ผลของเมทฟอร์มินต่อการแสดงออกระดับยีนของมัลติดรักรีซิสแทนซ์แอสโซซิเอตเต็ดโปรตีน 2 (เอ็มอาร์พี 2) ในเซลล์เอ็มซีเอฟ-7

นางสาวพลอย วรรณภากร

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาเภสัชศาสตรมหาบัณฑิต
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ปีการศึกษา 2559

EFFECT OF METFORMIN ON GENE EXPRESSION OF MULTIDRUG RESISTANCE ASSOCIATED PROTEIN 2 (MRP2) IN MCF-7 CELLS

Miss Ploy Wannapakorn

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science in Pharmacy Program in Pharmacology and Toxicology
Department of Pharmacology and Physiology
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Thesis Title	EFFECT OF METFORMIN ON GENE EXPRESSION OF MULTIDRUG RESISTANCE ASSOCIATED PROTEIN 2 (MRP2) IN MCF-7 CELLS
By	Miss Ploy Wannapakorn
Field of Study	Pharmacology and Toxicology
Thesis Advisor	Associate Professor Suree Jianmongkol, Ph.D.

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พลอย วรรณภากร : ผลของเมทฟอร์มินต่อการแสดงออกระดับยีนของมัลติดรักรีซิสแทนซ์แอสโซซิเอตเต็ดโปรตีน 2(เอ็มอาร์พี 2) ในเซลล์เอ็มซีเอฟ-7 (EFFECT OF METFORMIN ON GENE EXPRESSION OF MULTIDRUG RESISTANCE ASSOCIATED PROTEIN 2 (MRP2) IN MCF-7 CELLS) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. ภญ. ดร.สุรีย์ เจียรณ์มงคล, 47 หน้า.

เมทฟอร์มินเป็นยารักษาเบาหวาน กลุ่มไบกวาไนด์ ที่มีฤทธิ์ทางเภสัชวิทยาหลากหลาย รวมถึงฤทธิ์ในการต้านมะเร็ง งานวิจัยนี้มีวัตถุประสงค์เพื่อศึกษาผลของเมทฟอร์มิน ที่มีต่อการทำงานและการแสดงออกของโปรตีนตัวขนส่งยา MRP2 ในเซลล์มะเร็งเต้านม MCF-7 โดยการทำงานของ MRP2 ตรวจวัดจากการสะสมของสารเรืองแสง CDCF ภายในเซลล์ ซึ่งผลการทดลองพบว่าเมทฟอร์มินไม่มีผลเปลี่ยนแปลงระดับของ CDCF ภายในเซลล์ หลังจากที่ให้นาน 30 นาทีแต่อย่างใด อย่างไรก็ตามการให้เมทฟอร์มินในช่วงความเข้มข้น 1-5 mM แก่เซลล์ MCF-7 เป็นระยะเวลานาน 24-48 ชั่วโมง พบว่าปริมาณของ MRP2 mRNA ที่ตรวจวัดด้วยวิธี RT-PCR ลดลงอย่างมีนัยสำคัญ ทั้งนี้ผลของเมทฟอร์มินในการลดระดับการแสดงออกของ MRP2 mRNA ขึ้นกับความเข้มข้นที่เซลล์ได้รับยาดังกล่าว การศึกษายังพบว่าเซลล์ MCF-7 ที่ได้รับเมทฟอร์มิน ความเข้มข้น 5 mM มีระดับของ phosphorylated ERK1/2 (p-ERK1/2) และ phosphorylated p38 (p-p38) ซึ่งเป็น active form ของ ERK1/2 และ p38 ลดลงเมื่อเปรียบเทียบกับกลุ่มควบคุมที่ไม่ได้รับเมทฟอร์มิน ผลการวิจัยนี้แสดงให้เห็นว่าเมทฟอร์มิน สามารถยับยั้งการทำงานของ ERK และ p38 ใน MAPK pathway ได้ นอกจากนี้เมื่อให้ compound C ความเข้มข้น 10 μM เพื่อยับยั้งการกระตุ้น AMPK ในเซลล์ที่ได้รับเมทฟอร์มิน พบว่า compound C มีผลยับยั้งการลดปริมาณลงของ MRP2 mRNAและยับยั้งการทำงานของ MAPK ในเซลล์ดังกล่าว ดังนั้นจึงสรุปได้ว่าเมทฟอร์มินอาจมีผลลดระดับ MRP2 mRNA ในเซลล์มะเร็งเต้านม MCF-7 ผ่านทางการยับยั้ง MAPK signaling pathway ซึ่งอาจมีส่วนสัมพันธ์กับความสามารถของเมทฟอร์มิน ในการกระตุ้นการทำงานของ AMPK pathway นอกจากนี้ควรต้องศึกษาผลของเมทฟอร์มินที่มีต่อการแสดงออกของ MRP2 ในระดับโปรตีนเป็นลำดับต่อไป

ภาควิชา เภสัชวิทยาและสรีรวิทยา ลายมือชื่อนิสิต .....

สาขาวิชา เภสัชวิทยาและพิษวิทยา ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

ปีการศึกษา 2559

5776119733 : MAJOR PHARMACOLOGY AND TOXICOLOGY
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PLOY WANNAPAKORN: EFFECT OF METFORMIN ON GENE EXPRESSION OF MULTIDRUG RESISTANCE ASSOCIATED PROTEIN 2 (MRP2) IN MCF-7 CELLS. ADVISOR: ASSOC. PROF. SUREE JIANMONGKOL, Ph.D., 47 pp.

Metformin is a biguanide anti-diabetic drug with various pharmacological activities including anti-carcinogenic action. This study was to determine the effects of metformin on function and expression of MRP2 in human breast cancer MCF-7 cells. The activity of MRP2 was assessed by measuring intracellular accumulation of CDCF fluorochrome. The results showed that metformin had no direct effect on CDCF accumulation after 30-min treatment. Prolong treatment of MCF-7 cells with metformin (1-5 mM) for 24-48 hr resulted in significant reduction of MRP2 mRNA levels, as measured by RT-PCR assay. Metformin down-regulated expression of MRP2 mRNA in concentration-dependent manners. Moreover, the cells treated with metformin (5 mM) had lower levels of phosphorylated ERK (p-ERK) and p38 (p-p38) than those of the control groups. These results suggested that metformin could inhibit basal activities of ERK and p38 in the MAPK pathway. The presence of an AMPK inhibitor compound C (10 μM) could prevent down-regulation of MRP2 mRNA as well as reduction of basal MAPK activities caused by metformin. These findings suggested that metformin might decrease MRP2 mRNA expression in the MCF-7 cells via inhibition of MAPK signaling pathway. The metformin-mediated alteration of MAPK signaling might relate to activation of AMPK pathway. Further determination of MRP2 protein level in the metformin-treated cells should be pursued.

Department: Pharmacology and Physiology
Field of Study: Pharmacology and Toxicology
Academic Year: 2016

Student's Signature .....
Advisor's Signature .....

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LIST OF ABBREVIATIONS

AAF	= Acetylaminofluorene
ABC	= Adenosine triphosphate binding cassette
ADP	= Adenosine diphosphate
AMPK	= AMP-activated protein kinase
ANOVA	= Analysis of variance
ATP	= Adenosine triphosphate
BCRP	= Breast cancer resistance protein
BSA	= Bovine serum albumin
ºC	= Degree celsius
CDCF-DA	= 5(6)-Carboxy-2',7'-dichlorofluorescein diacetate
CO_2	= Carbon dioxide
DMSO	= Dimethyl sulphoxide
DNA	= Deoxyribonucleic acid
EDTA	= Ethylenediamine tetraacetic acid
ERK	= Extracellular signal regulated kinase
FBS	= Fetal bovine serum
5-FU	= 5-fluorouracil
GSH	= Glutathione
HBSS	= Hanks' balanced salt
HCl	= Hydrochloric acid
HRP	= Horseradish peroxidase
hr	= hour
JNK	= c-Jun NH2-terminal kinase
kDa	= Kilodalton
LXR	= The liver X receptor
MAPK	= Mitogen-activated protein kinase
MDR	= Multidrug resistance
MDR1	= Multidrug resistance protein 1
MRP2	= Multidrug resistance associated protein 2
mTOR	= mammalian Target of Rapamicin

MTT	= 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide
Ml	= milliliter
mM	= millimolar
NF-kB	= Nuclear factor kappa-light-chain-enhancer of activated B cells
PBS	= Phosphate buffered saline
PI3K	= Phosphoinositide 3-kinase
PTEN	= Phosphatase and tensin homologue
PVDF	= Polyvinylidene fluoride
P-gp	= P-glycoprotein
RIPA	= Radioimmunoprecipitation assay
RT-PCR	= Reverse transcription polymerase chain reaction
SDS	= Sodium dodecyl sulfate
SEM	= Standard error of mean
SREBP	= Sterol regulatory element binding protein
µg	= microgram
µl	= microliter
µM	= micromolar

