phosphate buffer solution
P-gp	=	Permeability glycoprotein
pH	=	Measure of the acidity or basicity of a solution
RN-C	=	rhinacanthin-C
rpm	=	round per minute
SEM	=	Standard error of mean
µl	=	microlitre
µM	=	micromolar
v/v	=	volume by volume

CHAPTER I
INTRODUCTION

Rhinacanthus nasutus Kurz. has been known in Thai traditional medicine for treatment of various diseases such as skin diseases, hepatitis, hypertension, and cancer (Siripong et al., 2006a). Rhinacanthin-C (Fig 1) is one of the bioactive naphthoquinones isolated from the leaves and roots of this plant. This compound demonstrated its potential therapeutic value as an anticancer agent with the cytotoxic, antiproliferative, antitumor, and pro-apoptotic activities (Kongkathip et al., 2004; Siripong et al., 2006b).

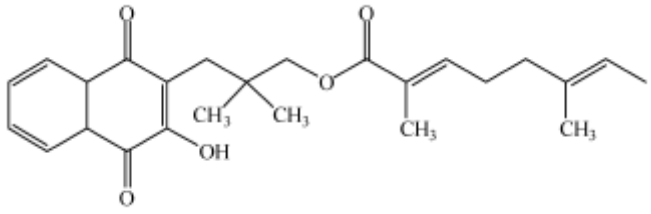


Figure 1. Chemical structure of rhinacanthin-C.

It has been accepted that multidrug resistance (MDR) is one of the problems to cause chemotherapeutic failure in cancer treatment. The principle MDR mechanism has been linked to the overexpression of drug efflux transporters in particular the ATP-binding cassette superfamily (ABC-transporters) in these cancer cells, leading to the "less-than-toxic" intracellular level of the cytotoxic drug. Consequently, these cells become resistant to chemotherapy. Examples of the ABC efflux transporters include the P-glycoprotein (P-gp, *ABCB1* gene product), the multidrug resistance-associated protein 2 (MRP2, *ABCC2* gene product), the breast cancer resistance protein (BCRP, *ABCG2* gene product) (Fletcher et al., 2010). In addition to the cancer cells, these efflux pumps are also found in various normal tissues such as epithelium or endothelium of intestine, liver, kidney and blood brain barrier. Depending on their localization, the ABC-transporters involve in the pharmacokinetic behavior of their chemical substrates either by limiting the penetration into the organ systems or by enhancing the elimination from the tissues (Glavinas et al., 2004; Leslie, Deeley, and Cole, 2005; Takano, Yumoto, and

Murakami, 2006).

The substrates of ABC transporters are diverse in chemical structure (Sucre, 2007). Several drug groups and plant-derived compounds including anticancer drugs (e.g., vinblastine, doxorubicin), NSAIDs (e.g., indomethacin heptyl ester), calcium channel blocker (e.g., verapamil), lignans (e.g., eudesmin, diphyllin, deoxyschizandrin), piperine, capsaisin, flavonoid (myricetin, robinetin) are known to interact with P-gp or MRP2 either as being substrates or inhibitors (Morris and Zhang 2006; Lim et al., 2007; Marchetti et al., 2007; Yoo et al., 2007b; Sharom, 2008; Zrieki, Farinotti, and Buyse, 2008). Prediction of chemicals being substrates or inhibitors of the ABC transporters from structure activity relationship is uncertain. The functional testing for the capability of the compounds to interact with the transporter is inevitably needed. Chemicals with highly hydrophobic, planar aromatic structure with one protonable nitrogen potentially interact with the ABC transporters (Seelig, 1998; Sucre, 2007). Considering that rhinacanthin-C is an aromatic compound with lipophilic property, it might bind to the ABC drug efflux pumps, and modulate the pump activity. Consequently, its therapeutic value in highly expressed MDR-cancer cells would be compromised.

Alteration of either function or expression of the drug efflux transporters can affect drug efficacy and safety in therapeutic use. Any compounds with modulating potential on drug efflux pumps may alter the therapeutic and adverse effects of another drug substrate. For example, simultaneous treatment of quinidine and digoxin for 3 weeks significantly increased plasma digoxin and prolonged its half-life which was due to the inhibitory effect of quinidine on P-gp function (Doherty, 1982; Fromm et al., 1999). Moreover, Saint John's wort was reported to significantly decrease bioavailability of P-gp drug substrates such as indinavir, cyclosporine A, and digoxin through enhancement of P-gp expression (Johne et al., 1999; Piscitelli et al., 2000; Ruschitzka et al., 2000; Perloff et al., 2001). A number of anticancer drugs such as doxorubicin, vincristine and cisplatin are known P-gp modulators and inducers (Veneroni et al., 1994; Demeule, Brossard, and Beliveau, 1999; Hinoshita et al., 2000). Thus, it would be interesting to determine whether rhinacanthin-C was able to modulate either function or expression of

the ABC drug efflux pumps.

Currently, there are no reports of the effects of rhinacanthin-C on the function and expression of the ABC efflux transporters in particular P-gp and MRP2. Hence, this study aimed to investigate whether rhinacanthin-C affected the function and expression of P-gp and MRP2 transporters, using the *in vitro* model of Caco-2 cells. This information would be useful for the prediction of drug interaction that may cause by rhinacanthin-C through the mechanism involving P-gp and/or MRP2. In addition to the direct effect on the P-gp function, the effect of rhinacanthin-C on the P-gp expression after prolonged treatment was also examined.

Objectives

The objectives of this study were:

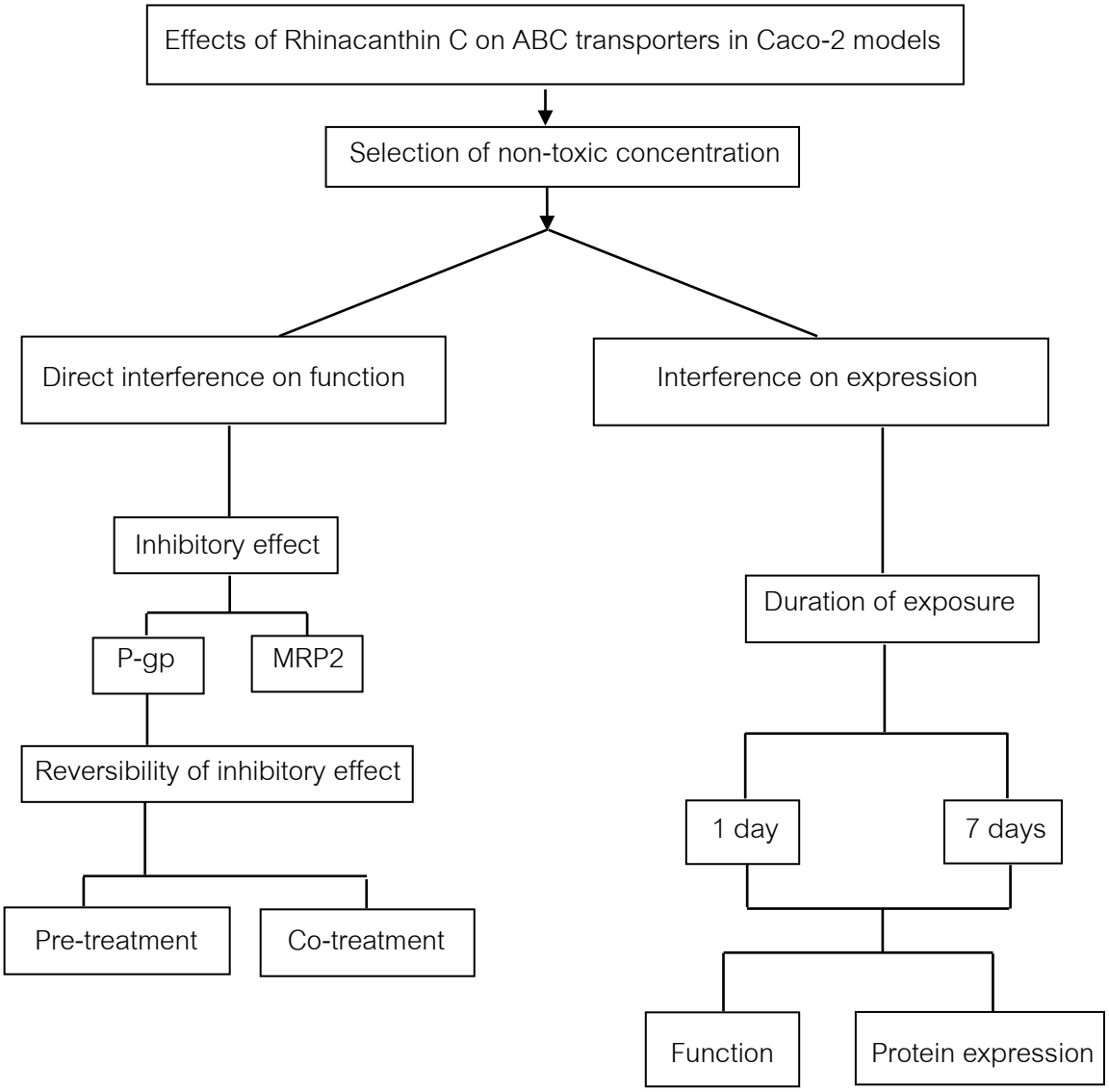
1. To examine the effect of rhinacanthin-C on function of P-gp and MRP2 transporters in Caco-2 cells

2. To determine the effect of rhinacanthin-C on expression of P-gp transporter in short and long term exposure

Scope of study

The Caco-2 cells are human colon adenocarcinoma cell line which can differentiate into enterocyte and express the ABC efflux transporters including P-gp and MRP2 (Sun et al., 2008). Under the specific condition, the Caco-2 cells cultured for 21 days express the appreciable levels of P-gp and MRP2 (Kamiyama et al., 2009). In this study, the effects of rhinacanthin-C on the function and expression of the specific drug efflux pumps in the Caco-2 monolayers were determined. In each experiment, the concentrations of rhinacanthin-C were non-cytotoxic and did not cause cell detachment. The functions of P-gp and MRP2 were determined in the Caco-2 monolayers cultured for 21 days with the uptake assay. In addition, the modulating effects of rhinacanthin-C on P-gp function were tested in various exposure conditions in order to suggest the reversibility of its action. Moreover, prolonged treatment the cells with rhinacanthin-C upto 7 days were also performed to determine its effect on the expression of P-gp.

Experimental design



CHAPTER II
LITERATURE REVIEW

The ATP-binding cassette transporters (ABC-transporters) are one of the defense mechanisms in the body to prevent chemical assaults. These efflux transporters are able to pump toxins or cytotoxic compounds out of the cells. Because the ABC transporters are found in the epithelium of intestine, liver, kidney and the endothelium of blood brain barrier, they have roles in restricting the penetration of chemicals into the tissue. Hence, the drug efflux pumps can determine the pharmacokinetic profiles of their substrates through influence on absorption, distribution and excretion (Glavinas et al., 2004; Leslie et al., 2005; Takano et al., 2006). In addition, the ABC transporters are highly expressed in the cancer cells, which may lead to multidrug resistance and therapeutic failure. Examples of the ABC efflux transporters include the P-glycoprotein (P-gp, *ABCB1* gene product), the multidrug resistance-associated protein 2 (MRP2, *ABCC2* gene product), the breast cancer resistance protein (BCRP, *ABCG2* gene product) (Fletcher et al., 2010). Two of the ABC transporters including P-glycoprotein (P-gp) and multidrug resistance-associated protein 2 (MRP2) were studied as the potential targets of rhinacanthin-C.

1. P-glycoprotein (P-gp)

1.1 Structure, tissue distribution and physiological function

P-gp is the efflux transporter in the *ABCB1* subfamily of ABC transporters. This transporter is a long chain of 1,300 amino acids consisting of two homologous of six transmembrane (TM) domains (Fig 2). The TM segments at 5, 6 and 11, 12 as shown in figure 2 are drug-binding domains. The two nucleotide binding domains are located in the linker region between two TM domains and at the C-terminal region (Haritova et al., 2006).

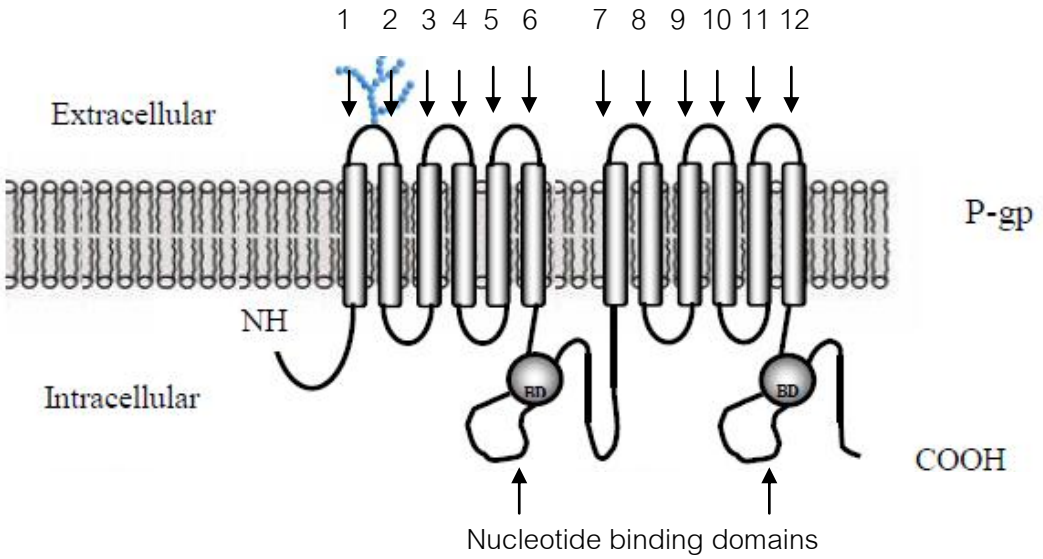


Figure 2. Topology of P-glycoprotein (P-gp) (Haritova et al., 2006).

P-gp is mostly expressed on the apical site of the epithelium tissue such as intestine, liver, kidney, lung, colon, placenta, and the endothelium of blood brain barrier (Leslie et al., 2005). The P-gp levels vary in each organ. For example, the numbers of P-gp found in colon and ileum are higher than those found in jejunum, duodenum and stomach (Takano et al., 2006). The main function of P-gp is to transport endogenous and exogenous compounds (its substrates) out of the cell. Hence, P-gp limits the absorption of compounds in the gastrointestinal tract, excretes the compounds out of the body through the kidney and liver. In addition, it also prevents the brain from exposure of toxic chemicals (Schinkel, 1997). The inhibition or induction of P-gp may lead to alteration of pharmacokinetic profiles of its substrates and contributes to the drug interaction problems (Endres et al., 2006). For example, quinidine increases the absorption of digoxin in the intestine and reduces the biliary and renal clearance of digoxin through inhibition of P-gp function in these organs. Furthermore, verapamil could increase the cyclosporine A level in the rat and human brains due to its inhibitory action on P-gp (Endres et al., 2006).

In addition, prolonged exposure of rifampin and St. John's Wort increased the expression of P-gp in the intestine, resulting in the absorption of digoxin in human (Greiner et al., 1999; Durr et al., 2000). The overexpression of P-gp after using P-gp

inducers contributes to the multidrug resistance in several drug groups such as anticancer, antidepressants, antiepileptic or anti-HIV agents, leading to the decrease of drug efficacy and safety (Sharom, 2008; Colabufo et al., 2010).

1.2 P-gp substrates, inhibitors and inducers

The compounds that affect P-gp activity can be divided into direct modulator of the function and modulator of protein expression. The compounds being transported by P-gp to extracellular membrane are classified as the P-gp substrate. The compounds that directly suppress P-gp activity are known as the P-gp inhibitor. Examples of the substrates and inhibitors of P-gp are listed in table 1 and 2 (Endres et al., 2006; Sharom, 2008). The compounds that can bind with P-gp are diverse in chemical structures (Sucre, 2007). Mostly, they are highly hydrophobic, planar aromatic structure with one protonable nitrogen, hydrogen bond interaction (Seelig, 1998; Sucre, 2007). Moreover, the expression of P-gp can be influenced by exposure to either inducers such as rifampin and St John's wort (SJW) or suppressors such as indomethacin heptyl ester and naproxen (Marchetti et al., 2007; Zrieki et al., 2008; Colabufo et al., 2010).

Table 1. Examples of drugs known as P-gp substrates and inhibitors (Endres et al., 2006; Sharom, 2008).

