The effect of N-*trans-p*-coumaroyl tyramine on indomethacin-/diclofenac-mediated-cytotoxicity in breast cancer cells.

Miss Angkana Wongsakul

A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy in Pharmacology
Inter-Department of Pharmacology
GRADUATE SCHOOL
Chulalongkorn University
Academic Year 2021

ผลของ N-*trans-p*-coumaroyl tyramine ต่อความเป็นพิษของอินโดเมทาซินหรือไดโคลฟีแนคในเซลล์มะเร็งเต้านม

น.ส.อังคณา วงษ์สกุล

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
สาขาวิชาเภสัชวิทยา (สหสาขาวิชา) สหสาขาวิชาเภสัชวิทยา
บัณฑิตวิทยาลัย จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2564

Thesis Title	The effect of N-*trans-p*-coumaroyl tyramine on indomethacin-/diclofenac-mediated-cytotoxicity in breast cancer cells.
By	Miss Angkana Wongsakul
Field of Study	Pharmacology
Thesis Advisor	Associate Professor SUREE JIANMONGKOL, Ph.D.

Accepted by the GRADUATE SCHOOL, Chulalongkorn University in Partial Fulfillment of the Requirement for the Doctor of Philosophy

..... Dean of the GRADUATE SCHOOL
(Associate Professor YOOTTHANA CHUPPUNNARAT, Ph.D.)

DISSERTATION COMMITTEE

..... Chairman
(Assistant Professor Frist Lieutenant NAOWARAT SUTHAMNATPONG, D.V.M., Ph.D.)

..... Thesis Advisor
(Associate Professor SUREE JIANMONGKOL, Ph.D.)

..... Examiner
(Associate Professor PIYANUCH WONGANAN, Ph.D.)

..... Examiner
(Associate Professor VARISA PONGRAKHANANON, Ph.D.)

..... External Examiner
(Associate Professor Pornpun Vivithanaporn, Ph.D.)

อังคณา วงษ์สกุล : ผลของ N-*trans-p*-coumaroyl tyramine ต่อความเป็นพิษของอินโดเมทาซินหรือไดโคลฟีแนคในเซลล์มะเร็งเต้านม. (The effect of N-*trans-p*-coumaroyl tyramine on indomethacin-/diclofenac-mediated-cytotoxicity in breast cancer cells.) อ.ที่ปรึกษาหลัก : รศ. ภญ.ดร.สุรีย์ เจียรณ์มงคล

ยาอินโดเมทาซิน (INDO) และ ไดโคลฟีแนค (DIC) คือ ยาต้านการอักเสบที่ไม่ใช่สเตียรอยด์ (NSAIDs) ได้ถูกรายงานว่ามีศักยภาพในการต้านมะเร็ง ความเป็นพิษต่อเซลล์ของยาอินโดเมทาซินและไดโคลฟีแนคอาจเพิ่มขึ้นเมื่อรวมกับสารธรรมชาติบางชนิด เช่น piperine ที่อยู่ในพืชตระกูลพริกไทย นอกจากนี้ยังพบ N-*trans-p*-coumaroyltyramine (TCT) (อนุพันธ์ของกรดคูมาริก) จำนวนมากในพืชชนิดนี้และพืชชนิดอื่น ๆ ที่ใช้กันทั่วไปในการรักษาแผนโบราณ ในการศึกษานี้เพื่อประเมินศักยภาพของ TCT ต่อ NSAIDs (INDO และ DIC) ที่เหนี่ยวนำความเป็นพิษในเซลล์มะเร็งเต้านม MCF-7 และกลไกที่เกี่ยวข้อง การรวมกันของ TCT กับ NSAIDs หรือ ER stressors ที่ความเข้มข้นไม่เป็นพิษต่อเซลล์สามารถลดการมีชีวิตของเซลล์ MCF-7 และเซลล์ MCF-7 ที่ดื้อต่อยาไมโตแซนโทรน หลังจากให้เป็นเวลา 48 ชั่วโมง ในการศึกษานี้เซลล์ MCF-7 ที่ดื้อต่อยาไมโตแซนโทรน มีการแสดงออกของตัวขนส่งสารออกนอกเซลล์ (BCRP) ที่สูงกว่าเซลล์ MCF-7 ผลการศึกษาได้แสดงว่า TCT ไม่มีผลในการยับยั้งการทำงานของตัวขนส่งสารออกนอกเซลล์ ซึ่งชี้ให้เห็นว่าศักยภาพของ TCT ในการเพิ่มความเป็นพิษต่อเซลล์ไม่ได้ขึ้นกับตัวขนส่งสารออกนอกเซลล์ นอกจากนี้การรวมกันของ TCT กับ NSAIDs หรือ ER stressorsสามารถลด mitochondria membrane potential (MMP) ได้อย่างมีนัยสำคัญ และในทำนองเดียวกันพบว่าสัดส่วนของ Bax/BcL-2 และการตายของเซลล์แบบอะพอพโทซิสในเซลล์ MCF-7 ทั้งสองชนิดเพิ่มขึ้น นอกจากนี้การรวมกันของ TCT กับ NSAIDs หรือ ER stressors จะส่งผลต่อการลดการแสดงออกของโปรตีนที่เกี่ยวข้องกับการอยู่รอดของเซลล์ p-Nrf-2/HO-1 และเพิ่มการกระตุ้นวิถีสัญญาณความเครียดของเอนโดพลาสมิคเรติคูลัม PERK/eIF-2a/ATF-4/CHOP ดังนั้นการศึกษานี้สรุปว่า TCT สามารถเพิ่มความเป็นพิษของยาอินโดเมทาซินหรือไดโคลฟีแนคของเซลล์ MCF-7 โดยการกระตุ้นการตายของเซลล์แบบอะพอพโทซิสที่เกี่ยวข้องกับความเครียดของเอนโดพลาส มิคเรติคูลัมในเซลล์มะเร็งเต้านม MCF-7

สาขาวิชา	เภสัชวิทยา (สหสาขาวิชา)	ลายมือชื่อนิสิต .....
ปีการศึกษา	2564	ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

5987812920 : MAJOR PHARMACOLOGY

KEYWORD: N-trans-p-Coumaroyltyramine; NSAIDs; ER stress; Apoptosis; Potentiation; Breast cancer cells

Angkana Wongsakul : The effect of N-*trans-p*-coumaroyl tyramine on indomethacin-/diclofenac-mediated-cytotoxicity in breast cancer cells.. Advisor: Assoc. Prof. SUREE JIANMONGKOL, Ph.D.

Indomethacin (INDO) and diclofenac (DIC) are non-steroidal anti-inflammatory (NSAIDs) have been reported their anticancer potential. Their cytotoxicity could be increased when combining with certain natural compounds such as piperine in *Piper* plants. N-*trans-p*-coumaroyltyramine (TCT) (a coumaric acid derivative) was also found at significant amount in these plants and other commonly used in traditional medicine. This study determined the potentiative effect of TCT on NSAIDs (INDO and DIC) induced cytotoxicity in MCF-7 breast cancer cells, and the underlying mechanisms. Combination of TCT with either NSAIDs or various ER stressors at their non-cytotoxic concentrations were able to reduce cell viability in MCF-7 and mitoxantrone-resistant MCF-7/MX cells after 48 h treatment. In this study, MCF-7/MX cells expressed higher activities of efflux transporter (BCRP) than the parental MCF-7 cells. The results showed that TCT had no inhibitory effect on their activities, suggesting that the potentiation was independent of transporters. Evidently, combination of TCT with either NSAIDs or ER stressors significantly reduced mitochondria membrane potential (MMP) in concurrent with the rising of Bax/BcL-2 ratio and apoptosis in both MCF-7 cell types. Furthermore, the combination also resulted in decreased expression of survival proteins p-Nrf-2/HO-1, and increased activation of the endoplasmic reticulum (ER) stress signaling PERK/eIF-2a/ATF-4/CHOP pathways. In conclusion, TCT could increase INDO or DIC cytotoxicity of MCF-7 cells by promoting ER stress-dependent apoptosis in MCF-7 breast cancer cells.

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

Academic Year: 2021 Advisor's Signature .....

ACKNOWLEDGEMENTS

I would like to express the deepest appreciation to my thesis advisor, Associate Professor Suree Jianmongkol, Ph.D. for their invaluable advice, attention, motivation, encouragement and patience throughout this study.

I would like also to thank Associate Professor Rutt Suttisri, Ph.D., Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Chulalongkorn University for providing N-tran-p-coumaroyltyramine.

I would like to thank Chulalongkorn University Drugs and Health Products Innovation Promotion Center and Pharmaceutical Research Instruments, Faculty of Pharmaceutical science for providing facilities.

This work was supported by the CU Graduate School Thesis Grant of Chulalongkorn University

I am grateful to all staff members of the Department of Pharmacology, Pharmaceutical science for their help. The special thanks are also extended to all my friends for their suggestion, encouragement and assistance.

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

Angkana Wongsakul

TABLE OF CONTENTS

LIST OF TABLES

Page

LIST OF FIGURES

Page

LIST OF ABBREVIATIONS

ABC	= Adenosine triphosphate binding cassette
ANOVA	= Analysis of variance
ATCC	= American Type Culture Collection
ATP	= Adenosine triphosphate
ATF-4	= Activating transcription factor-4
ATF-6	= Activating transcription factor-6
Bax	= BCL2-associated X
Bcl-2	= B-cell lymphoma 2
Bip	= Binding of immunoglobulin protein
BSA	= Bovine serum albumin
°C	= degree Celsius
Calcein-AM	= Calcein acetoxymethyl ester
CDCF	= Carboxy-dichlorofluorescein
CDCFH-DA	= 2′,7′-Dichlorofluorescin diacetate
CHOP	= C/EBP homologous transcription factor
CO_2	= Carbon dioxide
CPT	= Camptothecin
CsA	= Cyclosporine A
DIC	= Diclofenac
EDTA	= Ethylenediaminetetraacetic acid
eIF-2α	= eukaryotic Initiation factor-2α
ER	= Endoplasmic reticulum
FBS	= Fetal bovine serum
GAPDH	= Glyceraldehyde 3-phosphate dehydrogenase
h	= hour

HBSS	= Hanks' balanced salt
H_2O_2	= Hydrogen peroxide
HO-1	= Heme oxygenase-1
IC_{50}	= Inhibitory Concentration 50
INDO	= Indomethacin
IRE-1α	= Inositol-requiring enzymes-1α
JNK1/2	= c-Jun N-terminal kinases1/2
MCF-7	= Human breast adenocarcinoma cell line
MCF-7/MX	= Human breast adenocarcinoma mitoxantrone resistant cell line
MDR	= Multidrug Resistance
mg	= milligram
ml	= milliliter
mM	= millimolar
MMP	= Mitochondrial membrane
MTT	= 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide
MX	= Mitoxantrone
Na_3VO_4	= Sodium orthovanadate
NaCl	= Sodium chloride
NaF	= Sodium fluoride
Nrf-2	= Nuclear factor erythroid 2-related factor 2
NSAIDs	= Nonsteroidal anti-inflammatory drugs
p	= phosphorylation
PBS	= Phosphate buffered saline
PERK	= Pancreatic-like ER kinase
P-gp	= P-glycoprotein

PIP	= Piperine
PMSF	= Phenylmethylsulfonyl fluoride
PVDF	= Polyvinylidine fluoride
ROS	= Reactive oxygen species
ROT	= Rotenone
RPMI-1640	= Roswell Park Memorial Institute -1640 medium
SDS	= Sodium dodecyl sulfate
SEM	= Standard Error of Mean
TAM	= Tamoxifen
TCT	= N-*trans-p*-coumaroyltyramine
TBS-T	= Tris Buffered Saline - Tween 20
TGN	= Thapsigargin
TMRE	= Tetramethylrhodamine
Tris-HCl	= Tris hydrochloride
TUN	= Tunicamycin
V	= Voltage
VBL	= Vinblastine
µg	= microgram
µl	= microliter
µM	= micromolar

CHAPTER I
INTRODUCTION

1.1 Background and rationale

High dose of anticancer drugs often causes unendurable adverse effects to the breast cancer patients which lead to failure cancer treatment (Nurgali et al., 2018; Wang et al., 2019). Addition of pharmacoenhancers to anticancer regimens is one approach to boost therapeutic outcomes in cancer treatment without titrating up the dose of anticancer drugs may not be needed, and patients can consequently better tolerate chemotherapy. Several natural compounds such as flavonoids, naphthoquinone, terpenes, and alkaloids have been reported their ability to enhance cytotoxicity of anticancer agents through various mechanism such as inhibition of efflux transporters, increased cellular stress and induction of apoptosis (Chaisit et al., 2017; T. Chen et al., 2019). For example, a naphthoquinone rhinacanthin-C at its non-cytotoxic concentrations (0.04 or 0.1 μM) significantly enhanced doxorubicin-mediated cytotoxicity by 38 fold in MCF-7 cell after 48 h treatment, possibly through inhibition of MRP2 and P-gp activities (Chaisit et al., 2017). Treatment colorectal cancer HCT116 and LoVo cells with piperlongumine (2 μM) significantly enhanced oxaliplatin (10 μM) mediated cytotoxicity by 2-fold after 1 h treatment through ER stress-mediated apoptotic cell death (T. Chen et al., 2019).

Indomethacin (INDO) and diclofenac (DIC) are non-steroidal anti-inflammatory drugs (NSAIDs) known mainly for pain relief via inhibition of cyclooxygenase. (Poku et al., 2020; Zhang et al., 2018). It has shown their anticancer activities both cancer cell model and animal model (Boonyong et al., 2020a, b; Feng et al., 2016; Rana et al., 2015; Wong, 2019). The underlying mechanisms of INDO/DIC induced cytotoxicity has been linked to increase production of reactive oxygen species (ROS) and accumulation of unfolded proteins in endoplasmic reticulum (ER stress) (Kim et al.;

Mazumder et al., 2019; Wang et al., 2018; Zeeshan et al., 2016). As known high levels of accumulated unfolded protein exceeding UPR results in activation of three signaling proteins sensors are PERK (protein kinase RNA (PKR)-like ER kinase), IRE-1α (inositol-requiring protein-1α), and ATF-6 (activating transcription factor). It has been reported PERK and IRE-1α are initiate ER stress sensors and related to apoptotic cell death (Bhattarai et al., 2021; Hetz and Papa, 2018; Ishiwata-Kimata et al., 2018; Rellmann et al., 2021). Several studies have been reported that both INDO and DIC were able to activation of PERK and IRE-1α signaling pathways in various cancer cells which leading to cell death such as Caco-2 cells, BT-474 cells, and lung A549 cells (Boonyong et al., 2020a; Guo et al., 2015; Park et al., 2018). Moreover, using NSAIDs in combination with certain phytochemicals such as resveratrol, curcumin, piperine or naringenin effectively increased cancer cell death both *in vitro* and *in vivo* (Boonyong et al., 2020a, b; Feng et al., 2016; Rana et al., 2015; Redondo-Blanco et al., 2017).

In addition to promoting stress-mediated cell death, the chemo-sensitizing effects of phytochemicals may be correlated to their interference with the activities of the ATP binding cassette (ABC) drug efflux transporters, particularly P-glycoprotein (P-gp), multi-drug resistance proteins 1 (MRP1) and 2 (MRP2), and breast cancer resistance protein (BCRP) (Dobiasova et al., 2020; Li et al., 2016; Tinoush et al., 2020). The suppression of drug efflux pumps could increase the accumulation of their cytotoxic drug substrates to effective levels within the target cells. For examples, the natural chemo-sensitizers are alkaloids (e.g. lobeline, stemofoline), flavonoids (e.g. naringenin), and polyphenols (e.g. resveratrol, rhinacanthin-C) (Chaisit et al., 2017; Cocetta et al., 2021; Iriti et al., 2017; Umsumarng et al., 2017; Ye et al., 2019).

N-*trans-p*-coumaroyltyramine (TCT) is a coumaric acid derivative with an amide found in the stems and fruits of *Piper wallichii* (Miq.) Hand.-Mazz (family Piperaceae). *P. wallichii* have been used medicinally in the treatment of gastro-intestinal, inflammatory and circulatory diseases in China, India and Thailand (Daorueang, 2020; Salehi et al.). Although this compound can also be found as a constituent of several other plants, few reported of its pharmacological activities have been done. TCT was reported as displaying anti-trypanosomal activity and inhibitory action against acetylcholinesterase and α-glucosidase (Kim and Lee; Moradi-Afrapoli et al., 2012; Nishioka et al., 2014). It has been reported that amide alkaloid from *Piper* plants such as piperine and piperlongumine were capable of potentiating the cytotoxicity of oxaliplatin and placlitaxel through mechanism involving ROS-mediated ER stress and inhibition of drug efflux transporters (W. Chen et al., 2019; Rawat et al., 2020; Syed et al., 2017; Yaffe et al., 2015). Regarding this, we studied the chemo-enhancing potential of TCT on NSAIDs-mediated cytotoxicity in cancel models. The information on the potentiation effect and the underlying mechanisms of the TCT and NSAIDs (INDO and DIC) combination have not been demonstrated.

In this study, we demonstrated for the first time that TCT was able to enhance the ER stress-associated apoptosis induced by INDO or DIC in MCF-7 breast cancer cells and mitoxantrone-resistant MCF-7 cells (MCF-7/MX). The potentiation mechanisms were mainly related to ER stress induction via activation of PERK and IRE-1α signaling pathways. In addition, drug transporter dependent mechanism of cytotoxic potentiation is also evaluated.

1.2 Hypothesis

TCT could enhance INDO- and DIC-cytotoxicity in breast cancer MCF-7 cells and the underlying mechanism of TCT may involve ER stress induction which leading to apoptotic cell death.

1.3 Objectives

The objectives of this work were

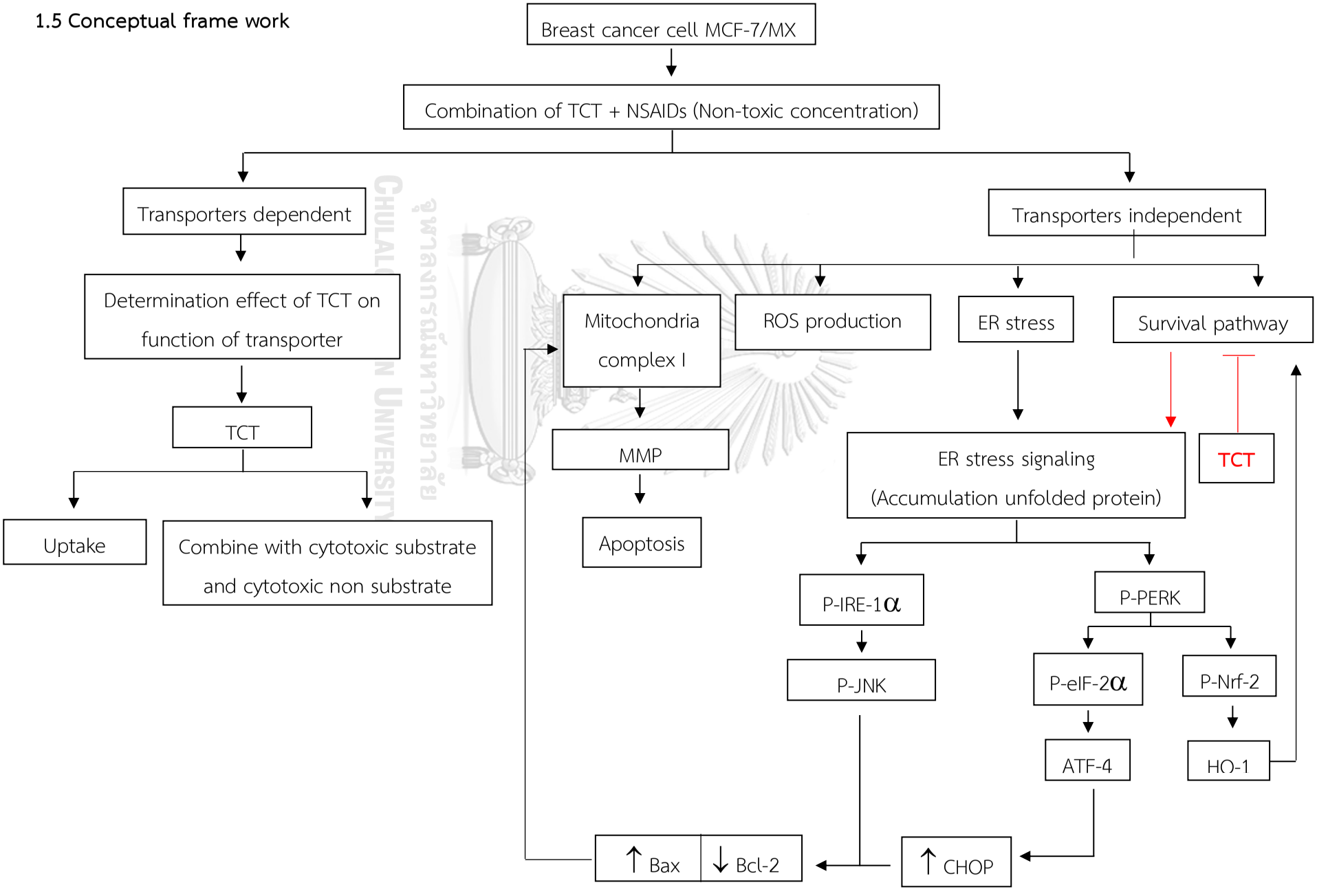
1. To evaluate the potentiation effect of TCT when combining with either INDO or DIC on the cytotoxicity in MCF-7 breast cancer cells.
2. To elucidate the mechanisms of TCT in enhancing NSAIDs-mediated cytotoxicity and the involvement of ER stress response in MCF-7 breast cancer cells.

1.4 Scope of study

The scope of this study demonstrated the potential of TCT as a pharmacoenhancer when combining with INDO and DIC in MCF-7 cancer cells and its mechanism of potentiation. The study was divided into 2 subtopics following:

1. Determination of the potentiation effect of TCT when combining with either INDO or DIC on cytotoxicity in MCF-7 breast cancer cells.
2. Investigation of underlying mechanism of TCT in enhancing INDO and DIC cytotoxicity in MCF-7 breast cancer cells through various mechanism such as inhibition of efflux transporters, increased cellular stress and induction of apoptosis.