CHAPTER I

INTRODUCTION

Cancer is a disease involving abnormal uncontrollable cell division which can become malignant with very poor prognosis. The main types of cancer treatment include surgery, radiation therapy, hormone therapy, immunotherapy, targeted therapy and chemotherapy. Chemotherapy is a treatment of choice for cancer, nonetheless the efficacy of chemotherapeutic drugs can be confined by multidrug resistance phenomenon. The multidrug resistance toward chemotherapy occurs through various mechanisms including up/down-regulation of proteins in apoptosis cell death pathway (Baguley, 2010), suppression of phosphatase and tensin homolog function (PTEN) associated with PI3K function (Baguley, 2010) and increasing drug efflux transporters (Tsuruo et al., 2003). The drug efflux transporters in particular those in ATP binding cassette (ABC) family such as P-glycoprotein (P-gp), multidrug resistance associated protein 1 (MRP1), multidrug resistance associated protein 2 (MRP2) reduce intracellular accumulation of chemotherapy agents by pumping the drugs out of the cells (Leonard et al., 2003). Hence, therapeutic efficacy of these chemotherapeutic drugs apparently reduces, leading to the need of higher doses given to patients. Consequently, drug toxicity increases. The ABC transporters in particular MRP2 is intrinsically expressed in several types of cancer cells including breast cancer MCF-7 cells (Chaisit et al., 2016). The expression of MRP2 is also reported in HepG2 liver cancer cells (Cantz et al., 2000), kidney cancer cells derived from patient specimens (Schaub et al., 1999). Expression of P-gp and MPR2, has been responsible for intrinsic cellular resistance toward treatment with cytotoxic agents (Kovalchuk et al., 2008; Choi et al., 2007). In addition, changing in expression levels of MRP2 has been reported in the cells exposed with a number of compounds such camptothecin, cisplatin (Ke et al., 2013), tamoxifen (Choi et al., 2007). For example, the MCF-7 cells treated with tamoxifen for 9 months developed drug resistance via up-regulation of functional MRP2 level (Choi et al., 2007). Changing in MRP2 expression could be associated with number of signaling pathways such as MAPK and LXR-SREBP signaling pathways (Kobayashi et al., 2013; Xiao et al.,

2012). It was demonstrated that activation of LXR-SREBP signaling pathway enhanced mRNA and protein levels of MRP2 in HepG2 cells (Kobayashi et al., 2013). In addition, several transcription factors in survival adaptation of cancer cells such as AP-1, FOXO, NF-kB are also reported to involve with regulation of MRP2 at transcription process (Wagner and Nebreda, 2009).

Metformin (N, N dimethylbiguanide hydrochloride) is a biguanide anti-diabetic drug with various pharmacological activities including anti-carcinogenic effect. Metformin has been reported to inhibit proliferation of various cancer cells such as prostate cancer, colon cancer, ovarian cancer and breast cancer (Ben Sahra et al., 2010). In addition, metformin could inhibit P-gp (MDR1) expression at gene and protein levels in breast cancer cells (Kim et al., 2010). These down-regulation effects was corrected with activation of AMPK signaling pathway and inhibition of NF-KB function (Kim et al., 2010). Currently, there are no reports about the effect of metformin on MRP2 function and expression. Although both P-gp and MRP2 belong to the ABC transporters family, they are encoded by different genes. Hence, mechanisms regulating expression of P-gp and MRP2 can be different. It was demonstrated that changing of P-gp and MRP2 protein levels in the liver of Wistar rats given 2-acetylaminofluorene (AAF) 20 mg / kg daily for 3 days was incomparable (Tang et al., 2000). In addition, AAF (10-40 μM) up-regulated expression of P-gp mRNA in HepG2 cells after treatment for 8 hr (Kuo et al., 2002). This up-regulation might be mediated through activation of phosphoinositide 3-kinase (PI3K) pathway and NF-kB function (Kuo et al., 2002). Furthermore, AAF increased the extents of MRP2 mRNA in HepG2 cells and in hepatoma cell lines (Hepa 1-6) via an activation of the NF-E2-related factor 2 (Nrf2) pathway (Vollrath et al., 2006).

This study aimed to study the effects of metformin on MRP2 function and expression in human breast cancer MCF-7 cells. Its direct inhibitory action as well as its potential to affect MRP2 expression after prolong treatment were evaluated. Moreover, the possible mechanism of metformin on altering MRP2 expression at transcription level was also investigated.

Hypothesis

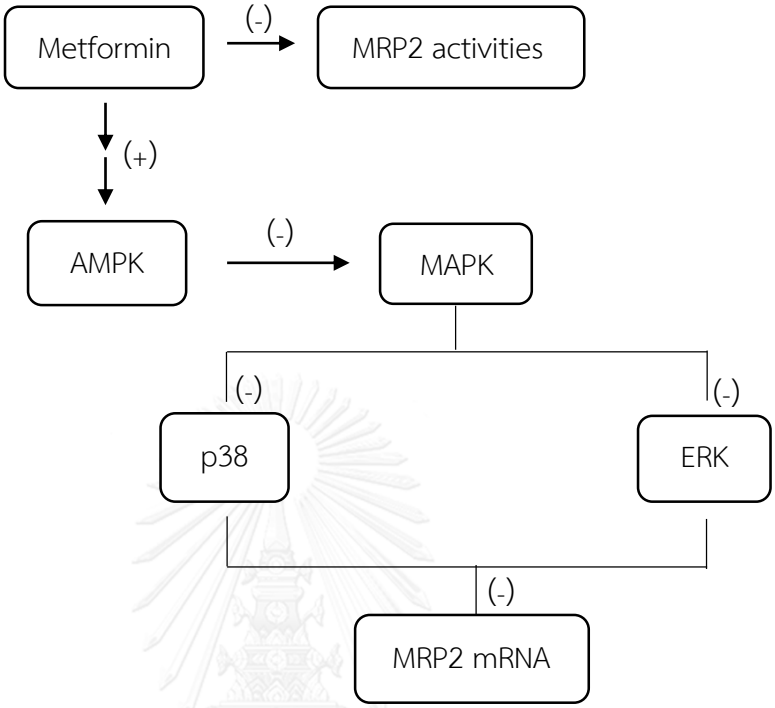
Metformin could directly interfere with MRP2 function as well as affect the expression of MRP2 mRNA in MCF-7 cells after prolong treatment. It was possible that metformin-mediated alteration of MRP2 mRNA involved with AMP-activated protein kinase (AMPK) activation, leading to suppression of mitogen-activated kinase (MAPK) signaling pathway.

Objective

The objectives of this study were as follows.

1. To study the direct effect of metformin on of MRP2 function in breast cancer cells (MCF-7 cells).
2. To study the effects of metformin on the expression of MRP2 mRNA after prolong treatment.
3. To investigate an involvement of AMPK and MAPK signaling pathways in metformin-mediated alteration of MRP2 expression at transcription level.

Conceptual framework



CHAPTER II
LITERATURE REVIEW

1. Breast cancer

Breast cancer is the leading cause of cancer death in female. There are several types of cancer treatment including surgery, radiation therapy, hormone therapy, targeted therapy and chemotherapy. Effectiveness of each treatment has been linked to types and stages of cancer. Certain cancers such as adrenal cancer and renal cancer may have intrinsic resistance toward the use of chemotherapeutic agents such as doxorubicin, mitoxantrone, paclitaxel, docetaxel, cyclophosphamide and capecitabine (Dean et al., 2005; Hassan et al., 2010). Some cancers may develop chemotherapeutic resistance later (Dean et al., 2005; Baguley, 2010). The cancer resistance to chemotherapeutic drugs is most likely non-specific to one drug group. Usually, the resistance phenomenon cause cancer insensitive toward chemicals in various unrelated structure known as multidrug resistance (MDR).

Development of MDR can be caused by several mechanisms including alteration of protein expression in apoptotic cell death pathway (Sharma et al., 2006; Olson and Hallahan, 2004), suppression of phosphatase and tensin homolog function (PTEN) associated with PI3K function (Baguley, 2010), and increasing expression of the ATP binding cassette (ABC) transporters. The ABC transporters reportedly having crucial role in MDR include P-glycoprotein (P-gp), breast cancer resistance protein (BCRP), multidrug resistance associated protein (MRPs) subfamily such as MRP1, MRP2 (Baguley, 2010; Fletcher et al., 2010). These transporters reduce intracellular accumulation of chemotherapy agents by actively pumping the drugs out of the cells with the use of (Baguley, 2010; Choi, 2005). Hence, therapeutic efficacy of these chemotherapeutic drugs apparently decreases, leading to the need of higher doses given to patients. Consequently, untolerated drug toxicity increases. Breast cancer cells intrinsically express certain ABC transporters such as MRP1, MRP2 and MRP3 (Faneyte et al., 2004). In addition, these cancer cells may increase the expression of the drug efflux pumps under certain conditions. It was reported that the expression of P-gp increased in

breast cancer treated with taxol such as paclitaxel and docetaxel (Hassan et al., 2010). Long-term treatment of doxorubicin caused up-regulation of function and expression of P-gp in human breast cancer MCF-7 cells (Chaisit et al., 2016). Moreover, overexpression of BCRP was reported in several of mitoxantrone-resistant cell lines such as colon carcinoma (S1 and HT29), gastric carcinoma (EPG85-257), fibrosarcoma (EPF86-079) and human breast carcinoma (MCF-7) (Doyle and Ross, 2003; Robey et al., 2001).

Hence, it is likely that interference on either function or expression of the ABC drug efflux transporters may overcome MDR, and increase chemotherapeutic efficacy in cancer treatment.

2. Function and expression of MRP2 in cancer

The ATP-binding cassette (ABC) transporters (e.g., P-gp, BCRP, MRP1, MRP2) are implicated in active transport of diverse compounds being their substrates across plasma membrane (Fletcher et al., 2010). The ABC transporters have been found in various normal tissues as well as in cancer tissues. These transporters can be determinant of drug absorption and disposition, depending on the tissues they express (Szakács et al., 2008). For example, P-gp is found in the apical surface of small and large intestine having a role in limiting drug absorption (Sparreboom et al., 1997; Johnstone et al., 2000). In cancer, these transporters play a cellular protective role against xenobiotics or chemical threats through limiting the intracellular accumulation of the threats (Chan et al., 2004).