Drug group	Drug
Anticancer drugs	Vinblastine, vincristine, doxorubicin, paclitaxel, mitoxantrone, topotecan, etoposide
Cardioactive drugs	Verapamil, digoxin, quinidine
HIV protease inhibitors	Saquinavir, ritonavir, nelfinavir
Antifungals	Ketoconazole, itraconazole
Antihistamines	Fexofenadine, cetirizine
Antibiotics	Erythromycin, valinomycin, gramicidin D
Antiepileptics	Felbamate, topiramate
Immunosuppressant	Cyclosporine A, tacrolimus

Table 2. Examples of natural compounds known as P-gp substrates and inhibitors.

Natural compound	Compound	Reference
Flavonoids	Diosmin, naringin, quercetin, piperine, capsaicin	Morris and Zhang, 2006; Han, Tan, and Lim, 2006; Yoo et al., 2007a
Lignans	Deoxyschizandrin, diphyllin, eudesmin	Lim et al., 2007; Yoo et al., 2007b
Alkaloids	Voacamine, thaliblastine, cyclopamine, tomatidine	Chen, Ramachandran, and Krishan, 1993; Lavie et al., 2001; Meschini et al., 2005
Terpenoids	Glycyrrhetic acid, (R)-(+)-citronellal, ophiobolin A	Yoshida et al., 2006

1.3 Roles of P-gp in drug interaction

Interference on the function and expression of P-gp can affect pharmacokinetics of the drug substrates, leading to alteration in drug efficacy and safety (Marchetti et al., 2007). For example, the presence of talinolol (a P-gp inhibitor) increased the bioavailability of digoxin after oral administration (Westphal et al., 2000). It was demonstrated that ritonavir decreased the renal clearance of digoxin via inhibition of P-gp (Ding et al., 2004). Furthermore, quinidine was found to increase loperamide level in the brain and subsequently caused drug toxicity (Sadeque et al., 2000). The natural compounds are also reported to cause drug interaction through interference of P-gp activity. For instance, piperine, a major compound in black pepper, inhibited the activity of P-gp, leading to an increase in the plasma concentration of phenytoin and rifampin (Bhardwaj et al., 2002).

The effects on protein expression can lead to the changes in activity of P-gp and involve with the drug interaction issues. For example, rifampin (a P-gp inducer) reduced the bioavailability of fexofenadine (Hamman et al., 2001). It has been reported that St John's wort decreased the plasma level of cyclosporine A after prolong administration (Bauer et al., 2003). In addition, the increased P-gp expression has been linked to the drug resistance and therapeutic failure in chemotherapy (Fletcher et al., 2010). It has been found that drug resistant tumor cells had a higher number of P-gp which could protect them from cytotoxic drugs by reduction of intracellular drug concentration (Krishna and Mayer, 2000).

2. Multidrug resistance-associated protein 2 (MRP2)

2.1 Structure, tissue distribution and physiological function

Multidrug resistance-associated protein 2 (MRP2) belongs to the human *ABCC2* subfamily of ABC transporters. The MRP2 transporter is a long chain of 1,545 amino acids with three membrane-spanning domains (MSD) and two cytosolic nucleotide binding domains (Fig 3) (Haritova et al., 2006). The TM segments at 6, 9, 16 and 17 of this protein as shown in figure 3 are drug binding sites of MRP2 (Fardel et al., 2005).

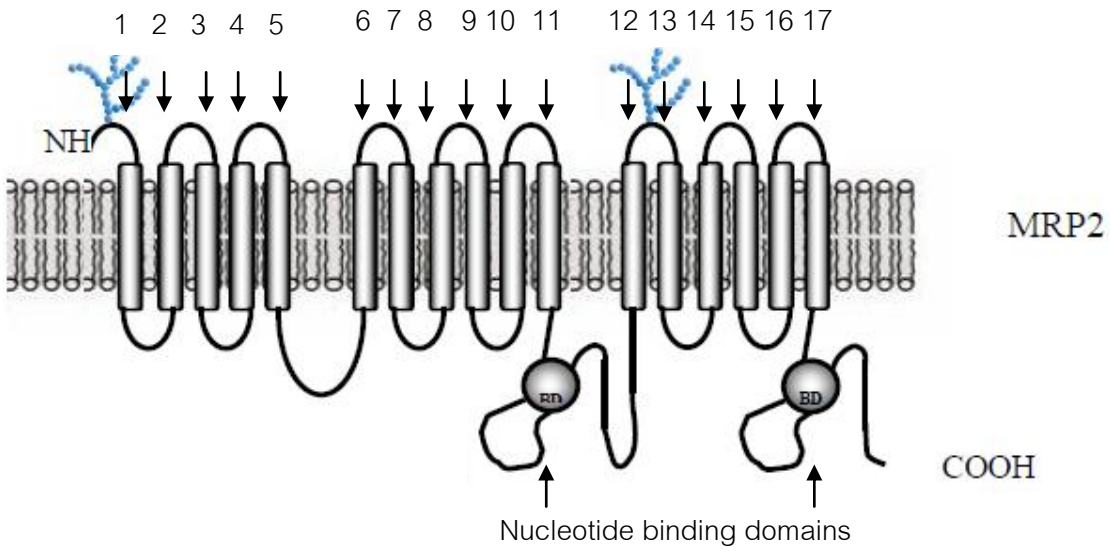


Figure 3. Topology of Multidrug resistant-associated protein 2 (MRP2) (Haritova et al., 2006).

The MRP2 expression is found mainly on the hepatocyte canalicular membrane and also on the apical membrane of renal proximal tubule cell and the intestine. In addition, MRP2 is also found in human placenta to excrete toxins and endogenous conjugates from the fetus. Conversely, the present of MRP2 is low in stomach, lung and blood-testis barrier (Jedlitschky, Hoffmann, and Kroemer, 2006). Similar to P-gp, the function of MRP2 is to limit penetration of drugs, toxins and carcinogens into the cells (Fardel et al., 2005).

2.2 MRP2 substrates, inhibitors and inducers

MRP2 substrates include lipophilic or cationic compounds (eg. vincristine, vinblastine) and conjugated xenobiotics with glucuronide, sulfate and glutathione (Jedlitschky et al., 2006). Examples of substrates and inhibitors of MRP2 are listed in Table 3 and 4. Like P-gp, the compounds that interact with MRP2 are diverse in chemical structures. In addition, certain compounds can interact with both P-gp and MRP2. For example, diltiazem and taurolithocholic acid could be transported via both P-gp and MRP2 (Matsson et al., 2009). It has been proposed that the specific binding sites of rat MRP2 contain two hydrophobic sites and the two electrostatically positive sites (Hirono et al., 2005). Moreover, the binding with MRP2 requires hydrogen bond acceptors (Coburger et al., 2010). It has been demonstrated that the biphenyl substituted herterocyclic compounds specific interacted with MRP2 (Xing, Hu, and Lai, 2009). An increase of torsion angles of substituted biphenyl ring increased specific binding of the compounds to MRP2 (Lai et al., 2007; Xing et al., 2009). In addition, the expression of MRP2 can be changed by exposure to either inducers such as chemical carcinogen 2-acetylaminofluorene (AAF), rifampin and nifedipine or suppressors such as nocodazole and trichostatin A (Fardel et al., 2005).

2.3 Roles of MRP2 in drug interaction

Inhibition of MRP2 can change the drug pharmacokinetics (Fardel et al., 2005). For example, it was reported that probenecid (an MRP2 inhibitor) increased the bioaviability and reduced the biliary excretion of SN-38 glucoronide (prodrug of irinotecan) (Horikawa et al., 2002). Furthermore, heavy metals, chemical carcinogen and drugs (rifampin, phenytoin) can upregulate the expression of MRP2, leading to alteration of drug efficacy and safety (Fardel et al., 2005). For example, exposure to arsenic for 2 days increased the expression of MRP2 in rat and human primary hepatocytes, leading to reduction of the efficacy of phenytoin (an MRP2 drug substrate) (Vernhet et al., 2001; Potschka, Fedrowitz, and Loscher, 2003). Moreover, the overexpression of MRP2 was related to cisplatin resistance in the treatment of human colorectal cancer (Hinoshita et al., 2000).

Table 3. Examples of drugs known as MRP2 substrates and inhibitors (Fardel et al., 2005; Endres et al., 2006; Jedlitschky et al., 2006).

MRP2 substrate	MRP2 inhibitor
Methotrexate	MK571
2,4-dinitrophenyl-S-glutathione	Furosemide
Vinblastine	Indomethacin
Vincristine	Probenecid
Etoposide	Cyclosporin A
Cisplatin	Glibenclamide
Irinotecan	Phenobarbital
Ampicillin	
Saquinavir	
Pravastatin	
Acetaminophen	

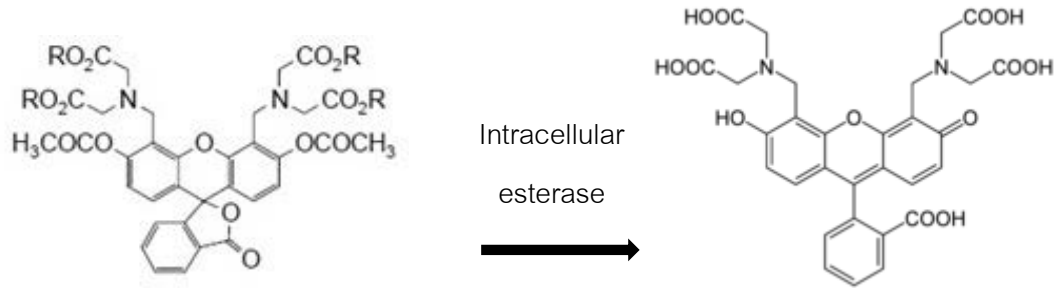
Table 4. Examples of mycotoxins, dietary and environmental toxins known as MRP2 substrates (Jedlitschky et al., 2006; Morris and Zhang, 2006; Yoshida et al., 2008).

Myricetin	Ochratoxin A	Arsenic triglutathione
Robinetin	Chelerythrine-SG	Mercury diglutathione
Glycyrrhetic acid	2-amino-1-methyl-6-	
Abietic acid	phenylimidazo[4, 5-b] pyrindine	
Curcumin	(PhIP)	

Evaluation of the function of efflux drug transporters

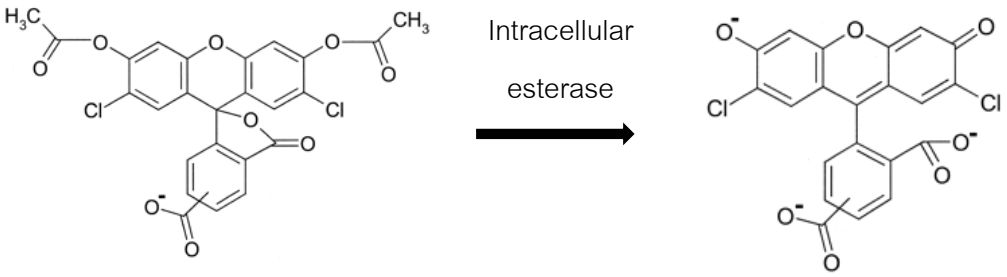
The transporters function test of *in vitro* systems used in this study is the uptake assay which detects the translocation of the amounts of efflux transporters substrates (Glavinas et al., 2004). The P-gp and MRP2 specific substrates are calcein-AM and CDCFDA, respectively (Essodaigui, Broxterman, and Garnier-Suillerot, 1998; Haimeur et al., 2004). Both of them are non-fluorescent and hydrophobic which can diffuse passively into the lipid bilayer. Then calcein-AM and CDCFDA are hydrolyzed to fluorescent products, calcein and CDCF by intracellular esterase enzyme, respectively (Fig 4). These fluorescent probes are hydrophilic which is unable to move out of cells passively. In the presence of efflux transporter inhibitors, the intracellular substrate retentions faster increase. The accumulations of fluorescents are measured by spectrofluorometer (Glavinas et al., 2004).

a)



Calcein-am (non-fluorescent) Calcein (fluorescent)

b)



CDCFDA (non-fluorescent) CDCF (fluorescent)

Figure 4. Specific substrates of efflux drug transporters and their fluorescent derivertives: a) P-gp substrate, b) MRP2 substrate.

Caco-2 cells

The Caco-2 cells have been commonly used as an *in vitro* model for paracellular, transcellular pathway of human intestinal drug absorption and intestinal metabolism (Meunier et al., 1995; Breemen and Li, 2005). The cells are a human colon adenocarcinoma cell line which is similar to the human intestinal mucosa (Meunier et al., 1995). During the culture, the cells differentiate to form tight junctions and express drug transporters and metabolic enzymes. The expressions of drug transporters on the apical side of Caco-2 cells are influx transporters and efflux pumps such as organic anion transporting polypeptides (OATP), organic cation transporters (OCT), P-gp, MRP2. The intestinal basolateral side also expresses the transporters increasing the drug absorption such as MRP3, MRP4, MRP6 and organic solute transporters (OST) (Fig 5) (Sun et al., 2008).

In order to obtain the appreciable level of P-gp and MRP2 expression, the Caco-2 cells have to be cultured in an optimal condition. Several factors affect the expression of these transporters in particularly P-gp such as passage numbers, age and trypsinization of the cells. For example, high passage numbers in Caco-2 cells are linked to the less expression of transporter proteins (Yu, Cook, and Sinko, 1997). In addition, it has been demonstrated that trypsinization before the cells reaching confluence (around 70% confluence) significantly increases the P-gp expression levels (Anderle et al., 1998). Moreover, the Caco-2 cells need 17 to 27 days after seeding to obtain the P-gp with fully function (Hosoya, Kim, and Lee, 1996).

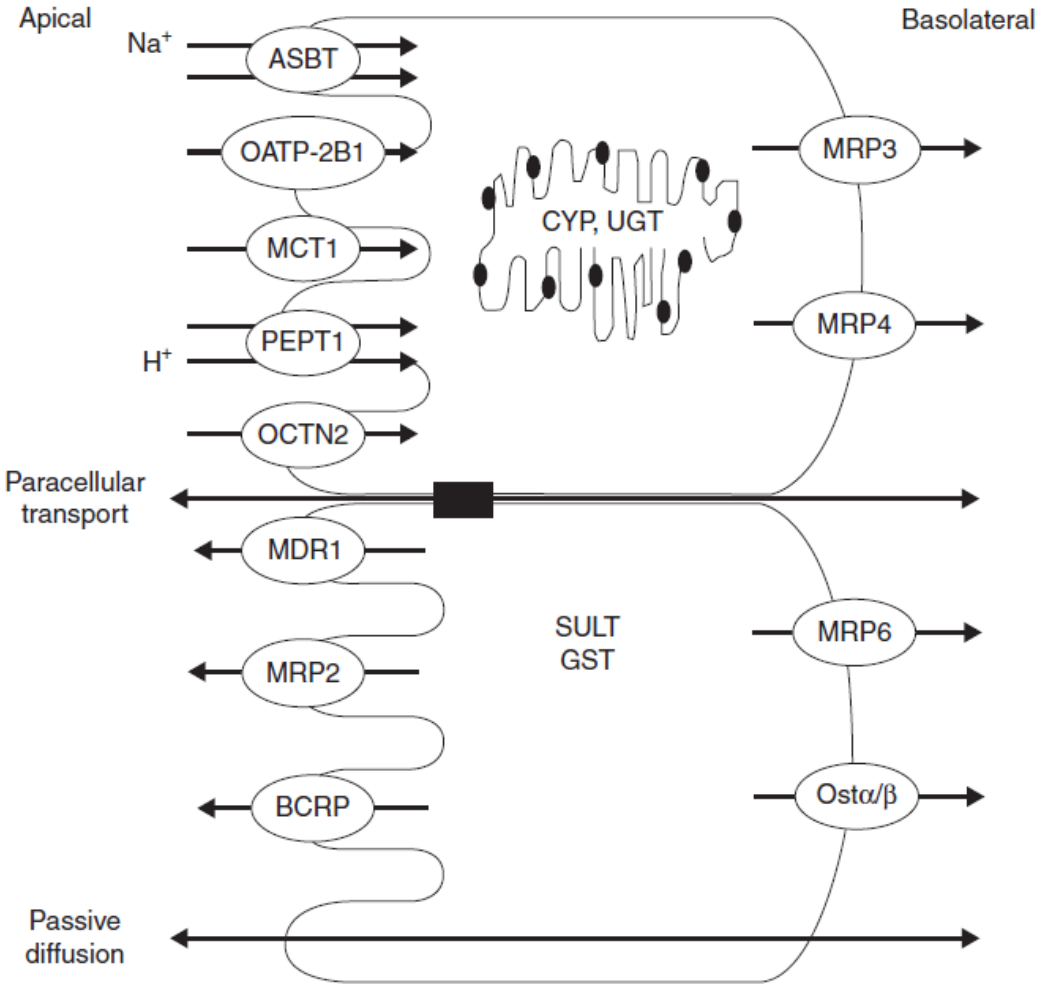


Figure 5. Drug transporters and metabolic enzymes in the Caco-2 model (Sun et al., 2008).