1.5 Conceptual frame work



CHAPTER II

LITERATURE REVIEW

2.1 Nonsteroidal anti-inflammatory drugs (NSAIDs) in cancer treatment

Non-steroidal anti-inflammatory drugs (NSAIDs) such as indomethacin (INDO), diclofenac (DIC) are anti-inflammatory, analgesics, and antipyretics drugs through inhibition of prostaglandin (PG) biosynthesis (Gunaydin and Bilge, 2018). In addition, INDO and DIC have been reported their potential in cancer treatment. It was demonstrated that either DIC (18 mg/kg /twice a week, for 4 week) or INDO (2.5 mg/kg/day, for 6 week) significantly reduced tumor volume by 33% and 22% in nude mice injected with ovarian adenocarcinoma HEY cells, compared to those of control groups (Valle et al., 2013). INDO (3 mg/kg) given to HCT116/nude mice for 4 weeks significantly decreased HCT116 tumor volume by approximately 2-fold (Wang and Zhang, 2005). DIC (18 mg/kg) significantly decreased tumor volume in nude mice with A549 non-small lung cancer by 2-fold (Moody et al., 2010). It is likely that, in addition to COX-dependent mechanism, NSAIDs might induce direct cytotoxicity toward cancer cells.

2.2 The mechanisms of INDO- and DIC-induced cytotoxicity

The mechanisms of INDO and DIC induced cytotoxicity have been linked to inhibition of mitochondria complex I and increasing intracellular ROS level (Mazumder et al., 2019; Sandoval-Acuña et al., 2012). INDO and DIC at the concentration ranging from 500 nM to 1000 μM generated cytotoxicity in various cell models such as Caco-2, AGS cells, 4T1EM3 cells (Boonyong et al., 2020b; Mazumder et al., 2019; Terasaki et al., 2019). It was reported that INDO (20 μM) increased ROS production from 5-fold to 10-fold after 24 h-treatment, resulting in apoptotic cell death in metastatic breast 4T1E/M3 cancer cell line (Terasaki et al., 2019). INDO and

DIC (1000 μM) were able to increase intracellular ROS production by 3-fold in Caco-2 cells after 2-h treatment (Boonyong et al., 2020b). In addition, INDO and DIC at 250 μM were able to inhibit mitochondrial complex I activity in Caco-2 and AGS cells, leading to ROS-mediated cells death (Mazumder et al., 2019; Sandoval-Acuña et al., 2012).

Increase of intracellular ROS levels was associated with increased unfolded proteins in endoplasmic reticulum (ER). As known, unfolding protein response (UPR) involves in the control of protein homeostasis (Boonyong et al., 2020a; Lindholm et al., 2017). High levels of accumulated unfolded proteins lead to activation of three signaling proteins sensors which are PERK (protein kinase RNA (PKR)-like ER kinase), IRE-1α (inositol-requiring protein-1α), and ATF-6 (activating transcription factor). These proteins sensors normally form complex with chaperone Binding immunoglobulin protein (BiP) in the ER. Upon increasing amount of these unfold proteins, their dissociation from BiP-sensors complexes occurs. Subsequently, these free sensors (i.e., PERK, IRE-1α and ATF-6) turn into their active phosphorylated forms by autophosphorylation process (Figure 1) (Almanza et al., 2019; Bhattarai et al., 2021; Hetz and Papa, 2018). Activation of PERK and IRE-1α is related to increasing of Bax (pro-apoptotic) and decreasing of Bcl-2 (anti-apoptotic) expression. This process was reportedly dependent on up-regulation of CHOP (C/EBP homologous transcription factor) protein (Boonyong et al., 2020a; Li et al., 2019; Riaz et al., 2020). In addition, autophosphorylated PERK leads to up-regulation of a detoxification enzyme heme oxygenase-1 (HO-1) and increasing of survival response through Nrf2 activation (Consoli et al., 2021; Fujiki et al., 2019; Gáll et al., 2019). The phosphorylated IRE-1α involves with apoptotic cell death by activation of JNK pathway (Fu et al., 2021; Li et al., 2019; Riaz et al., 2020). Activation of three UPR

signaling proteins sensors at ER membrane has been reported to associate with cancer cell death (Figure 1) (Fu et al., 2021; Limonta et al., 2019).

INDO (1000 μM) were able to activate PERK and IRE-1α pathway of the UPR in CaCo-2 cells, leading to increase of CHOP-dependent expression of the apoptotic protein (Bax and BcL-2) after 48 h exposure (Boonyong et al., 2020a).

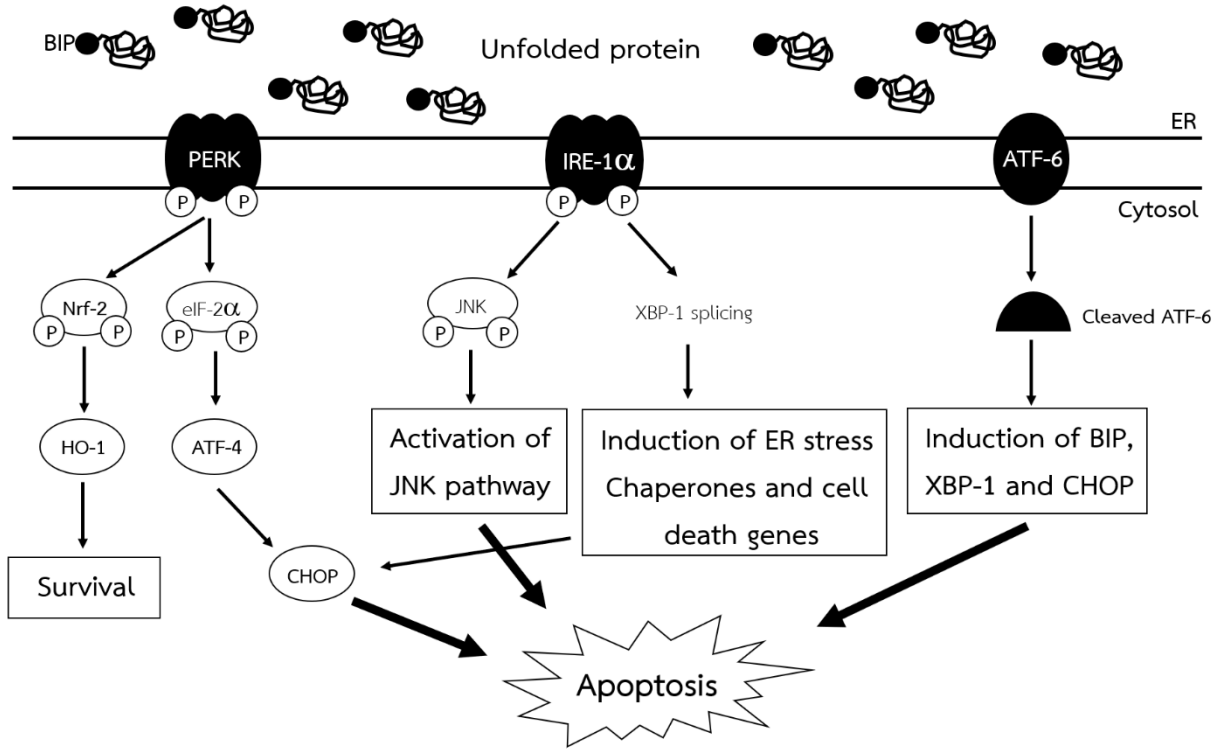


Figure 1. Unfolded protein response-mediated ER stress pathways (Modified from (Di Fazio et al., 2012)

2.3 Pharmacoenhancer in cancer treatment

One approach to boost therapeutic outcomes in breast cancer treatment is the addition of pharmacoenhancers in anticancer regimens. Several mechanisms leading to the loss of drug effectiveness include activation of detoxifying systems (e.g. HO-1, NQO1), enhanced repair of drug induced DNA damage, evasion of drug-induced apoptosis, increases of ATP-binding cassette (ABC) efflux transporters including P-glycoprotein (P-gp), breast cancer resistance protein (BCRP), multidrug

resistance-associated proteins (MRPs) etc. (Gillet and Gottesman, 2010b; Mansoori et al., 2017) (Figure 2). Hence, any compounds interfering with such mechanism might increase cell susceptibility to anticancer agents.

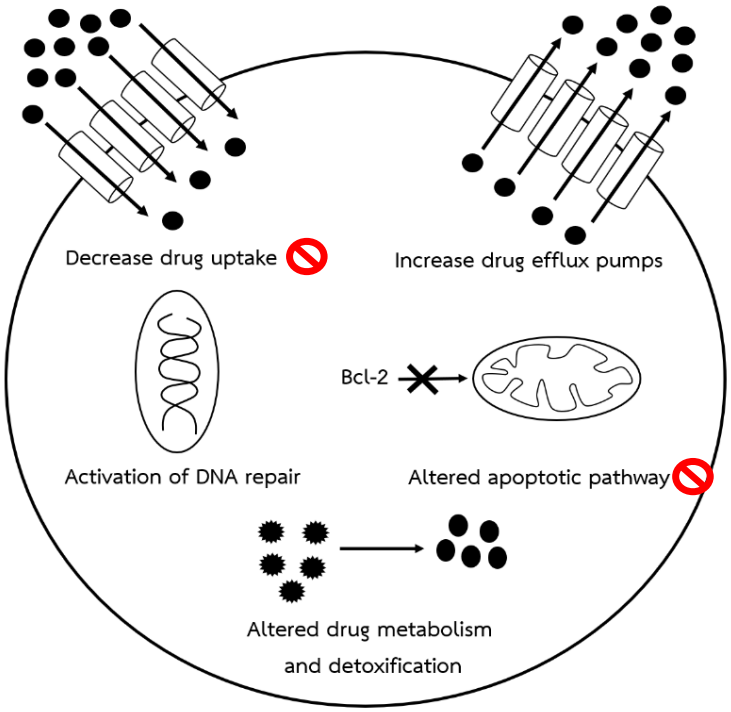


Figure 2. The mechanisms of multidrug resistance in cancer cells (Modified from (Yergeri et al., 2014)

It has been reported that the combinatorial approach has much more effectiveness in cancer treatment than the use of cytotoxic drugs alone (Bayat Mokhtari et al., 2017; Nurgali et al., 2018; Wang et al., 2019). Currently, several natural compounds have been reported their ability to potentiate cytotoxicity of anticancer agents through transporter-dependent mechanism and transporter-independent mechanisms (Cao et al., 2019; Chaisit et al., 2017; T. Chen et al., 2019; Pérez-Soto et al., 2019).

2.3.1 Transporter-dependent mechanism

High expression level of the ATP binding cassette (ABC) efflux transporters, particularly P-glycoprotein (P-gp), breast cancer resistance protein (BCRP), and multidrug resistance-associated proteins (MRPs), has been largely responsible for multi-drug resistance in cancer. As known, the ABC transporters could limit intracellular accumulation of their cytotoxic substrates. Consequently, intracellular concentration of those cytotoxic drugs could not reach their cytotoxic threshold (Figure 2) (Chaisit et al., 2017; Choi and Yu, 2014; Robey et al., 2018). Examples of cytotoxic drug substrates are doxorubicin (P-gp and MRP2 substrate), and mitoxantrone (a specific substrate of BCRP) (Austin Doyle and Ross, 2003; Chaisit et al., 2017; Kawahara et al., 2020). In addition, INDO and DIC are also reported as substrate of MRPs and BCRP efflux transporters (Lagas et al., 2008; Zhang et al., 2016).

Hence, direct inhibition of the activities of these ABC efflux transporters could increase intracellular accumulation of cytotoxic drugs, and increased cytotoxicity of those drugs (Chaisit et al., 2017; Choi and Yu, 2014; Robey et al., 2018). Several plant derived compounds such as naphthoquinones, alkaloids have been demonstrated their enhancing effects through this transporter related mechanism. For example, rhinacanthin-C (naphthoquinone) at its non-cytotoxic concentrations (0.04 or 0.1 μM) significantly increased doxorubicin-mediated cytotoxicity by approximately 38-fold in MCF-7 cell after 48-h treatment through inhibition of MRP2 and P-gp activities (Chaisit et al., 2017). In addition, piperine (alkaloid) at non-cytotoxic concentrations (30-65 μM) could enhance cell sensitivity toward doxorubicin treatment in CEM/ADR 5000 resistance cell (overexpress p-gp) and Caco-2 cell via inhibition of P-gp activities (H. Li et al., 2018).

2.3.2 Transporter-independent mechanism

Ineffectiveness of cytotoxic compounds might stem from survival adaptation of cellular processes. It was demonstrated that certain natural compounds such as alkaloids increased cellular stress and mitochondria dysfunction, leading to apoptosis of cancer cells (Figure 2) (Cadenas, 2004; Liou and Storz, 2010). For example, piperlongumine (alkaloid) at 2 μM caused a 2-fold increase of intracellular ROS levels, and significant apoptotic death of the HCT-116 and LoVo cells treated with oxaliplatin, compared with oxaliplatin alone (Chen et al., 2009). Similarly to piperlongumine, a terpene alantolactone was able to enhance oxaliplatin cytotoxicity via increased ROS production in HCT-116 and RKO cells after 24-h exposure (Cao et al., 2019). In addition, combination of alantolactone (10 mg/kg) and oxaliplatin (2 mg/kg) reduced tumor growth of HCT-116 cells in nude mice after 13-days treatment (Cao et al., 2019). Moreover, an alkaloid isoliensinine significantly enhanced cellular uptake of cisplatin, and increased ROS-mediated apoptosis in colorectal HCT-15 cancer cells (Manogaran et al., 2019).

2.4 Phytochemicals in cancer treatment

Several bioactive phytochemicals such as flavonoids, anthocyanidins, carotenoids, terpenoids, alkaloids have been reported their capability to induce apoptotic cell death through ROS-mediated ER stress *in vitro* and *in vivo* models (Cao et al., 2019; T. Chen et al., 2019; Gu et al., 2015; Park et al., 2018). For example, an antraquinone chrysophanol (10-100 μM) increased ROS generation after 48-h exposure in BT-474 and MCF-7 cells, leading to phosphorylation of PERK, eIF-2α, CHOP and IRE-1α proteins (Park et al., 2018). Resveratrol was able to increase arsenic trioxide cytotoxicity through ROS production and up-regulation of pro-apoptotic Bax protein in lung A549 cells (Gu et al., 2015). Berberine a major alkaloid

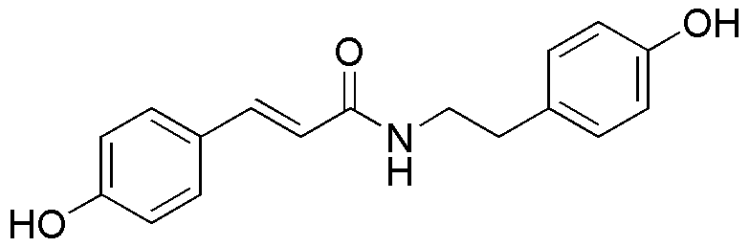
in *Berberis aquifolium* caused ROS-mediated ER stress in C6 rat glioma cells (Chen et al., 2009). Treatment breast cancer MCF-7 cells with platycodin D at 10-30 μM for 1 h increased intracellular ROS, followed by ER stress response (Yu and Kim, 2010). In addition, piperlongumine (30 mg/kg/day) intraperitoneal injected to xenograft nude mice for 3 weeks significantly reduced tumor weight by 2-fold, possibly through ROS production, ER stress and PARP cleavage in tumors (Karki et al., 2017).

Combination of NSAIDs with certain phytochemicals such as resveratrol, curcumin, piperine or naringenin has been demonstrated their effectiveness in enhancing cancer cell death both *in vitro* and *in vivo* (Boonyong et al., 2020a, b; Feng et al., 2016; Rana et al., 2015; Redondo-Blanco et al., 2017). Co-treatment of resveratrol (10-30 μM) with INDO (30 μM) for 48-h significantly induced apoptotic cell death in colon cancer HCA-17 and SW480 cells, compared with INDO alone (Feng et al., 2016). The combination of DIC (8 mg/kg) and curcumin (50 mg/kg) caused apoptotic cell death in 1,2-dimethylhydrazine dihydrochloride (DMH)-induced colorectal cancer in Sprague-Dawley rats after 6 week-treatment by increasing ROS generation and decreasing the mitochondria membrane potential (Rana et al., 2015). Moreover, the combination of DIC (1000 μM) with either curcumin (50 μM), naringenin (100 μM) or piperine (10 μM) significantly enhanced apoptotic cell death in Caco-2 cells (Boonyong et al., 2020a, b).

2.5 N-*trans-p*-coumaroyltyramine (TCT)

N-*trans-p*-coumaroyltyramine (amide alkaloid compound) is a coumaric acid derivative, the chemical structure of TCT consists of coumaric acid and tyramine linking by amide bond (Figure 3) (Kim and Lee, 2003; Park et al., 2002). TCT was abundantly found in *Piper wallichii* (Miq.) Hand.-Mazz. (Thai common name: Sa-kan) and others in. Piperaceae family including white pepper (*Piper nigrum),* bell pepper (*Capsinum annuum),* eggplant (*Solanum melongena*). In addition to TCT, piperine

(PIP) was also found in Sa-kan. Sa-kan, is an herbal plant commonly found in the northern and northeastern part of Thailand. It has been used as carminative, antiflatulent and tonic element in several traditional medicines combatting gastrointestinal discomfort (Daorueang, 2020; Salehi et al., 2019; Sireeratawong et al., 2012).



TCT ($C_{17}H_{17}NO_3$) MW: 283.3

Figure 3. Structure of N-*trans-p*-coumaroyltyramine (Kim and Lee, 2003)

A few pharmacological activities of TCT have been reported, including anti-trypanosomal activity and inhibit of some enzymes such as acetylcholinesterase and α-glucosidase (Kim and Lee, 2003; Moradi-Afrapoli et al., 2012; Nishioka et al., 2014). Recently, a TCT derivative, N-coumaroyl tyramine diacetate was reported to increase cytotoxicity of TNF-Related Apoptosis Inducing Ligand (TRAIL) in dose-dependent manner in Kelly and TRIAL-resistant Hela cells after 12-h exposure (Yashaswini and Salimath, 2017). In addition, mice given N-coumaroyl tyramine diacetate (2.85 mg/kg) in concurrent with radiation had less microvessel density than the control group (Yashaswini and Salimath, 2017). However, the information of TCT on mechanisms of anticancer activity and its potential enhancement effect is very limit. Moreover, effects of a combination between TCT and NSAIDs on cytotoxicity in cancer model have not been reported.

CHAPTER III

MATERIALS AND METHODS

3.1 Materials

3.1.1 Compounds

N-*trans-p*-coumaroyltyramine (TCT) was kindly provided by Associate Professor Rutt Suttisri, Ph.D., Department Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Thailand. TCT was isolated from vines plant of *Piper wallichii* (Miq.) Hand.-Mazz or Sa-kan (Thai common name). The dried plant material was extracted with methanol (3 times) at room temperature and filtered. The filtrate was evaporated to residue prior to purification, as previously described (Kim and Lee, 2003). All methanolic extract was purified by silica gel column chromatography. The structure was identified by spectroscopic techniques (Kim and Lee, 2003).

A stock solution of 100 mM of TCT was prepared in dimethyl sulfoxide (DMSO) 99.9% and stored at -20ºC until analysis. The final concentration of TCT were prepared by serial dilution with RPMI medium to concentrations of 10 and 100 µM and the percentage of DMSO was less than 0.1% (v/v).

3.1.2 Chemicals and reagents

Diclofenac (DIC) and indomethacin (INDO), and other compounds such as bovine serum albumin (BSA), bromophenol blue, calcein acetoxymethyl (Calcein-AM), camptothecin (CAM), carboxy-dichlorofluorescein (CDCF), 2′,7′-dichlorofluorescein diacetate (CDCF-DA), cyclosporine A (CsA), dimethyl sulfoxide (DMSO), ethylenediaminetetraacetic acid (EDTA), glycerol, Hanks' balanced solution (HBSS), Hoechst 33342, hydrogen peroxide (H_2O_2), KO143, β-mercaptoethanol,

mitoxantrone (MX), non-essential amino acid, paraformaldehyde, penicillin, piperine (PIP), phenylmethylsulfonyl fluoride (PMSF), protease inhibitor cocktails, rotenone (ROT), sodium chloride (NaCl), sodium dodecyl sulfate (SDS), sodium fluoride (NaF), sodium orthovanadate (Na_3VO_4), streptomycin sulfate, tamoxifen (TAM), tetramethylrhodamine (TMRE), thapsigargin (TGN), tris hydrochloride (Tris-HCl), triton X-100, trypsin tunicamycin (TUN), tween 20, and vinblastine (VBL) were purchased from Sigma Chemical Co. (St Louis, MO, USA). RPMI-1640 medium, L-glutamine, fetal bovine serum (FBS) and 3-(4,5-Dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were purchased from Gibco Life Technologies (Grand Island, NY, USA). RPMI-1640 without phenol red medium were purchased from Corning (NY, USA). Pierce™ BCA protein assay kit was purchased from Thermo Fisher Scientific (Waltham, MA, USA). The Immobilon-P PVDF membranes was purchased from Merck Milliporen (Darmstadt, Germany). Super signal West Pico chemiluminescent substrates is purchased from Pierce Biotechnology (Rockford, IL, USA). The mouse monoclonal anit-Bax, anti-Bcl-2, anti-HO-1, anti-Nrf-2 antibodies were purchased from Santa Cruz Biotechnology, Inc. (Dallas, Texas, USA). The mouse monoclonal anti-ATF-4, anti-CHOP, anti-IRE-1α, anti-p-IRE-1α, anti-PERK, were purchased from Cell Signaling Technology (Beverly, MA, USA). The mouse monoclonal anti-GAPDH and secondary goat anti-mouse IgG (H&L) horseradish peroxidase (HRP) were purchased from Calbiochem (San Diego, CA, USA). Rabbit monoclonal anti-JNK, anti-p-JNK, anti-p-Nrf-2, anti-p-PERK and rabbit monoclonal HRP-conjugated anti-(rabbit-IgG) antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA).