P-gp is the most studied ABC transporters in MDR development. In addition to P-gp, several cancer cells such as liver cancer, breast cancer and renal cancer have been shown high levels of MRP2 expression (Cantz et al., 2000; Faneyte et al., 2004; Schaub et al., 1999). The expression of MRP2 resulted in resistance to chemotherapeutic drug substrate of MRP2 such as methotrexate (Hooijberg et al 1999), cisplatin, vinblastine, camptothecin (Evers et al., 2000). The expressed levels of the ABC transporters can be affected by several factors such as culture condition, cancer cell type or drug/chemical exposure (Fardel et al., 2005). For example, atorvastatin and pitavastatin could increase mRNA and protein levels of MRP2 in HepG2 cells after

24 hr exposure (Kobayashi et al., 2013). Another example is sulforaphane. This drug could increase MRP2 protein level in HepG2 cells, A549 cells and Caco2 cells after treatment for 48 hr (Harris and Jeffery, 2007). Sodium arsenic (ASIII) was able to up-regulate MRP2 mRNA and protein in both primary rat hepatocytes and normal human hepatocytes via activation of the mitogen-activated protein kinase (MAPK) pathway (Vernhet et al., 2001). However, sodium arsenic (ASIII) had no effect on MRP1 and MRP3 mRNA levels (Vernhet et al., 2001). Hence, regulation of gene expression of each ABC transporter may be different. It has been report tetramethylpyrazine reduced both P-gp and MRP2 levels in doxorubicin-resistant liver cancer cells (BEL-74022/dox cells) (Wang et al., 2010). However, lipopolysaccharide had the opposite effect on extents of P-gp mRNA and MRP2 mRNA in rat liver. The P-gp mRNA levels in liver tissues from Wistar rats given lipopolysaccharide 2 mg / kg increased significantly, as compared with that of the control group. On the contrary, the level of MRP2 mRNA in the rat liver decreased (Vos et al., 1998).

3. Mechanisms involving an expression of MRP2

Changing in expression levels of MRP2 has been reported in the cells exposed with a number of compounds such camptothecin, cisplatin (Ke et al., 2013), tamoxifen (Choi et al., 2007). For example, the MCF-7 cells treated with tamoxifen for 9 months developed drug resistance via up-regulation of functional MRP2 level (Choi et al., 2007). Changing in MRP2 expression is reported to be associated with number of signaling pathways such as MAPK and LXR-SREBP signaling pathways (Jakubikova et al., 2005; Kobayashi et al., 2013; Xiao et al., 2012). Proteins in the MAPK signaling pathway include extracellular signal regulated kinase (ERK), p38 and c-Jun NH2-terminal kinase (JNK) which involving of protein kinase cascades (Zhang and Liu, 2000). These proteins have been related to regulation of cell proliferation, differentiation, and cell survival adaptation (McCubrey et al., 2007). Activation of this MAPK pathway can result in activation of other transcription factors such as c-Jun (AP-1), ATF2, p53 (Kim and Choi, 2010) and NF-kB (Tsai et al., 2003).

An involvement of MAPK pathway in MRP2 up-regulation has been demonstrated. For example, gemcitabine at the concentrations of 11.4 and 114 μg /

ml increased the extent of MRP2 mRNA in pancreatic cancer cells (Bxpc-3 cells), which was related to a rising of RAF/ERK mRNA (Xiao et al., 2012). A tyrosine kinase inhibitor gefitinib (5 μM) could suppress expression of MRP2 mRNA in gemcitabine-resistant pancreatic cancer cells (Xiao et al., 2012). Furthermore, the suppressive effect of IL-1 β on MRP2 mRNA level in HepG2 cells involved with an inhibition of MAPK pathway (Hisaeda et al., 2004). In addition, it was reported that a p38 inhibitor (SB203580) could reduce MRP2 protein expression in Caco2 cells (Vinette et al., 2015). It should be nothing that several transcription factors in survival adaptation of cancer cells such as AP-1, FOXO, NF-kB are also reported to involve with regulation of MRP2 at transcription process (Wagner and Nebreda, 2009).

4. Metformin and anti-cancer activities

Metformin (N, N dimethylbiguanide hydrochloride) is a biguanide anti-diabetic drug with various pharmacological activities including anti-carcinogenic effect. The mechanism of metformin action is related to its ability to inhibit the mitochondrial respiratory chain complex I, leading to reduction of ATP synthesis. Consequently, AMP-activated protein kinase (AMPK) is activated, causing a decrease in hepatic glucose production as well as inhibition of cancer cells division (Viollet et al., 2012). Metformin has been reported to inhibit proliferation of various cancer cells such as prostate cancer, colon cancer, ovarian cancer and breast cancer via AMP-activated protein kinase (AMPK) / mammalian Target of Rapamicin (mTOR) pathway (Ben Sahra et al., 2010) as well as via activation of the MAPK pathway (Hwang et al., 2013). At the concentration of 10 mM, metformin could cause cell cycle arrest in G0-G1 phase and apoptosis in the MCF-7 cells after 24-72 hr treatment, possibly due to inhibition of MAPK/ERK phosphorylation (Queiroz et al., 2014). The similar effect of metformin on cell cycle and apoptosis was also observed in HTh74 thyroid cancer, doxorubicin-resistant thyroid cancer (HTh74Rdox) and triple negative breast cancer cells (ER/PR/Her-2 negative) (Chen et al., 2012; Liu et al., 2009). Metformin produced apoptosis in HTh74 and HTh74Rdox cells through inhibition of ERK phosphorylation (Chen et al., 2012), whereas its apoptotic effect in ER/PR/Her-2 negative involved activation of AMPK pathway. Activation of AMPK resulted in phosphorylation of p53,

a tumor suppressor gene (Liu et al., 2009; Jones et al., 2005). It was also reported that activation of AMPK signaling pathway caused suppression of MAPK/ERK pathway (Tosca et al., 2010; Hwang et al., 2013).

5. Effect of metformin on expression of the ABC transporters

Currently, there are no reports about the effect of metformin on MRP2 function and expression. Metformin at 10 mM was shown to reduce MDR1 (P-gp) expression at gene and protein levels in doxorubicin-resistant MCF-7 breast cancer cells (Kim et al., 2010). This down-regulation effects was correlated to activation of AMPK signaling pathway and inhibition of NF-kB function (Kim et al., 2010). Moreover, metformin in the concentrations ranging from 1 mM to 10 mM decreased mRNA and protein level of P-gp in concentration dependent manner in 5-fluorouracil resistant hepatoma cell line (Bel-7402 cells). This result was associated with activation of the AMPK / mTOR pathway and the inhibition of HIF1-α, a transcription factor associated P-gp expression (Ling et al., 2014). In addition, treatment the doxorubicin-resistant K562 cells with metformin at concentration 1 mM and 5 mM for 48 hr reduced mRNA and protein levels of P-gp via the inhibition of MAPK / ERK pathway (Xue et al., 2016). Metformin-mediated suppression of HIF1-α in Cholangiocarcinoma cells (RBE and HCCC-9810 cells) might be responsible to a decreased MRP1 protein level (Ling et al., 2014). As known, HIF1-α is transcription factor involving in regulation of several genes including MDR1 (P-gp), ABCC1 (MRP1), ABCC2 (MRP2) (Lv, 2015; Wang and Minko, 2004). Hence, it is likely that metformin affect the expression ABC transporters via different signaling mechanisms. As shown in Figure 1, metformin decrease MRP2 mRNA expression through AMPK activation leading to inhibition of mitogen-activated kinase (MAPK) signaling pathway in breast cancer cells.

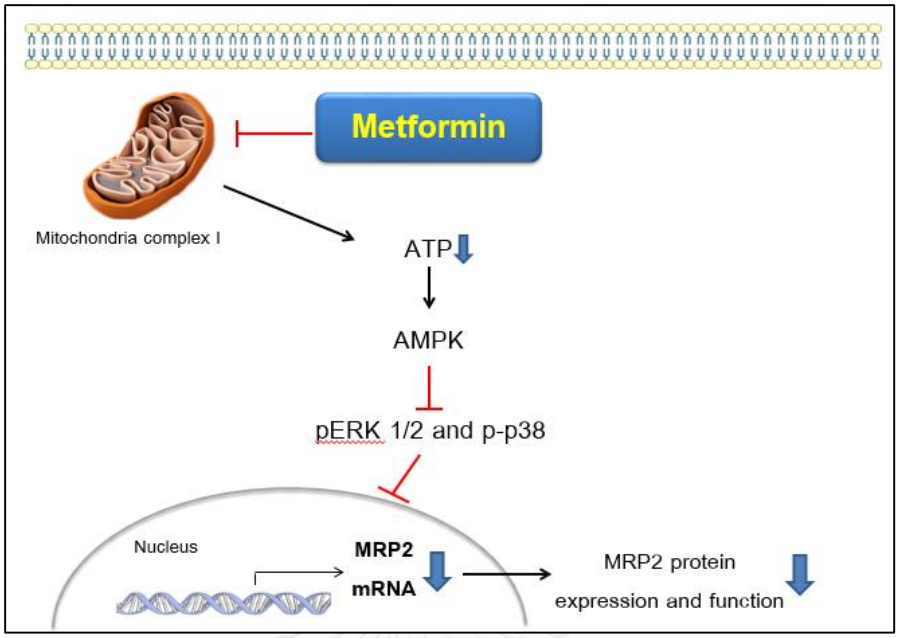


Figure 1 Proposed signal transduction pathways involving with MRP2 expression in breast cancer cells.

CHAPTER III

MATERIALS AND METHODS

1. Materials

1.1 Chemicals and reagents: Metformin was obtained from Aurolab (Tamil Nadu, India).

Other chemicals including bovine serum albumin (BSA), ethylenediamine tetraacetic acid (EDTA), 3-(4,5dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT reagent), penicillin G sodium, protease inhibitor cocktail, streptomycin sulfate, Triton X-100, trypsin, 5(6)-carboxy-2′,7′-dichlorofluorescein diacetate (CDCFDA), indomethacin were obtained from Sigma Chemical Co. (St Louis, MO, USA). Compound C (an AMPK inhibitor) was purchased from Calbiochem (San Diego, CA, USA). Super signal West Pico chemiluminescent substrates and TRIzol® Reagent were purchased from Pierce Biotechnology (Rockford, IL, USA).