Rhinacanthus nasutus Kurz.

Figure 6. *Rhinacanthus nasutus* Kurz.

Rhinacanthus nasutus Kurz. (*R. nasutus* Kurz.) is one of the herbal plants in the Acanthaceae family. This plant is known as "Thong Pan Chang" in Thai name. It is widely found throughout Thailand (Siripong et al., 2006a). *R. nasutus* Kurz. is a small erect shrub with the height of 70 – 200 cm. Leaves are spreading opposite with white petal of flowers. The corolla tube is divided to one upper lip erect and three lower lips (Fig 6) (Panichayupakaranant, Chatkrapunt, and Supavita, 2006).

R. nasutus Kurz. has been used as Thai traditional medicine for treatment in various diseases such as eczema, pulmonary tuberculosis, hepatitis, diabetes, hypertension and cancers (Siripong et al., 2006a). Isolation of this plant provides in many naphthoquinones ester derivatives, as shown in figure 7, including rhinacanthins (A-D, G-Q), rhinacanthone, lignan, sterols and triterpenoid (Siripong et al., 2006a). Rhinacanthin-C is the main bioactive naphthoquinone compound substituted with aliphatic ester (Fig 1) (Siripong et al., 2006b). Because of its many therapeutic effects,

rhinacanthin-C is also synthesized chemically in many routes (Awai et al., 1995; Gotoh et al., 2004).

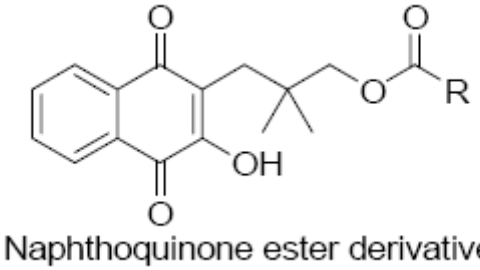


Figure 7. Chemical main structure of naphthoquinone ester.

Pharmacological action of rhinacanthin-C

1.) Antitumor activity

Rhinacanthin-C, N and Q isolated from the roots of *R. nasutus* Kurz. have a potential apoptotic effect on the growth of human cervical carcinoma cells line (HeLaS3). Induction of apoptosis in HeLaS3 cells may involve the activation of caspase-3 pathway (Siripong et al., 2006b). In agreement with Siriwatanametanon and colleagues (2010), it was reported that the petroleum ether and ethyl acetate extract of *R. nasutus* Kurz. leaves show the highest cytotoxicity against HeLa cells. In addition, the methanolic extract of *R. nasutus* Kurz. leaves exhibited more specifically cytotoxic in leukemia cells overexpressing *MDR1* gene (CEM/ADR5000) than normal leukemia cells (CCRF-CEM). Moreover, three naphthoquinone esters (C, N and Q) solubilized in liposome form showed the suppression of the tumor growth in tumor implanted mice (Meth-A sarcoma-bearing BALA/c mice) after injected intraperitoneally (i.p.) with 5 mg/kg/d for 10 days (Siripong et al., 2006c). However, the water extract of *R. nasutus* Kurz. roots had no preventive effect in the mouse colon carcinogenesis model induced by azoxymethane (AOM) with dextran sodium sulfate (DSS) after administered either in the initiation or promotion phase (Kupradinun et al., 2009).

2.) Antiproliferative activity

Rhinacanthin-C isolated form ethanol extract of *R. nasutus* Kurz. roots has antiproliferative activity against cells overexpressing MDR1 and several cancer cells such as human cervical carcinoma cell line (Hela), human prostate carcinoma cell line (PC-3 cells) and human bladder carcinoma cell line (T24). In addition, this compound could reduce cancer cells in mice cancer model (Sarcoma 180-bearing mice) after given orally rhinacanthin-C once a day for 14 days (Gotoh et al., 2004). In the liposomal form, rhinacanthin-C, -N and –Q also demonstrated significant inhibition of proliferation in HeLaS3 cells (Siripong et al., 2006c).

3.) Antimicrobial activity

The minimum inhibitory concentration of rhinacanthin-C in methanolic extract of *R. nasutus* Kurz. leaves showed antibacterial effect on *S. mutans* in 4 µg/ml and *S. epidermis*, *P. acnes* and *S. aureus* in 8-16 µg/ml (Puttarak, Charoonratana, and Panichayupakaranant, 2010). Its antimicrobial effect is only found in gram-positive bacteria (Sattar et al., 2004).

4.) Antiviral activity

Rhinacanthin-C and rhinacanthin-D exhibited antiviral activity against human cytomegalovirus (CMV) with EC_{50} values of 0.02 and 0.22 g/ml, respectively (Sendl et al., 1996).

5.) Anti-inflammatory activity

Three naphthoquinone derivatives consisting of rhinacanthin-C, -D, -N isolated from *R. nasutus* Kurz. leaves demonstrated the anti-inflammatory activity in murine macrophage-like RAW264.7 cells though inhibit nitric oxide and prostaglandin E_2 (PGE_2) production after lipopolysaccharide (LPS) induction. Rhinacanthin-C was the most potent inhibition on PGE_2 production among in three naphthoquinones. By the mechanism of transcription level, rhinacanthin-C also inhibited iNOS and COX-2 gene expression. However, three compounds had no effect on release of tumor necrosis factor (TNF-α) (Tewtrakul, Tansakul, and Panichayupakaranant, 2009).

6.) Antifungal activity

Aqueous ethanolic extract of *R. nasutus* Kurz. produced the antifungal activity in dose dependent against *Candida albicans* and *Trichophyton mentagorphytes* in the fungal strains tested (Sattar et al., 2004). Moreover, *R. nasutus* Kurz.leaf extract also inhibited various species of dermatophytes. It showed the fungistatic activity at concentration less than 13.6 mg/ml and fungicidal activity at concentration more than 13.6 mg/ml (Darah and Jain, 2001).

CHAPTER III
MATERIALS AND METHODS

1. Materials

1.1 Test compound

Rhinacanthin-C was kindly provided by Dr. Pongpun Siripong, National Cancer Institute, Bangkok, Thailand. The purification and identification were described in Siripong and colleagues (2006b) (Appendix A).

Rhinacanthin-C was dissolved in 99.9% dimethyl sulfoxide (DMSO) and then diluted to the desired concentrations with the final concentration of DMSO at less than 0.5% (v/v) in all experiments. At this concentration, DMSO had no cytotoxic effect on the Caco-2 cells (Violante et al., 2002).

1.2 Chemicals

Dulbecco's Modified Eagle's Medium (DMEM) and L-glutamine were purchased from Gibco Life Technologies (Grand Island, NY, USA).

Fetal bovine serum (FBS) was purchased from Biochrome AG (Germany).

Sodium bicarbonate, sodium chloride, potassium chloride and sodium phosphate monobasic were purchased from Ajax Finechem (Australia, New Zealand).

Dimethyl sulfoxide was purchased from Lab-scan Asia (Ireland).

The following chemicals were purchased from Sigma chemical company (St. Louis, MO, USA); trypan blue, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), trypsin, ethylenediaminetetraacetic acid (EDTA), penicillin, streptomycin, non-essential amino acid (NEAA), diacetate ester of 5(6)-carboxy-2', 7'-dichlorofluorescein (CDCFDA), Hank's balanced salt solution (HBSS), calcein acetoxymethyl ester (calcein-AM), verapamil, probenecid, indomethacin, triton X-100, sodium phosphate dibasic.

Doxorubicin hydrochloride was purchased from Calbiochem (Merck, Germany).

FITC mouse anti-Human P-glycoprotein antibody was purchased from BD biosciences (USA).

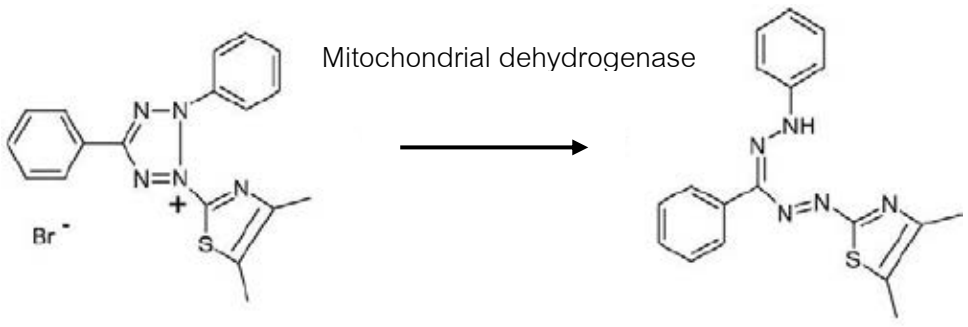
1.3 Cell Cultures

The Caco-2 cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). The Caco-2 cells (passage 48 to 72) were seeded at the density 6.7–8x10^3 cells/cm^2. The cells were maintained in complete culture medium (DMEM supplemented with 10% heat-inactivated FBS, 1% penicillin-streptomycin, 1% NEAA, and 2mM L-glutamine) at 37°C in a humidified atmosphere of 5% CO_2 (Appendix C). The culture medium was replaced every other day. The cells were subcultured at 70% confluency with trypsinization. In brief, after removing the medium from T75 culture flasks, the cells were washed with PBS and added 2 ml of 0.25% trypsin and 0.038% EDTA solution. The cells were incubated in CO_2 incubator at 37°C until the detachment was observed. Then, a 6 ml of complete medium was added to stop the trypsinization. Subsequently, the cells were collected by centrifugation at 1,500 rpm for 4 minutes.

2. Methods

2.1 Determination of cell viability

The cell viability was tested by an MTT assay. The principle of this assay is to measure the activity of mitochondrial dehydrogenase existing only in the viable cells. This enzyme catalyzes the reaction that converts a yellow water-soluble MTT to violet formazan crystals (Fig 8). Subsequently, the amount of formazan crystals can be measured spectrophotometrically at the absorbance of 570 nm (Mosmann, 1983; Gonzalez and Tarloff, 2001).



MTT (Yellow water-soluble tetrazolium dye)

Formazan products (Violet water-insoluble crystals)

Figure 8. Conversion of a yellow water-soluble MTT to formazan crystal by mitochondrial dehydrogenase.

In the experiment, the Caco-2 cells were cultured on a 24-well plate at the seeding density of 1.3×10^4 cells/cm^2 for 14 days. The complete medium was replaced every other day. Then, the cells were treated with various concentrations of rhinacanthin-C (upto 400 μM), and further cultured for another 1 or 7 days. After treatment, the medium containing rhinacanthin-C was changed every other day. At the end of the treatment period, the medium was replaced with a 350 μl of an MTT solution (0.4 mg/ml), and the cells were further incubated in the CO_2 incubator for another 4 hours. Then, the MTT solution was discarded carefully. A 350 μl of DMSO was added

to dissolve violet formazan crystals. The optical density (OD) of soluble formazan was measured at the absorbance of 570 nm by the microplate reader (Wallac 1420 VICTOR 3, PerkinElmer Inc., USA). The percentage of cell viability was calculated using the equation as shown below. The untreated groups were used as control at 100% cell viability.

$$\% \text{ cell viability} = \frac{(OD_{\text{treated (Average)}})}{OD_{\text{control(Average)}}} \times 100$$

2.2 Determination of P-gp and MRP2 activities

The functions of P-gp and MRP2 were determined via the substrate accumulation assay (or the uptake assay). The principle of this assay is to determine the differences of intracellular substrate accumulation in the presence and absence of inhibitors. The specific substrates and positive inhibitors of efflux drug transporters in this study were listed in Table 5.

Table 5. Specific substrates and inhibitors of efflux drug transporters

Efflux drug transporter	Substrate	Inhibitor	Reference
P-gp	Calcein-AM	Verapamil (100 μM)	Ampasavate et al., 2010
MRP2	CDCFDA	Indomethacin(500 μM), Probenecid (500 μM)	Fan et al., 2009; Siissalo et al., 2009

In the study, the cells were seeded in the 24-well plate at a density of $1.3x10^4$ cells/cm^2 and cultured for 21 days. During the maintenance, the medium was changed every other day. On the day of an experiment, the medium was discarded and the cells were washed twice with HBSS. Then, the cells were treated with rhinacanthin-C in HBSS at various concentrations (ranging from 0.625 to 100 μM) for 30 minutes at 37°C. Subsequently, the medium was replaced with the fresh HBSS containing rhinacanthin-C and a specific substrate of either P-gp (calcein-AM at 0.4 μM) or MRP2 (CDCFDA at 5 μM). The cultures were further incubated for another 30 minutes. Then, the solution was removed and the cells were washed gently three times with cold phosphate buffer solution (PBS) on ice to stop the activity of transporters. In order to determine the intracellular accumulation of the substrate, the cells were lysed with 0.1% Triton X-100, followed by centrifugation at 10,000 rpm for 4 minutes. The supernatant was transferred to 96 well black microplates. The fluorescent intensity of calcein or CDCF was determined with the microplate reader (Wallac 1420 VICTOR 3, PerkinElmer Inc., USA) at an excitation wavelength and an emission wavelength of 485nm and 535

nm, respectively. In addition, the inhibitory effect of rhinacanthin-C on P-gp function was determined in another two treatment conditions. One was the condition which the cells were treated with rhinacanthin-C for 30 minutes, followed by replacement with calcein-AM solution. The other was the condition that the cells were treated with only the mixture containing rhinacanthin-C and calcein-AM for 30 minutes. After treatment with rhinacanthin-C, the cells were lysed and measured for the intracellular accumulation of calcein as mentioned above.

2.3 Determination of P-gp expression

The expression of P-gp was determined with the use of immunofluorescent probe against P-gp expressed on the plasma membrane. In this experiment, the cells were seeded at the density of $1.3x10^4$ cells/cm^2 and grown in the 24-well plate for 14 days. Then, the cells were treated with rhinacanthin-C in complete medium at the non-cytotoxic concentrations for either 1 or 7 days. At the end of the treatment period, the activity of P-gp and its expression were determined. In this study, doxorubicin, a known P-gp inducer, was used as positive control. The activity of P-gp was carried out by the uptake assay in the presence and absence of verapamil as mentioned above.

In order to determine the expression of P-gp, the medium containing the test compound were discarded, and the cells were washed twice with PBS. Then, the cells underwent the trypsinization with trypsin-EDTA solution (200 µl) at 37°C to produce single cells. Subsequently, an 800 µl of PBS containing 2% FBS was added in each well to stop the trypsinization process. The cell suspensions were centrifuged at 4°C at 5,000 rpm for 4 minutes. Then, the supernatant was discarded and the cells were resuspened with fresh cold PBS (100 µl), followed by addition of a 20 µl of P-gp antibody conjugated with fluorescein isothiocyanate (FITC). After 30 minutes incubation period at 4°C, a 900 µl of PBS containing 2% FBS was added, and the cells were resuspended carefully. Subsequently, the cell suspension was centrifuged at 4°C at 5,000 rpm for 4 minutes. The precipitated cells were collected and fixed with a 500 µl of 1% paraformaldehyde in PBS. Afterwards, the FITC conjugated with P-gp antibody on the cell surface was analyzed by a flow cytometer (BD FACSCalibur, BD Biosciences, USA). The fluorescent intensity was measured by counting 10,000 events at an excitation wavelength of 488 nm and an emission wavelength of 535 nm.