3.1.3 Experimental instruments

1. Autoclave: Hirayama, Saitama, Japan
2. Bio-Rad Incubators Electrophoresis Power Supply: Cambridge Scientific Corp, USA
3. Fluorescence microscope: BX-FLA, Olympus, Tokyo, Japan
4. GE ImageQuant LAS 4000: GE Healthcare Life-Science Ltd., Branch, Taiwan
5. Hot air oven: MEMMERT, Buchenbach, Germany
6. Humidified carbon dioxide incubator: Forma Scientific, Marietta, OH, USA
7. Image J program: NIH, Bethesda, MD, USA
8. Inverted microscope: Axivert 135, Ziess, Konstanz, Germany
9. Microplate reader: Wallac 1420 VICTOR 3, PerkinElmer Inc., Hopkinton, MA, USA
10. Multi-well plates: Corning, New York, NY, USA
11. Laminar air flow hood: BossTech, Bangkok, Thailand
12. pH meter: CG 842, Schott, Hofheim, Germany
13. Refrigerated centrifuge Z 383K: Hermle Labortechink, Burtadingen, Germany
14. Trans-blot® SD semi-dry transfer cell: Missouri, TX, USA
15. Tissue culture flasks: Corning, New York, NY, USA
16. Vortex mixer: mode K550-GE. Scientific Industries, New York, NY, USA
17. Water bath: WB22, Memmert, Hannover, Germany

3.1.4 Cell culture

Cell culture were used in this study including human breast adenocarcinoma MCF-7 (ATCC® HTB-22™) was obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). The cells were subcultured at 80-90% confluence, using 0.25% trypsin solution containing 1 mM EDTA. The cells were maintained in RPMI-1640 supplemented with 10% FBS and 1% penicillin–streptomycin at 37 ℃ in a humidified atmosphere of 5% CO_2.

The mitoxantrone-resistant subline MCF-7/MX cells were in-house developed from the MCF-7 cells by culturing the cells in RPMI-1640 medium containing mitoxantrone (MX). The cells were further developed into the MX-resistant subline MCF-7/MX cells by stepwise selection method upon increasing concentrations of MX at 0.01 μM until the maximum concentration of 0.7 μM (Ji et al., 2010). Then, MCF-7/MX cells were maintained in RPMI-1640 complete medium containing mitoxantrone 0.7 μM. The cells were cultured in MX-free RPMI-1640 complete medium for 1 weeks before experiments.

3.2 Methods

3.2.1 Cell viability assay

Cell viability was determined using by MTT assay. The cells were seeded into 96 well plates at the density of 5×10^3 cells/well for 24 h. The cells were treated with non-toxic concentration of the test compounds (i.e., TCT 10, 100 μM, PIP 10, 100 μM, INDO 10 μM and DIC 10 μM) for 48 h. After 48-h treatment, the cells were further incubated with MTT solution (0.5 mg/ml) in the CO_2 incubator at 37 ˚C for 4 h. The formazan crystals were dissolved in 100 μl DMSO and then quantified spectrophotometrically at 570 nm using a microplate reader.

3.2.2 Apoptosis assay

Apoptotic cell death was detected by Hoechst 33342 staining method (Crowley et al., 2016). The cells were seeded into 96-well plates at 5×10^3 cells/well overnight prior to treatment with the test compounds (i.e., TCT 100 μM, PIP 100 μM, INDO 10 μM, DIC 10 μM, TGN 0.0125 μM, TUN 0.25 μg/ml and H_2O_2 10 μM) for 48 h. After treatment, cells were washed three times with PBS and stained with Hoechst 33342 (10 μM) for 30 min. Then, the cells were fixed 4% paraformaldehyde for 30 min at 4°C, followed by washing three times with ice-cold PBS. The cells were visualized under a fluorescence microscope (20X, original magnification) at wavelengths at 350/461 nm (excitation/emission). The apoptotic cells are considered by chromatin condensation or nuclei fragmentation.

3.2.3 Uptake assay

Inhibitory effects of the test compound on activities of the major ABC efflux transporters (i.e., P-gp, MRP1, MRP2, and BCRP) were demonstrated using specific substrate accumulation assay (Chaisit et al., 2017). Cells (2×10^5 cells/ well) were seeded onto 24-well plates overnight prior to pre-treat with a known pump inhibitor (i.e., CsA 50 μM, INDO 500 μM, KO143 10 μM, TCT 10, 100 μM, PIP 10, 100 μM) for 30 min at 37 °C followed by treatment with the specific substrate of each transporter (i.e., calcein-AM 0.4 μM (P-gp), DCDF 5.2 μM (MRP1), CDCFDA 5 μM (MRP2) and pheophobide A 10 μM (BCRP) was added for another 30 min. At the end of this co-incubation period, the cells were lysed with 1% Triton X-100 and read the fluorescent intensity of each substrate with a microplate reader at 485/535 nm (excitation/emission) for calcein, DCDF and CDCF; and at 488/ 675 nm (excitation/emission) for pheophorbide A.

3.2.4 Determination of reactive oxygen species (ROS)

To measure ROS production, cells (2×10^4 cells/well) were grown overnight in 96-well plates prior to DCFH-DA assay (Boonyong et al., 2020b). Cells were incubated with a fluorescent probes DCFH-DA 100 μM for 30 min, followed by addition of the test compounds (i.e., TCT 100 μM, INDO 10 μM, DIC 10 μM) for 2 h. Rotenone at 20 μM was used as a positive control group. At the end of this co-incubation period, the cells were lysed with 1% Triton X-100 and measured fluorescent intensity with a microplate reader at 485/535 nm (excitation/emission).

3.2.5 Determination of mitochondrial function

Mitochondrial function was evaluated by TMRE assay (Boonyong et al., 2020a). The cells were seeded into 96-well plates at 5×10^3 cells/ well overnight, followed by incubation with the test compounds (i.e., TCT 100 μM, INDO 10 μM and DIC 10 μM) for 48 h. Then, cells were washed three times with PBS and TMRE 1 μM was added for another 30 min, followed by wash three times with ice-cold PBS and the fluorescent intensity of TMRE in mitochondrial matrix was read with a microplate reader at 549/575 nm (excitation/emission).

3.2.6 Western blot analysis

The expression of ER stress signaling proteins such as PERK, IRE-1α and apoptotic regulatory protein such as Bax and BcL-2 were determined by western blot analysis. After treatment, cell lysates were prepared in RIPA lysis buffer (50 mM Tris-HCl pH 6.8, 150 mM NaCl, 1% Tritron-X, 0.5% sodium deoxycholate, 0.1% SDS, 1mM EDTA, 1 mM PMSF, 1 mM NaF, 1 mM, Na_3VO_4 and protease inhibitor cocktails) for protein extraction. Cell lysate was then centrifuged at 12000 x g for 20 min at 4 °C and collected the supernatant for blotting analysis. Protein concentration was quantified using Pierce BCA protein assay kit according to the manufacturer's instructions.

Equal amounts of proteins (40 µg) was added into sample buffer (60 mM Tris-HCl, 2% SDS, 10% glycerol, 5% β-mercaptoethanol and 0.01% bromophenol blue). Then, the proteins were denatured at 95 ℃ or 5 min and loaded onto 7-12% SDS-PAGE, and separated at constant voltage of 100 V for 3 h. The proteins were transferred to PVDF membrane using constant voltage of 10 V for 1. After blocking with 5% skim milk in TBS-T for 30 min at room temperature, the membranes were probed with primary antibodies at for anti-Bax (1:1000), anti-Bcl-2 (1:1000), anti-CHOP (1:1000), anti-PERK (1:1000), anti-p-PERK (1:1000), anti-ATF-4 (1:1000), anti-eIF-2α (1:1000), anti-p-eIF-2α (1:1000), anti-HO-1 (1:1000), anti-IRE-1α (1:1000), anti-p-IRE-1α (1:1000), anti-JNK1/2 (1:1000), anti-p-JNK1/2 (1:1000), anti-Nrf-2 (1:1000), anti-p-Nrf-2 (1:1000), and GAPDH (1:2000) with TBS-T containing 3% non-fat dry milk at 4 ℃ overnight, followed by incubation with the HRP-conjugated secondary antibodies (1:2000). Protein bands were then developed using the Super signal West Pico chemiluminescent substrates. The immunoblots were visualized and quantified by a GE ImageQuant LAS 4000 (GE Healthcare Life-Sciences Ltd., Branch, Taiwan). Protein expression levels were normalized to those of GAPDH (an internal control for proteins loaded onto the gel).

3.2.7 Statistical analysis

Data are presented as mean ± SEM and obtained from least three separated experiments (n = 3). The differences between treatments groups were determined by One-way analysis of variance (ANOVA), followed by the Bonferroni post-hoc test. The statistical significance between untreated and treated groups is considered at $p < 0.05$.

CHAPTER IV
RESULTS

4.1 Effects of TCT on drug-induced cytotoxicity in MCF-7 cells

4.1.1 Enhancement effect of TCT on INDO- and DIC-mediated cytotoxicity

As shown in Table 1, the IC_{50} values of INDO, DIC and mitoxantrone (MX) in MCF-7/MX cells were approximately 2.3, 5.1, 11.7 times less sensitive than those in MCF-7/WT cells. The IC_{50} values of PIP in sensitive and resistant MCF-7 cell after 48-h exposure were comparable. The concentration ranges of TCT in this study (10-100 μM) were non-cytotoxic in both cell types, therefore its IC_{50} values could not be estimated.

. TCT and PIP 10 μM significantly increased both INDO and DIC by approximately 1.5-2.5 times in MCF-7/WT and MCF-7/MX cells. Upon increasing the concentration of both compounds in combination with NSAIDs to 100 μM, the cytotoxicity increased significantly as shown in Figure 4A-D. Moreover, both TCT and PIP (at 10 and 100 μM) were able to significantly increase cytotoxicity of MCF-7/WT cells exposed to MX (at the IC_{50} value of 2 μM) as shown in Figure 4A and B. However, their sensitization effect was not observed in MCF-7/MX cells exposed to MX (at the IC_{50} of 24 μM) as shown in Figure 4C and D. Effects of PIP-MX combination and PIP alone on cytotoxicity of MCF-7/MX cells were not statistically different. It was worth noting that, at 10 and 100 μM, TCT had no effect on cell viability, whereas PIP significantly reduced the viability of MCF-7/WT and MCF-7/MX cells to approximately 38% (Figure 4B and D).

Compounds	IC_{50} (μM) ± SEM. (48 h)	
	MCF-7/WT	MCF-7/MX
INDO	111.97 ± 0.03	254.10 ± 0.02
DIC	77.41 ± 0.02	393.13 ± 0.01
MX	2.08 ± 0.02	24.32 ± 0.03
PIP	55.47 ± 2.02	66.25 ± 2.32
TCT	N.D.	N.D.

Table 1 The IC_{50} Values of Indomethacin (INDO), Diclofenac (DIC), Mitoxantrone (MX) Piperine (PIP) and N-*trans-p*-coumaroyltyramine (TCT) in MCF-7 and MCF-7/MX cells. N.D. = Not determined

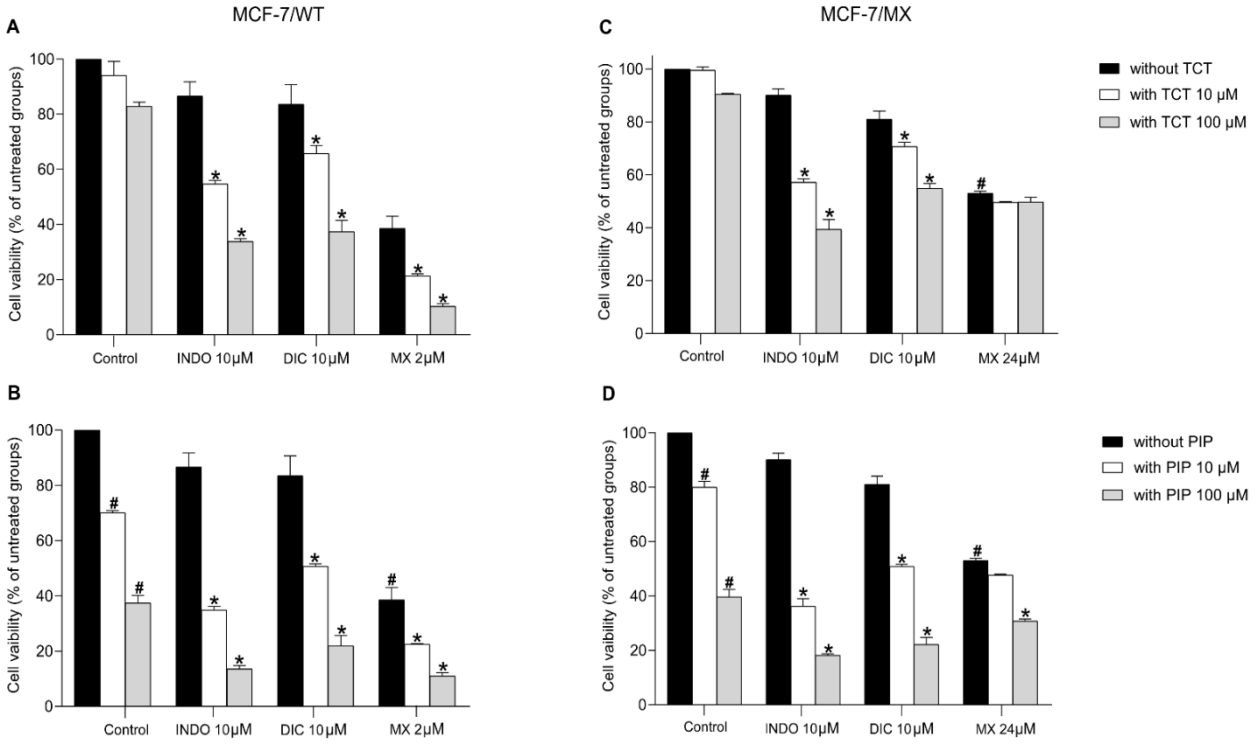
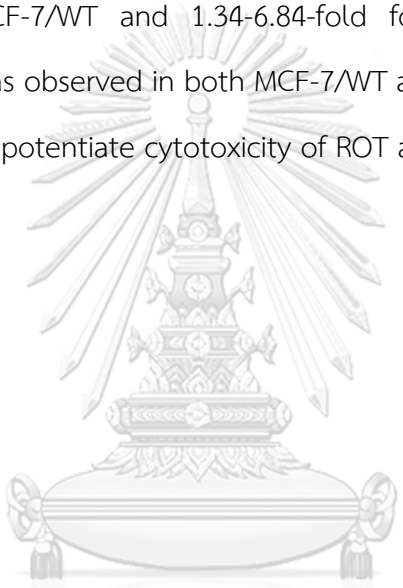


Figure 4. Effects of TCT or PIP on drug-induced cytotoxicity in MCF-7/WT (A and B) and MCF-7/MX cells (C and D)after 48-h exposure to INDO, DIC, and MX. Each bar represents mean ± SEM (n=3). $^{*}P< 0.05$ compared with the drug-treated group in the absence of the test compound. $^{\#}P< 0.05$ compared with the untreated control.

4.1.2 Enhancement effect of TCT on other cytotoxic agents

As shown in Figure 5A-D neither TCT nor PIP increased cell sensitivity to the cytotoxic agents including tamoxifen 10 μM (TAM), vinblastine 1 nM (VBL), and camptothecin 0.5 nM (CPT) after 48-h exposure. In contrast, as shown in Figure 5A and C the combination of TCT (at 10 and 100 μM) with certain ER stressors including hydrogen peroxide 10 μM (H_2O_2), rotenone 1 μM (ROT), thapsigargin 0.0125 μM (TGN), and tunicamycin 0.25 μg/ml (TUN) significantly increased cell death approximately 1.23-10.27-fold for MCF-7/WT and 1.34-6.84-fold for MCF-7/MX. PIP-mediated sensitization to H_2O_2 was observed in both MCF-7/WT and MCF-7/MX cells. However, PIP did not significantly potentiate cytotoxicity of ROT and TUN in the resistant cells.



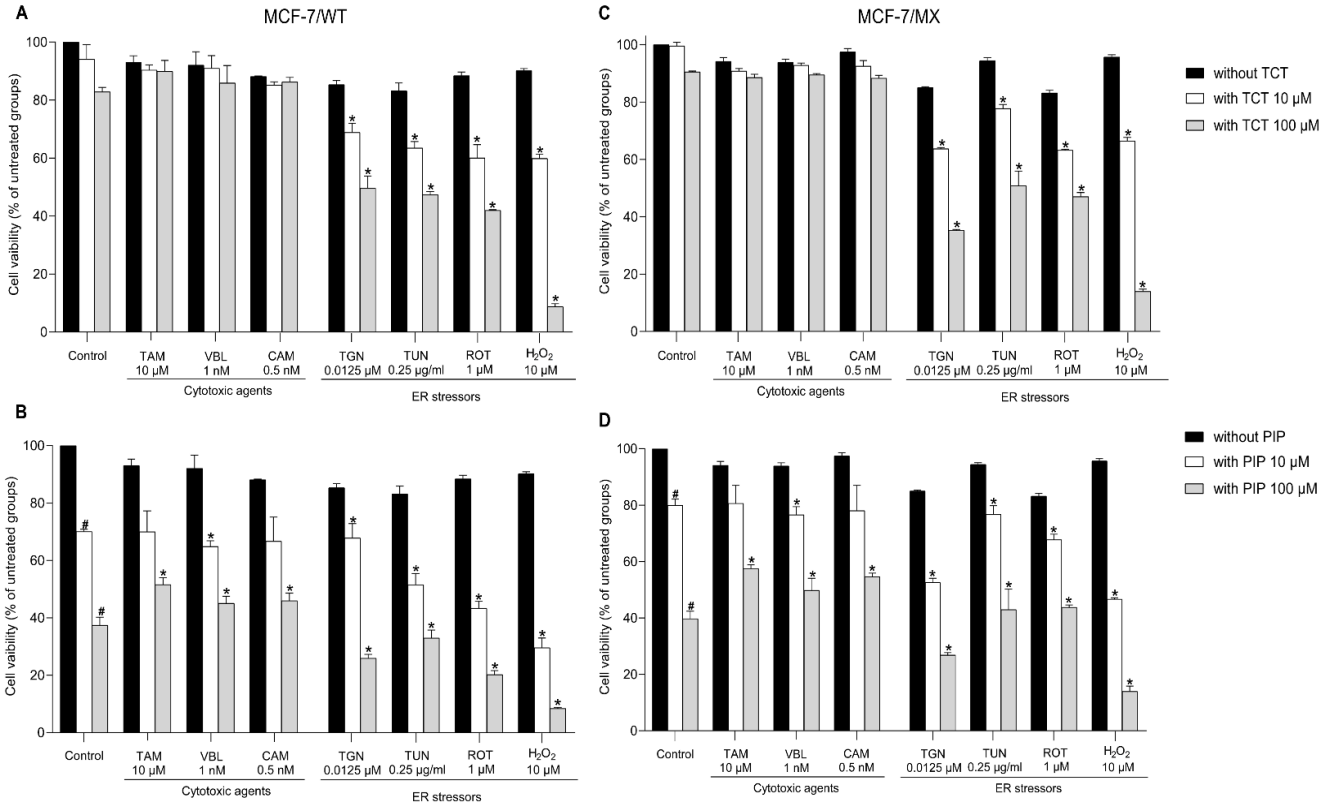


Figure 5. Effects of TCT or PIP on the viability of MCF-7/WT (A and B) and MCF-7/MX cells (C and D) after 48-h exposure to either cytotoxic anti-cancer agents (TAM, VBL, and CPT) or ER stressors (H_2O_2, ROT, TGN, and TUN). Each bar represents mean ± SEM (n=3). $^{*}P< 0.05$ compared with the drug-treated group in the absence of the test compound. $^{\#}P< 0.05$ compared with the untreated control.

4.2 Effect of TCT on drug efflux transporters

4.2.1 Function expression of efflux transporter in MCF-7 cells

As shown in Figure 6A and B, MCF-7/WT and MCF-7/MX cells expressed functionally active MRP1 and MRP2. INDO 500 μM (an MRP1/MRP2 inhibitor) significantly increased intracellular accumulation of the MRP1 substrate DCDF and the MRP2 substrate CDCF. Moreover, MCF-7/MX cells also showed an appreciable level of BCRP activity. The presence of KO143 10 μM (a BCRP inhibitor) significantly increased the BCRP substrate pheophorbide A in MCF-7/MX cells by approximately 2.4 times. Neither MCF-7/WT nor MCF-7/MX cells exhibited significantly increased level of intracellular calcein in the presence of cyclosporine A 50 μM (a P-gp inhibitor), suggesting the absence of P-gp function.

4.2.2 Effect of TCT on efflux transporter activity

As shown in Figure 7A and B, neither TCT nor PIP at 10 and 100 μM was able to significantly increase accumulation of DCDF, CDCF, and pheophorbide A after 30 min exposure in both cells, suggesting the lack of inhibitory action against MRP1, MRP2, and BCRP activities. Hence, TCT and PIP exerted their potential chemo-sensitizing activity to INDO and DIC via mechanisms independent of drug efflux transporters.