RPMI-1640 medium and fetal bovine serum (FBS) were purchased from Gibco Life Technologies (Grand Island, NY, USA).

Polyvinylidine difluoride (PVDF) membranes were purchased from Pall Gelman Laboratory (Pensacola, FL, USA).

ImProm-II™ reverse transcription system was purchased from Promega (Madison, WI, USA).

BCA Protein Assay Kit was purchased from Thermo Scientific™ (Rockford, IL, USA)

1.2 Antibodies: The mouse monoclonal anti-ERK1/2 and anti-phosphorylated ERK1/2, the rabbit monoclonal anti-p38 and anti-phosphorylated p38, secondary goat anti-rabbit IgG (H&L) horseradish peroxidase (HRP) were purchased from Cell Signaling Technology (Beverly, MA, USA). Secondary goat anti-mouse IgG (H&L) horseradish peroxidase (HRP) was purchased from Calbiochem (San Diego, CA, USA).

1.3 Experimental instruments

1. Autoclave: Hirayama, Saitama, Japan

2. GE ImageQuant LAS 4000: GE Healthcare Life-Sciences Ltd., Branch, Taiwan
3. Hot air oven: MEMMERT, Buchenbach, Germany
4. Humidified carbon dioxide incubator: Forma Scientific, Marietta, OH, USA
5. Inverted microscope: Axiovert 135, Zeiss, Konstanz, Germany
6. Microplate reader: Wallac 1420 Perkin-Elmer Victor 3, Perkin Elmer lnc., Waltham, Massachusetts, USA
7. Multiwell plates: Corning, New York, USA
8. NanoDrop™ 2000/2000c Spectrophotometers: Thermo Scientific, Wilmington, UK
9. OmniPAGE mini vertical systems: Cleaver Scientific, Warwickshire, UK
10. Orbital shaker: OS-20, Biosan, Riga, Latvia
11. pH meter: CG 842, Schott, Hofheim, Germany
12. Refrigerated centrifuge: Z 383K, Hermle Labortechink, Burladingen, Germany
13. Tissue culture flasks: Corning, New York, USA
14. Vortex mixer: mode K550-GE. Scientific Industries, New York, USA
15. Water bath: WB22, Memmert, Hannover, Germany

1.4 Cell culture

Human breast adenocarcinoma MCF-7 cell line was obtained from American Type Culture Collection (ATCC® HTB22™). The cells (passage between 160-180) were grown in RPMI-1640 (supplement with 10% heat-inactivated fetal bovine serum and 1% penicillin-streptomycin) in humidified 37 ℃, 5% CO_2 and 95% air. The culture medium were changed every other day. The cells were sub-cultured by trypsin-EDTA at 80% confluence.

2. Methods

2.1 MTT assay

Cell viability was determined by an MTT assay. The cells were seeded onto 96 well-plates at the density of $3.1x10^4$ cells/cm^2 and cultured for 2 days. Then, the cells were treated with metformin at various concentrations ranging from 1 mM to 20 mM for 24 and 72 hr. At the end of each treatment period, the cells were washed and further incubated with MTT reagent (0.5 mg/ml) for another 4 hr. The formazan crystals generated inside the cells were dissolved with DMSO, and measured the absorbance at 570 nm with a microplate reader (Wallac 1420 VICTOR 3, PerkinElmer Inc., USA). The concentrations of metformin that produced cell death of less than 20% were considered non-cytotoxic.

2.2 Substrate accumulation assay

The MRP2 activity was determined by a substrate accumulation assay. The cells were seeded onto 24 well-plates at 1.16×10^5 cells/cm^2, and cultured for 2 days. On the day of experiment, the cells were washed with Hank's balanced salts solution (HBSS) and incubated with metformin (at a concentration ranging from 1 to 5 mM) for 30 min. Subsequently, an MRP2 substrate 5(6)-Carboxy-2',7'-dichlorofluorescein diacetate (CDCF-DA) at a concentration of 5 μM was added into the assay system for another 30 min. In this study, indomethacin, a known MRP2 inhibitor at a concentration of 500 μM was used as a positive control group. At the end of the incubation period, cells were washed with ice-cold PBS and lysed with 0.1% Triton X-100. The fluorescent intensity of dichlorofluorescein CDCF was determined with a microplate reader (Wallac 1420 Perkin-Elmer Victor 3; Perkin Elmer Inc.) at an excitation wavelength 485 nm and emission wavelength 535 nm. The protein was quantified by BCA Protein Assay Kit (Thermo Scientific™, USA) at 570 nm.

Furthermore, changing in MRP2 function in the MCF-7 cells after prolonged treatment with metformin was also determined with a substrate accumulation assay.

In this experiment, the cells were treated with metformin (up to 5 mM) for 24 and 48 hr. At the end of each treatment period, the cells were washed 3 times prior to measurement of MRP2 activity with the uptake assay as abovementioned. The MRP2 activity was estimated by the % increase in intracellular CDCF in the presence of indomethacin (500 μM).

2.3 Reverse transcriptase polymerase chain reaction (RT-PCR) assay

The mRNA contents of ABCC2 (MRP2), MDR1 (P-gp), ABCG2 (BCRP) and beta actin were determined by an RT-PCR technique. The cells were seeded onto 6 well-plates at the density of 1.05 $x10^5$ cells/cm^2 and cultured for 2 days. On the day of experiment, the cells were lysed and extracted for total RNA using TRIzol ® reagent according to the manufacturer's instructions (Gibco Life Technologies, Grand Island, NY, USA) and kept at -80 °C. The amounts of RNA samples were quantified using a NanoDrop™ 2000/2000c spectrometer at the wavelengths 260 and 280 nm. RNA was reversely transcribed to cDNA by ImProm-II TM reverse transcription system (Promega, USA), with specific primers as follows: 5′-CAG ACA GCA GGA AAT GAA GTT GAA-3′ and 5′-ACC AAC TCA CAT CCT GTC TGA-3′ for P-gp, 5′-ACT TGT GAC ATC GGT AGC ATG C-3′ and 5′-GTG GGC GAA CTC GTT TTG-3′ for MRP2, 5′-TGA CAT TAA GGA GAA GCT GTG CTA-3′ and 5′-GAG TTG AAG GTA GTT TCG TGG ATG-3′ for beta actin and 5′-ATC CCC AGG CCT CTA TAG CT-3′ and 5′-GAG ATT GAC CAA CAG ACC ATC A-3′ for BCRP (The Gemini Singapore Science Park II, Singapore). Then, PCR amplification was conducted with the PCR conditions as follows: (1) for P-gp determination: an initial denaturing at 95°C for 5 minutes, 30 cycles of denaturing at 95°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 30 s; (2) for MRP2 and beta actin determinations: an initial denaturing at 95°C for 5 minutes, 40 cycles of denaturing at 94°C for 30 s, annealing at 60°C for 30 s and extension at 72°C for 40 s; and (3) for BCRP determination: an initial denaturing at 95°C for 5 minutes, 30 cycles of denaturing at 95°C for 30 s, annealing at 58°C for 30 s and extension at 72°C for 40 s. The PCR products were determined by gel electrophoresis (1.5% agarose) and detected by the reporter dye (SYBR) Green under a luminescence-Image analyzer (ImageQuant™ LAS 4000, GE Healthcare Bio-sciences, Japan).

Effects of metformin on mRNA levels of MRP2, BCRP and P-gp were also examined in metformin-treated cells. The cells were treated with metformin (at concentration ranging from 1 to 5 mM) for either 24 or 48 hr. Subsequently, the cells were washed and determined for mRNA via the RT PCR technique described above. In addition, an involvement of AMPK inhibitor compound C (10 μM) into the medium during the metformin treatment period (24 hr).

2.4 Western blot analysis

The amount of protein expression was determined by western blot analysis. In this study, activities of ERK1/2 and p-38 were determined from changing in their phosphorylated forms, which can be detected by the western blot technique. The cells were seeded onto 6 well-plates at the density of 1.05 x10^5 cells/cm^2 and cultured for 2 days. The cells were treated with metformin 5 mM either in the presence or absence of an AMPK inhibitor Compound C at a concentration of 10 μM for 24 hr. Then, the cells were washed with PBS three times prior to treatment with ice-cold RIPA lysis buffer (50 mM Tris-HCl pH 6.8, 150 mM NaCl, 1% Tritron-X, 0.5% sodium deoxycholate and 0.1% SDS) supplemented with protease inhibitor cocktails (1:100). The cell lysate was centrifuged at 16,000x g for 15 min at 4 °C. The precipitated portion was collected. The amount of protein in precipitated samples was quantified using BCA Protein Assay Kit and measured the absorbance spectrophotometrically at 570 nm.

Subsequently, each of protein samples (at the amount of 80 μg) was boiled in sample buffer (60 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol and0.01% bromophenol blue) at 95 °C for 5 min. After the denaturation process, the protein samples were run on a 12% SDS-polyacrylamide gel electrophoresis at the constant voltage of 100 V for 120 min, followed by electrical transfer to PVDF membrane at the constant voltage of 60 V for 120 min. Then, the membranes were blocked with 5% BSA in TBS-T (50 mM Tris-base, 150 mM NaCl and 0.05% Tween 20) for 30 min at room temperature. After the blocking process, the membranes were probed with primary antibodies for ERK1/2 (1:2000), p-ERK1/2 (1:1000), p-38 (1:2000), p-p-38 (1:1000) or beta actin (1:1000) at 4 °C overnight. Subsequently, the membranes

were washed for 7 min with TBS-T three times and incubated with corresponding HRP-conjugated secondary antibody in TBS-T for 60 min at room temperature. After incubation, the membranes were washed and developed using the Super signal West Pico chemiluminescent substrates. The membranes were visualized by a GE ImageQuant LAS 4000. The activities of ERK1/2 and p-38 were normalized to beta actin and shown as relative fold induction.