2.4 Data analysis

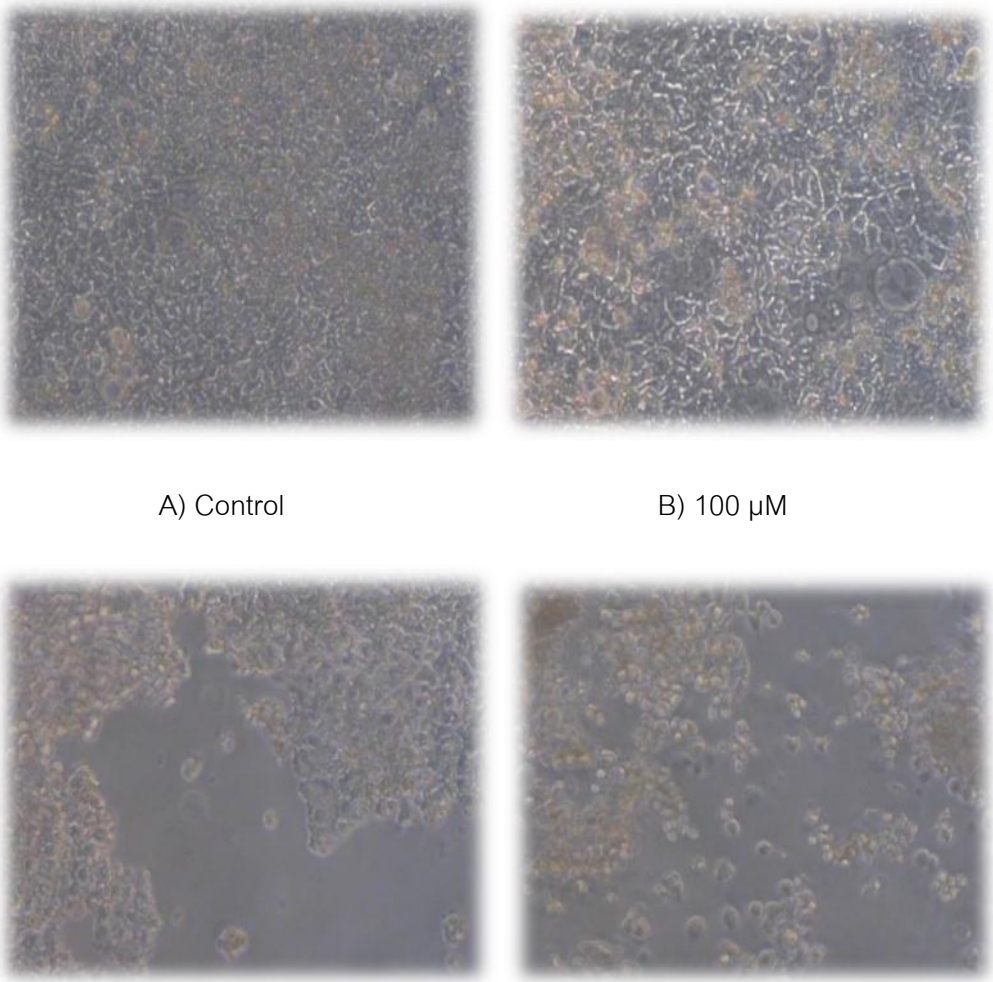
The results were calculated and expressed as the mean ± SEM obtained from at least three separated experiments. Data were presented as the percentage in relative to those of the control group in each experiment (100%). Statistical analysis was tested either by unpaired T-test or one-way analysis of variance (ANOVA) followed by post-hoc Scheffe's analysis, where appropriate. Significant value was considered at $P < 0.05$.

CHAPTER IV
RESULTS

1. The effects of rhinacanthin-C on cell viability

After 1 day exposure to rhinacanthin-C at the concentration of 200 μM, the cells were detached from monolayer and floated in medium, as shown in figure 9. The detachment of the cells increased when the concentration of rhinacanthin-C increased to 400 μM. At the concentration up to 100 μM, rhinacanthin-C did not cause cell detachment after 1 day exposure.

The concentrations of rhinacanthin-C (ranging from 25-100 μM) had no effect on the viability of the Caco-2 cells after treatment for 1 day, as measured by the MTT assay (Fig 10). However, the cytotoxic effect of this compound increased when the treatment period was extended to 7days. The concentration of rhinacanthin-C had to be reduced by at least 150 folds in order to maintain cell survival. At the 7 day-treatment period, the non-cytotoxic concentration of rhinacanthin-C in this study was less than 0.625 μM (Fig 11).



A) Control B) 100 μM

C) 200 μM D) 400 μM

Figure 9. The Caco-2 monolayers (passage 66) after treatment with rhinacanthin-C for 1 day. The concentrations of rhinacanthin-C were at A) 0 μM (control group), B) 100 μM, C) 200 μM, and D) 400 μM. The pictures were taken under an inverted microscope (Axiovert 135, Zeiss, Germany) at the magnification of x100.

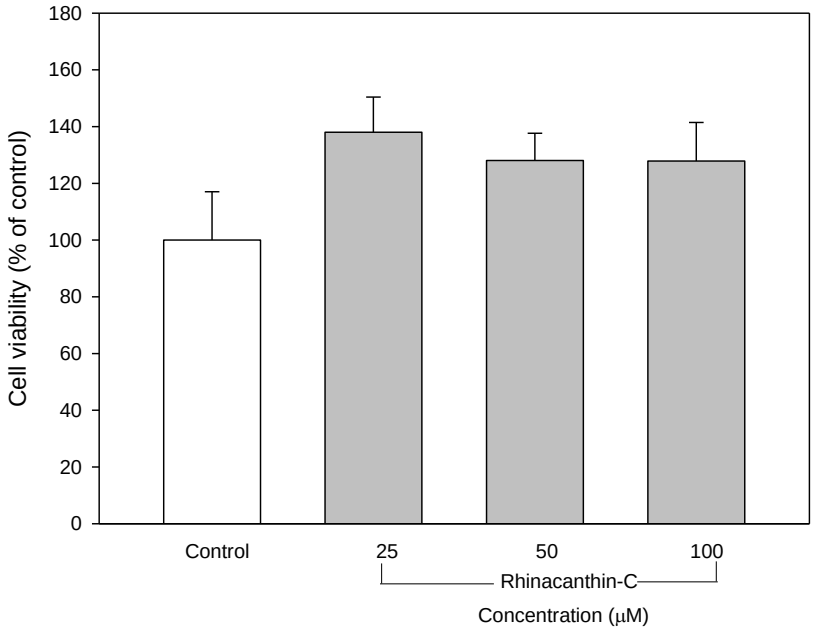


Figure 10. The viability of the Caco-2 cells after treatment with rhinacanthin-C for 1 day. The cell viability was determined by the MTT assay and expressed as the percentage of control (untreated group). Data represented the mean ± SEM (n=3). * $P < 0.05$ *vs* control.

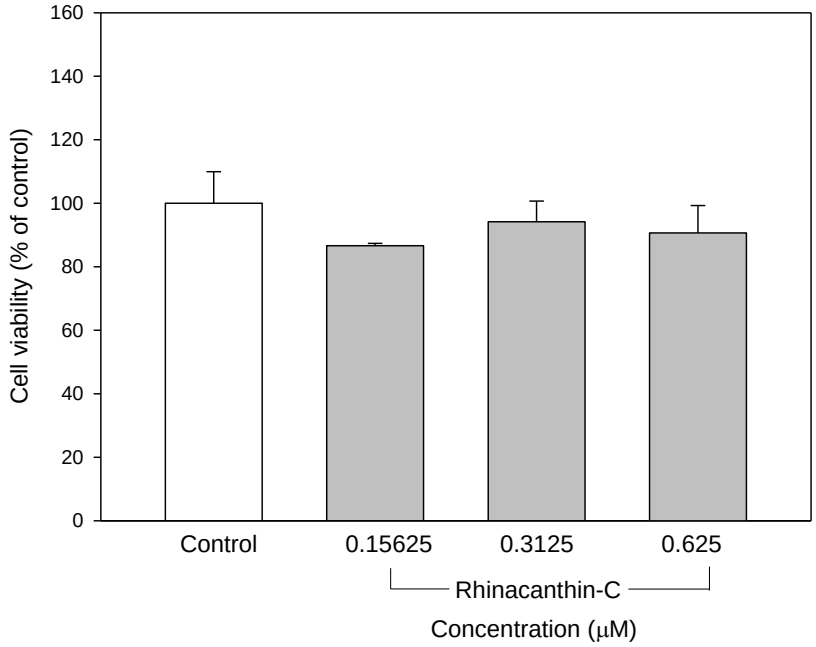


Figure 11. The viability of the Caco-2 cells after treatment with rhinacanthin-C for 7 days. The cell viability was determined by the MTT assay and expressed as the percentage of control (untreated group). Data represented the mean ± SEM (n=3-4). * $P < 0.05$ *vs* control.

2. The effects of rhinacanthin-C on P-gp and MRP2 activities

In this study, the presence of 100 μM verapamil (a known P-gp inhibitor) increased intracellular accumulation of calcein by 3.23 folds in the 21 day-cultured Caco-2 cells (Fig 12). In addition, the presence of probenecid and indomethacin at 500 μM (two known MRP2 inhibitors) increased intracellular CDCF retention by 2.11 and 2.66 folds, respectively (Fig 13). These results suggested that the Caco-2 cells after cultured for 21 days contained the active P-gp and MRP2 at the appreciable level. Moreover, rhinacanthin-C had no influence on the intracellular fluorescence intensity of calcein in the uptake assay (Appendix D).

The effect of rhinacanthin-C on P-gp function was shown in figure 14. Rhinacanthin-C was able to increase the intracellular calcein retention, suggesting its inhibitory action against P-gp activity. In addition, rhinacanthin-C significantly elicited its inhibitory effect against P-gp at the concentration of 2.5 μM, as evidenced by an increase in intracellular calcein retention by approximately 1.74 folds. The inhibitory effects of rhinacanthin-C increased in the concentration-dependent manner and reached the saturated maximal level at 100 μM. The intracellular calcein increased by approximately 3.2 folds, which was quite comparable to the action of verapamil (100 μM) (Fig 14).

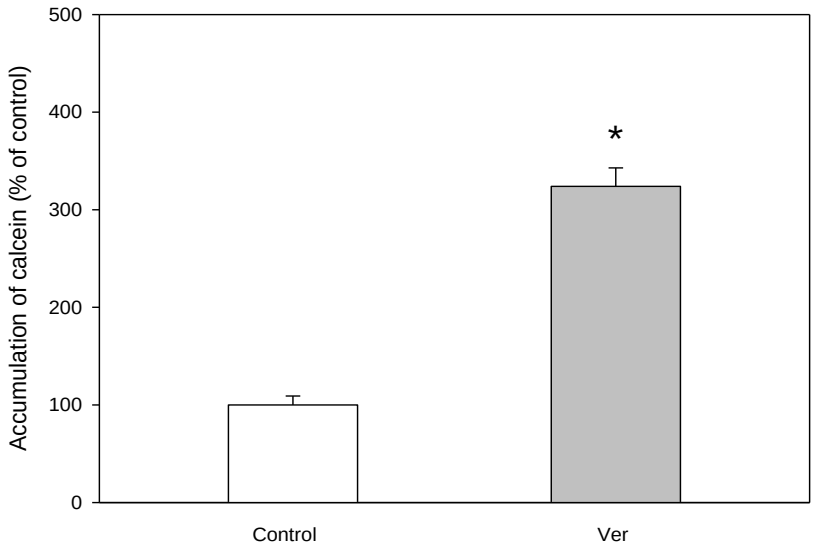


Figure 12. Effect of verapamil on P-gp function in the Caco-2 cells. In this experiment, verapamil (Ver), a known P-gp inhibitor, at the concentration of 100 μM was used as the positive control group. The calcein accumulation was expressed as the percentage of control. Values represented the mean ± SEM (n=3-6). * $P < 0.05$ *vs* control.

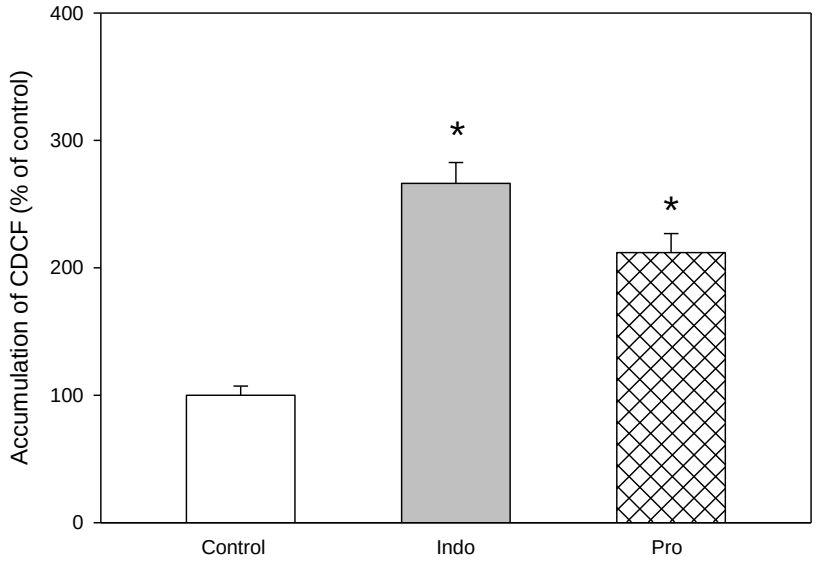


Figure 13. Effects of indomethacin and probenecid on MRP2 function in the Caco-2 cells. In this experiment, two MRP2 inhibitors, indomethacin (Indo) and probenecid (Pro), at the concentration of 500 μM was used as the positive control groups. The CDCF accumulation was expressed as the percentage of control. Values represented the mean ± SEM (n=3-5). * $P < 0.05$ *vs* control.

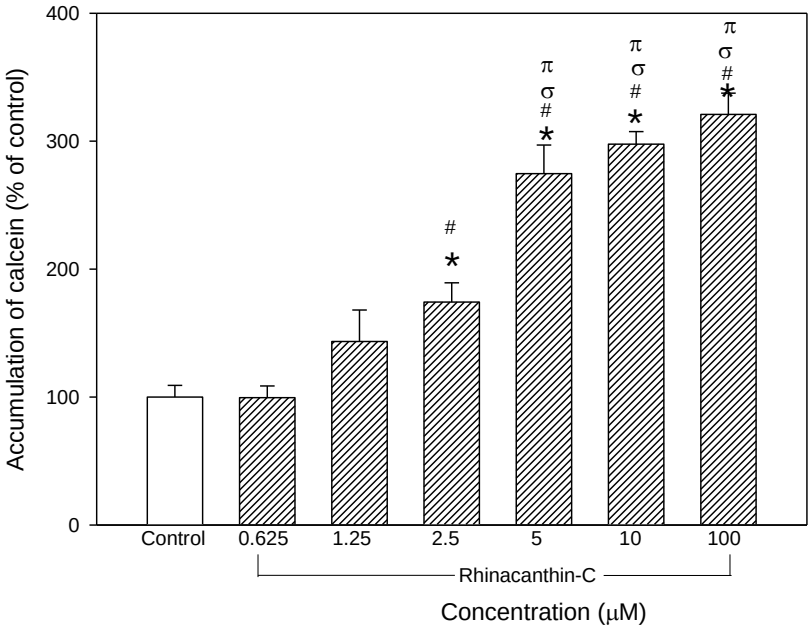


Figure 14. Effect of rhinacanthin-C on P-gp function in the Caco-2 cells. The calcein accumulation was expressed as the percentage of control. Values represented the mean ± SEM (n=3-6). * $P < 0.05$ *vs* control. $^{\#}$ $P < 0.05$ *vs* 0.625 μM. $^{\sigma}$ $P < 0.05$ *vs* 1.25 μM. $^{\pi}$ $P < 0.05$ *vs* 2.5 μM.

The rhinacanthin-C-mediated inhibition of P-gp activity was further investigated for its reversibility. The effects of rhinacanthin-C on intracellular calcein retention were determined in another two conditions as described in Materials and Methods. In these experiments, verapamil, a known reversible inhibitor of P-gp function, was also used as the positive control group. As shown in figure 15, rhinacanthin-C elicited its maximal inhibitory action against P-gp in the condition where the pretreatment and cotreatment with substrate was performed. Under this condition, rhinacanthin-C was able to increase an accumulation of intracellular calcein by approximately 4.34 folds. The withdrawal of rhinacanthin-C during the pretreatment period reduced calcein retention within cells from 4.34 folds to 2.88 folds (Fig 15). Interestingly, the inhibition against P-gp activity was not observed when rhinacanthin-C was present only in the pretreatment period. As shown in figure 15, the effects of rhinacanthin-C on P-gp activity in each assay condition were similar to those observed with the use of verapamil as the positive inhibitor.