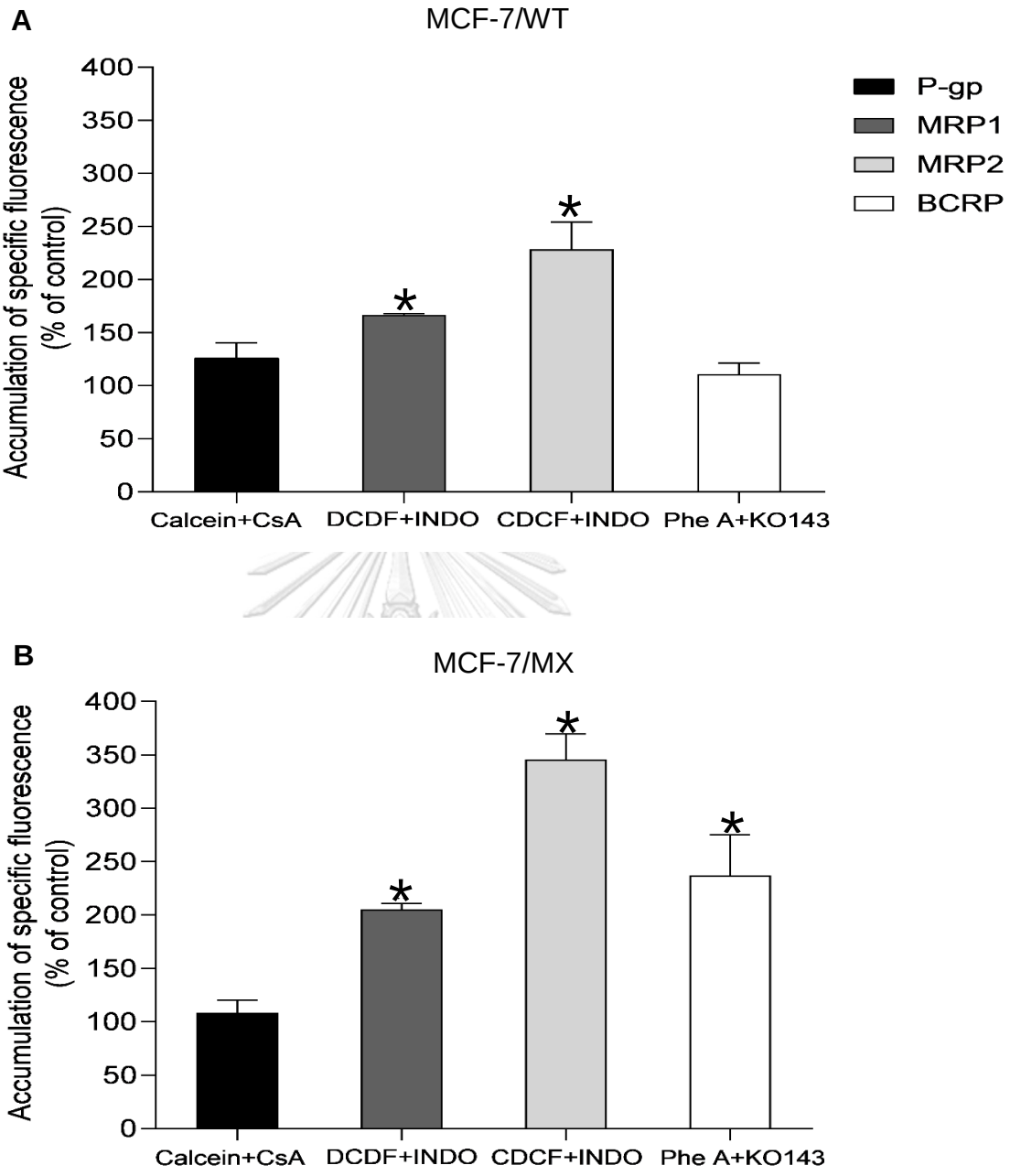


Figure 6. Basal activities of P-gp, MRP1, MRP2, and BCRP in MCF-7/WT (A) and MCF-7/MX (B). Each bar represents mean ± SEM (n=3). $^{*}P < 0.05$ compared with the control group (without inhibitor).

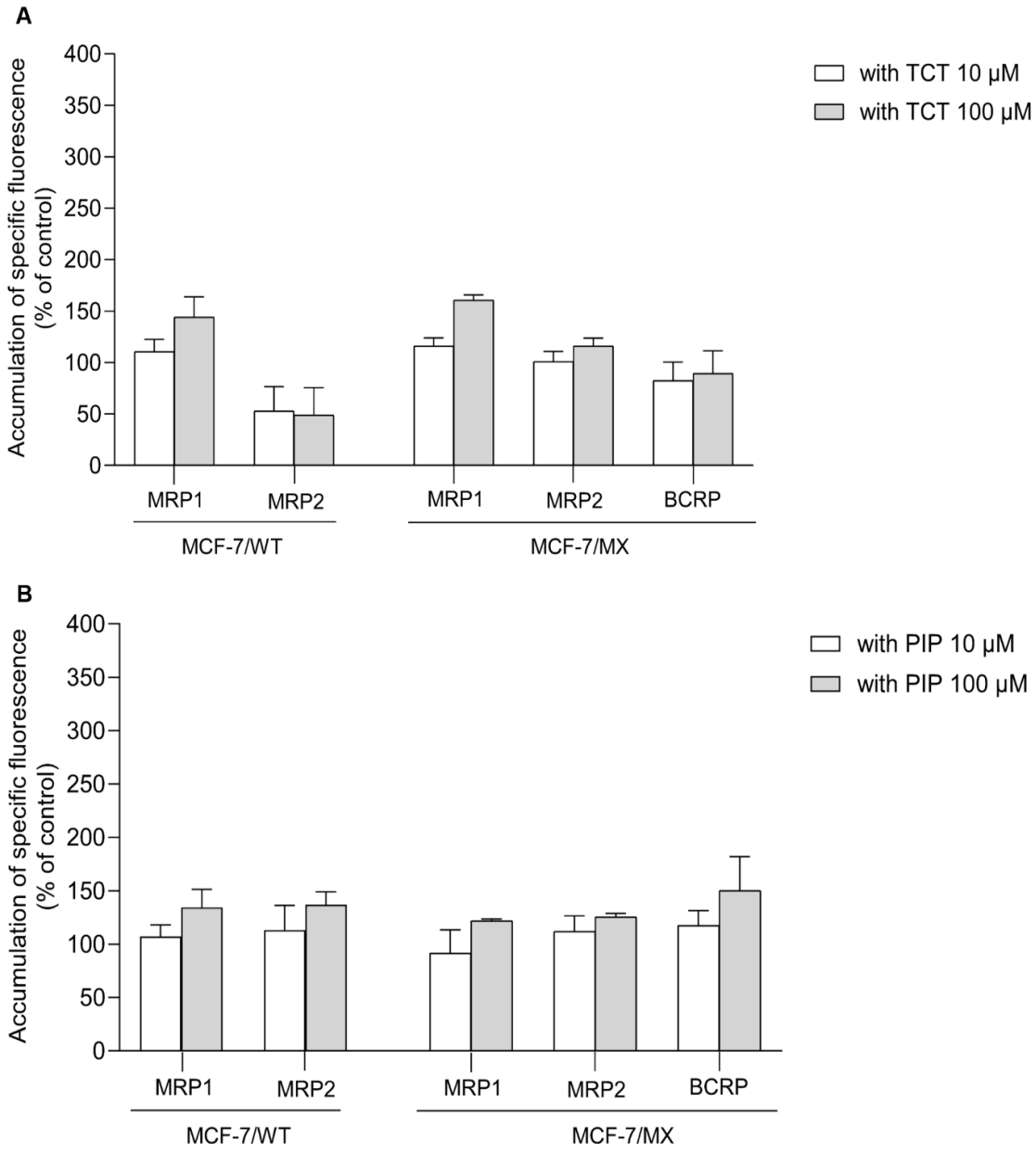
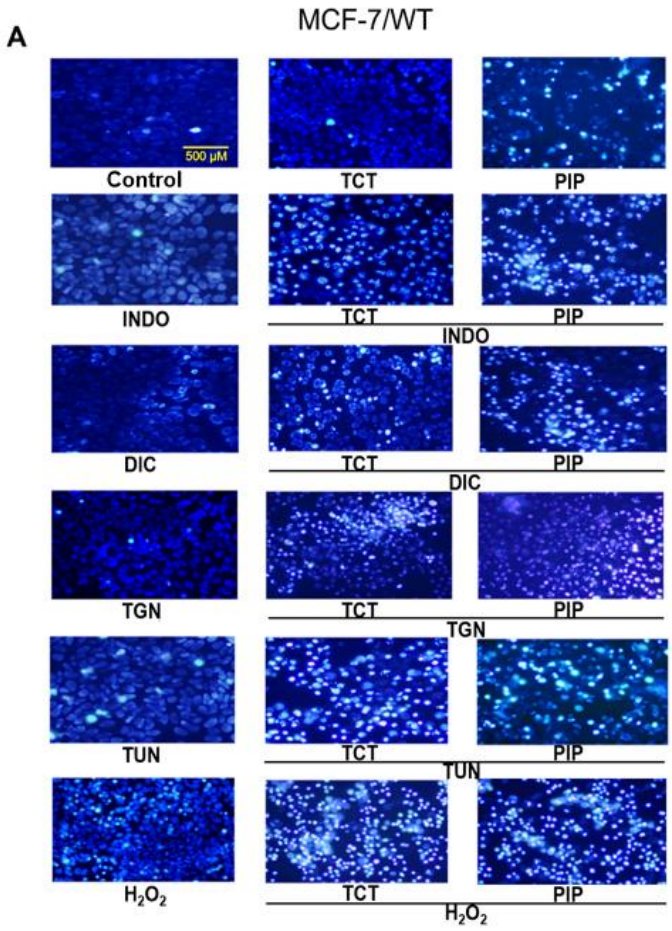
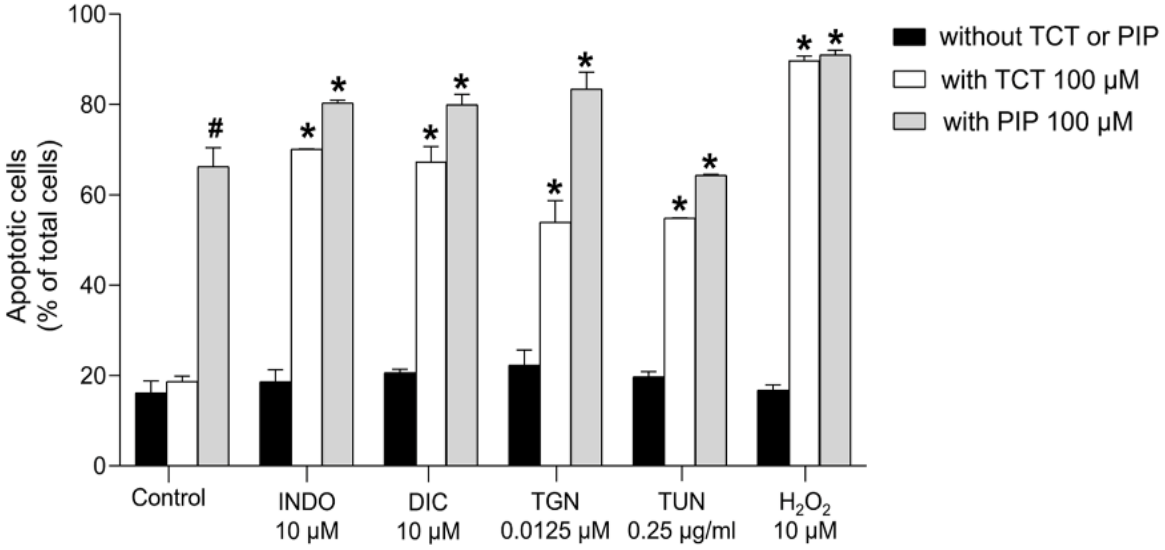


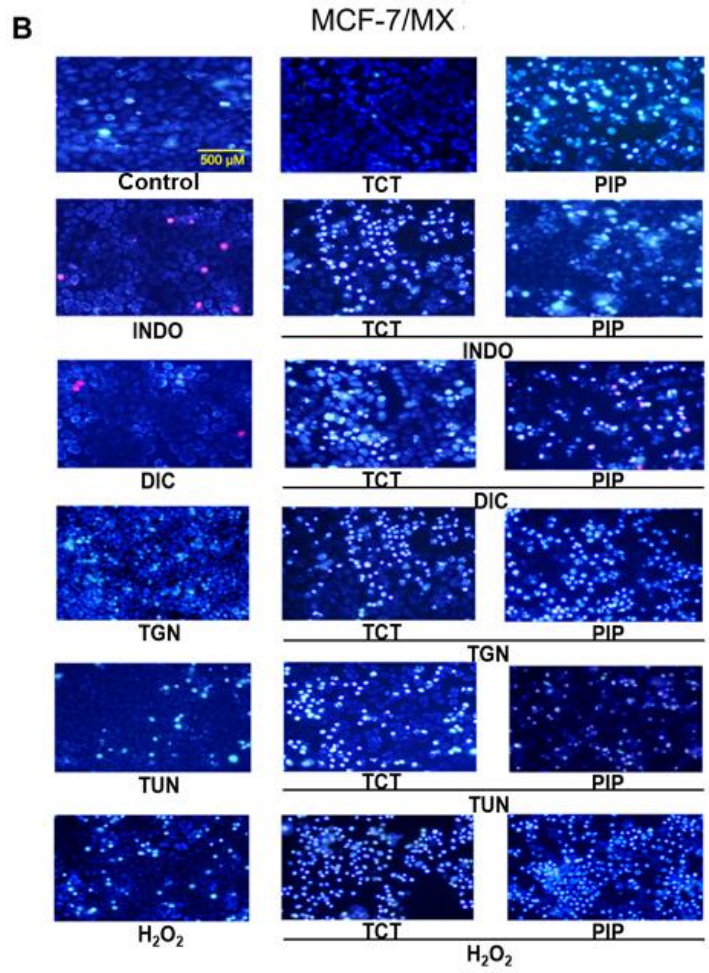
Figure 7. Activities of the drug efflux transporters in MCF-7/WT and MCF-7/MX cells. Intracellular accumulation of specific fluorescent substrates of each transporter (DCDF, CDCFDA and Phe A) in the presence of TCT (A) and PIP (B). Each bar represents mean ± SEM (n=3). *P < 0.05 compared with the control group (without inhibitor).

4.3 Effect of TCT on enhances of INDO- and DIC- mediated apoptotic cell death

The apoptotic cells were characterized by cell shrinkage, chromatin condensation, and nuclei fragmentation, using Hoechst 33342 dyes staining. As shown in Figure 8A and B, both MCF-7/WT and MCF-7/MX cells that were treated with 100 μM of TCT in combination with 10 μM of INDO and DIC or ER stressors (TGN; 0.0125 μM, TUN; 0.25 μg/ml, and H_2O_2; 10 μM) after 48-h exposure showed higher number of apoptotic cells, compared to those treated with single compound. The percentage of drug-induced apoptotic MCF-7/WT and MCF-7/MX cells significantly increased, by approximately 4-fold, in the presence of 100 μM TCT (Figure 8A and B). At 100 μM, PIP alone increased apoptotic cell death by 4-5 folds, compared to the untreated MCF-7/WT and MCF-7/MX groups. Apparently, when 100 μM PIP was used in combination with INDO, DIC or ER stressors, PIP itself was mainly responsible for the observed apoptotic cell death after 48-h treatment. It should be noted that the sensitivity of MCF-7/WT and MCF-7/MX cells to each treatment was comparable.


A
MCF-7/WT
500 μM
Control
TCT
PIP
INDO
TCT
PIP
INDO
DIC
TCT
PIP
DIC
TGN
TCT
PIP
TGN
TUN
TCT
PIP
TUN
H2O2
TCT
PIP
H2O2


Apoptotic cells
(% of total cells)
100
80
60
40
20
0
without TCT or PIP
with TCT 100 μM
with PIP 100 μM
Control
INDO
10 μM
DIC
10 μM
TGN
0.0125 μM
TUN
0.25 μg/ml
H2O2
10 μM


B
MCF-7/MX
500 µM
Control
TCT
PIP
INDO
TCT
PIP
INDO
DIC
TCT
PIP
DIC
TGN
TCT
PIP
TGN
TUN
TCT
PIP
TUN
H2O2
TCT
PIP
H2O2

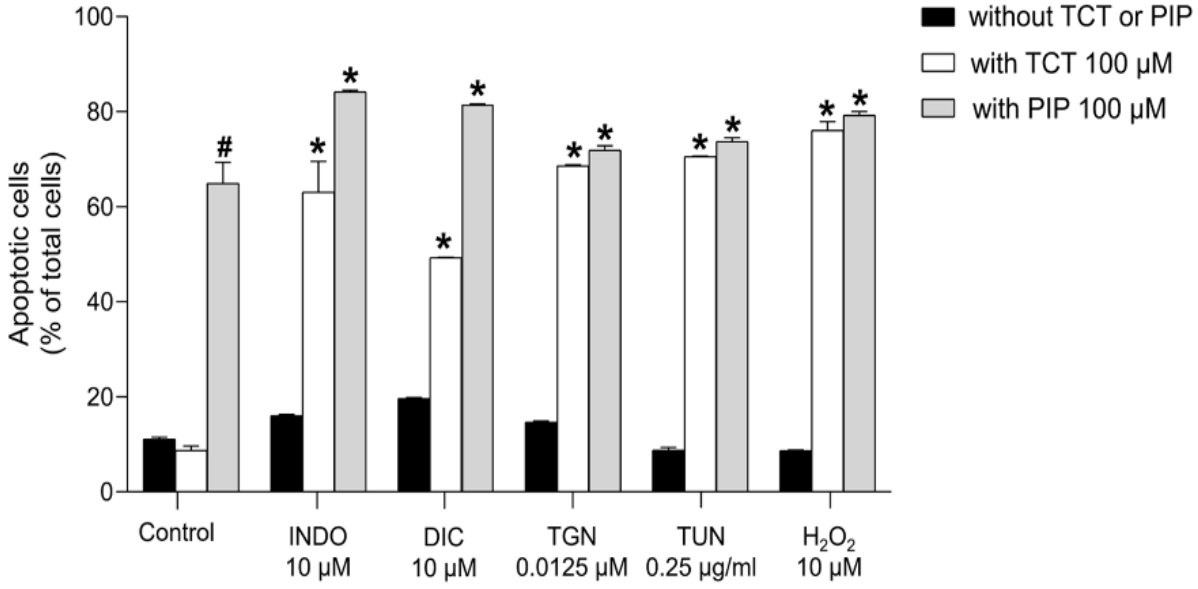

without TCT or PIP
with TCT 100 µM
with PIP 100 µM
Apoptotic cells
(% of total cells)
100
80
60
40
20
0
Control
INDO
10 µM
DIC
10 µM
TGN
0.0125 µM
TUN
0.25 µg/ml
H2O2
10 µM

Figure 8. Fluorescent imaging of Hoechst 33342 staining MCF-7/WT (A) and MCF-7/MX cells (B) after 48-h exposure to ER stressors (INDO, DIC, TGN, TUN, and H_2O_2) in the presence and absence of TCT or PIP. (20x magnification; scale bar = 500 μm). Apoptotic cells having condensed chromatin and/or fragmented nuclei were counted and expressed relative to total cells. Each bar represents mean ± SEM (n = 3). $^{*}P<$ 0.05 compared with the ER stressor-treated group in the absence of the test compound. $^{\#}P<$ 0.05 compared with the untreated control.

4.4 Effects of TCT on ROS production in MCF-7 cells.

As shown in Figure 9A and B, INDO and DIC at 500 μM were significantly increased intracellular ROS levels by approximately 1.5 times in both MCF-7/WT and MCF-7/MX cells after exposure for 2 h. Compared to NSAIDs alone group, combination with TCT at 100 μM did not increase ROS levels in both cell types. In addition, TCT could not enhance rotenone-mediated ROS generation. The results suggested that TCT did not possess intrinsic activity to enhance ROS production in the NSAIDs-treated cells.

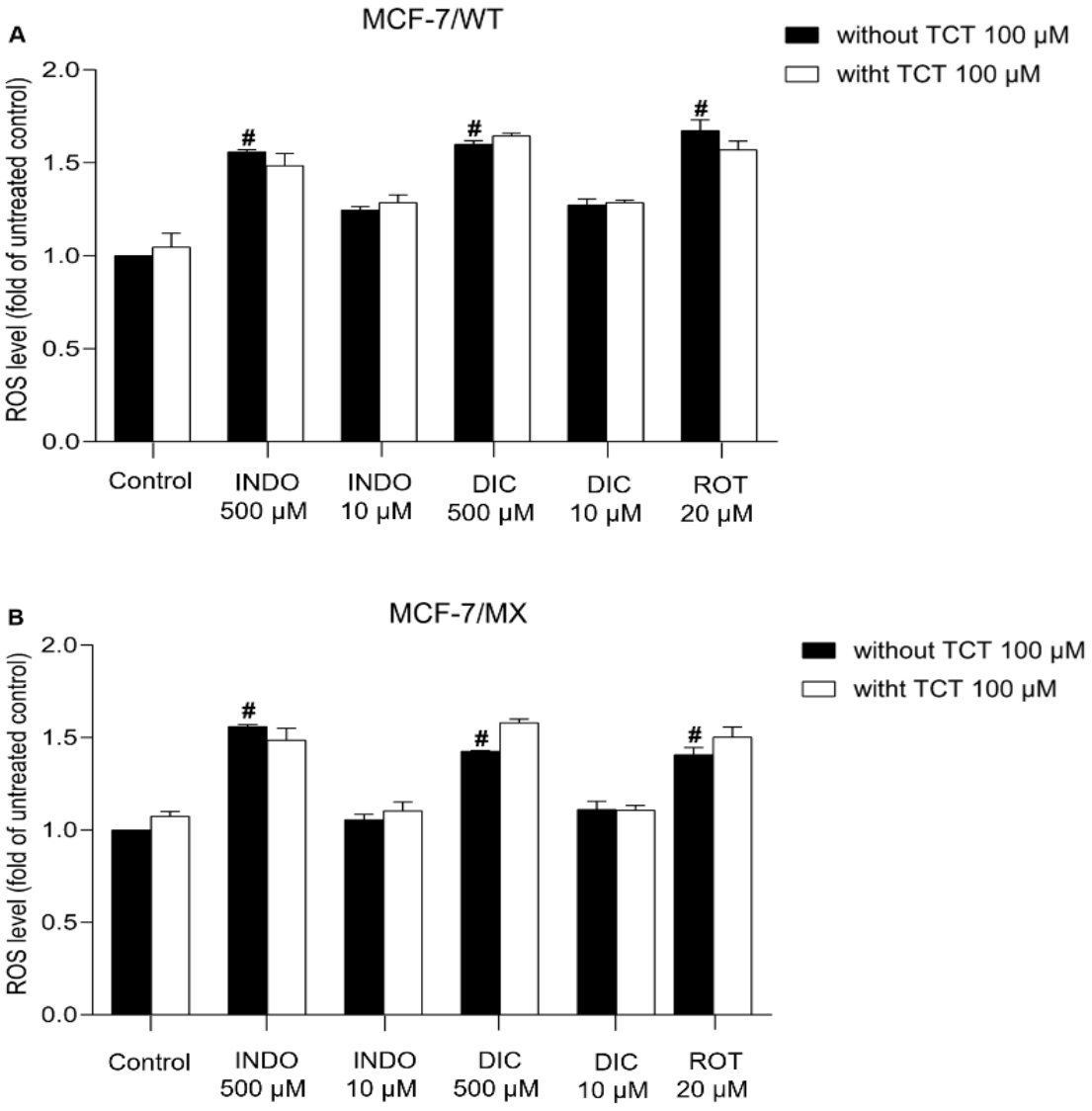


Figure 9. Reactive oxygen species (ROS) production in MCF-7/WT (A) and MCF-7/MX cells (B) after 2-h exposure to INDO, DIC and ROT in the presence and absence of 100 μM TCT. Each bar represents mean ± SEM ***(***n = 3***).*** $^{*}P< 0.05$ compared with the drug-treated group in the absence of TCT. $^{\#}P< 0.05$ compared with the untreated control.