2.5 Data analysis

Data were expressed as mean ± SEM obtained from three separated experiments (n=3). Statistical analyses were performed by one-way analysis of variance (ANOVA), followed by the post-hoc Dunnett's test. Differences were considered statistically significance when $p < 0.05$.

CHAPTER IV

RESULTS

4.1 Effect of metformin on cell viability

Metformin caused cytotoxicity to the MCF-7 cells after treatment for either 24 or 72 hr in concentration dependent manner. The apparent cytotoxic concentrations of metformin in this study were greater than 10 mM at 24 hr-treatment and 5 mM at 72 hr-treatment, respectively (Figure 2).

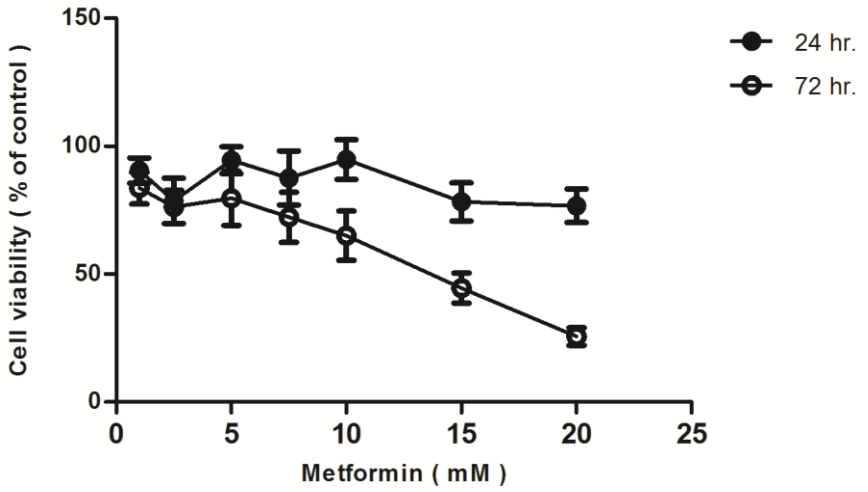


Figure 2 Cell viability after treatment with metformin (1-20 mM) for 24 and 72 hr.

4.2 Direct effect of metformin on MRP2 function

Basal MRP2 activity in MCF-7 cells was determined by the fold-increase of intracellular CDCF in the presence of MRP2 specific inhibitor. In this study, addition of an MRP2 inhibitor indomethacin (500 μM) increased intracellular CDCF accumulation by approximately 2.38 fold (Figure 3). Regarding to this, the MCF-7 cells grown under my experimental condition contained MRP2 function at appreciable level. Metformin at the concentrations upto 5 mM had no significant effect on intracellular CDCF levels,

comparing with the untreated control group (without metformin) (Figure 3). The results suggested that metformin had no inhibitory effect against MRP2 activity.

The alteration of MRP2 activities after prolonged treatment (either 24 or 48 hr) the cells with metformin was also assessed with the use of indomethacin mediated fold-increase of intracellular CDCF. As shown in Figure 4, indomethacin-mediated intracellular CDCF accumulation in the cells treatment with metformin (upto 5 mM) for 24 hr did not increase significantly, comparing to those of the control group. Upon extending the metformin treatment period to 48 hr, metformin at the non-cytotoxic concentration of 5 mM was able to cause a significant increase of intracellular CDCF accumulation in the presence of indomethacin by approximately 2.22 fold. The results suggested that metformin at high concentration (5 mM) could increase basal MRP2 activity in MCF-7 cells.

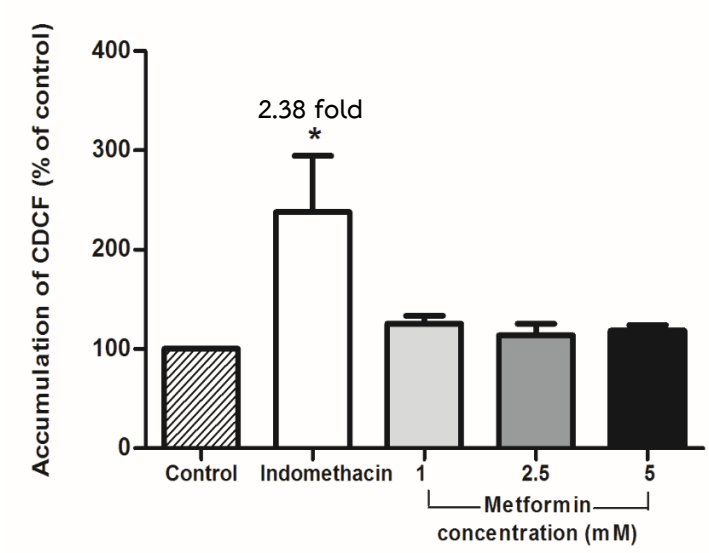
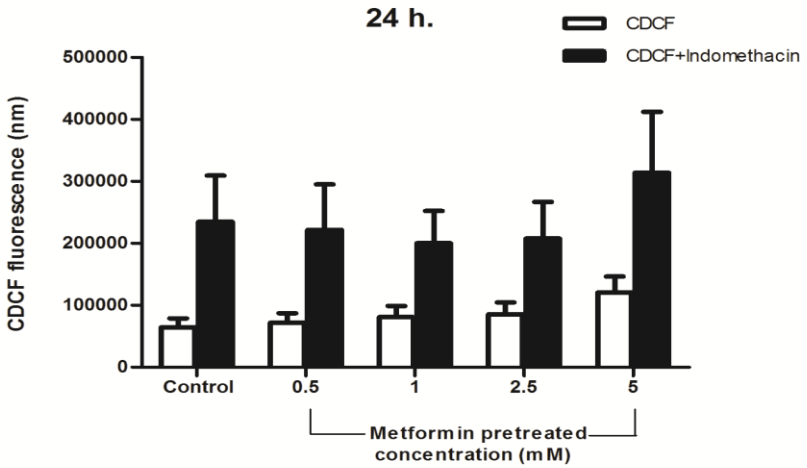


Figure 3 Effect of metformin at the concentration upto 5 mM and indomethacin (500 μM) on intracellular CDCF accumulation in the MCF-7 cells after 30 min incubation. Data were calculated and expressed as the percentage (%) of untreated control group. *$p < 0.05$ vs control (n=3 separated experiments).

A.



B.

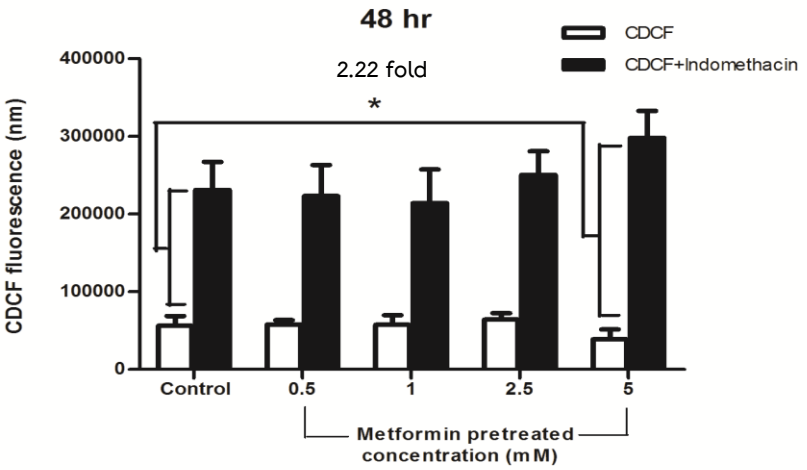


Figure 4 Basal MRP2 activity in the metformin-treated MCF-7 cells. The cells were pretreated with metformin for either 24 hr A) and 48 hr B) prior to determining intracellular CDCF accumulation in the presence and absence of indomethacin (500 μM). Significant increase in intracellular CDCF in the presence of indomethacin (500 μM) in relative to those in the absence of indomethacin indicated the basal MRP2 activity. Each bar represents the mean ± SEM obtained from 3 separated experiments (n=3). *$p < 0.05$ compared with the control group.

4.3 Effect of metformin on expression of the ABC transporters in MCF-7 cells.

Baseline mRNA levels of certain ABC transporters including MRP2, P-gp and BCRP in specific subtypes of MCF-7 cells were examined as follows: MRP2 mRNA in parental MCF-7 cells (wildtype; MCF-7/WT), P-gp mRNA in doxorubicin resistant MCF-7 cells (MCF-7/dox cells) and BCRP mRNA in mitoxantrone resistant cells (MCF-7/MX cells). The mRNA extents of these specific ABC transporters were clearly shown in Figure 5.

As shown in Figure 6 and 7 treatment the cells with metformin either for 24 or 48 hr reduced MRP2 mRNA in concentration dependent manner. This suppressive effect was statistically significant at the concentrations of 2.5 and 5 mM for 24 hr-treatment period. Upon extending the treatment period to 48 hr, metformin at the concentration of 1 mM was able to reduce MRP2 mRNA significantly (Figure 7).

Furthermore, the effects of metformin on P-gp mRNA and BCRP mRNA levels were further examined in MCF-7/dox cells and MCF-7/MX cells, respectively. As shown in Figure 8 and 9, metformin (5 mM) significantly decreased the relative amount of P-gp mRNA in MCF-7/dox cells, but not BCRP mRNA in MCF-7/MX cells after 24 hr treatment.