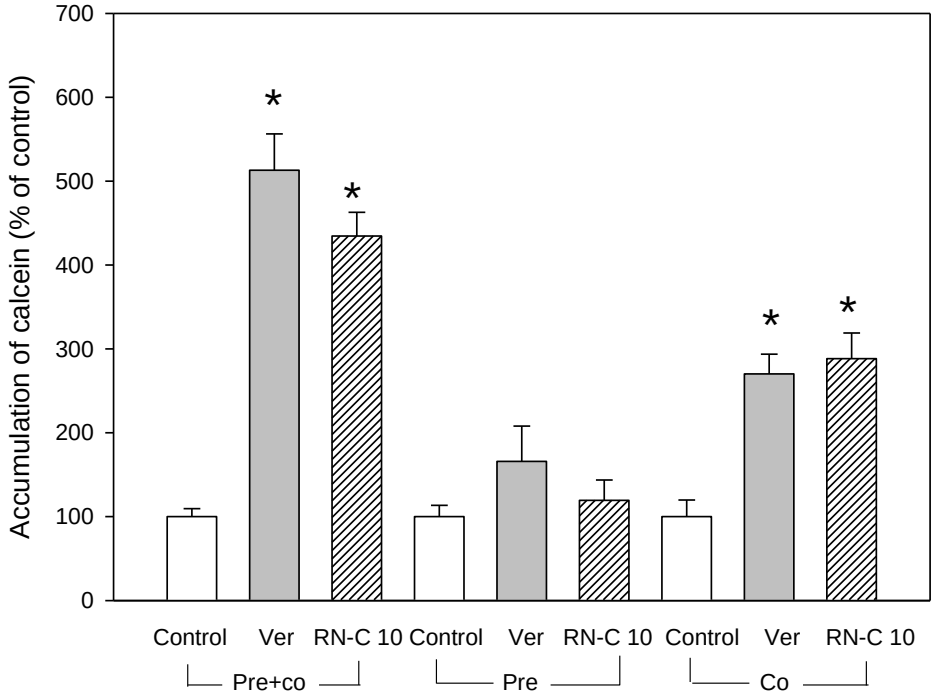


Figure 15. Effect of rhinacanthin-C at 10 μM on P-gp function in the Caco-2 cells under different conditions: pre-and co-treatment (Pre+co), pre-treatment (Pre) and co-treatment (Co) as described in Materials and Methods. In this experiment, verapamil (Ver) at the concentration of 100 μM was used as the positive control group. The calcein accumulation was expressed as the percentage of control. Values represented the mean ± SEM (n=3). * $P < 0.05$ *vs* control.

The effect of rhinacanthin-C on MRP2 function was shown in figure 16. Rhinacanthin-C at the concentration up to 100 μM was able to increase the accumulation of CDCF in the Caco-2 cells. The maximum inhibitory effect of this compound against MRP2 activity was approximately 1.79 folds, which was less than the effect of probenecid and indomethacin (Fig 16).

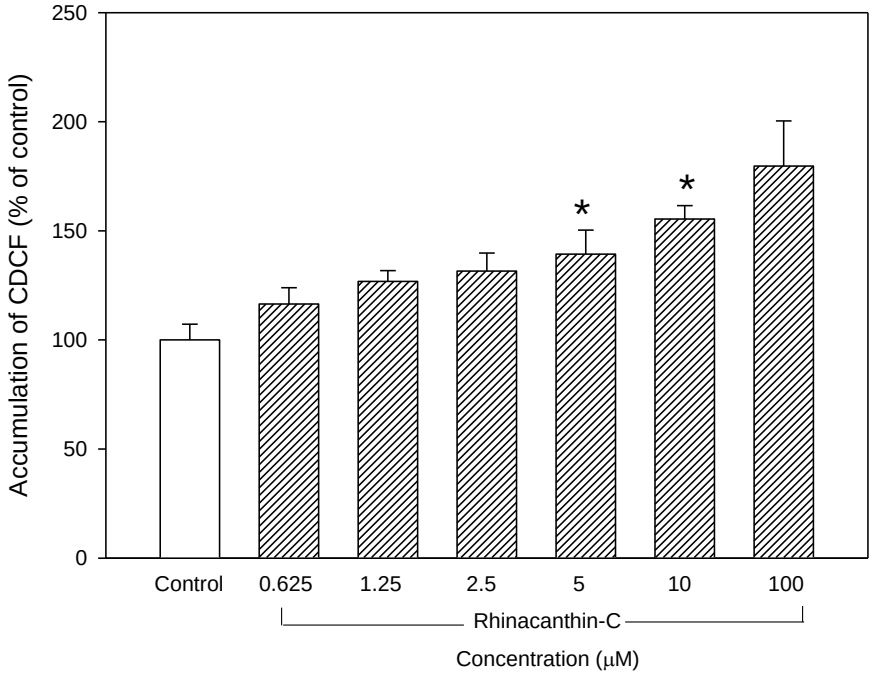


Figure 16. Effect of rhinacanthin-C on MRP2 function in the Caco-2 cells. Most of the values represented the mean ± SEM (n=3-5). The data in rhinacanthin-C 100 μM group were obtained from 2 separated experiments (n=2). * $P < 0.05$ *vs* control.

3. The effect of rhinacanthin-C on P-gp expression

3.1 Protein expression

In this study, the effects of rhinacanthin-C on the expression of P-gp after either 1 day or 7 days treatment were determined with the use of selective FITC-labeled P-gp antibody. The antibody specifically bound to membrane P-gp, subsequently the FITC intensity was quantified by flow cytometry. As shown in figure 17, the FITC labeled Caco-2 cells showed an increase of the fluorescence intensity in comparison with the cells without FITC labeling. This result suggested that the Caco-2 cells at the age of 21 days expressed P-gp on the plasma membrane. In this experiment, doxorubicin, a known P-gp inducer, was used as the positive control. All of the concentrations used in this experiment were non-cytotoxic (Appendix E). Treatment the cells on day 14 after seeding with doxorubicin for 1 day increased the extent of P-gp expression as compared with the untreated group (control) (Fig 18). Increase of doxorubicin concentration from 3 μM to 10 μM resulted in a significant increase of fluorescent intensity of FITC-labeled cells, suggesting a higher number of P-gp levels in the 10 μM treated group. The similar profile was also observed in the cells treated with doxorubicin at the concentration of 1 and 3 μM for 7 days (Fig 19). The number of expressed P-gp in the 3 μM-treated group was higher than those in the 1 μM-treated group. The result indicated that the effect of doxorubicin on the increased P-gp expression was concentration dependent. Furthermore, the inductive effect of doxorubicin on P-gp expression was also attributed to the exposure period. As shown in figure 20, the expression levels of P-gp on day 15 and day 21 after seeding were not comparable. Treatment of the cells with doxorubicin (3 μM) for 1 day increased the expression of P-gp significantly. The prolonged treatment of 7 days period resulted in a further increase of the P-gp levels, suggesting the time-dependent effect of doxorubicin exposure (Fig 20, 21).

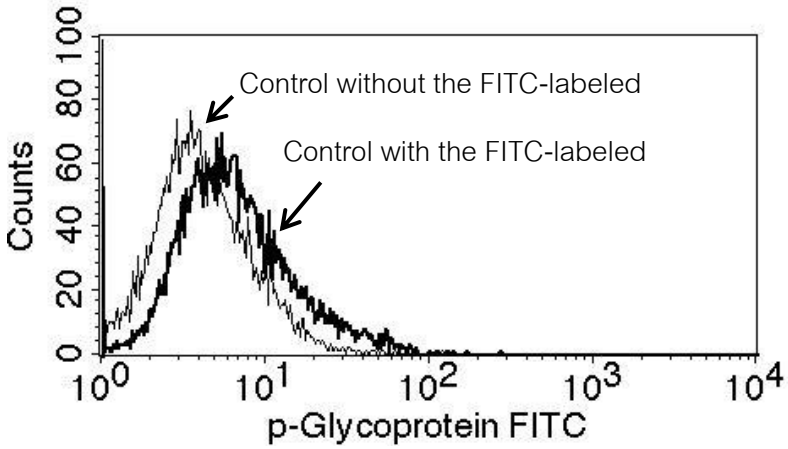


Figure 17. Flow cytometric analysis of P-gp expression in the Caco-2 cells cultured for 21 days. The data was expressed as the relative fluorescence intensity. Control indicated the cells that labeled with the FITC-labeled P-gp antibody and without the FITC-labeled.

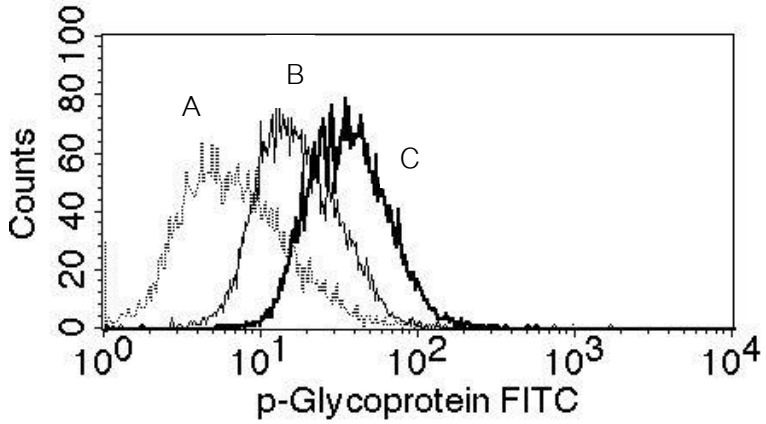


Figure 18. Flow cytometric analysis of P-gp expression in the Caco-2 cells treated with doxorubicin for 1 day. The concentrations of doxorubicin were at A) 0 μM (control group), B) 3 μM, and C) 10 μM. The data were expressed as the relative fluorescence intensity.

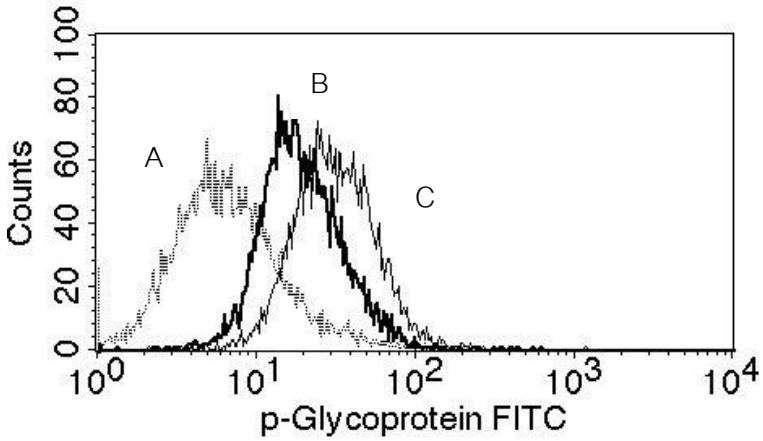


Figure 19. Flow cytometric analysis of P-gp expression in the Caco-2 cells treated with doxorubicin for 7 days. The concentrations of doxorubicin were at A) 0 μM (control group), B) 1 μM, and C) 3 μM. The data were expressed as the relative fluorescence intensity.

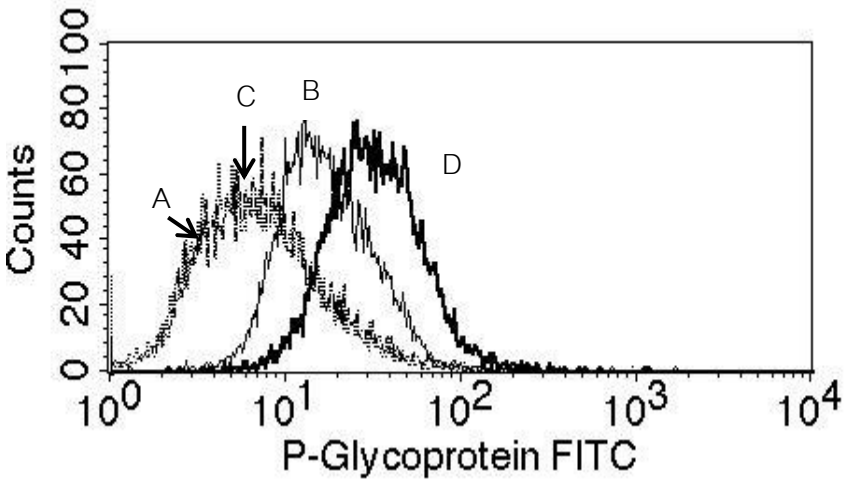


Figure 20. Flow cytometric analysis of P-gp expression in the Caco-2 cells after 1 day and 7 days exposure to doxorubicin at 3 μM. The treatment included A) 0 μM for 1 day (control group), B) 3 μM for 1 day, C) 0 μM for 7 days (control group), and D) 3 μM for 7 days. The data were expressed as the relative fluorescence intensity.

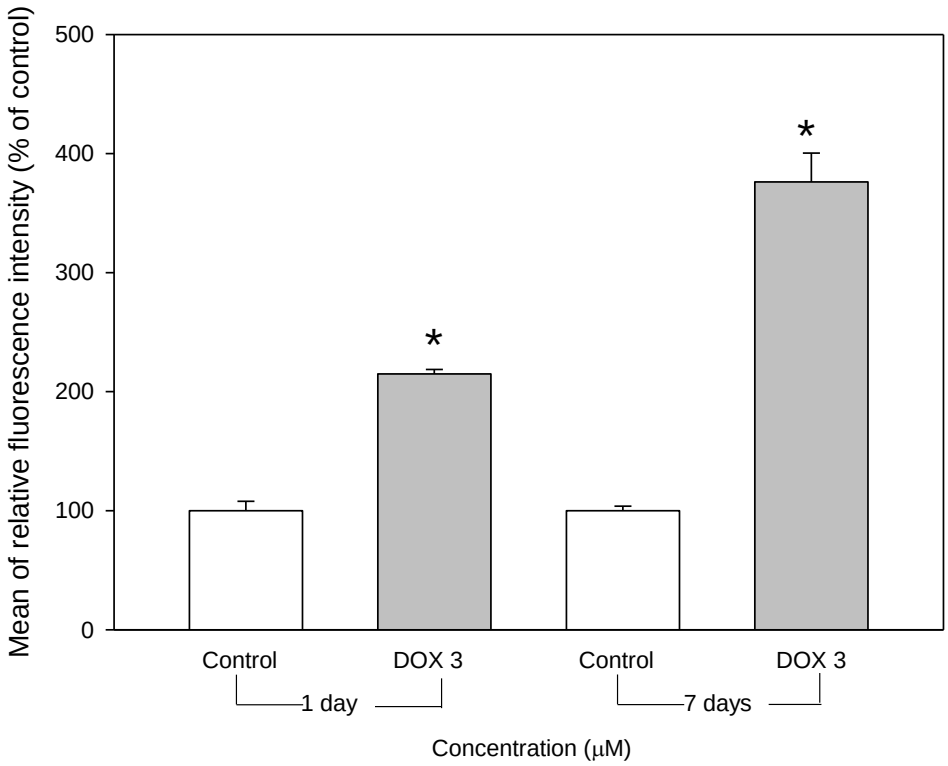


Figure 21. Effect of doxorubicin on P-gp expression in the Caco-2 cells after 1 day and 7 days treatment. The data were expressed as the percentage of the control group (untreated groups) at equivalent cultured age. Values represented the mean ± SEM (n=3-6). * $P < 0.05$ *vs* control.

The 14 day cultured Caco-2 cells treated with rhinacanthin-C (100 μM) for 1 day expressed the higher P-gp levels than the control group did. The number of P-gp increased gradually when the concentration of rhinacanthin-C increased (Fig 22). The maximum level of P-gp expression induced by rhinacanthin-C was up to approximately 1.74 folds of the untreated group (Fig 23). Nevertheless, its maximum level of expression was significant lower than that induced by doxorubicin (3 μM and 10 μM). As shown in figure 23, doxorubicin at the concentration of 3 μM and 10 μM caused an induction of P-gp expression by 2.15 and 4.2 folds, respectively.