4.5 Effects of TCT on mitochondria function in MCF-7 cells.

As shown in Figure 10A and B, either INDO, DIC at 10 μM or ER stressors (TGN; 0.0125 μM, TUN; 0.25 μg/ml, and H_2O_2; 10 μM) alone did not cause the loss of mitochondria membrane potential (MMP) after 48 h exposure. The combination of either INDO, DIC or ER stressor with TCT at 100 μM for 48 h were significantly reduced MMP function by approximately 35-55% in both MCF-7/WT and MCF-7/MX cells when comparing with NSAIDs or ER stressor alone.

The reduction of MMP in the cells exposed to either INDO, DIC at 10 μM or ER stressors (TUN at 0.25 μg/ml) in the presence of 100 μM TCT for 48 h was well correlated to increment of apoptotic Bax/ Bcl-2 proteins and apoptotic cell death. As shown in Figure 11A-D, after 48-h incubation period, either INDO, DIC at 10 μM or TUN at 0.25 μg/ml had no significant effect on the expression levels of pro-apoptotic Bax and antiapoptotic Bcl-2 proteins both MCF-7/WT and MCF-7/MX. While combining TCT with INDO, DIC or TUN significantly increased pro-apoptotic Bax and decreased anti-apoptotic Bcl-2 protein levels when compared with groups without TCT in MCF-7/WT and MCF-7/MX. The ratio of Bax/Bcl-2 of the combination of either INDO, DIC or TUN after 48 h treatment increased by approximately 2.5-3-fold in MCF-7/WT and MCF-7/MX.

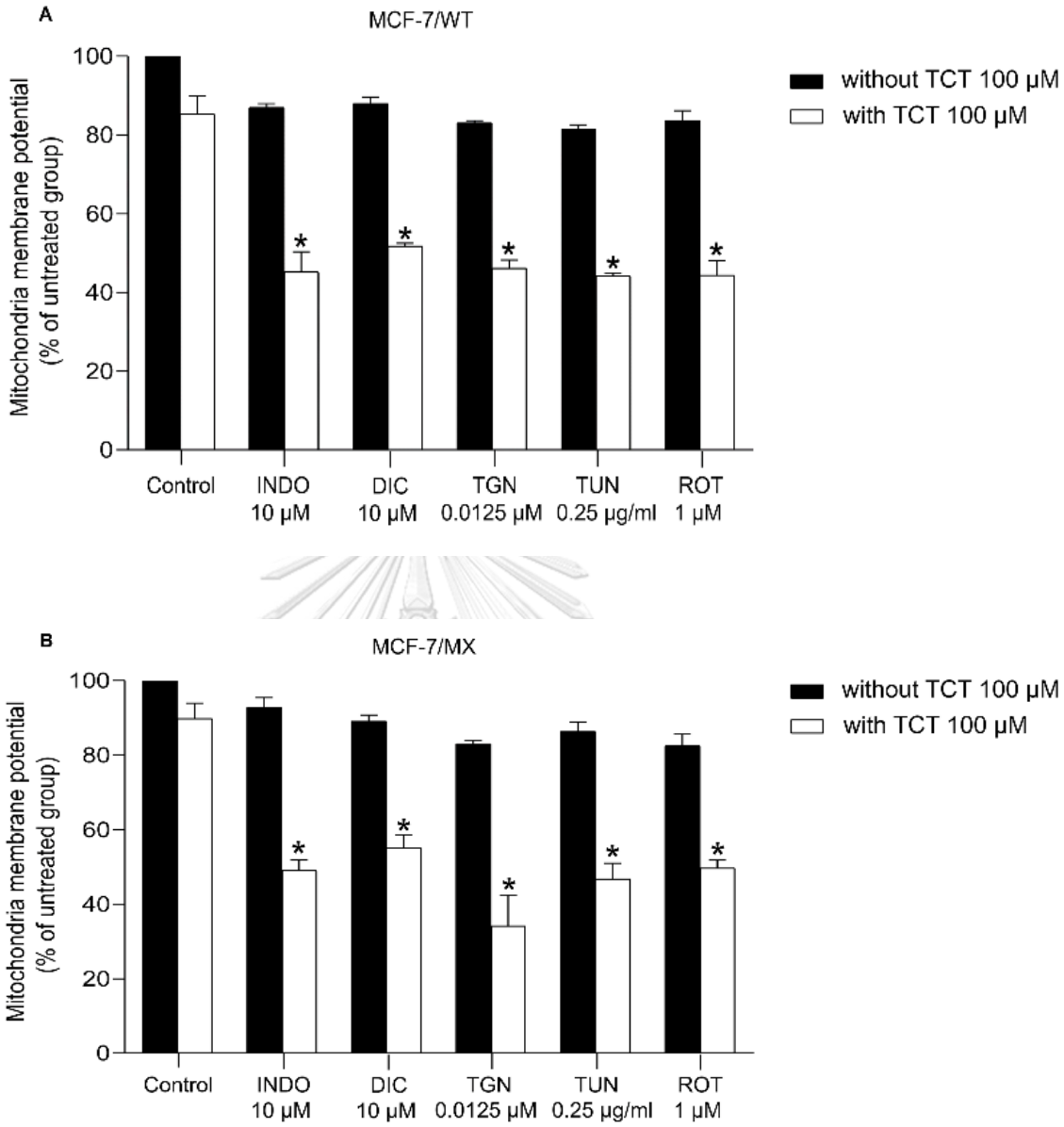
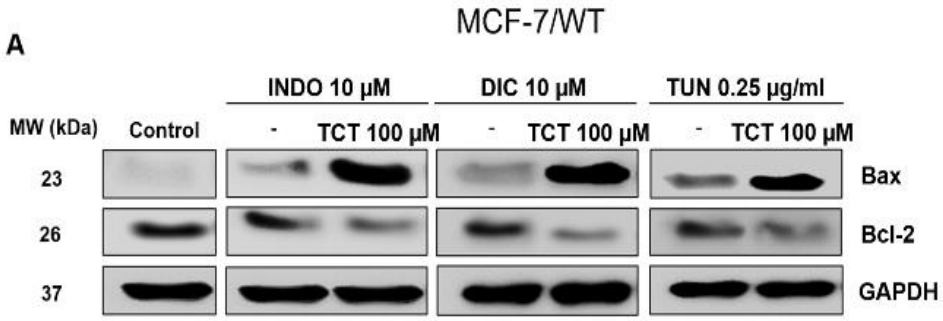
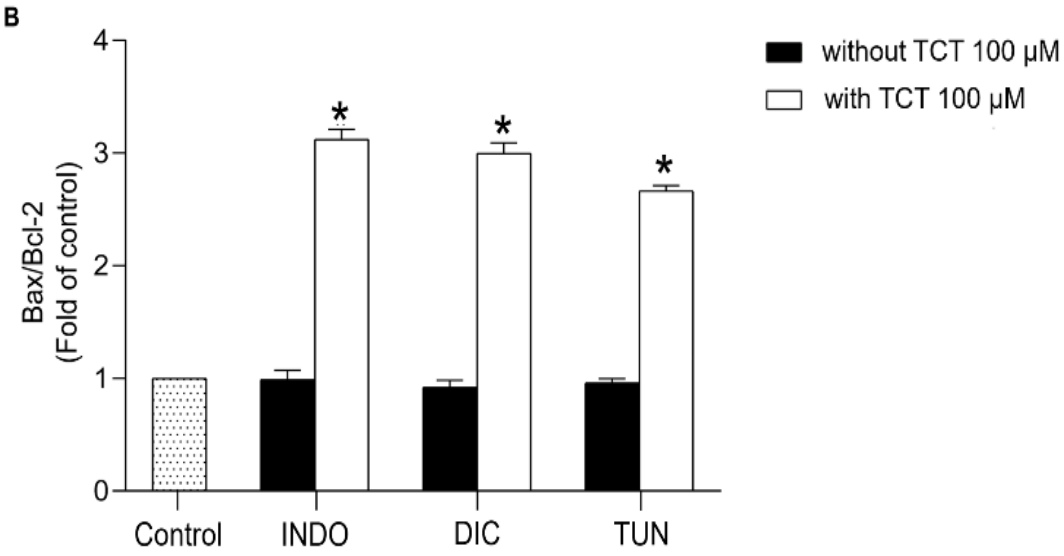


Figure 10. Effect of TCT on mitochondrial membrane potential (MMP) in MCF-7 (A) and MCF-7/MX cells (B) after 48-h exposure to ER stressors (INDO, DIC, TGN, TUN, and H_2O_2). Each bar represents mean ± SEM (n = 3). $^{*}P< 0.05$ compared with the stressor-treated group without TCT. $^{\#}P< 0.05$ compared with the untreated control.


A
MCF-7/WT
INDO 10 μM
DIC 10 μM
TUN 0.25 μg/ml
MW (kDa)
Control
-
TCT 100 μM
-
TCT 100 μM
-
TCT 100 μM
23
26
37
Bax
Bcl-2
GAPDH


B
without TCT 100 μM
with TCT 100 μM
Bax/Bcl-2
(Fold of control)
0
1
2
3
4
*
*
*
Control
INDO
DIC
TUN

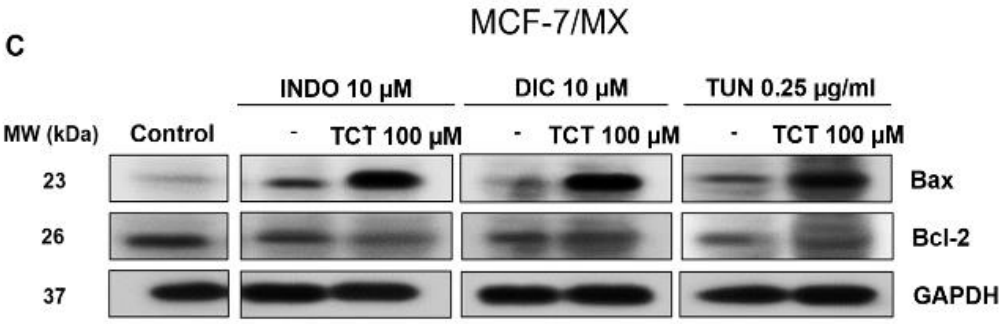

C
MCF-7/MX
INDO 10 μM
DIC 10 μM
TUN 0.25 μg/ml
MW (kDa)
Control
-
TCT 100 μM
-
TCT 100 μM
-
TCT 100 μM
23
26
37
Bax
Bcl-2
GAPDH

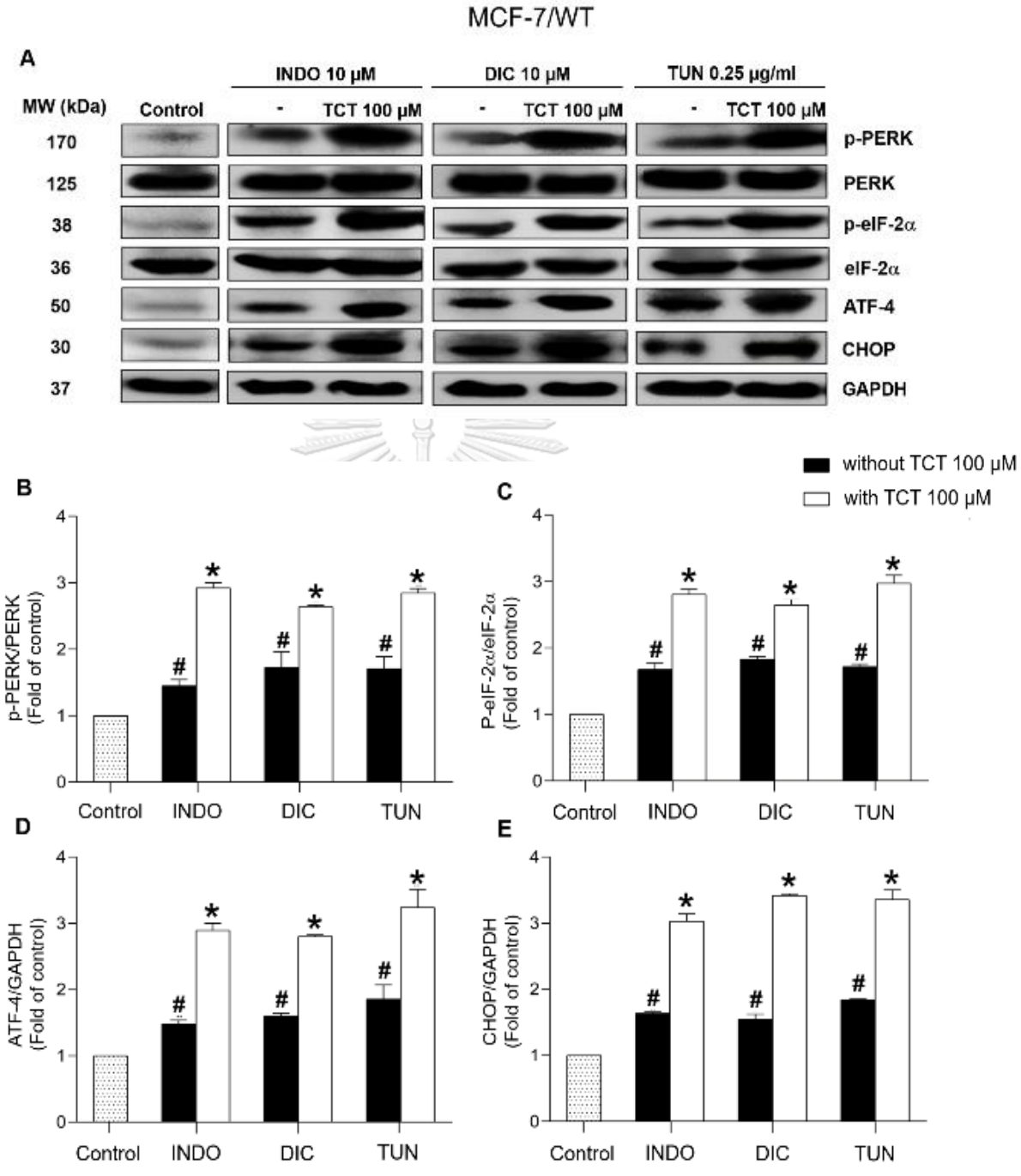
Figure 11. Immunoblots and their densitometrical analysis of Bax and Bcl-2 proteins in MCF-7 (A, B) and MCF-7/MX cells (C, D) after 48-h exposure to ER stressors (INDO, DIC, and TUN) in the presence and absence of TCT. Each bar represents mean ± SEM (n=3). $^{*}P< 0.05$ compared with the stressor-treated group without TCT. $^{\#}P< 0.05$ compared with the untreated control.

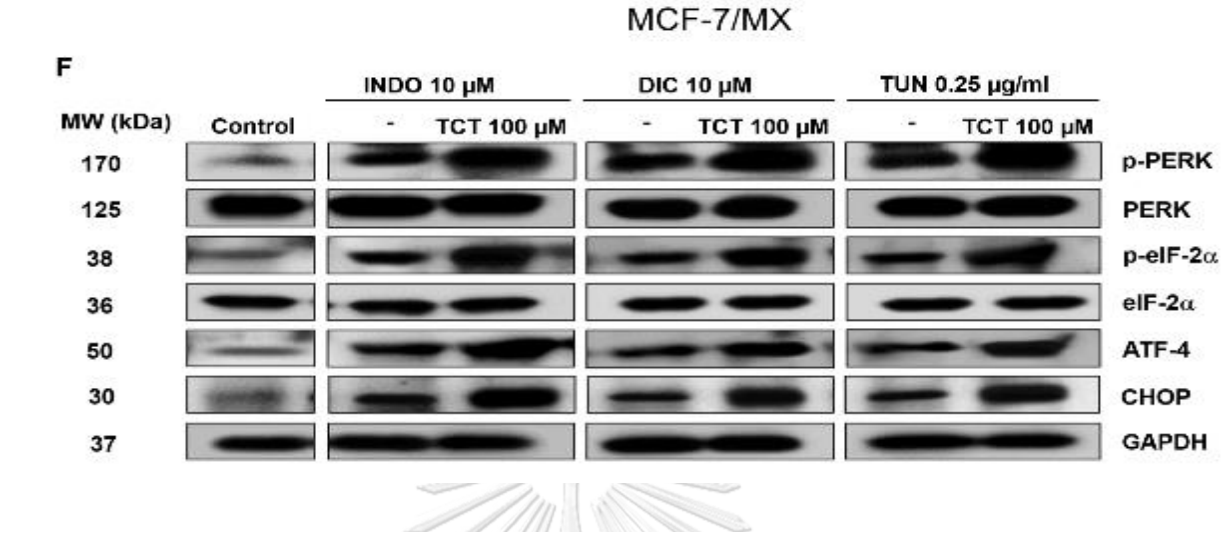
4.6 Involvement of ER stress signaling pathways in TCT enhancing effect on INDO- and DIC-mediated cytotoxicity.

INDO and DIC have been reported to produce ER stress-mediated cytotoxicity via activation of PERK and IRE-1α stress sensors in various cancer cell lines (Boonyoung et al., 2020; Chang et al., 2020; Park et al., 2018; Gu et al., 2015). This study investigated the effect of TCT on promotion breast cancer cell sensitivity to both NSAIDs via ER stress-dependent mechanism.

4.6.1 Effects of TCT on ER stress-mediated PERK/eIF-2α/ATF-4/CHOP signaling pathways.

Activation of PERK proteins results in the stimulation eIF-2α/ATF-4/CHOP signaling cascades which associated with apoptotic cell death through changes in the expression levels of pro-apoptotic Bax and anti-apoptotic Bcl-2 proteins (Fujiki et al., 2019; Zhang et al., 2019; Hu et al., 2019). As shown in Figure 12A-J, either INDO, DIC at 10 μM or TUN at 0.25 μg/ml alone significantly increased the phosphorylation form of PERK (p-PERK) and eIF2-α (p-eIF-2α) protein expressions as well as the extents of ATF-4 and CHOP protein expressions in MCF-7/WT and MCF-7/MX after 48-h exposure. Furthermore, the presence of 100 μM TCT in combination with INDO, DIC or TUN significantly increased protein expression levels of p-PERK/ p-eIF-2α/ ATF-4/CHOP protein when compared to drug alone group.