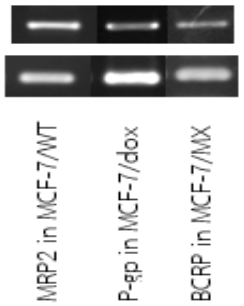
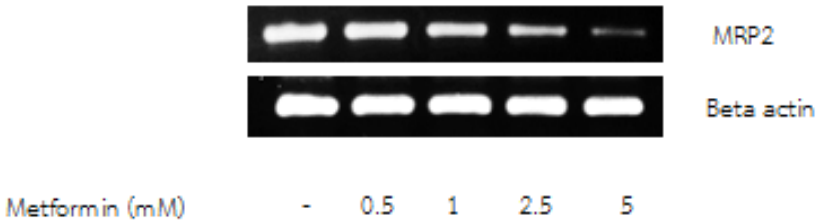


Figure 5 Basal mRNA level of MRP2 in MCF-7 cells, P-gp in MCF-7/dox cells and BCRP in MCF-7/MX cells.

A.



B.

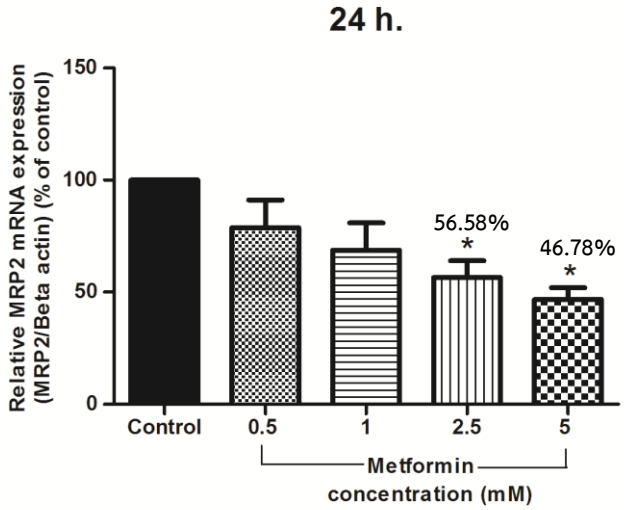


Figure 6 Relative MRP2 mRNA level in the metformin treated MCF-7 cells for 24 hr. A) Representative profile of MRP2 mRNA. B) Bar graph represents the mean ± SEM (n=3) of MRP2 mRNA level after normalized with beta actin mRNA (MRP2/beta actin mRNA). Data are expressed as the percentage of the control group. *$p < 0.05$ indicated statistically significant difference from the control group.

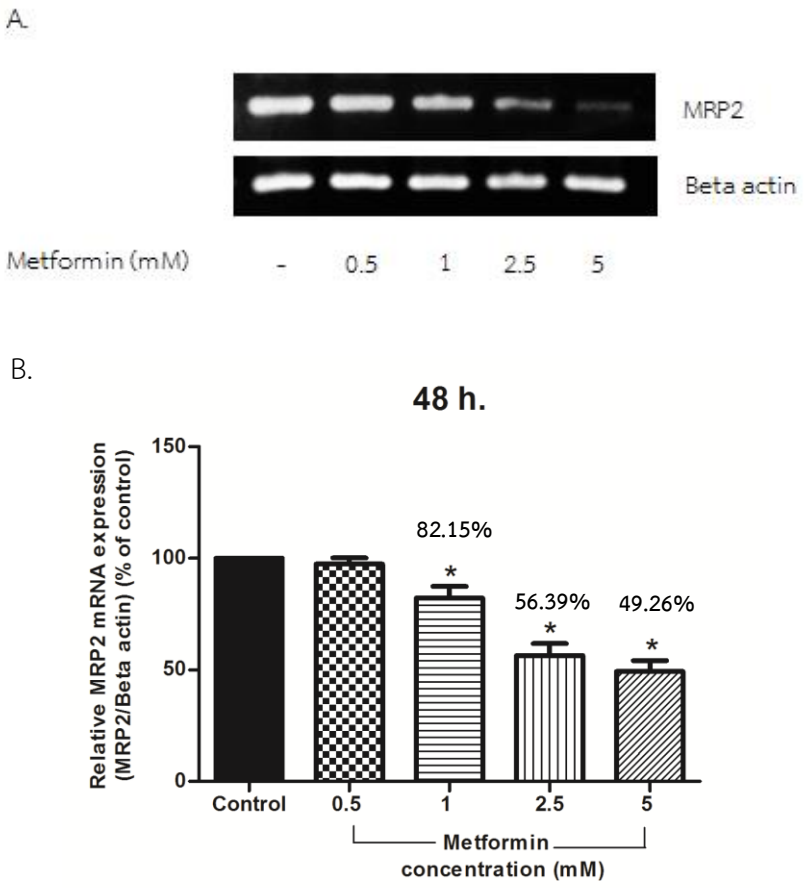
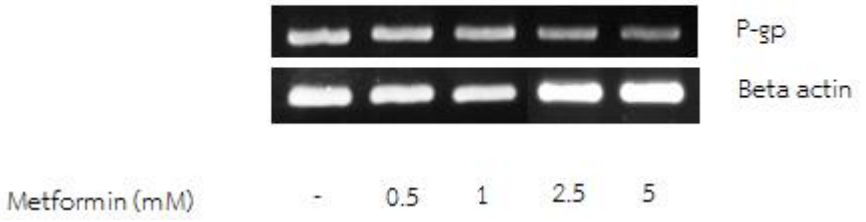


Figure 7 Relative MRP2 mRNA level in the metformin treated MCF-7 cells for 48 hr. A) Representative profile of MRP2 mRNA. B) Bar graph represents the mean ± SEM (n=3) of MRP2 mRNA level after normalized with beta actin mRNA (MRP2/beta actin mRNA). Data are expressed as the percentage of the control group. *$p < 0.05$ indicated statistically significant difference from the control group.

A



B.

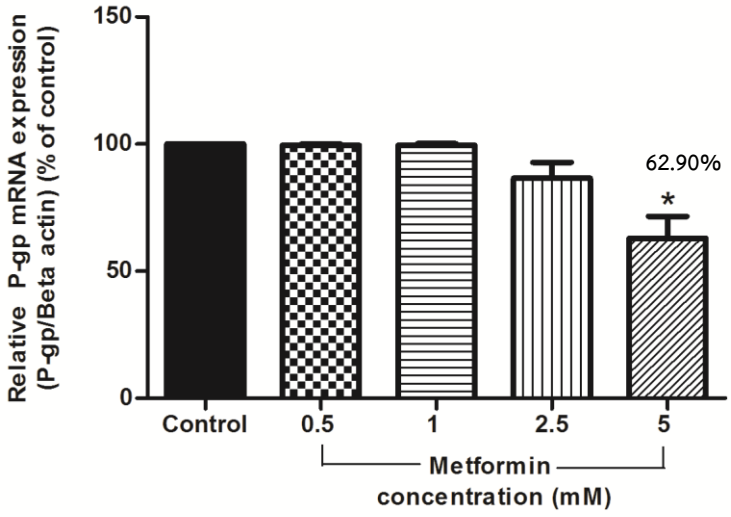


Figure 8 Relative P-gp mRNA level in the metformin treated MCF-7/dox cells for 24 hr. A) Representative profile of P-gp mRNA. B) Bar graph represents the mean ± SEM (n=3) of P-gp mRNA level after normalized with beta actin mRNA (P-gp/beta actin mRNA). Data are expressed as the percentage of the control group. *$p < 0.05$ indicated statistically significant difference from the control group.

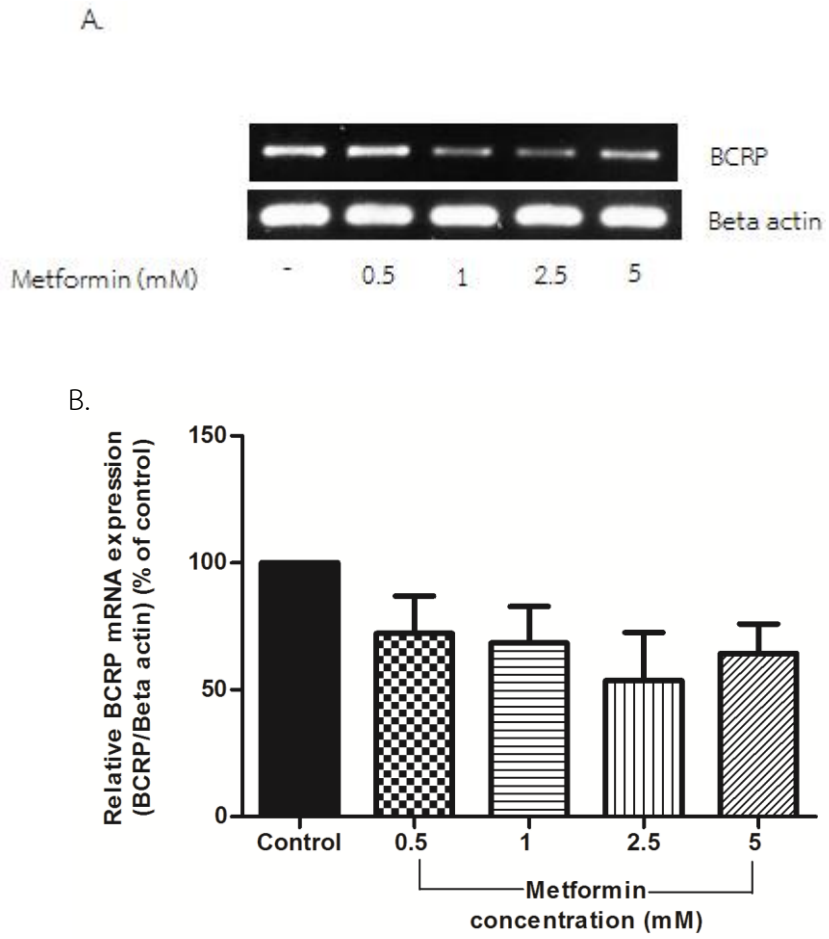


Figure 9 Relative BCRP mRNA level in the metformin treated MCF-7/MX cells for 24 hr. A) Representative profile of BCRP mRNA. B) Bar graph represents the mean ± SEM (n=3) of BCRP mRNA level after normalized with beta actin mRNA (BCRP/beta actin mRNA). Data are expressed as the percentage of the control group. *$p < 0.05$ indicated statistically significant difference from the control group.