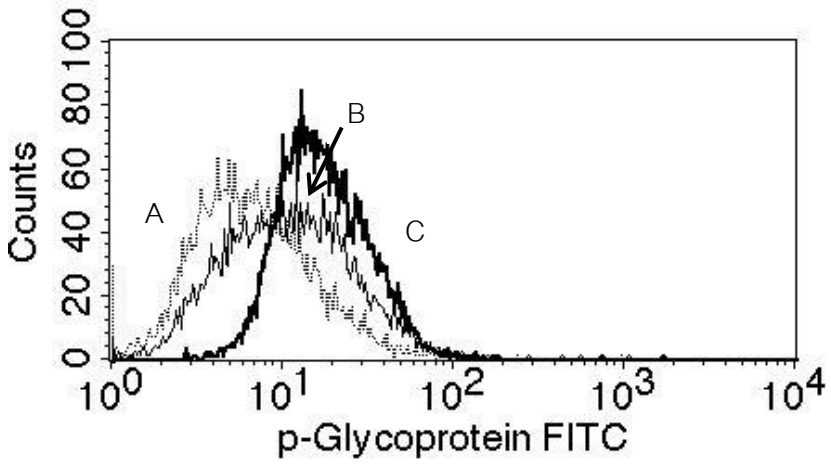


Figure 22. Flow cytometric analysis of P-gp expression in the Caco-2 cells after treatment with rhinacanthin-C and doxorubicin for 1 day. A) untreated cells (control), B) rhinacanthin-C 100 μM, and C) doxorubicin 3 μM. The data were expressed as the relative fluorescence intensity.

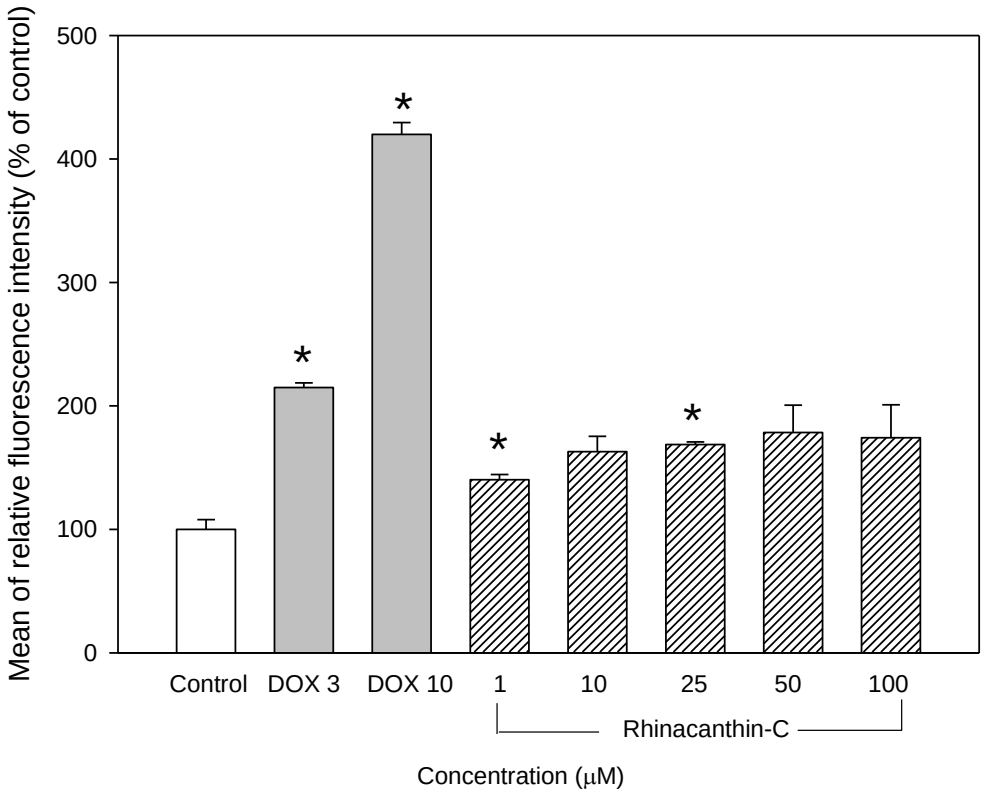


Figure 23. Effect of rhinacanthin-C on P-gp expression in the Caco-2 cells after 1 day treatment. Doxorubicin (DOX) (3 and 10 μM) was used as the positive control groups. The data were expressed as the percentage of control. Values represented the mean ± SEM (n=3-6). * $P < 0.05$ *vs* control.

Treatment the cells with rhinacanthin-C at the non-cytotoxic concentration of 0.156-0.625 μM for 7 days had no effect on the P-gp levels as compared to the untreated control group (Fig 24, 25). These results demonstrated that rhinacanthin-C at the low concentration (less than 0.625 μM) had no effect on P-gp expression after prolong exposure.

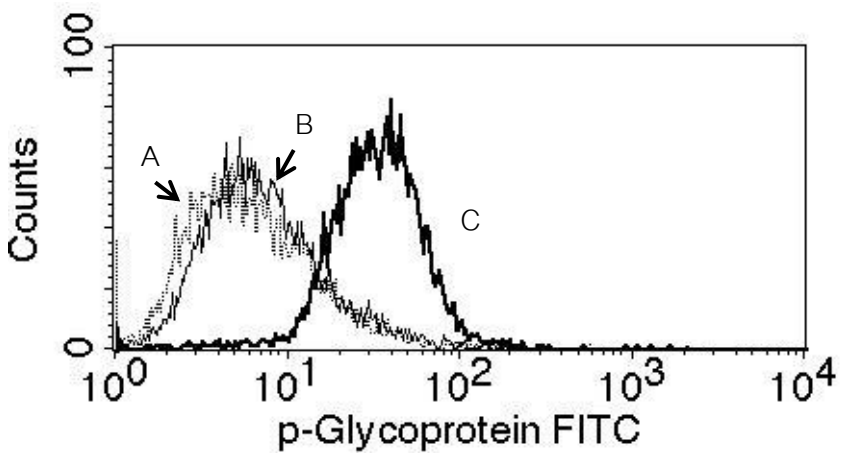


Figure 24. Flow cytometric analysis of P-gp expression in the Caco-2 cells treated with either rhinacanthin-C or doxorubicin for 7 days. A) untreated cells (control), B) rhinacanthin-C 0.625 μM, and C) doxorubicin 3 μM. The data were expressed as the relative fluorescence intensity.

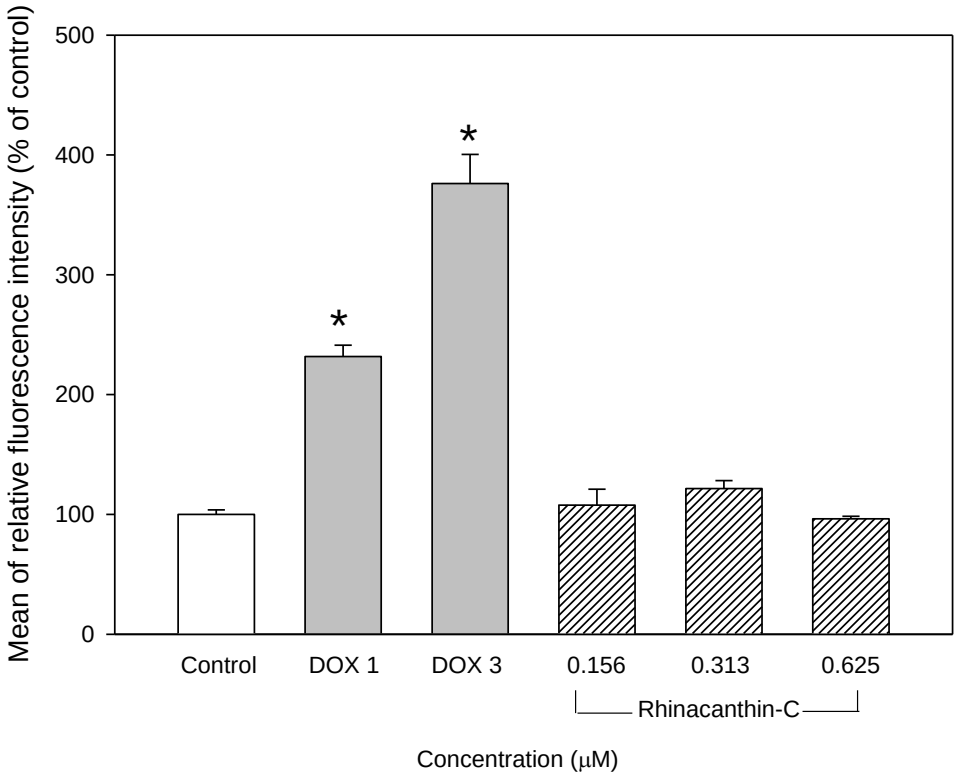


Figure 25. Effect of rhinacanthin-C on P-gp expression in the Caco-2 cells after 7 days treatment. In this experiment, doxorubicin (DOX) (1 and 3 μM) was used as the positive control groups. The data were expressed as the percentage of control. Values represented the mean ± SEM (n=3). * $P < 0.05$ *vs* control.

3.2 P-gp activity

In addition to the expression of P-gp, the activity of P-gp was also assessed after the prolonged treatment with doxorubicin for 1 day and 7 days. The activity was demonstrated as the fold increase of intracellular calcein accumulation in the presence and absence of verapamil (Fig 26). The activities of P-gp in the cells treated with doxorubicin (3 and 10 μM) for 1 day were not significantly different from those of the untreated cells (Table 6). The prolonged treatment of doxorubicin (3 μM) for 7 days was able to slightly increase the intracellular calcein retention by 3.39 folds (Table 6). Treatment the cells with rhinacanthin-C for either 1 or 7 days had no effect on the activity of P-gp as measured by calcein accumulation assay (Fig 27, 28, Table 7, 8). As shown in the table 7 and 8, the fold increases of P-gp function in the cells treated with rhinacanthin-C at the non-cytotoxic concentrations (1-100 μM for 1 day treatment and 0.156-0.625 μM for 7 days treatment) were not different from those of the untreated control groups. It was likely that rhinacanthin-C was unable to upregulate the function of P-gp after exposure to 1 day and 7 days.

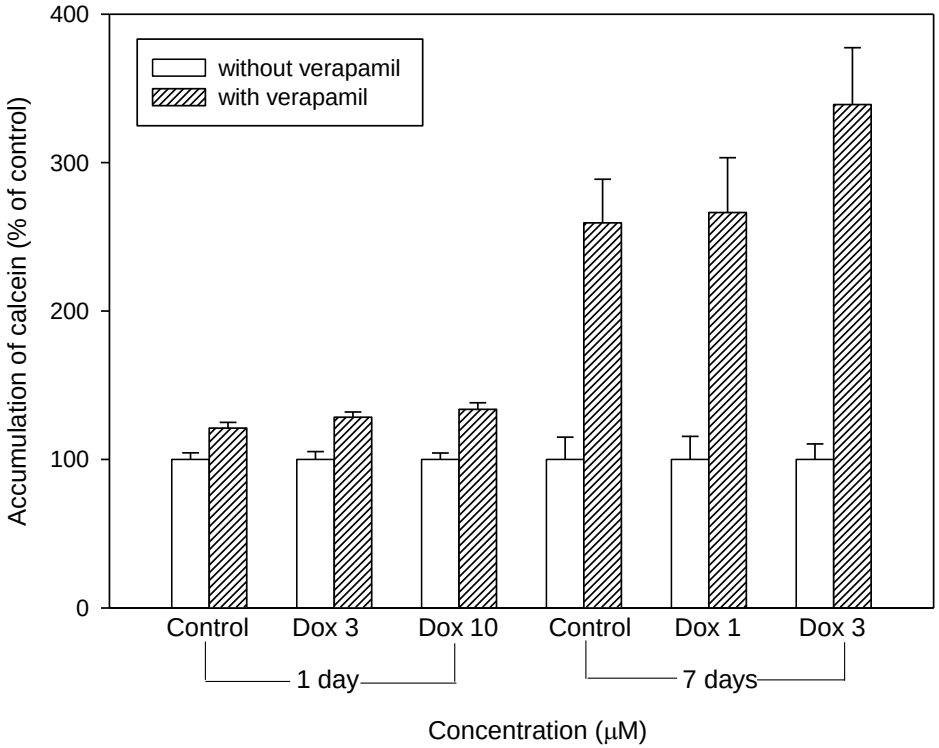


Figure 26. Effect of doxorubicin on P-gp function in the Caco-2 cells after 1 day and 7 days treatment. The intracellular calcein accumulation in the presence of verapamil was expressed as the percentage of those in the absence of verapamil at equivalent cultured age. Values represented the mean ± SEM (n=3-6).

Table 6. The folds increase in intracellular calcein retention in the presence and absence of verapamil after treatment with doxorubicin. The cells were evaluated for P-gp function after treatment with doxorubicin for 1 day and 7 days.

Groups	Fold of P-gp function *	
	1 day treatment	7 days treatment
Untreated control group	1.21	2.59
Doxorubicin 1 μM	-	2.66
Doxorubicin 3 μM	1.28	3.39
Doxorubicin 10 μM	1.34	-
* calculated from intracellular calcein in the presence of verapamil/ those in the absence of verapamil		

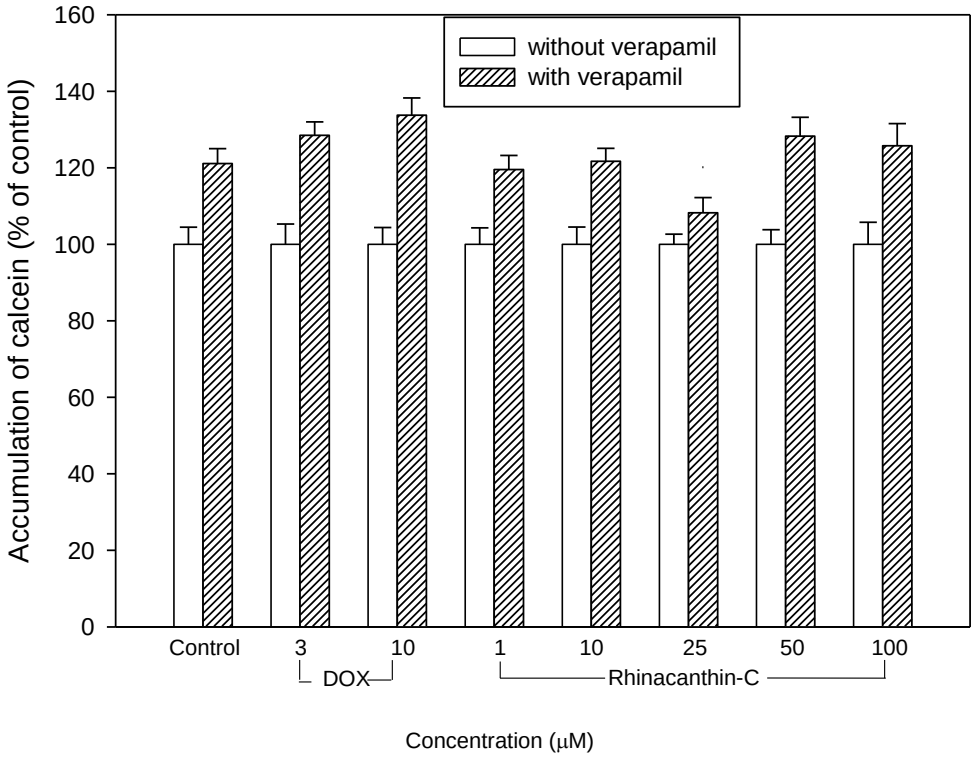


Figure 27. Effect of rhinacanthin-C on P-gp function in the Caco-2 cells after 1 day treatment. In this experiment, doxorubicin (DOX) (3 and 10 μM) was used as the positive control groups. The intracellular calcein accumulation in the presence of verapamil was expressed as the percentage of those in the absence of verapamil. Values represented the mean ± SEM (n=3-7).