MCF-7/WT
A
INDO 10 μM
DIC 10 μM
TUN 0.25 μg/ml
MW (kDa)
Control
- TCT 100 μM
- TCT 100 μM
- TCT 100 μM
170
125
38
36
50
30
37
p-PERK
PERK
p-eIF-2α
eIF-2α
ATF-4
CHOP
GAPDH
without TCT 100 μM
with TCT 100 μM
B
p-PERK/PERK (Fold of control)
C
P-eIF-2α/eIF-2α (Fold of control)
D
ATF-4/GAPDH (Fold of control)
E
CHOP/GAPDH (Fold of control)
Control
INDO
DIC
TUN


MCF-7/MX
F
INDO 10 μM
DIC 10 μM
TUN 0.25 μg/ml
MW (kDa)
Control
-
TCT 100 μM
170
125
38
36
50
30
37
p-PERK
PERK
p-eIF-2α
eIF-2α
ATF-4
CHOP
GAPDH

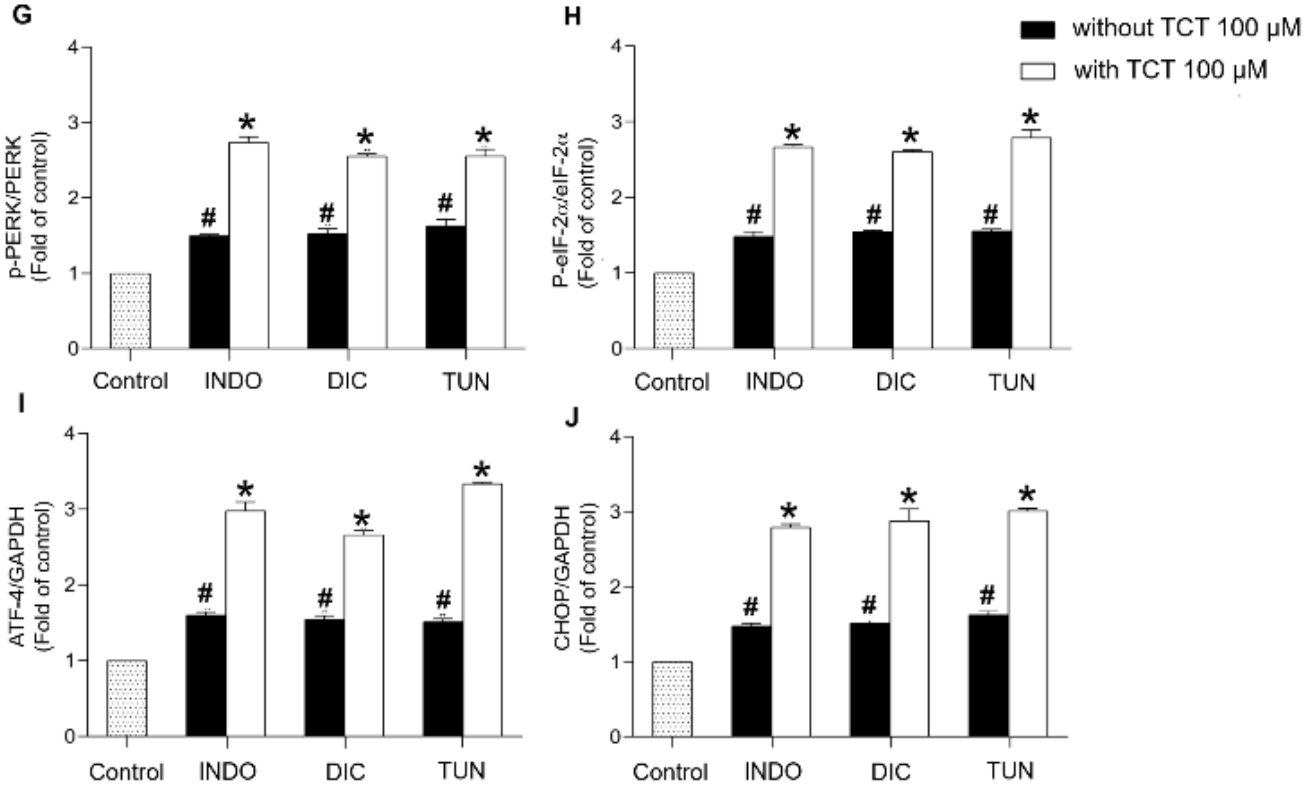
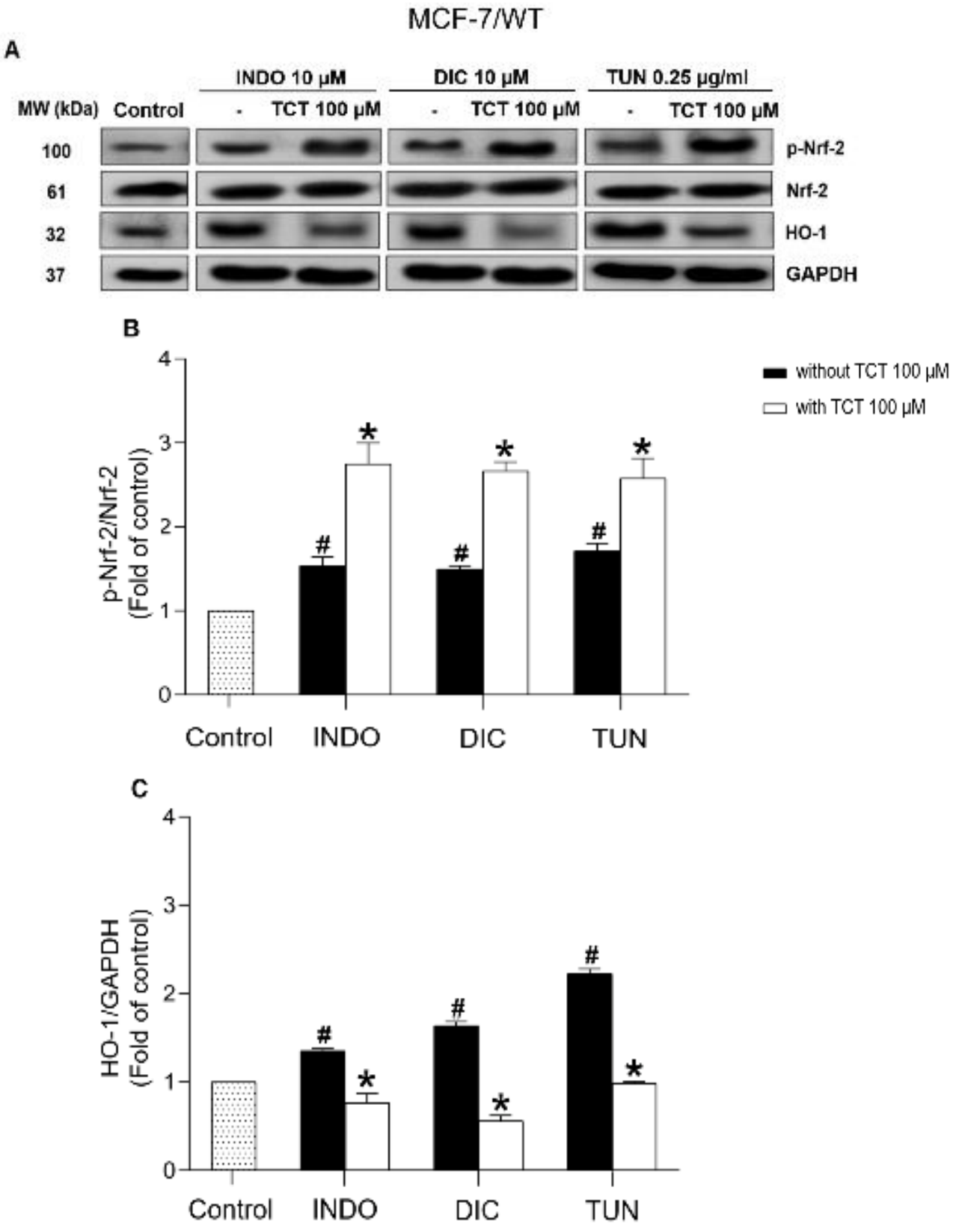

G
H
without TCT 100 μM
with TCT 100 μM
p-PERK/PERK (Fold of control)
P-eIF-2α/eIF-2α (Fold of control)
I
J
ATF-4/GAPDH (Fold of control)
CHOP/GAPDH (Fold of control)
Control
INDO
DIC
TUN

Figure 12. Expression of PERK/ eIF-2α/ ATF-4/ CHOP proteins and their phosphorylated forms in MCF-7/WT (A-E) and MCF-7/MX cells (F-J) after 48-h exposure to ER stressors (INDO, DIC, and TUN) in the presence and absence of TCT. Data from densitometrical analysis are expressed as the ratios of p-PERK to PERK (B, G), p-eIF-2α to eIF-2α (C, H), ATF-4 to GAPDH (D, I) and CHOP to GAPDH (E, J). Each bar represents mean ± SEM (n=3). $^{*}P< 0.05$ compared with the stressor-treated group without TCT. $^{\#}P< 0.05$ compared with the untreated control.

4.6.2 Effects of TCT on ER stress-mediated Nrf-2/HO-1 signaling pathway.

Activation of PERK also stimulates cell survival mechanism, particularly the phosphorylation of transcription factor Nrf-2, resulting in the up-regulation of detoxifying enzymes including HO-1 providing cell survival mechanism against INDO, DIC, and other ER stressors (Boonyong et al., 2020; Fujiki et al., 2019; Dong et al., 2020; Wang et al., 2020; Bellezza et al., 2017). In this study, either INDO, DIC at 10 μM or TUN at 0.25 μg/ml alone significantly increased phosphorylated form of Nrf-2 (p-Nrf-2) protein expressions in MCF-7/WT and MCF-7/MX cells after 48-h treatment as shown in Figure 13A-F. Similarly, the presence of 100 μM TCT in combination with INDO, DIC or TUN significantly increased protein expression levels of p-Nrf-2 in both cell types, but the protein expression level of HO-1 decreased significantly when compared to when compared to drug alone group.


MCF-7/WT
A
INDO 10 μM
DIC 10 μM
TUN 0.25 μg/ml
MW (kDa)
Control
-
TCT 100 μM
-
TCT 100 μM
-
TCT 100 μM
100
61
32
37
p-Nrf-2
Nrf-2
HO-1
GAPDH
B
without TCT 100 μM
with TCT 100 μM
p-Nrf-2/Nrf-2
(Fold of control)
0
1
2
3
4
#
*
Control
INDO
DIC
TUN
C
HO-1/GAPDH
(Fold of control)
0
1
2
3
4
#
*
Control
INDO
DIC
TUN

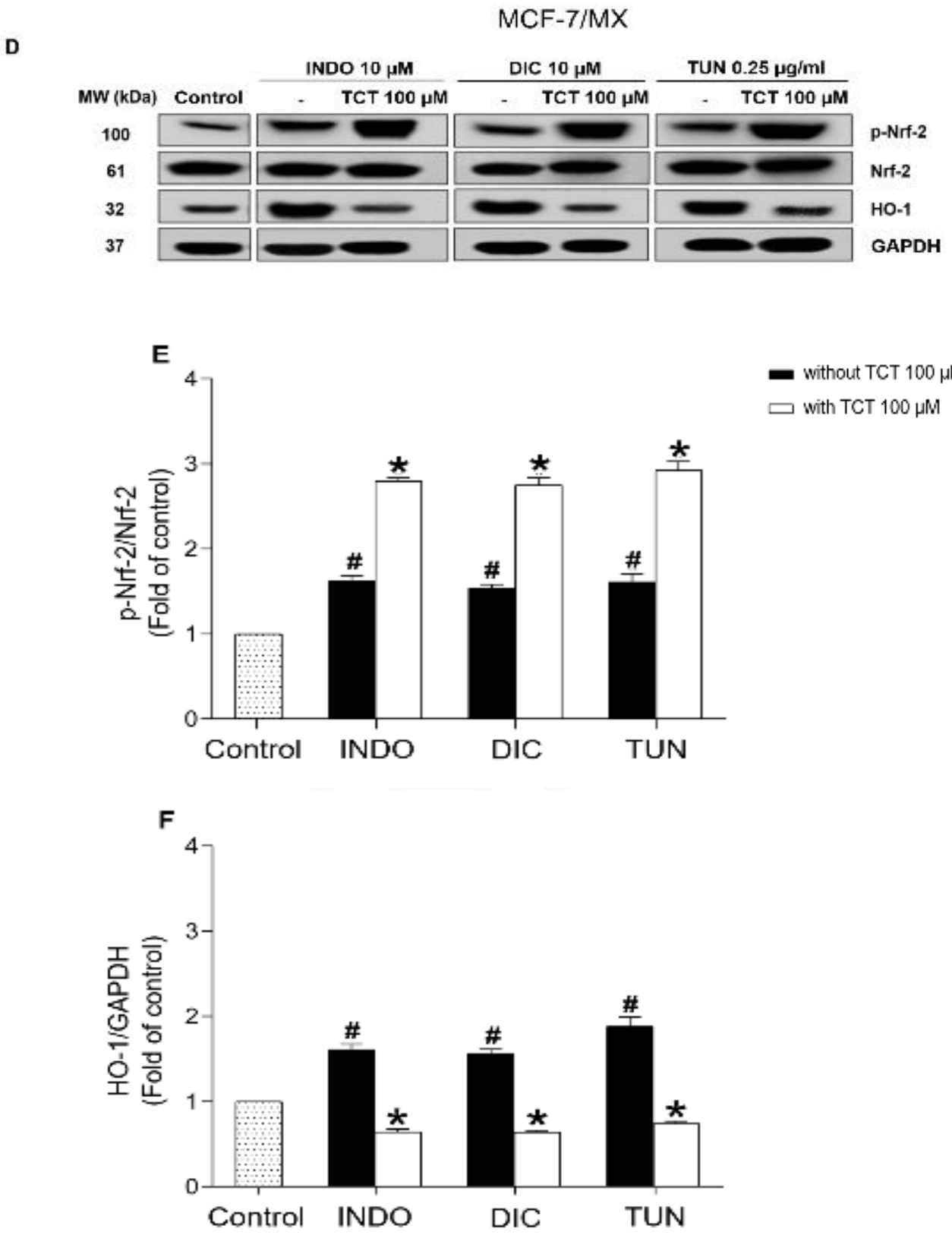
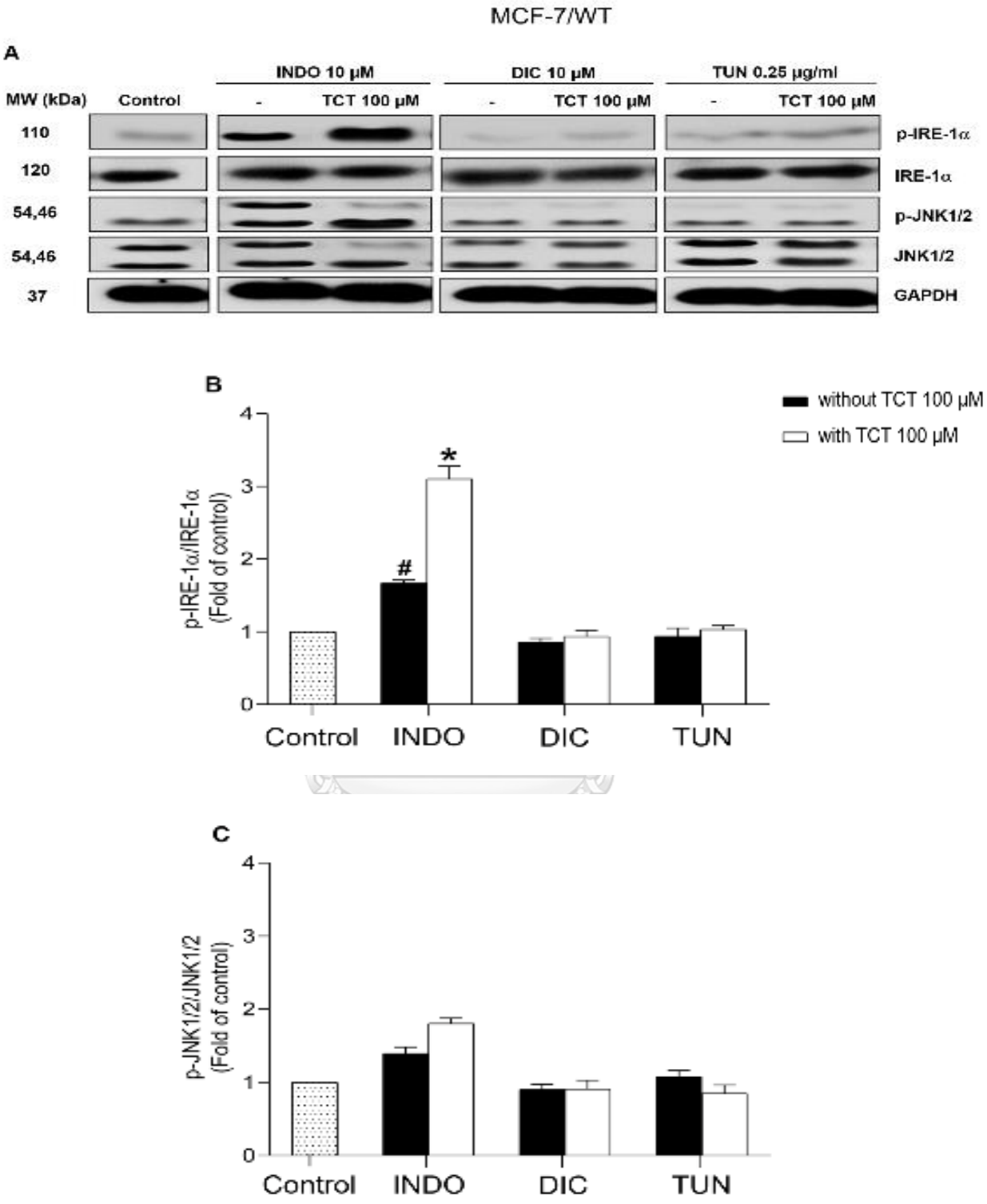

D
MCF-7/MX
INDO 10 μM
DIC 10 μM
TUN 0.25 μg/ml
MW (kDa)
Control
-
TCT 100 μM
-
TCT 100 μM
-
TCT 100 μM
100
61
32
37
p-Nrf-2
Nrf-2
HO-1
GAPDH
E
without TCT 100 μM
with TCT 100 μM
p-Nrf-2/Nrf-2
(Fold of control)
0
1
2
3
4
#
*
Control
INDO
DIC
TUN
F
HO-1/GAPDH
(Fold of control)
0
1
2
3
4
#
*
Control
INDO
DIC
TUN

Figure 13. Expression of Nrf-2 and HO-1 proteins in MCF-7/WT (A-C) and MCF-7/MX cells (D-F) after 48-h exposure to ER stressor (INDO, DIC, and TUN) in the presence and absence of TCT. Data from densitometrical analysis are expressed as the ratios of p-Nrf-2 to Nrf-2 (B, E) and HO-1 to GAPDH (C, F). Each bar represents mean ± SEM (n = 3). $^{*}P< 0.05$ compared with the stressor-treated group without TCT. $^{\#}P< 0.05$ compared with the untreated control.

4.6.3 Effects of TCT on ER stress-mediated IRE-1α/JNK1/2 signaling pathway.

Activation of IRE-1α/JNK1/2 signaling, leading to direct an increase of Bax/ Bcl-2 ratio and apoptotic cell death in cancer cells (Chang et al., 2020; Boonyong et al., 2020). As shown in Figure 14A-F, INDO (10 μM), but not DIC (10 μM), or TUN (0.25 μg/ ml) significantly increased phosphorylated form of IRE-1α (p-IRE-1α) protein expression in MCF-7/WT and MCF-7/MX cells when compared to the untreated groups after 48-h treatment. However, INDO had no significant effect on the phosphorylation of JNK1/2 (p-JNK1/ 2) protein expression in both cell types. In addition, combination of TCT at 100 μM and INDO at 10 μM significantly increased p-IRE-1α protein expression when compared to without TCT groups while had no effect on p-JNK1/2 protein expression in both cells (Figure 14A-F).


MCF-7/WT
A
INDO 10 μM
DIC 10 μM
TUN 0.25 μg/ml
MW (kDa)
Control
-
TCT 100 μM
-
TCT 100 μM
-
TCT 100 μM
110
120
54,46
54,46
37
p-IRE-1α
IRE-1α
p-JNK1/2
JNK1/2
GAPDH
B
without TCT 100 μM
with TCT 100 μM
p-IRE-1α/IRE-1α
(Fold of control)
#
*
Control
INDO
DIC
TUN
C
p-JNK1/2/JNK1/2
(Fold of control)
Control
INDO
DIC
TUN

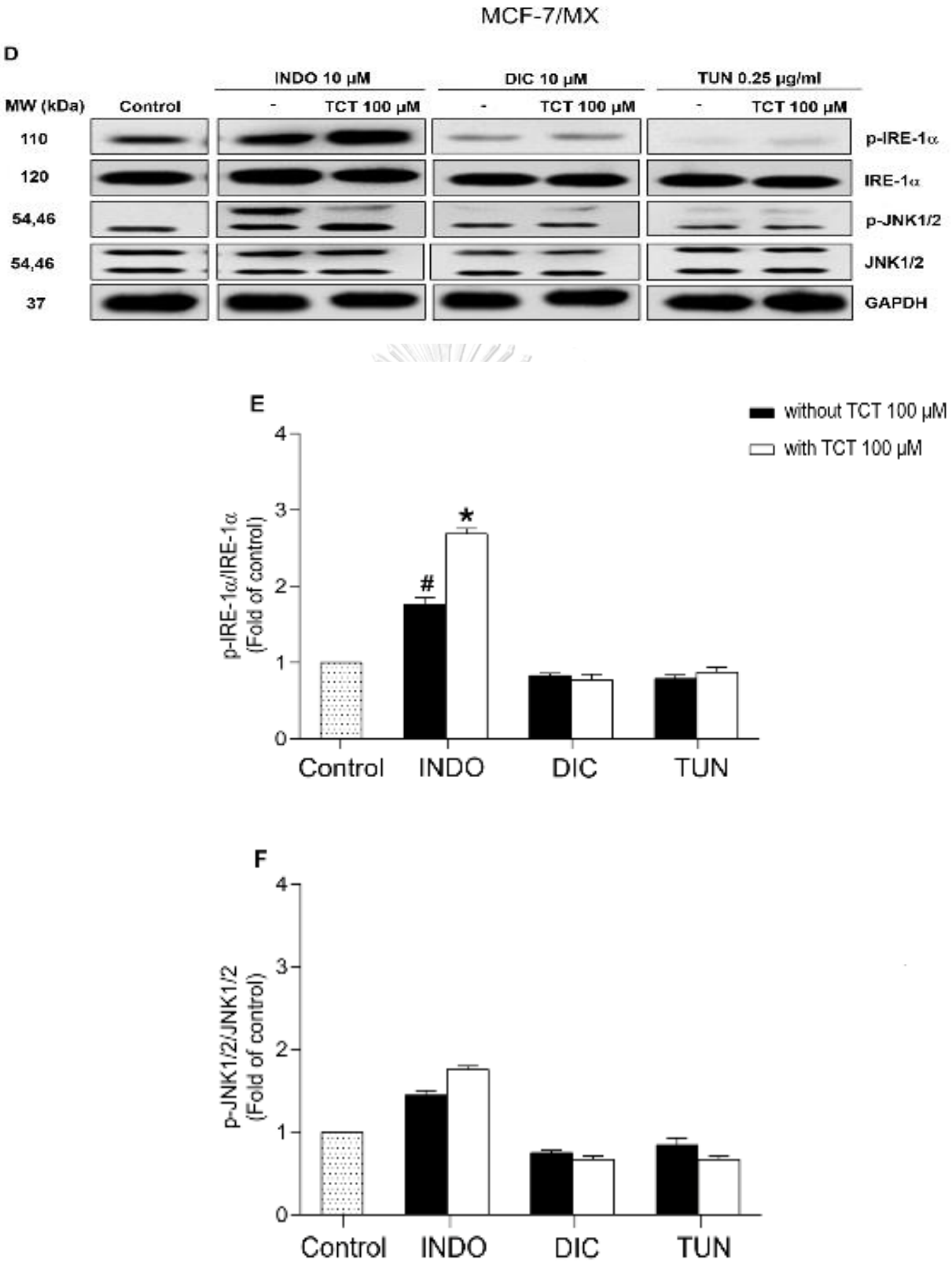

MCF-7/MX
D
INDO 10 μM
DIC 10 μM
TUN 0.25 μg/ml
MW (kDa)
Control
-
TCT 100 μM
-
TCT 100 μM
-
TCT 100 μM
110
120
54,46
54,46
37
p-IRE-1α
IRE-1α
p-JNK1/2
JNK1/2
GAPDH
E
without TCT 100 μM
with TCT 100 μM
p-IRE-1α/IRE-1α
(Fold of control)
#
*
Control
INDO
DIC
TUN
F
p-JNK1/2/JNK1/2
(Fold of control)
Control
INDO
DIC
TUN

Figure 14. Expression of IRE-1α/JNK1/2 and their phosphorylated forms in MCF-7/WT (A-C) and MCF-7/MX cells (D-F) after 48-h exposure to ER stressors (INDO, DIC, and TUN) in the presence and absence of TCT. Data from densitometrical analysis are expressed as the ratios of p-IRE-1α to IRE-1α (B, E) and p-JNK1/2 to JNK1/2 (C, F). Each bar represents mean ± SEM (n = 3). $^{*}P< 0.05$ compared with the stressor-treated group without TCT. $^{\#}P< 0.05$ compared with the untreated control.