4.4 An involvement of the AMP-activated protein kinase (AMPK) pathway in metformin-mediated reduction of MRP2 mRNA expression in MCF-7 cells.

An involvement of AMPK pathway in metformin-mediated reduction of MRP2 mRNA levels in MCF-7 cells was further investigated. In this study, an AMPK activator AICAR was able to reduce MRP2 mRNA level in the MCF-7 cells after 24-hr treatment. However, extents of MRP2 mRNA in the cells treated with AICAR and compound C were comparable to those in the untreated control group (Figure 10). These results suggested that an activation of AMPK could result in down-regulation of MRP2 at transcription level. Treatment the cells with metformin (5 mM) for 24 hr significantly reduced MRP2 mRNA level (Figure 9). Addition of compound C in the culture medium could prevent the suppressive action of metformin on MRP2 mRNA expression. The MRP2 mRNA extents in the metformin-treated MCF-7 cells increased significantly from 33.22 % to 75.18 % (by approximately 2.26 fold) in the presence of compound C (Figure 10). These findings suggested that metformin might decrease MRP2 mRNA expression at the transcription level through activation of AMPK pathway.

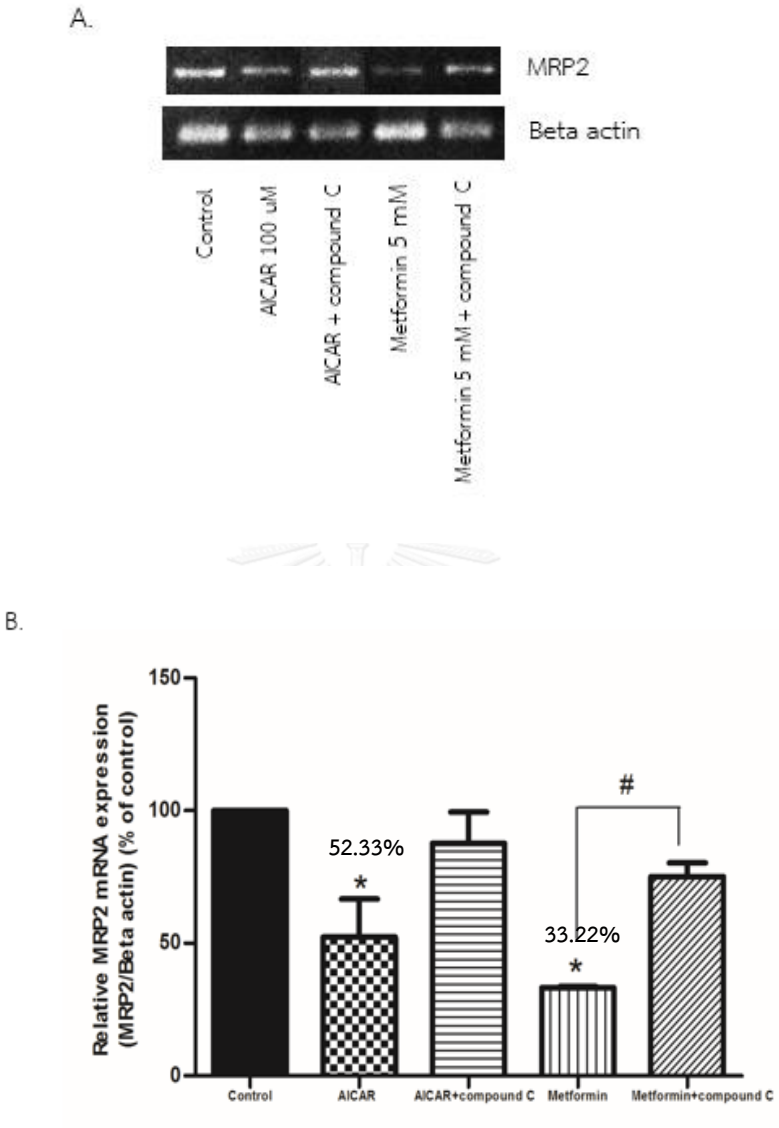


Figure 10 Relative MRP2 mRNA level in the MCF-7 cells treated with metformin for 24 hr. A) Representative profile of MRP2 mRNA. B) Bar graph represents the mean ± SEM (n=3) of MRP2 mRNA level after normalized with beta actin mRNA (MRP2/beta actin mRNA). Data are expressed as the percentage of the control group. *$p < 0.05$ indicated statistically significant difference from the control group; #$p < 0.05$ indicated statistically significant difference between treatment in the presence and absence of compound C.

Furthermore, the inter-relationship between AMPK and MAPK pathways was also investigated in this study. Activation of MAPK family including ERK and p38 has been related to an increased expression of MRP2 mRNA. In this study, compound C caused higher phosphorylated p38 protein (p-p38) level, as compared with that of the control group. However, it had no effect on ERK activation (Figure 11). Treatment the MCF-7 cells with metformin (5 mM) for 24 hr reduced the phosphorylated form of ERK and p38, as compared with those of the untreated control groups. Moreover, compound C could prevent the suppressive effect of metformin on either ERK or p38 activation (Figure 12).

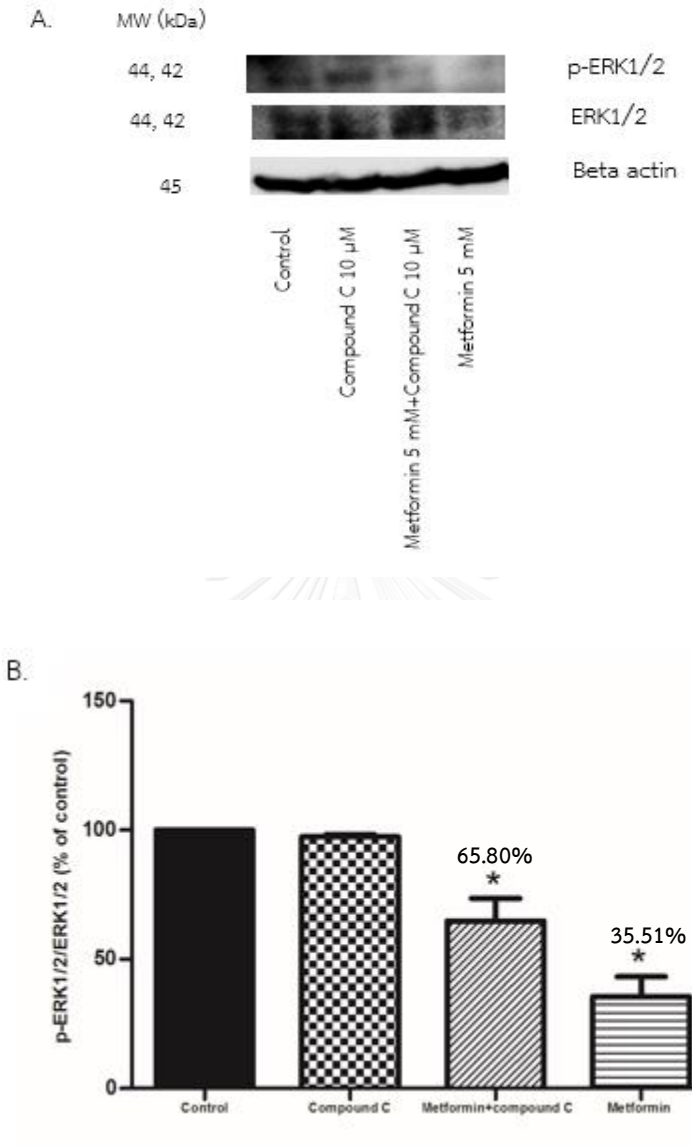


Figure 11 Effects of metformin on the expression of ERK1/2 and its phosphorylated form (p-ERK1/2) in MCF-7 cells. (A) Immunoblots of ERK and phosphorylated ERK. (B) Densitometrical analysis of immunoblots calculated as the ratio of phosphorylated ERK to ERK. Each bar represents the mean ± SEM obtained from separated experiments (n=3). *$p < 0.05$ compared with the control group.