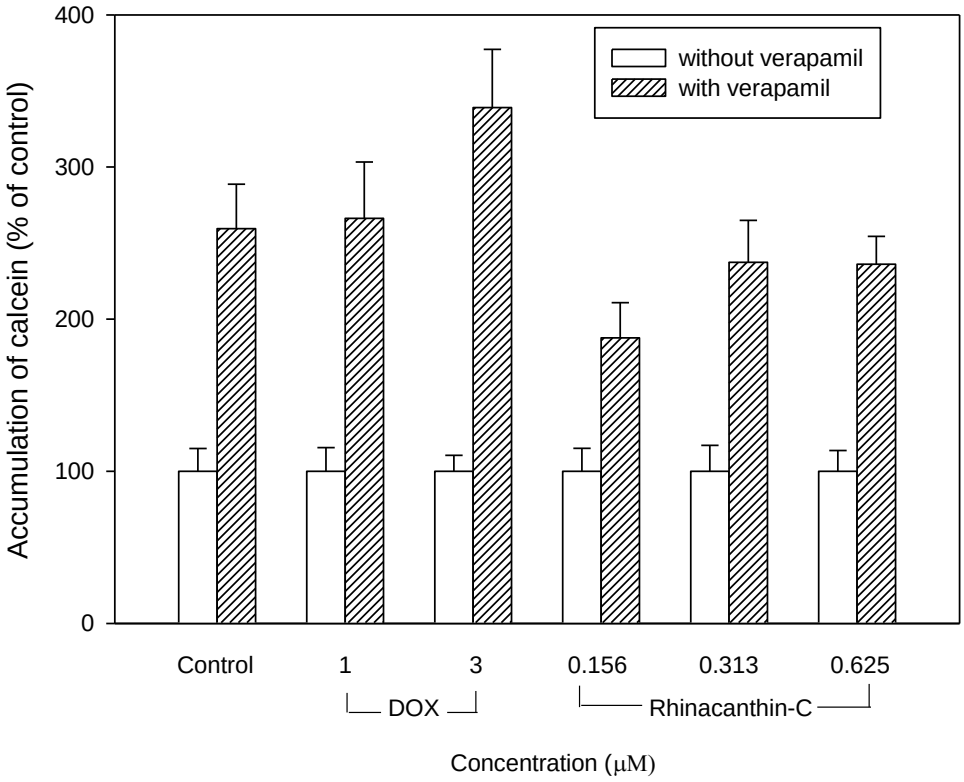


Figure 28. Effect of rhinacanthin-C on P-gp function in the Caco-2 cells after 7 days treatment. In this experiment, doxorubicin (DOX) (1 and 3 μM) was used as the positive control groups. The intracellular calcein accumulation in the presence of verapamil was expressed as the percentage of those in the absence of verapamil. Values represented the mean ± SEM (n=3).

Table 7. The folds increase in intracellular calcein retention in the presence and absence of verapamil after 1 day treatment with either doxorubicin or rhinacanthin-C. The cells were evaluated for P-gp function after treatment with either doxorubicin or rhinacanthin-C for 1 day.

Groups	Fold of P-gp function *
Untreated control group	1.21
Doxorubicin 3 μM	1.28
Doxorubicin 10 μM	1.34
Rhinacanthin-C 1μM	1.20
Rhinacanthin-C 10 μM	1.22
Rhinacanthin-C 25 μM	1.08
Rhinacanthin-C 50 μM	1.28
Rhinacanthin-C 100 μM	1.26
* calculated from intracellular calcein in the presence of verapamil/ those in the absence of verapamil	

Table 8. The folds increase in intracellular calcein retention in the presence and absence of verapamil after 7 days treatment with either doxorubicin or rhinacanthin-C. The cells were evaluated for P-gp function after treatment with either doxorubicin or rhinacanthin-C for 7 days.

Groups	Fold of P-gp function *
Untreated control group	2.59
Doxorubicin 1 μM	2.66
Doxorubicin 3 μM	3.39
Rhinacanthin-C 0.156 μM	1.88
Rhinacanthin-C 0.313 μM	2.37
Rhinacanthin-C 0.625 μM	2.36
* calculated from intracellular calcein in the presence of verapamil/ those in the absence of verapamil	

CHAPTER V
DISCUSSION AND CONCLUSION

In this study, the Caco-2 cells cultured for 21 days were used as the *in vitro* model for investigation of the function of two membrane transporters (P-gp and MRP2). As known, the Caco-2 cell cultured in specific conditions for 21 days could differentiate into enterocytes with expression of P-gp and MRP2 on the apical side along with other transporters (Sun et al., 2008). Several factors can influence the expression of the transporters including cultured period, passage number, trypsinization and cell density (Hosoya et al., 1996; Yu et al., 1997; Anderle et al., 1998; Volpe, 2008). The cultured conditions of Caco-2 cells in this study were suitable to enable the expression of the P-gp and MRP2 at the appreciable levels. Based upon the substrate accumulation assays, the presence of verapamil (a known inhibitor of P-gp) increased intracellular calcein retention by 3.23 folds whereas indomethacin (a known inhibitor of MRP2) increased the intracellular CDCF accumulation by 2.66 folds.

In this study, rhinacanthin-C was demonstrated to interfere with the function of P-gp and MRP2 efflux pumps in the uptake assays. Thus, this compound could interact with both P-gp and MRP2 with the potential to be either substrate or inhibitor. Because the uptake assay cannot distinguish substrate from inhibitor, the transport study of this compound might be needed to identify the intrinsic action of rhinacanthin-C on these two transporters (Zhang et al., 2006; Siissalo et al., 2009). Nevertheless, it has been established that various chemical structures can interact with the ABC efflux pumps (Matsson et al., 2009). Some of these compounds have less degree of specificity, leading to its capability to bind with multiple drug efflux pumps (Matsson et al., 2009). The structure activity relationship suggests that any compound with highly lipophilicity, planar aromatic regions and hydrogen bond can possibly interact with efflux transporters (in particular P-gp and MRP2) (Seelig, 1998; Sucre, 2007; Matsson et al., 2009). The lipophilic property enables the compound to dissolve into the bilayer membrane and readily reaches the embedded binding sites of P-gp and MRP2 (Raviv et al., 1990; Seelig et al., 2003; Fardel et al., 2005). In addition, the hydrogen bond

interaction between the compound and the recognition sites of each transporter contributes to the binding compatibility (Seelig et al., 2003; Hirono et al., 2005). Considering its chemical structure (Fig 1), rhinacanthin-C has one planar aromatic region, showing the lipophilicity, with long chain aliphatic ester containing hydrogen bond acceptors and donors. Thus, this compound could be readily bind to the active site of both P-gp and MRP2, and interfere their function in the uptake assay.

The inhibitory effect of rhinacanthin-C was apparently more potent toward P-gp activity than that of MRP2. The results showed that rhinacanthin-C (100 μM) increased the CDCF accumulation by 1.79 folds, comparing to its ability to increase the calcein accumulation upto 3.2 folds. It was likely that the molecular structure of rhinacanthin-C was more compatible for its interaction with P-gp than MRP2. Although, it has been reported that certain compounds can be substrate of either P-gp or MRP2, the ligand binding sites of these two transporters are not identical. It was proposed that the primary binding sites of rat MRP2 contained the two hydrophobic sites and the two electrostatically positive sites (Hirono et al., 2005). In addition, MRP2 in the Caco-2 cells also perfered the compound with high steric bulkiness of ortho-substitued biphenyl ring (Lai et al., 2007). It was suggested that the MRP2 required the higher number of aromatic bonds than P-gp did for the binding (Pedersen et al., 2008). The numbers of aromatic bonds of MRP2 specific inhibitors were approximately 6-18 aromatic bonds, in comparison with the 0-16 aromatic bonds observed in the P-gp specific inhibitors (Matsson et al., 2009). Even though the structure of rhinacanthin-C consists of hydrogen acceptors and donors with 11 aromatic bonds, it has only one hydrophobic regions (Fig 1). This may cause less compatibility of rhinacanthin-C on binding to MRP2 than to P-gp.

The effect of rhinacanthin-C on P-gp inhibition was further investigated on its reversibility with the application of assay condition. The inhibitory action of rhinacanthin-C on P-gp increased significantly in the pre-and co-treatment condition where the compound was present in the assay medium both in the period before and after addition of calcein-AM. The inhibitory effect was not observed in the pretreatment condition

where rhinacanthin-C was replaced with calcein-AM. Hence, the inhibitory effect of rhinacanthin-C was apparently transient. The transporter could recover its fully function in the absence of this compound. These findings were also observed in the experiment with the use of verapamil, a known reversible inhibitor of P-gp. In contrast, the irreversible inhibitors such as inostamycin, alkylating modulator (fluphenazine-mustard, trans-fluphenazine-mustard) were reported to have long lasting inhibition against P-gp activity, even when the inhibitor were removed from the assay by washing with PBS many times (Kawada and Umezawa, 1995; Yang et al., 1997). Hence, it was likely that rhinacnathin-C reversibly interacted with P-gp.

In comparison with the standard pre-and co-treatment condition, the withdrawal of rhinacanthin-C during the pretreatment period caused a decrease in the intracellular calcein retention. The result suggested that less amount of calcein-AM entered the cells in the co-treatment condition. It was possible that calcein-AM was able to reach and occupy the P-gp binding sites faster than rhinacanthin-C. This might be due to the different membrane permeability of each compound passing through the cell membrane (Doppenschmitt et al., 1999). Compounds with high permeability are likely to have higher concentration in the membrane than compounds with low permeability, leading to an increase in their number to interact with P-gp (Sharom et al., 1999). It could be anticipated that the presence of rhinacanthin-C for 30 min prior to addition of calcein-AM increased its availability at the P-gp binding sites in the cellular membrane.

It was well established that the expression of P-gp in the Caco-2 cells was time-dependent (Hosoya et al., 1996; Volpe, 2008). The expression increased markedly after cell differentiation and reached the highest level around 14 to 17 days after seeding (Zrieki et al., 2008). In this study, the effects on the expression were studied in the cells aged 14 days after seeding. Hence, it could be assumed that the extent of P-gp protein was at maximum level in the plateau state. The results were also demonstrated that the levels of expressed P-gp in the cells on day 15 and day 21 after seeding were not comparable. Therefore, the effects of the treatment either with doxorubicin or with rhinacanthin-C were unlikely to result from alteration of intrinsic P-gp level in the Caco-2

cells.

In agreement with other studies, doxorubicin could increase the activity of P-gp through upregulation of the protein (Maitra et al., 2001). In addition, the effect of doxorubicin on the expression of P-gp in the Caco-2 cells was related to duration of exposure and its concentration (Silva et al., 2011). In addition to the upregulation, xenobiotics could suppress the expressed of P-gp, leading to the loss of P-gp activity in the cells (Maitra et al., 2001). For example, nonsteroidal anti-inflammatory drugs (NSAIDs) such as indomethacin heptyl ester, nimesulide and naproxen decreased P-gp expression and activity after prolong exposure for 4 passages in the Caco-2 cells (Zrieki et al., 2008). Interestingly, alteration of the amount of expressed protein might not always result in the change of activities. As shown in this study, treatment the cells with doxorubicin for 1 day and 7days significantly increased the P-gp expression up to 2.15 and 3.76 folds; however, the P-gp activity did not increased correspondingly. At 1 day treatment with doxorubicin, the P-gp activity was indifferent from that of the untreated group. In addition, the activity slightly increased to 1.31 fold when the cells were treated for 7 days. These findings were in agreement with the results from other studies (Silva et al., 2011). The poor correlation between the number of P-gp expression and activity was probably related to the underdeveloped function of the new P-gp proteins that expressed on the surface of the Caco-2 cells (Silva et al., 2011). It was reported that it took around 10 days for the expressed P-gp to obtain its full function (Hosoya et al., 1996). Additionally, it could be anticipated that the immunofluorescent method for protein determination was more sensitive than the uptake assay for protein activity, leading to a poor correlation. Moreover, calcein is a substrate of MRP2 (Prime-Chapman et al., 2004). Therefore, the interpretation of intracellular calcein retention can be linked to the activity of either P-gp or MRP2 (Eneroth et al., 2001; Masereeuw et al., 2003). In the case that the MRP2 was also upregulated, calcein might be pumped out of the cells increasingly, leading to the less accumulated calcein retention within the cells. In this study, the expression of MRP2 could not be excluded. Further investigation on this matter may be needed. It should be noted that the level of P-gp

expression could vary in each passage (Yu et al., 1997). In this study, the activities of P-gp in the cells aged 21 days (passage 48-72), as estimated by the effect of verapamil in the uptake assay, were in the range of 2.46 to 5.93 folds.

The effects of rhinacanthin-C on the expression of P-gp were also observed in this study. Rhinacanthin-C (at the concentration of 1-100 μM for 1 day-treatment) was able to induce P-gp expression but its effect to increase the function was not observed. It was possibly that the induced P-gp could not function fully at this state. Increasing the treatment period from 1 day to 7 days could not be done at the high concentration of 1-100 μM, due to its cytotoxicity. At the highest non-cytotoxic concentration of rhinacanthin-C at 7 days treatment had no effect on both expression and function of P-gp.

In conclusion, rhinacanthin-C could affect the function of P-gp and MRP2 efflux pumps. The inhibitory effect of rhinacanthin-C was more potent toward P-gp than toward MRP2 activities, as measured by the substrate accumulation assay. Its inhibitory action against P-gp activity was reversible. Moreover, rhinacanthin-C at the high concentration after 1 day treatment was able to induce the P-gp expression without any appreciable change in its function. Combination of rhinacanthin-C and other P-gp drug substrates should be investigated further to evaluate the efficacy and safety of its uses.

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APPENDICES

APPENDIX A

Rhinacanthin-C

Rhinacanthin-C was kindly provided by Dr. Pongpun Siripong, National Cancer Institute, Bangkok, Thailand. The preparation and purification processes were described in Siripong et al. (2006b). Briefly, the roots of *R. nasutus* Kurz. were ground and extracted by methanol before this extract was separated by *n*-hexane and further with chloroform and methanol. To obtain rhinacanthin-C, the chloroform extract was purified by column chromatography and provided this bioactive compound as red oil, using chloroform, methanol and *n*-hexane. Rhinacanthin-C was identified by UV, IR, ^{1}H- and ^{13}C-NMR and MS.

APPENDIX B

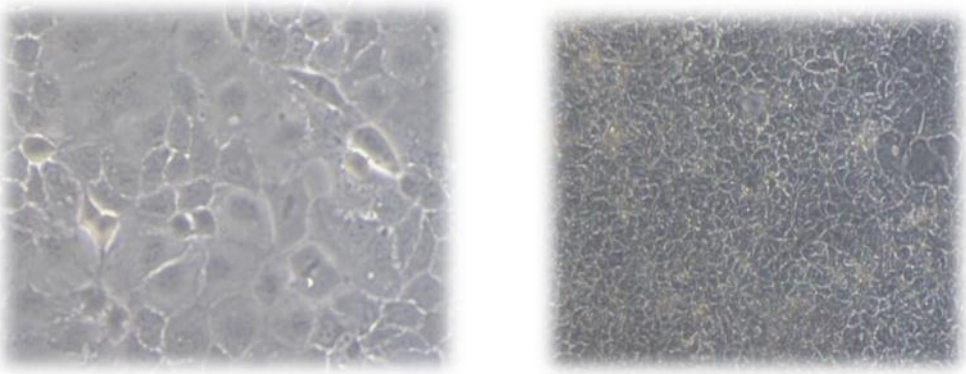
List of experimental instruments

1. Centrifuge model EBA 20: Hettich, Germany
2. Centrifuge model MIKRO 120: Hettich, Germany
3. Refrigerated centrifuge: Z383K, Hermle Labortechink, Burladingen, Germany
4. Laminar air flow hood: Hepaco, U.S.A.
5. Humidified carbon dioxide incubator: Forma Scientific, OH, USA
6. Vortex mixer: model K550-GE, Scientific Industries, NY, USA
7. Fluorescence microplate reader, Wallac 1420 Victor 3: Perkin-Elmer, USA
8. PH meter: Schott, Germany
9. Inverted microscope: Axiovert 135, Zeiss, Germany
10. Sonicator: Clifton, England
11. Flow cytometry: BD FACSCalibur, USA
12. Hot air oven: Memmert, Germany
13. Autoclave: Hirayama, Japan
14. Water bath: Memmert, Germany
15. Flask-trap aspirator: Biosan, Latvia
16. Serological pipettes: Thermo Scientific, USA
17. Repetitive pipette: Rainin, Germany
18. Adjustable pipette 2.5, 10 µl: Eppendorf, Germany
19. Adjustable pipette 20, 200, 1000 µl: Pipetman, Gilson, USA
20. Option 3 water purifier: ELGA, UK
21. Balance 7 positions: Mettler Toledo, Switzerland

APPENDIX C

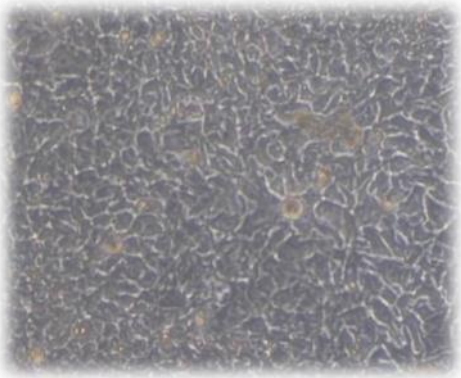
Cells culture

The Caco-2 cell line was obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). The morphology of the Caco-2 cells was observed by the inverted microscope with phase contrast optics in combination (Axiovert 135, Zeiss, Germany) connected with microcomputer-assisted image capture system (Pinnacle 8, Pinnacle system, Germany). The images were demonstrated the morphology of 3, 14 and 21 days (Figure 29).



a) Caco-2 cells 3 days

b) Caco-2 cells 14 days



c) Caco-2 cells 21 days

Figure 29. The morphology of the Caco-2 cells after being cultured for a) 3 days, b) 14 days, and c) 21 days under experimental condition. The pictures were taken under an inverted microscope (Axiovert 135, Zeiss, Germany) at the magnification of x100.