CHAPTER V
DISCUSSION

Combination of non-selective COX inhibitors (e.g. INDO and DIC) and certain natural compounds (e.g. resveratrol, curcumin, piperine and naringenin) have been reported its effectiveness in enhancing cancer cell death both *in vitro* and *in vivo* models (Boonyong et al., 2020a, b; Feng et al., 2016; Rana et al., 2015; Redondo-Blanco et al., 2017). In addition, a number of alkaloid compounds (e.g. piperine, piperlongumine and isoliensinine) have been reported their ability to increase cytotoxic effect of various anticancer agents (e.g. oxaliplatin, paclitaxel and cisplatin) through several mechanisms such as inhibition of efflux transporters, increased cellular stress and induction of apoptosis (Chaisit et al., 2017; T. Chen et al., 2019; Do et al., 2013; Manogaran et al., 2019). In this study, INDO and DIC were selected as model drugs to investigate the potentiation effect of an alkaloid TCT on NSAIDs cytotoxicity in MCF-7 breast cancer cells and its mitoxantrone-resistant subline.

It has been well established that high expression levels of the ATP binding cassette (ABC) transporters, particularly P-glycoprotein (P-gp), breast cancer resistance protein (BCRP), multidrug resistance-associated proteins (MRPs), lead to failure of several cytotoxic anti-cancer drugs such as mitoxantrone, doxorubicin in breast cancer treatment (Choi and Yu, 2014; Gillet and Gottesman, 2010a; Sun et al., 2012). Taken that INDO and DIC are substrate of MRPs and BCRP efflux transporter (Zhang et al., 2016), the mitoxantrone resistant MCF-7 subline with the high expression levels of BCRP efflux transporter was employed in this study. The results showed that the MCF-7/MX cells were more resistant to mitoxantrone (MX) (a specific substrate of BCRP) by approximately 11.7 times, compared with the MCF-7/WT cell. These results were corresponded to high activities of BCRP efflux transporter observed in the resistant subline. Both cell types expressed MRP1 and MRP2 activities. Neither MCF-7

nor MCF-7/MX cells exhibited P-gp function. Apparently, MCF-7 cell line was more sensitivity to INDO and DIC than MCF-7/MX subline. It was likely that their different sensitivities toward INDO and DIC cytotoxicity could be related to the different expression levels of the efflux transporters. The capability of TCT to potentiate NSAIDs cytotoxicity was first determined in both MX-sensitive and MX-resistant MCF-7 cells. At the concentration of 10 μM, INDO and DIC were not toxic to both cell types. Concurrent treatment these two MCF-7 cell types with NSAIDs and TCT at their non-cytotoxic concentrations significantly increased cell death, as measured by MTT assay. Notably, the potentiative effect of TCT in the combination was concentration dependent. The results also indicated that the combinatorial effects were similar in both cell types. In addition to TCT, piperine (PIP) was also found in *Piper* plants. It has been reported PIP was capable of potentiating the cytotoxic drugs such as doxorubicin in Caco-2 and CEM/ADR 5000 resistant cells, leading to cancer cell death (H. Li et al., 2018). Thus, PIP was also tested in parallel to TCT. The combination of PIP with NSAIDs had no significant effect on cell death when compared to PIP alone in both cell types. It was likely that TCT was able to potentiate NSAIDs cytotoxicity better than PIP.

Both TCT and PIP significantly increased the sensitivity of MCF-7 cells toward MX toxicity. However, the potentiation effect of both compounds was not observed in MCF-7/MX cells. In addition, combination of either TCT or PIP with other cytotoxic agents (i.e., tamoxifen (TAM), vinblastine (VBL), and camptothecin (CPT)) were also investigated. TCT and PIP could not increase the cytotoxicity of these cytotoxic drugs in both cell types.

The ability to enhance cytotoxicity effects of natural compounds may be related to their interference with the activities of ABC drug efflux transporters (Dobiasova et al., 2020; Li et al., 2016; Tinoush et al., 2020). For example, a naphthoquinone rhinacanthin-C at its non-cytotoxic concentrations (0.04 or 0.1 μM)

significantly enhanced doxorubicin mediated cytotoxicity in MCF-7 cell after 48 h treatment through inhibition of MRP2 and P-gp activities (Chaisit et al., 2017). Thus, the inhibitory effect of TCT and PIP on MRP1, MRP2 and BCRP activities were determined. The results showed that TCT and PIP had no inhibitory effect on activities of MRP1, MRP2 and BCRP in both cell types. Hence, TCT and PIP exert their potential chemo-sensitizing activity to INDO and DIC via mechanisms independent of drug efflux.

The enhancement effect on cytotoxicity of natural compounds may be related to increase cellular stress (T. Chen et al., 2019). For example, tetrandrine (alkaloid) was able to potentiate hydrogen peroxide leading to apoptotic cell death in HaCaT Human keratinocyte cells after 12 h (Cheng et al., 2021). In this study, the combinatorial effect of TCT and various ER stressors [i.e., H_2O_2, ROT, TGN, and TUN] on cytotoxicity were also assessed. Similarly to the potentiative effects on NSAIDs cytotoxicity, both TCT and PIP were able to increase the death of MCF-7 cells caused by each ER stressor. However, at the same concentration, TCT had no effect on cell viability whereas PIP was able to kill MCF-7 and MCF-7/MX cells. Thus, PIP was excluded for further study. These data suggested that TCT might induce apoptotic cell death caused by INDO and DIC through ER stress-related mechanisms.

INDO and DIC have been reported to cause apoptotic cell death by inhibition of mitochondria complex I, increased intracellular ROS level and production of ER stress in several cancer cells such as Caco-2, Glioma H4, and 4T1EM3 cells (Boonyong et al., 2020b; Chang et al., 2020; Sandoval-Acuña et al., 2012; Terasaki et al., 2019). Evidently, combination of TCT with NSAIDs did not increase intracellular ROS level when compared to NSAIDs alone in both cell types. As such, it was unlikely that TCT could enhance ROS production in the NSAIDs-treated cells. Moreover, reduction of mitochondria activity was observed in both cell types after exposed to TCT in combination with either INDO, DIC or E R stressors. It should be noted that neither

INDO nor DIC alone had effect on mitochondria membrane potential (MMP). These data suggested that TCT might enhance either INDO, DIC or ER stressors cytotoxicity, in part, from the loss of MMP in MCF-7 and MCF-7/MX cells. In addition, combination of TCT with either INDO, DIC or ER stressors caused increase of Bax and decreased Bcl-2 expression in both cell types. It has been reported that the loss of MMP was correlated to increase Bax/Bcl-2 expression ratios, leading to disruption of the mitochondria function and apoptotic cell death (Iurlaro and Munoz-Pinedo, 2016; Liu et al., 2015; Liu et al., 2016).

INDO and DIC were demonstrated to produce ER stress-mediated cytotoxicity via activation of PERK/eIF-2α/ATF-4/CHOP signaling pathways. The activation of PERK protein (stress sensors) is related to apoptotic cell death in various cancer cell lines including Caco-2, Glioma, H4BT-474, MCF-7, A549 cells (Boonyong et al., 2020a; Chang et al., 2020; Gu et al., 2015; Park et al., 2018). In this study, INDO and DIC were able to significantly increase phosphorylation of PERK (p-PERK) as well as ER stressor (TUN) by approximately 1.4 to 1.7 times. The activation of p-PERK subsequently triggers phosphorylated eIF-2α (p-eIF-2α), ATF-4, and CHOP expression (Fujiki et al., 2019; Zhang et al., 2019). Combination of TCT with either INDO, DIC or TUN were able to significantly increase protein expression of p-PERK/p-eIF-2α/ATF-4/CHOP by approximately 2 times when compared to those without TCT in both cell types. It has been reported that CHOP is a nuclear transcription factor that regulates the expression of proteins involved in controlling cell homeostasis and stress responses such as Nrf-2 signaling and apoptotic BCl-2 proteins (Hu et al., 2018; Huang et al., 2021). Increased CHOP expression could trigger cell death and promote apoptosis through changing in the expression levels of Bax and Bcl-2 proteins. Thus, these data suggested that TCT was able to enhance apoptotic cell death either INDO, DIC or ER stressor via activation of PERK/eIF-2α/ATF-4/CHOP pathway.

The activation of PERK also stimulates cell survival mechanism, particularly Nrf-2/HO-1 pathway, in various cell models such as Caco-2, HL-7702, mpkCCD and NCMs cardiomyocytes cells (Boonyong et al., 2020a; Dong et al., 2020; Fujiki et al., 2019; Wang et al., 2020). Phosphorylation of Nrf-2 results in the up-regulation of detoxifying enzymes including HO-1, providing cell survival mechanism against INDO, DIC, and other ER stressors (Bellezza et al., 2017; Boonyong et al., 2020a). In addition, several natural compounds such as procyanidins (flavonoid) were reported to protect apoptosis through the activation of PERK/ Nrf-2/ HO-1 pathway (Dong et al., 2020; Tan et al., 2020). Interestingly, combination of TCT with either INDO, DIC or TUN was able to significantly increase protein expression level of p-PERK and p-Nrf-2 whereas the expression level of HO-1 protein was decreased in both cell types. These data suggested that TCT were able to enhance the cytotoxicity mediated by INDO, DIC or TUN through the suppression of HO-1 expression. It has been demonstrated that the *HO-1* gene is a cytoprotective molecule against stress-induced apoptosis (Consoli et al., 2021). Phosphorylation of Nrf-2 leads to its dissociation from Kelch-like erythroid cell-derived protein with CNC homology-associated protein 1 (Keap-1), allowing its translocation into nucleus to initiate *HO-1* transcription (Loboda et al., 2016; Yu and Xiao, 2021). Hence, it might be possible that TCT could interfere with nuclear translocation of Nrf-2. Several previous reports demonstrated that certain compounds such as trigonelline (alkaloid), retinoic acid were induced apoptosis through the inhibition of Nrf-2 transport into nucleus and the reduction of HO-1 expression in various cancer cell lines cells (Arlt et al., 2013; Kim et al., 2015; Valenzuela et al., 2014). However, the effect of TCT on *HO-1* transcription, independent of Nrf-2 inhibition, could not be excluded. Regulation of HO-1 expression has been associated with several transcription factors, such as nuclear factor kappa-light-chain-enhancer of activated B cells (NF-**κ**B) and activator protein-1 (AP-1), binding to antioxidant response elements (AREs) and promoting *HMOX1*

transcription (Hahn et al., 2020; Medina et al., 2020). For example, the combination of TNF-α (15 ng/mL) and Bay11-7082 (NF-κB inhibitor) at 10 μM were able to decrease *HO-1* expression which leading to increase apoptotic cell death in DLBCL cells (Huang et al., 2016; X. Li et al., 2018). Taken together, these data indicated that the molecular mechanism underlying of TCT-mediated downregulation of HO-1 require further investigation.

INDO has been reported to activate IRE-1α/JNK1/2 signaling, leading to an increase of Bax/ Bcl-2 ratio and apoptotic cell death in cancer cells such as Glioma H4, Huh-7 and CaCo-2 cells (Boonyong et al., 2020a; Chang et al., 2020; Franceschelli et al., 2011). In this study, INDO but not DIC or TUN alone was able to increase phosphorylation of IRE-1α (p-IRE-1α). In addition, either INDO, DIC or TUN had no effect on the phosphorylation of JNK1/2 in both cell types. However, this increment of phosphorylated IRE-1α could not subsequently lead to the activation of JNK1/2 signaling. These data suggested that the downstream of IRE-1α were not mediated by JNK1/2 signaling in MCF-7 and MCF-7/MX cells. The activation of IRE-1α has been linked to the phosphorylation of JNK1/2 protein and X box-binding protein 1 (XBP1) mRNA splicing (Junjappa et al., 2018; Salminen et al., 2020). IRE-1α activity-dependent XBP1 mRNA splicing leads to increasing CHOP expression and changes in Bax and Bcl-2 expression, resulting in mitochondrial apoptotic cell death in cancer cells (Comitato et al., 2016; Hu et al., 2018; Lv and Qiao, 2018; Shi et al., 2013). Certain compounds such as Saxifragifolin D (triterpenoid) were reported to up-regulate IRE-1α, XBP-1 and CHOP protein expression, resulting to apoptotic cell death in MCF-7 and MDA-MB-231 cells (Shi et al., 2013). It was possible that TCT could potentiate INDO-mediated apoptosis in both cell types via activation of IRE-1α /XBP-1 signaling pathway. The role of XBP-1 in the enhancing effects of TCT on INDO toxicity in breast cancer cells should be investigated further.

In conclusion, TCT at non-toxic concentration was able to enhance INDO- and DIC-mediated apoptosis both MCF-7 wild type and resistance cell by suppression of survival proteins p-Nrf-2/HO-1, leading to promotion of the ER stress signaling PERK/eIF-2α/ATF-4/CHOP pathways.

REFERENCES

Almanza, Carlesso, Chintha, Creedican, Doultsinos, Leuzzi, Luís, McCarthy, Montibeller, More, Papaioannou, Püschel, Sassano, Skoko, Agostinis, Belleroche, Eriksson, Fulda, Gorman, Healy, Kozlov, Muñoz-Pinedo, Rehm, Chevet, and Samali. (2019). Endoplasmic reticulum stress signalling - from basic mechanisms to clinical applications. The FEBS Journal 2(286): 241-278.

Arlt, A., Sebens, S., Krebs, S., Geismann, C., Grossmann, M., Kruse, M. L., Schreiber, S., and Schafer, H. (2013). Inhibition of the Nrf2 transcription factor by the alkaloid trigonelline renders pancreatic cancer cells more susceptible to apoptosis through decreased proteasomal gene expression and proteasome activity. Oncogene 32(40): 4825-4835.

Austin Doyle, L., and Ross, D. D. (2003). Multidrug resistance mediated by the breast cancer resistance protein BCRP (ABCG2). Oncogene 22(47): 7340-7358.

Bayat Mokhtari, R., Homayouni, T. S., Baluch, N., Morgatskaya, E., Kumar, S., Das, B., and Yeger, H. (2017). Combination therapy in combating cancer. Oncotarget 8(23): 38022-38043.

Bellezza, I., Scarpelli, P., Pizzo, S. V., Grottelli, S., Costanzi, E., and Minelli, A. (2017). ROS-independent Nrf2 activation in prostate cancer. Oncotarget 8(40): 67506-67518.

Bhattarai, K. R., Riaz, T. A., Kim, H. R., and Chae, H. J. (2021). The aftermath of the interplay between the endoplasmic reticulum stress response and redox

signaling. Exp Mol Med 53(2): 151-167.

Boonyong, C., Vardhanabhuti, N., and Jianmongkol, S. (2020a). Modulation of non-steroidal anti-inflammatory drug-induced, ER stress-mediated apoptosis in Caco-2 cells by different polyphenolic antioxidants: a mechanistic study. J Pharm Pharmacol 72(11): 1574-1584.

Boonyong, C., Vardhanabhuti, N., and Jianmongkol, S. (2020b). Natural polyphenols prevent indomethacin-induced and diclofenac-induced Caco-2 cell death by reducing endoplasmic reticulum stress regardless of their direct reactive oxygen species scavenging capacity. J Pharm Pharmacol 72(4): 583-591.

Cadenas, E. (2004). Mitochondrial free radical production and cell signaling. Mol Aspects Med 25(1-2): 17-26.

Cao, J., Spielmann, M., Qiu, X., Huang, X., Ibrahim, D. M., Hill, A. J., Zhang, F., Mundlos, S., Christiansen, L., Steemers, F. J., Trapnell, C., and Shendure, J. (2019). The single-cell transcriptional landscape of mammalian organogenesis. Nature 566(7745): 496-502.

Chaisit, T., Siripong, P., and Jianmongkol, S. (2017). Rhinacanthin-C enhances doxorubicin cytotoxicity via inhibiting the functions of P-glycoprotein and MRP2 in breast cancer cells. Eur J Pharmacol 795(15): 50-57.

Chang, C. Y., Li, J. R., Wu, C. C., Wang, J. D., Liao, S. L., Chen, W. Y., Wang, W. Y., and Chen, C. J. (2020). Endoplasmic reticulum stress contributes to indomethacin-induced glioma apoptosis. Int J Mol Sci 21(2): 1-13.

Chen, T., Zhu, J., Wang, Y. H., and Hang, C. H. (2019). ROS-mediated mitochondrial dysfunction and ER Stress contribute to compression-induced neuronal injury. Neuroscience 416(15): 268-280.

Chen, T. C., Lai, K. C., Yang, J. S., Liao, C. L., Hsia, T. C., Chen, G. W., Lin, J. J., Lin, H. J., Chiu, T. H., Tang, Y. J., and Chung, J. G. (2009). Involvement of reactive oxygen species and caspase-dependent pathway in berberine-induced cell cycle arrest and apoptosis in C6 rat glioma cells. Int J Oncol 34(6): 1681-1690.

Chen, W., Lian, W., Yuan, Y., and Li, M. (2019). The synergistic effects of oxaliplatin and piperlongumine on colorectal cancer are mediated by oxidative stress. Cell Death Dis 10(8): 1-12.

Cheng, Y. C., Kuo, C. L., Hsu, S. Y., Way, T. D., Cheng, C. L., Chen, J. C., Liu, K. C., Peng, S. F., Ho, W. J., Chueh, F. S., and Huang, W. W. (2021). Tetrandrine Enhances H(2)O(2)-Induced apoptotic cell death through caspase-dependent pathway in human keratinocytes. In Vivo 35(4): 2047-2057.

Choi, Y. H., and Yu, a. A.-M. (2014). ABC transporters in multidrug resistance and pharmacokinetics, and strategies for drug development. Curr Pharm Des. 20(5): 793-807.

Cocetta, V., Quagliariello, V., Fiorica, F., Berretta, M., and Montopoli, M. (2021). Resveratrol as chemosensitizer agent: state of art and future perspectives. Int J Mol Sci 22(4): 1-20.

Comitato, R., Guantario, B., Leoni, G., Nesaretnam, K., Ronci, M. B., Canali, R., and Virgili, F. (2016). Tocotrienols induce endoplasmic reticulum stress and apoptosis in

cervical cancer cells. Genes & Nutrition 11(1): 32.

Consoli, V., Sorrenti, V., Grosso, S., and Vanella, L. (2021). Heme oxygenase-1 signaling and redox homeostasis in physiopathological conditions. Biomolecules 11(4): 1-23.

Crowley, L. C., Marfell, B. J., and Waterhouse, N. J. (2016). Analyzing cell death by nuclear staining with Hoechst 33342. Cold Spring Harb Protoc 2016(9): 778-781.

Daorueang, D. (2020). Antioxidant activity and effect of Sa-khan (Piper, Piperaceae) on the normal human proximal tubular epithelial cell line.

Di Fazio, P., Ocker, M., and Montalbano, R. (2012). New drugs, Old fashioned ways: ER stress induced cell death. Curr Pharm Biotechnol. 44(35): 2228-2234.

Do, M. T., Kim, H. G., Choi, J. H., Khanal, T., Park, B. H., Tran, T. P., Jeong, T. C., and Jeong, H. G. (2013). Antitumor efficacy of piperine in the treatment of human HER2-overexpressing breast cancer cells. Food Chem 141(3): 2591-2599.

Dobiasova, S., Rehorova, K., Kucerova, D., Biedermann, D., Kanova, K., Petraskova, L., Koucka, K., Vaclavikova, R., Valentova, K., Ruml, T., Macek, T., Kren, V., and Viktorova, J. (2020). Multidrug resistance modulation activity of silybin derivatives and their anti-inflammatory potential. Antioxidants 9(5): 1-22.

Dong, Y., Xing, Y., Sun, J., Sun, W., Xu, Y., and Quan, C. (2020). Baicalein alleviates liver oxidative stress and apoptosis induced by high-level glucose through the activation of the PERK/Nrf2 signaling pathway. Molecules 25(3): 599.

Feng, M., Zhong, L. X., Zhan, Z. Y., Huang, Z. H., and Xiong, J. P. (2016). Resveratrol treatment inhibits proliferation of and induces apoptosis in human colon cancer cells. Med Sci Monit 22(4): 1101-1108.

Franceschelli, S., Moltedo O Fau - Amodio, G., Amodio G Fau - Tajana, G., Tajana G Fau - Remondelli, P., and Remondelli, P. (2011). In the Huh7 hepatoma cells diclofenac and indomethacin activate differently the unfolded protein response and induce ER stress apoptosis. Open Biochem J 5: 45-51.

Fu, X., Cui, J., Meng, X., Jiang, P., Zheng, Q., Zhao, W., and Chen, X. (2021). Endoplasmic reticulum stress, cell death and tumor: Association between endoplasmic reticulum stress and the apoptosis pathway in tumors (Review). Oncol Rep 45(3): 801-808.

Fujiki, T., Ando, F., Murakami, K., Isobe, K., Mori, T., Susa, K., Nomura, N., Sohara, E., Rai, T., and Uchida, S. (2019). Tolvaptan activates the Nrf2/HO-1 antioxidant pathway through PERK phosphorylation. Sci Rep 9(1): 9245.