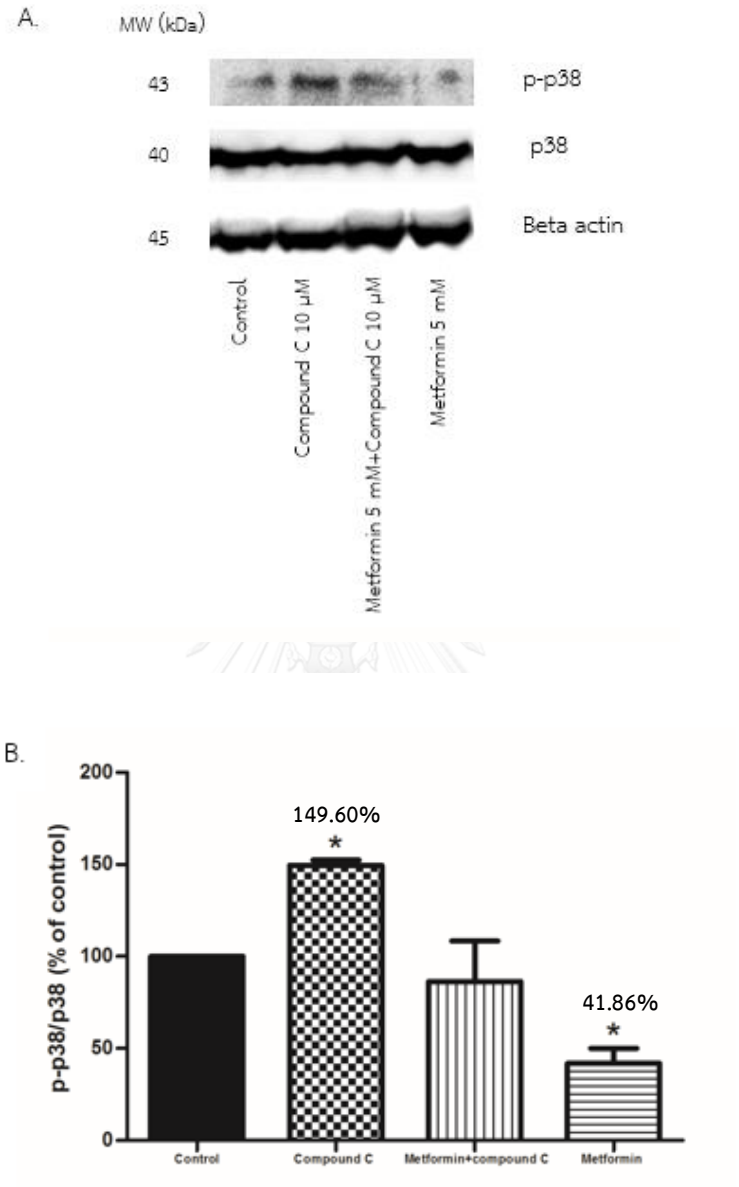


Figure 12 Effects of metformin on the expression of p38 and its phosphorylated form (p-p38) in MCF-7 cells. (A) Immunoblots of p38 and phosphorylated p38. (B) Densitometrical analysis of immunoblots calculated as the ratio of phosphorylated p38 to p38. Each bar represents the mean ± SEM obtained from separated experiments (n=3). *$p < 0.05$ compared with the control group

CHAPTER V
DISCUSSION

Metformin is a biguanide anti-diabetic drug with cytotoxic property against various cancer cell lines such as human pancreatic cancer cells (Bao et al., 2012), thyroid cancer cells (Chen et al., 2012), human glioblastoma multiform cells (Ucbek et al., 2014), prostate cancer cells (Ben et al., 2008) and colon cancer cells (Buzzai et al., 2007). Expression of the ABC efflux transporters in particular P-gp and MRP2 may cause MDR in cancer cells. It would be interesting whether metformin could overcome MDR by interfering these drug efflux pumps. In this study, effects of metformin on function and expression of MRP2 transporter were determined in the living breast cancer MCF-7 cells. Hence, it was crucial to select the non-cytotoxic concentrations of metformin for conducting transporter experiments. In this study, the concentrations that produced cell death of less than 20% were considered non-cytotoxic. The cytotoxicity of metformin toward MCF-7 cells was concentration-and time- dependent. The maximal non-cytotoxic concentrations of metformin in the MCF-7 cells were 10 mM at 24 hr-treatment and 5 mM at 72 hr-treatment.

Apparently, metformin had no direct interfering effect on MRP2 activity. However, the result from the functional studies of MRP2 suggested that metformin (at 5 mM) might be able to increase MRP2 function after prolonged 48 hr treatment. It could be hypothesized that an increased activity involved with up-regulation of mRNA or protein levels. However, there were reports that metformin (1-10 mM) decreased P-gp activities in MCF-7/dox cells after 2 days treatment.

Although P-gp and MRP2 belong to the superfamily of ABC transporters, they are encoded by different gene. P-gp is encoded by MDR1 gene whereas MRP2 is an ABCC2 gene product. These two transporters may be under different gene regulation pathway. Changing in mRNA or protein expression of each transporter in response to chemical treatment could vary and not in agreeable direction. It was reported that the extent of P-gp mRNA in hepatocytes isolated from lipopolysaccharide-treated rats

increased whereas the extent of MRP2 mRNA decreased (Vos et al., 1998). Hence, it might be anticipated that metformin had different effects on P-gp and MRP2 expression.

High expression of drug efflux transporters including P-gp and MRP2 has been widely accepted as a mechanism responsible for multidrug-resistance (MDR) in cancer, leading to chemotherapeutic failure (Baguley, 2010). Although P-gp is the most studied efflux transporter in MDR phenomenon, expression of MRP2 has been linked to drug resistance in various cancer cells such as human colorectal carcinoma and breast cancer cell line (Hinoshita et al., 2000; Choi et al., 2007). It was demonstrated that MRP2 overexpression caused cisplatin-resistance in human colorectal carcinoma (Hinoshita et al., 2000), and tamoxifen-resistance in breast cancer cells (Choi et al., 2007). Any compounds with abilities to suppress MRP2 function and/or expression might also be useful in reversing MDR in cancer.

Based on the RT-PCR analysis, human breast cancer MCF-7 cell line used in this study expressed high level of MRP2 mRNA. Moreover, the baseline MRP2 activity in the wildtype MCF-7 cells was at appreciable level as suggested by 3.5 fold-increase of intracellular CDCF in the presence of an MRP2 inhibitor indomethacin (500 μM). In addition to the wildtype MCF-7 cell line, the acquired MDR/MCF-7 cells types were also used in this study. They were doxorubicin resistant MCF-7 cells (MCF-7/dox) which expressed high P-gp level and mitoxantrone resistant MCF-7 cells (MCF-7/MX) which expressed high BCRP level. In this study, metformin was able to reduce mRNA extents of MRP2 and P-gp in breast cancer cells (MCF-7; MCF-7/dox) after 1 day treatment. In addition, the effect of metformin on MRP2 expression was apparently greater than that on P-gp expression in MCF-7/dox. After 24 hr treatment, metformin at the concentration of 2.5 mM significantly suppressed MRP2 mRNA level by 56.58%, but not P-gp mRNA level. The effect of metformin on P-gp mRNA level could be observed at the concentration of 5 mM after 24 hr treatment. Moreover, the suppressive effect

of metformin on MRP2 expression at transcription level was concentration- and time-dependent. Furthermore, the down-regulation effect of metformin on BCRP mRNA level in MCF-7/MX cells was not statistically significant.

Mechanisms involving gene regulation of the ABC transporters superfamily have been reported. For example, mollugin decreased P-gp expression at the transcription and translation processes in doxorubicin resistant breast cancer cell lines through AMPK-mediated repression of NF-kB and CREB (Tran et al., 2013). In addition, vincristine could activate the PI3K/Akt pathway in lymphoma cell lines, resulting in down-regulation of P-gp expression (Garcia et al., 2009). Expression of MRP2 could be associated with survival signaling pathways including ERK and p-38 in MAPK signaling pathways (Dhillon et al., 2007). It was reported that IL-1β decreased MRP2 mRNA through inhibit ERK, leading to disruption of IRF3 binding to ISRE on the MRP2 promoter in human hepatoblastoma cells (Hisaeda et al., 2004). Moreover, sodium arsenic could increase MRP2 expression of mRNA and protein in both primary rat hepatocyte and normal human hepatocyte via JNK in the MAPK pathway (Vernhet et al., 2001). Hence, it was likely that inhibition of MAPK pathway was able to down-regulate mRNA and protein of MRP2.

The relationship between AMPK and MAPK signaling pathways has been demonstrated. Metformin inhibits mitochondrial respiratory complex I, leading to activates AMP-activated protein kinase (AMPK) (Viollet et al., 2012). Activation of AMPK has been linked to inhibition of the mammalian target of rapamycin (mTOR) pathway as well as MAPK pathway (Zoncu et al., 2011; Tosca et al., 2010). Inhibition of mTOR pathway can alter mRNA translation and protein synthesis, which possibly leads to inhibition of cancer cell growth (Zoncu et al., 2011). It has been reported that activation of AMPK pathway leads to suppression of MAPK activities in several cells such as skeletal muscle (Hwang et al., 2013), sensory neurons (Tillu et al., 2012) and bovine granulosa cells (Tosca et al., 2010).

In this study, mechanisms of metformin-mediated down-regulation of MRP2 were investigated in the human breast cancer cell line MCF-7. The results suggested that the down-regulation effect of metformin at transcription level involved AMPK pathway. Treatment the MCF-7 cells with an AMPK activator AICAR (100 μM) for 24 hr caused significant reduction of MRP2 mRNA level. The presence of an AMPK inhibitor compound C (10 μM) could prevent the AICAR-mediated decrease of MRP2 mRNA. The results further demonstrated that compound C could prevent the reduction of MRP2 mRNA in the cells treated with metformin (5 mM). Furthermore, metformin (5 mM) after 24 hr-treatment significantly reduced phosphorylated ERK and p38, suggesting the inhibitory action of metformin on basal MAPK activities. The presence of compound C could prevent the suppressive effect of metformin on both phosphorylated ERK and p38. Taken together, it was likely that metformin could decrease MRP2 mRNA expression at the transcription level through activation of AMPK pathway and suppression of ERK and p38 activities.

Moreover, the MCF-7 cells treated with metformin (5 mM) for 48 hr apparently had an increased MRP2 activity, despite reduction of mRNA level. It was possible that changing in extents of mRNA and protein levels might not be always correlated (Pascal et al., 2008; Maier et al., 2009). It has been reported that no significant correlation between mRNA and protein expression in lung adenocarcinomas (Chen et al., 2002). Further determination of MRP2 protein level should be pursued. Furthermore, activation of AMPK has been linked to an increase of Na^+, K^+ ATPase activity in skeletal muscle cells (Benziane et al., 2012). The inductive effect of metformin, a known AMPK activator, on MRP2 functionality via increasing ATPase activity should be also studied in the future.

In conclusion, metformin had no direct effect on MRP2 activity. However, prolonged exposure of the MCF-7 cells with metformin for 24-48 hr could result in down-regulation of MRP2 mRNA, possibly through AMPK mediated inhibition of MAPK signaling pathway.

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Poster Presentation

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