APPENDIX D

Fluorescence quenching test

In order to investigate the possible interference between the test compounds and fluorescent calcein, the mixture of calcein and either rhinacanthin-C or doxorubicin was prepared in 0.1% triton X-100 solution. The fluorescent intensity of the mixture was determined in comparison with calcein solution at an excitation/emission wavelength of 485/535 nm.

As shown in table 9 and 10, the interference between the test compounds and fluorescent calcein had no fluorescence effect at an excitation/emission wavelength of 485/535 nm. Therefore, the combination of those compounds may not influence on the intracellular fluorescence intensity.

Table 9. Results of the interference between doxorubicin and calcein.

Calcein Concentration (nM)	Fluorescence intensity		
	Control (0.5% DMSO)	Doxorubicin 3 μM	Doxorubicin 10 μM
1	168.5	10445	28661.5
10	3148.5	9761.5	26593.5
100	28863.5	9636.5	26665
1,000	246407	9948.5	25565.5
10,000	2754815	2651251.5	2315771.5

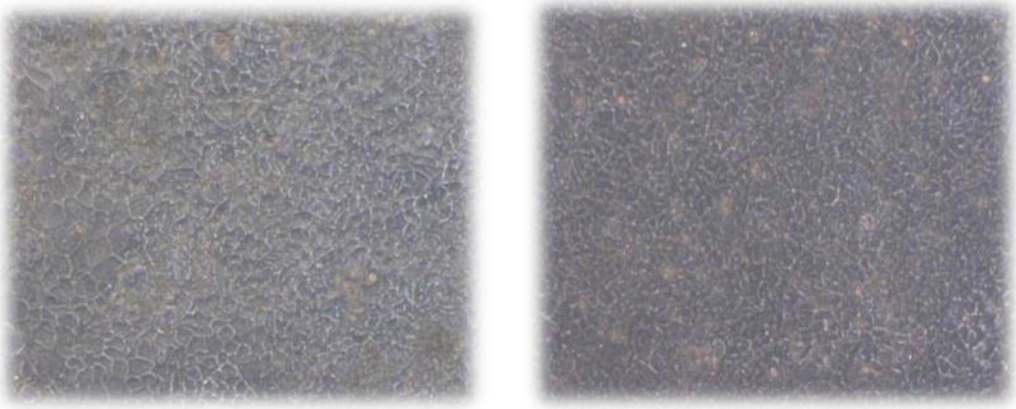
Table 10. Results of the interference between rhinacanthin-C and calcein.

Calcein Concentration (nM)	Fluorescence intensity		
	Control (0.5% DMSO)	Rhinacanthin-C 1.25 μM	Rhinacanthin-C 100 μM
1	401.5	460	646.5
10	3465	2213	3702.5
100	32170.5	24705	28794.5
1,000	315594.5	282028.5	296403
10,000	2504381	2481988	2422644

APPENDIX E

The viability of Caco-2 cells in doxorubicin

In this study, the cell detachment in the doxorubicin 10 μM was not observed compared with the control (Fig 30). In the MTT test, the concentrations of doxorubicin at lower 10 μM had no effect on the cell viability after treatment for 1 day and 7 days (Fig 31, 32). Thus, the range of 1 to 10 μM doxorubicin was used in the short and long term treatment.



A) Control B) 10 μM

Figure 30. The Caco-2 monolayers (passage 66) after treatment with doxorubicin for 1 day. The concentrations of doxorubicin were at A) 0 μM (untreated group), B) 10 μM. The pictures were taken under an inverted microscope (Axiovert 135, Zeiss, Germany) at the magnification of x100.

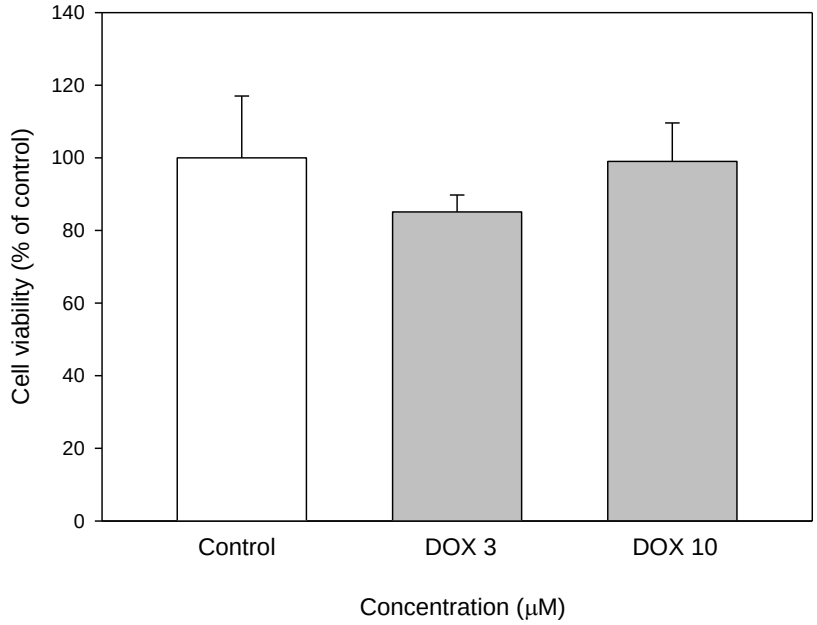


Figure 31. The viability of the Caco-2 cells after treatment with doxorubicin for 1 day. The cell viability was determined by the MTT assay and expressed as the percentage of control (untreated group). Data was represented as the mean ± SEM (n=3). * $P < 0.05$ *vs* control.

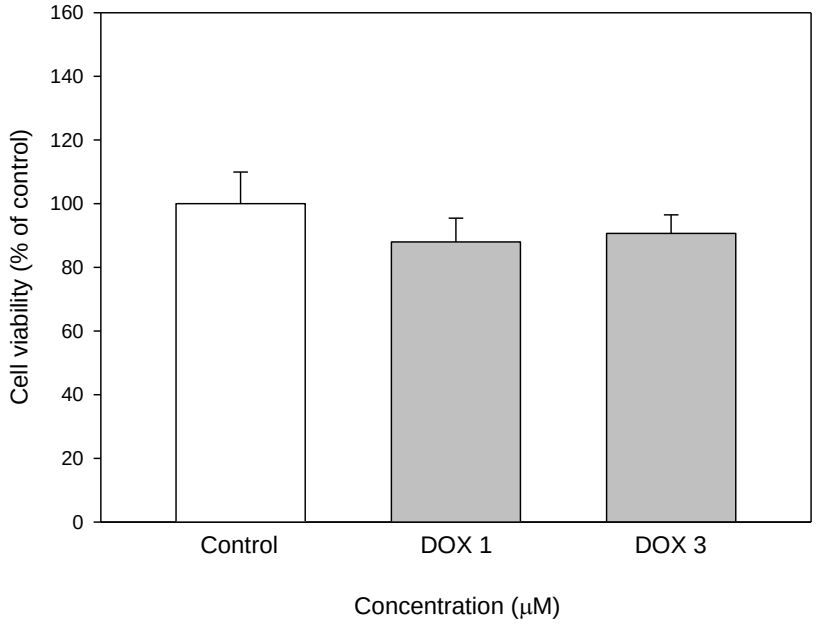


Figure 32. The viability of the Caco-2 cells after treatment with doxorubicin for 7 days. The cell viability was determined by the MTT assay and expressed as the percentage of control (untreated group). Data was represented as the mean ± SEM (n=3-4). * $P < 0.05$ *vs* control.

Table 11. Results of the viability of the Caco-2 cells after treatment with doxorubicin or rhincanthin-C for 1 day.

Groups	Optical density (OD)					
	n1	n2	n3	mean	SEM	mean (%)
Control	0.43	0.71	0.79	0.64	0.11	100
Doxorubicin 3 μM	0.49	0.55	0.60	0.55	0.03	89.44
Doxorubicin 10 μM	0.50	0.68	0.72	0.64	0.07	101.71
Rhincanthin-C 25 μM	0.70	0.91	0.97	0.86	0.08	138.03
Rhincanthin-C 50 μM	0.63	0.83	0.94	0.80	0.09	128.06
Rhincanthin-C 100 μM	0.66	0.82	0.90	0.79	0.07	127.82

Table 12. Results of the viability of the Caco-2 cells after treatment with doxorubicin or rhinacanthin-C for 7 days.

Groups	Optical density (OD)						
	n1	n2	n3	n4	mean	SEM	mean (%)
Control	0.68	0.36	0.41	0.60	0.51	0.05	100
Doxorubicin 1 μM	0.54	0.30	0.46	0.51	0.45	0.04	89.63
Doxorubicin 3 μM	0.51	0.35	0.45	0.54	0.46	0.03	93.38
Rhincanthin-C 0.156 μM	-	0.31	0.36	0.51	0.39	0.04	86.64
Rhincanthin-C 0.313 μM	-	0.30	0.44	0.56	0.43	0.05	94.16
Rhincanthin-C 0.625 μM	0.47	0.30	0.44	0.62	0.46	0.05	90.66

APPENDIX F

Data of the experimental results

Table 13. Results of rhinacanthin-C on calcein accumulation in the Caco-2 cells.

Groups	Accumulation of calcein (% of control)							
	n1	n2	n3	n4	n5	n6	mean	SEM
Control	105.74	72.33	131.42	82.81	143.98	63.71	100	9.10
Verapamil 100 μM	387.83	245.71	421.63	300.49	314.83	272.83	323.89	18.94
RN-C 0.625 μM	-	108.05	118.10	72.51	-	-	99.55	9.09
RN-C 1.25 μM	-	232.31	131.96	77.67	119.93	-	143.40	24.68
RN-C 2.5 μM	-	229.98	139.96	135.89	191.11	-	174.23	15.03
RN-C 5 μM	-	302.97	293.45	175.10	327.21	-	274.68	22.35
RN-C 10 μM	266.93	267.02	332.58	331.56	324.38	263.69	297.69	9.74
RN-C 100 μM	271.67	-	-	-	355.50	335.46	320.88	16.65

Table 14. Results of rhinacanthin-C on CDCF accumulation in the Caco-2 cells.

Groups	Accumulation of CDCF (% of control)						
	n1	n2	n3	n4	n5	mean	SEM
Control	72.97	110.20	111.39	80.12	125.32	100	7.20
Indomethacin 500 μM	243.45	352.70	248.83	209.14	277.48	266.32	16.34
Probenecid 500 μM	156.11	246.86	242.36	177.33	236.96	211.92	14.99
RN-C 0.625 μM	93.67	117.86	126.43	-	-	116.45	7.50
RN-C 1.25 μM	137.22	118.16	125.03	-	-	126.80	5.00
RN-C 2.5 μM	114.79	154.04	125.80	-	-	131.54	8.29
RN-C 5 μM	134.61	150.58	132.72	-	-	139.30	11.01
RN-C 10 μM	149.00	157.80	164.34	134.33	171.62	155.42	6.14
RN-C 100 μM	-	-	-	145.39	213.91	179.65	-

Table 15. Results of pre-treatment condition of calcein-AM uptake.

Groups	Accumulation of calcein (% of control)				
	n1	n2	n3	mean	SEM
Control	65.16	121.38	113.46	100	13.50
Verapamil 100 μM	68.99	138.23	290.32	165.85	42.05
RN-C 10 μM	64.91	189.04	104.49	119.48	24.20

Table 16. Results of co-treatment condition of calcein-AM uptake.

Groups	Accumulation of calcein (% of control)				
	n1	n2	n3	mean	SEM
Control	60.97	126.80	112.23	100	19.77
Verapamil 100 μM	208.74	335.20	266.54	270.16	23.61
RN-C 10 μM	194.83	352.77	317.49	288.36	30.62

Table 17. Results of pre-and co-treatment condition of calcein-AM uptake.

Groups	Accumulation of calcein (% of control)				
	n1	n2	n3	mean	SEM
Control	88.54	115.45	96.01	100	9.56
Verapamil 100 μM	379.14	590.54	569.38	513.02	43.18
RN-C 10 μM	366.43	420.53	516.92	434.63	28.12

Table 18. Results of P-gp expression in the Caco-2 cells after 1 day treatment with doxorubicin or rhinacanthin-C.

Groups	Mean of relative fluorescence intensity (% of control)							
	n1	n2	n3	n4	n5	n6	mean	SEM
Control	97.16	83.08	94.32	103.38	136.24	85.81	100	7.86
Doxorubicin 3 μM	-	-	-	216.70	220.31	207.75	214.92	3.73
Doxorubicin 10 μM	434.39	459.82	416.48	409.93	404.04	394.65	419.89	9.67
Rhinacanthin-C 1 μM	-	-	-	131.66	145.20	143.78	140.21	4.30
Rhinacanthin-C 10 μM	-	-	-	138.32	176.31	174.24	162.95	12.33
Rhinacanthin-C 25 μM	168.67	172.49	165.17	-	-	-	168.78	2.11
Rhinacanthin-C 50 μM	150.11	163.21	222.05	-	-	-	178.46	22.12
Rhinacanthin-C 100 μM	157.20	138.86	226.53	-	-	-	174.20	26.70

Table 19. Results of P-gp expression in the Caco-2 cells after 7 days treatment with doxorubicin or rhinacanthin-C.

Groups	Mean of relative fluorescence intensity (% of control)				
	N1	N2	N3	mean	SEM
Control	98.33	92.74	110.61	100	3.79
Doxorubicin 1 μM	247.74	220.01	211.56	231.76	9.54
Doxorubicin 3 μM	394.57	410.63	305.07	376.21	24.22
Rhinacanthin-C 0.156 μM	91.04	134.17	114.77	107.76	13.22
Rhinacanthin-C 0.313 μM	113.84	137.24	121.57	121.62	6.54
Rhinacanthin-C 0.625 μM	93.45	100.85	97.45	96.30	2.07

Table 20. Results of P-gp function in the Caco-2 cells after prolong exposure with doxorubicin or rhinacanthin-C.

Groups	% of Calcein accumulation							
	1 day treatment				7 days treatment			
	without verapamil		with verapamil		without verapamil		with verapamil	
	mean	SEM	mean	SEM	mean	SEM	mean	SEM
Control	100	4.45	121.10	3.90	100	15.01	259.45	29.31
Doxorubicin 1 μM	-	-	-	-	100	15.59	266.29	37.00
Doxorubicin 3 μM	100	5.28	128.46	3.53	100	10.52	339.14	38.27
Doxorubicin 10 μM	100	4.37	133.75	4.52	-	-	-	-
Rhinacanthin-C 0.156 μM	-	-	-	-	100	15.07	187.72	23.17
Rhinacanthin-C 0.313 μM	-	-	-	-	100	17.01	237.39	27.58
Rhinacanthin-C 0.625 μM	-	-	-	-	100	13.67	236.10	18.36
Rhinacanthin-C 1 μM	100	4.29	119.55	3.67	-	-	-	-
Rhinacanthin-C 10 μM	100	4.49	121.70	3.37	-	-	-	-
Rhinacanthin-C 25 μM	100	7.05	108.22	3.99	-	-	-	-
Rhinacanthin-C 50 μM	100	3.81	128.26	4.93	-	-	-	-
Rhinacanthin-C 100 μM	100	5.74	125.74	5.82	-	-	-	-

VITAE

Miss Ratjika Wongwanakul was born on May 21, 1984 in Bangkok, Thailand. She received her Bachelor's degree in Pharmacy from the Faculty of Pharmacy, Srinakharinwirot University, Bangkok, Thailand in 2008. Then, she entered the Master's degree program in the Faculty of Phamaceutical Science at Chulalongkorn University in 2009.

She has attended and presented a poster at the 34rd Pharmacological and Therapeutic Society of Thailand Meeting, March 22-24, 2012 at Chulalongkorn University, Bangkok, Thailand. The subject presented was effects of rhinacanthin-N on efflux drug transporters in Caco-2 cells. She also got the honorable mention presentation award from this title. The manuscript was published in Thai Journal of Pharmacology volume 34(1), 2012.