Gáll, T., Balla, G., and Balla, J. (2019). Heme, heme oxygenase, and endoplasmic reticulum stress—A new insight into the pathophysiology of vascular diseases. International Journal of Molecular Sciences 20(15): 3675.

Gillet, J. P., and Gottesman, M. M. (2010a). Mechanisms of multidrug resistance in cancer. Methods Mol Biol 596: 47-76.

Gillet, J. P., and Gottesman, M. M. (2010b). Mechanisms of multidrug resistance in cancer. (1940-6029 (Electronic)).

Gu, S., Chen, C., Jiang, X., and Zhang, Z. (2015). Resveratrol synergistically triggers apoptotic cell death with arsenic trioxide via oxidative stress in human lung adenocarcinoma A549 cells. Biol Trace Elem Res 163(1-2): 112-123.

Gunaydin, C., and Bilge, S. S. (2018). Effects of nonsteroidal anti-inflammatory drugs at the molecular level. Eurasian J Med 50(2): 116-121.

Guo, G., Meng, Y., Tan, W., Xia, Y., Cheng, C., Chen, X., and Gu, Z. (2015). Induction of apoptosis coupled to endoplasmic reticulum stress through regulation of CHOP and JNK in bone marrow mesenchymal stem cells from patients with systemic lupus erythematosus. J Immunol Res 2015(2015): 183738.

Hahn, D., Shin, S. A.-O., and Bae, J. A.-O. (2020). Natural antioxidant and anti-inflammatory compounds in foodstuff or medicinal herbs inducing heme oxygenase-1 expression. . Antioxidants (Basel) 9(12): 1191.

Hetz, C., and Papa, F. R. (2018). The unfolded protein response and cell fate control. Mol Cell 69(2): 169-181.

Hu, H., Tian, M., Ding, C., and Yu, S. (2018). The C/EBP homologous protein (CHOP) transcription factor functions in endoplasmic reticulum stress-induced apoptosis and microbial infection. Front Immunol 9(4): 3083.

Huang, J., Guo, P., Ma, D., Lin, X., Fang, Q., and Wang, J. (2016). Overexpression of heme oxygenase-1 induced by constitutively activated NF-**K**B as a potential therapeutic target for activated B-cell-like diffuse large B-cell lymphoma. Int J Oncol 49(1): 253-264.

Huang, J., Pan, H., Wang, J., Wang, T., Huo, X., Ma, Y., Lu, Z., Sun, B., and Jiang, H. (2021). Unfolded protein response in colorectal cancer. Cell Biosci 11(1): 26.

Iriti, M., Kubina, R., Cochis, A., Sorrentino, R., Varoni, E. M., Kabala-Dzik, A., Azzimonti, B., Dziedzic, A., Rimondini, L., and Wojtyczka, R. D. (2017). Rutin, a quercetin glycoside, restores chemosensitivity in human breast cancer cells. Phytother Res 31(10): 1529-1538.

Ishiwata-Kimata, Y., Le, G. Q., and Kimata, Y. (2018). Stress-sensing and regulatory mechanism of the endoplasmic-stress sensors Ire1 and PERK. Endoplasmic Reticulum Stress in Diseases 5(1): 1-10.

Iurlaro, R., and Munoz-Pinedo, C. (2016). Cell death induced by endoplasmic reticulum stress. FEBS J 283(14): 2640-2652.

Ji, N., Yuan, J., Liu, J., and Tian, S. (2010). Developing multidrug-resistant cells and exploring correlation between BCRP/ABCG2 over-expression and DNA methyltransferase. Acta Biochim Biophys Sin (Shanghai) 42(12): 854-862.

Junjappa, R. P., Patil, P., Bhattarai, K. R., Kim, H. R., and Chae, H. J. (2018). IRE1alpha implications in endoplasmic reticulum stress-mediated development and pathogenesis of autoimmune diseases. Front Immunol 9(2018): 1289.

Karki, K., Hedrick, E., Kasiappan, R., Jin, U.-H., and Safe, S. J. C. p. r. (2017). Piperlongumine induces reactive oxygen species (ROS)-dependent downregulation of specificity protein transcription factors. Cancer Prev Res (Phila). 10(8): 467-477.

Kawahara, I., Nishikawa, S., Yamamoto, A., Kono, Y., and Fujita, T. (2020). The impact of breast cancer resistance protein (BCRP/ABCG2) on drug transport across Caco-2 cell monolayers. Drug Metabolism and Disposition 48(6): 491-498.

Kim, D. K., and Lee, K. (2003). Inhibitory effect of trans-N-p-coumaroyl tryamine from the twigs of Celtis chinensis on the acetylcholinesterase. Arch Pharm Res. 26(9): 735-738.

Kim, J. K., Kang, K. A., Ryu, Y. S., Piao, M. J., Han, X., Oh, M. C., Boo, S. J., Jeong, S. U., Jeong, Y. J., Chae, S., Na, S. Y., and Hyun, J. W. Induction of endoplasmic reticulum stress via reactive oxygen species mediated by luteolin in melanoma cells. Anticancer Res. 36(5): 2281-2289.

Kim, S. R., Ha, Y. M., Kim, Y. M., Park, E. J., Kim, J. W., Park, S. W., Kim, H. J., Chung, H. T., and Chang, K. C. (2015). Ascorbic acid reduces HMGB1 secretion in lipopolysaccharide-activated RAW 264.7 cells and improves survival rate in septic mice by activation of Nrf2/HO-1 signals. Biochem Pharmacol 95(4): 279-289.

Lagas, J. S., van der Kruijssen Cm Fau - van de Wetering, K., van de Wetering K Fau - Beijnen, J. H., Beijnen Jh Fau - Schinkel, A. H., and Schinkel, A. H. (2008). Transport of diclofenac by breast cancer resistance protein (ABCG2) and stimulation of multidrug resistance protein 2 (ABCC2)-mediated drug transport by diclofenac and benzbromarone. Drug Metab Dispos. 37(1): 129-136.

Li, A., Song, N. J., Riesenberg, B. P., and Li, Z. (2019). The emerging roles of endoplasmic reticulum stress in balancing immunity and tolerance in health and diseases: mechanisms and opportunities. Front Immunol 10(11): 3154.

Li, H., Krstin, S., Wang, S., and Wink, M. (2018). Capsaicin and piperine can overcome multidrug resistance in cancer cells to doxorubicin. Molecules 23(3): 557.

Li, W., Zhang, H., Assaraf, Y. G., Zhao, K., Xu, X., Xie, J., Yang, D. H., and Chen, Z. S. (2016). Overcoming ABC transporter-mediated multidrug resistance: Molecular mechanisms and novel therapeutic drug strategies. Drug Resist Updat 27(2016): 14-29.

Li, X., He, Z., Cheng, B., Fang, Q., Ma, D., Lu, T., Wei, D., Kuang, X., Tang, S., Xiong, J., and Wang, J. (2018). Effect of BCLAF1 on HDAC inhibitor LMK-235-mediated apoptosis of diffuse large B cell lymphoma cells and its mechanism. Cancer Biol Ther 19(9): 825-834.

Limonta, P., Moretti, R. M., Marzagalli, M., Fontana, F., Raimondi, M., and Montagnani Marelli, M. (2019). Role of endoplasmic reticulum stress in the anticancer activity of natural compounds. Int J Mol Sci 20(4): 961.

Lindholm, D., Korhonen, L., Eriksson, O., and Koks, S. (2017). Recent insights into the role of unfolded protein response in ER stress in health and disease. Front Cell Dev Biol 5(10): 48.

Liou, G. Y., and Storz, P. (2010). Reactive oxygen species in cancer. Free Radic Res. 44(5): 1-31.

Liu, E., Liang T Fau - Wang, X., Wang X Fau - Ban, S., Ban S Fau - Han, L., Han L Fau - Li, Q., and Li, Q. (2015). Apoptosis induced by farrerol in human gastric cancer SGC-7901 cells through the mitochondrial-mediated pathway. Eur J Cancer Prev

24(5): 365-372.

Liu, H., Yang, J., Li, L., Shi, W., Yuan, X., and Wu, L. (2016). The natural occurring compounds targeting endoplasmic reticulum stress. Evid Based Complement Alternat Med 2016: 7831282.

Loboda, A., Damulewicz, M., Pyza, E., Jozkowicz, A., and Dulak, J. (2016). Role of Nrf2/HO-1 system in development, oxidative stress response and diseases: an evolutionarily conserved mechanism. Cell Mol Life Sci 73(17): 3221-3247.

Lv, S. X., and Qiao, X. (2018). Isovitexin (IV) induces apoptosis and autophagy in liver cancer cells through endoplasmic reticulum stress. Biochem Biophys Res Commun 496(4): 1047-1054.

Manogaran, P., Narasimha, M. B., Chih-Yang, H., and Viswanadha, V. P. (2019). Neferine and isoliensinine from nelumbo nucifera induced reactive oxygen species (ROS)-mediated apoptosis in colorectal cancer HCT-15 cells. African Journal of Pharmacy and Pharmacology 13(8): 90-99.

Mansoori, B., Mohammadi, A., Davudian, S., Shirjang, S., and Baradaran, B. (2017). The different mechanisms of cancer drug resistance: A brief review. Adv Pharm Bull 7(3): 339-348.

Mazumder, S., De, R., Debsharma, S., Bindu, S., Maity, P., Sarkar, S., Saha, S. J., Siddiqui, A. A., Banerjee, C., Nag, S., Saha, D., Pramanik, S., Mitra, K., and Bandyopadhyay, U. (2019). Indomethacin impairs mitochondrial dynamics by activating the PKCzeta-p38-DRP1 pathway and inducing apoptosis in gastric cancer and normal mucosal cells. J Biol Chem 294(20): 8238-8258.

Medina, M. V., Sapochnik, D., Garcia Sola, M., and Coso, O. (2020). Regulation of the expression of Heme Oxygenase-1: Signal transduction, gene promoter activation, and beyond. Antioxid Redox Signal 32(14): 1033-1044.

Moody, T. W., Switzer C Fau - Santana-Flores, W., Santana-Flores W Fau - Ridnour, L. A., Ridnour La Fau - Berna, M., Berna M Fau - Thill, M., Thill M Fau - Jensen, R. T., Jensen Rt Fau - Sparatore, A., Sparatore A Fau - Del Soldato, P., Del Soldato P Fau - Yeh, G. C., Yeh Gc Fau - Roberts, D. D., Roberts Dd Fau - Giaccone, G., Giaccone G Fau - Wink, D. A., and Wink, D. A. (2010). Dithiolethione modified valproate and diclofenac increase E-cadherin expression and decrease proliferation of non-small cell lung cancer cells. Lung Cancer. 68(2).

Moradi-Afrapoli, F., Yassa, N., Zimmermann, S., Saeidnia, S., Hadjiakhoondi, A., Ebrahimi, S. N., and Hamburger, M. (2012). Cinnamoylphenethyl amides from polygonum hyrcanicum possess anti-trypanosomal activity. Natural Product Communications 7(6): 1934578X1200700616.

Nishioka, T., Watanabe, J., Kawabata, J., and Niki, R. (2014). Isolation and activity of N-p-coumaroyltyramine, an α-glucosidase inhibitor in welsh onion (Allium fistulosum). Bioscience, Biotechnology, and Biochemistry 61(7): 1138-1141.

Nurgali, K., Jagoe, R. T., and Abalo, R. (2018). Editorial: adverse effects of cancer chemotherapy: anything new to improve tolerance and reduce sequelae? Front Pharmacol 9(22): 245.

Park, J. B., Schoene, N. J. B., and communications, b. r. (2002). Synthesis and characterization of N-coumaroyltyramine as a potent phytochemical which arrests human transformed cells via inhibiting protein tyrosine kinases. Biochem

Biophys Res Commun 292(4): 1104-1110.

Park, S., Lim, W., and Song, G. (2018). Chrysophanol selectively represses breast cancer cell growth by inducing reactive oxygen species production and endoplasmic reticulum stress via AKT and mitogen-activated protein kinase signal pathways. Toxicol Appl Pharmacol 360(1): 201-211.

Pérez-Soto, E., Estanislao-Gómez, C. C., Pérez-Ishiwara, D. G., Ramirez-Celis, C., del Consuelo Gómez-García, M. J. C.-D., Identification,, and Compounds, C. (2019). Cytotoxic effect and mechanisms from some plant-derived compounds in breast cancer. 45.

Poku, R. A., Jones, K. J., Van Baren, M., Alan, J. K., and Amissah, F. (2020). Diclofenac enhances docosahexaenoic acid-induced apoptosis in vitro in lung cancer cells. Cancers (Basel) 12(9): 2683.

Rana, C., Piplani, H., Vaish, V., Nehru, B., and Sanyal, S. N. (2015). Downregulation of PI3-K/Akt/PTEN pathway and activation of mitochondrial intrinsic apoptosis by Diclofenac and Curcumin in colon cancer. Mol Cell Biochem 402(1-2): 225-241.

Rawat, L., Hegde, H., Hoti, S. L., and Nayak, V. (2020). Piperlongumine induces ROS mediated cell death and synergizes paclitaxel in human intestinal cancer cells. Biomed Pharmacother 128: 110243.

Redondo-Blanco, S., Fernandez, J., Gutierrez-Del-Rio, I., Villar, C. J., and Lombo, F. (2017). New insights toward colorectal cancer chemotherapy using natural bioactive compounds. Front Pharmacol 8(14): 109.

Rellmann, Y., Eidhof, E., and Dreier, R. (2021). Review: ER stress-induced cell death in osteoarthritic cartilage. Cell Signal 78: 109880.

Riaz, T. A., Junjappa, R. P., Handigund, M., Ferdous, J., Kim, H. R., and Chae, H. J. (2020). Role of endoplasmic reticulum stress sensor IRE1alpha in cellular physiology, calcium, ROS signaling, and metaflammation. Cells 9(5): 1160.

Robey, R. W., Pluchino, K. M., Hall, M. A.-O. X., Fojo, A. T., Bates, S. E., and Gottesman, M. M. (2018). Revisiting the role of ABC transporters in multidrug-resistant cancer. Nat Rev Cancer 18(7): 452-464.

Salehi, B., Zakaria, Z. A., Gyawali, R., Ibrahim, S. A., Rajkovic, J., Shinwari, Z. K., Khan, T., Sharifi-Rad, J., Ozleyen, A., Turkdonmez, E., Valussi, M., Tumer, T. B., Monzote Fidalgo, L., Martorell, M., and Setzer, W. N. (2019). Piper species: A comprehensive review on their phytochemistry, biological activities and applications. Molecules 24(7): 1364.

Salminen, A., Kaarniranta, K., and Kauppinen, A. (2020). ER stress activates immunosuppressive network: implications for aging and Alzheimer's disease. J Mol Med (Berl) 98(5): 633-650.

Sandoval-Acuña, C., Lopez-Alarcón C Fau - Aliaga, M. E., Aliaga Me Fau - Speisky, H., and Speisky, H. (2012). Inhibition of mitochondrial complex I by various non-steroidal anti-inflammatory drugs and its protection by quercetin via a coenzyme Q-like action. Chem Biol Interact 199(1): 18-28.

Shi, J. M., Bai, L. L., Zhang, D. M., Yiu, A., Yin, Z. Q., Han, W. L., Liu, J. S., Li, Y., Fu, D. Y., and Ye, W. C. (2013). Saxifragifolin D induces the interplay between apoptosis

and autophagy in breast cancer cells through ROS-dependent endoplasmic reticulum stress. Biochem Pharmacol 85(7): 913-926.

Sireeratawong, S., Itharat, A., Lerdvuthisopon, N., Piyabhan, P., Khonsung, P., Boonraeng, S., and Jaijoy, K. J. I. S. R. N. (2012). Anti-inflammatory, analgesic, and antipyretic activities of the ethanol extract of Piper interruptum Opiz. and Piper chaba Linn. ISRN Pharmacol 2012(2012): 480265.

Sun, Y. L., Patel A Fau - Kumar, P., Kumar P Fau - Chen, Z.-S., and Chen, Z. S. (2012). Role of ABC transporters in cancer chemotherapy. Chin J Cancer 31(2): 51-57.

Syed, S. B., Arya, H., Fu, I. H., Yeh, T. K., Periyasamy, L., Hsieh, H. P., and Coumar, M. S. (2017). Targeting P-glycoprotein: Investigation of piperine analogs for overcoming drug resistance in cancer. Sci Rep 7(1): 7972.

Tan, J.-q., Li, P.-c., Li, Q., Tang, J.-t., and Xue, H.-k. (2020). Protective effect of procyanidin B2 on hydrogen peroxide (H2O2)-induced oxidative damage in MCF-7 cells. Applied Biological Chemistry 63(1): 58.

Terasaki, A., Kurokawa, H., Indo, H. P., Bando, H., Hara, H., Majima, H. J., Matsui, H., and Ito, H. (2019). Enhancement of PDT-cytotoxicity via ROS induced by indomethacin in metastatic breast cancer. Journal of Porphyrins and Phthalocyanines 23(11n12): 1440-1447.

Tinoush, B., Shirdel, I., and Wink, M. (2020). Phytochemicals: Potential lead molecules for MDR Reversal. Front Pharmacol 11: 832.

Umsumarng, S., Pitchakarn, P., Yodkeeree, S., Punfa, W., Mapoung, S., Ramli, R. A., Pyne,

S. G., and Limtrakul, P. (2017). Modulation of P-glycoprotein by Stemona alkaloids in human multidrug resistance leukemic cells and structural relationships. Phytomedicine 34(15): 182-190.

Valenzuela, M., Glorieux, C., Stockis, J., Sid, B., Sandoval, J. M., Felipe, K. B., Kviecinski, M. R., Verrax, J., and Buc Calderon, P. (2014). Retinoic acid synergizes ATO-mediated cytotoxicity by precluding Nrf2 activity in AML cells. Br J Cancer 111(5): 874-882.

Valle, B. L., D'Souza, T., Becker, K. G., Wood, W. H., 3rd, Zhang, Y., Wersto, R. P., and Morin, P. J. (2013). Non-steroidal anti-inflammatory drugs decrease E2F1 expression and inhibit cell growth in ovarian cancer cells. PLoS One 8(4): e61836.

Wang, H. M., and Zhang, G. Y. (2005). Indomethacin suppresses growth of colon cancer via inhibition of angiogenesis in vivo. World J Gastroenterol 11(3): 340-343.

Wang, J., Lu, L., Chen, S., Xie, J., Lu, S., Zhou, Y., and Jiang, H. (2020). PERK overexpression-mediated Nrf2/HO-1 pathway alleviates hypoxia/reoxygenation-induced injury in neonatal murine cardiomyocytes via improving endoplasmic reticulum stress. Biomed Res Int 2020: 6458060.

Wang, S., Binder, P., Fang, Q., Wang, Z., Xiao, W., Liu, W., and Wang, X. (2018). Endoplasmic reticulum stress in the heart: insights into mechanisms and drug targets. Br J Pharmacol 175(8): 1293-1304.

Wang, X., Zhang, H., and Chen, X. (2019). Drug resistance and combating drug resistance in cancer. Cancer Drug Resist 2: 141-160.

Wong, R. S. Y. (2019). Role of nonsteroidal anti-inflammatory drugs (NSAIDs) in cancer prevention and cancer promotion. Adv Pharmacol Sci 2019(31): 3418975.

Yaffe, P. B., Power Coombs, M. R., Doucette, C. D., Walsh, M., and Hoskin, D. W. (2015). Piperine, an alkaloid from black pepper, inhibits growth of human colon cancer cells via G1 arrest and apoptosis triggered by endoplasmic reticulum stress. Mol Carcinog 54(10): 1070-1085.

Yashaswini, B. M., and Salimath, B. P. (2017). Sensitization to TRAIL and radioprotective effect of N-coumaroyl tyramine diacetate from tinospora cordifolia induces apoptosis and inhibits neoangiogenesis to act as an anti-cancer compound. Paper presented at the (IOSR-JBB).

Ye, Q., Liu, K., Shen, Q., Li, Q., Hao, J., Han, F., and Jiang, R. W. (2019). Reversal of multidrug resistance in cancer by multi-functional Flavonoids. Front Oncol 9: 487.

Yergeri, M., kapse-Mistry, S., Govender, T., and Srivastava, R. (2014). Nanodrug delivery in reversing multidrug resistance in cancer cells. Front Pharmacol 5: 159.

Yu, C., and Xiao, J. H. (2021). The Keap1-Nrf2 system: A mediator between oxidative stress and aging. Oxid Med Cell Longev 2021(19): 6635460.

Yu, J. S., and Kim, A. K. J. J. o. m. f. (2010). Platycodin D induces apoptosis in MCF-7 human breast cancer cells. J Med Food 13(2): 298-305.

Zeeshan, H. M., Lee, G. H., Kim, H. R., and Chae, H. J. (2016). Endoplasmic reticulum

stress and associated ROS. Int J Mol Sci 17(3): 327.

Zhang, X., Ding, M., Zhu, P., Huang, H., Zhuang, Q., Shen, J., Cai, Y., Zhao, M., and He, Q. (2019). New insights into the Nrf-2/HO-1 signaling axis and its application in pediatric respiratory diseases. Oxid Med Cell Longev 2019(19): 3214196.

Zhang, Y., Han, Y. H., Putluru, S. P., Matta, M. K., Kole, P., Mandlekar, S., Furlong, M. T., Liu, T., Iyer, R. A., Marathe, P., Yang, Z., Lai, Y., and Rodrigues, A. D. (2016). Diclofenac and Its Acyl Glucuronide: Determination of In Vivo Exposure in Human Subjects and Characterization as Human Drug Transporter Substrates In Vitro. Drug Metab Dispos 44(3): 320-328.

Zhang, Z., Chen, F., and Shang, L. (2018). Advances in antitumor effects of NSAIDs. Cancer Manag Res 10(15): 4631-4640.

VITA

NAME	Miss Angkana Wongsakul
DATE OF BIRTH	9 July 1985
PLACE OF BIRTH	Bangkok