In Silico PREDICTION AND *In Vitro* EVALUATION OF CAFFEIC ACID AND ITS DERIVATIVES AS POTENTIAL EFFLUX PUMP INHIBITOR(S) IN *Pseudomonas aeruginosa*

NOOR ZAWANI BINTI ZAKARIA

UNIVERSITI SAINS MALAYSIA

2019

In Silico PREDICTION AND *In Vitro* EVALUATION OF CAFFEIC ACID AND ITS DERIVATIVES AS POTENTIAL EFFLUX PUMP INHIBITOR(S) IN *Pseudomonas aeruginosa*

by

NOOR ZAWANI BINTI ZAKARIA

Thesis submitted in fulfillment of the requirements

for the degree of

Master of Science

June 2019

ACKNOWLEDGEMENT

In the name of God, the Most Gracious, the Most Merciful

First and foremost, I would like to express my highest gratitude to Allah, the Most Beneficient, the Most Merciful for granting me the strength and will to finish this research. I would like to express my appreciation to my main supervisor, Dr Suriani Mohamad for all of her time, patience, support, advice and encouragement in guiding me to complete this research. Her guidance really helped me a lot in all time during the process of completing this research. My sincere thanks to all my co-supervisors; Dr Ezatul Ezleen Kamarulaman and Prof Amirin Sadikun, who's shared with me their expertise, experience and research insight. Besides that, I would like to extend my warmest thanks to all of the technical staffs from the School of Pharmaceutical Sciences, in particular to En Amir, En Anuar, En Zainuddin, Pn Mahani and Pn Salida for their continuous help and technical support. Not to forget my laboratory partners, Norshima and Nafiu, who always being there during ups and downs and giving a moral boosting and spirit lifting support during my research journey. I would like also to give special thanks to the past and present members of Pharmaceutical Design and Simulation (PhDS) for sharing their knowledge and opinion during this study. I am grateful to USM for granting me the USM fellowship scheme for the financial support during my study. My sincere appreciation to the most special and important persons in my life, my lovely husband, Nuaim Siddiqi Danial, my mother, Noraziah Abdul Rahman and my parents in law, who constantly support and always being there for me during the hard time of my study. Thank you and May God bless all of you!

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF SCHEMES

LIST OF ABBREVIATIONS

ADT	AutoDock Tools
CAPE	Caffeic acid phenethyl ester
CAPA	Caffeic acid phenethyl amide
CCCP	Carbonyl cyanide m-chlorophenyl hydrazine
dH_2O	Distilled water
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
E. coli	*Escherichia coli*
e.g.	exempli gratia (for example)
EPI	Efflux pump inhibitor
et al	*et alia* (and others)
EtBr	Ethidium bromide
FTIR	Fourier Transform Infrared
gpf	Grid parameter file
HCl	Hydrocholric acid
Hz	Hertz
i.e.	id est (in other words)
INT	p-iodonitrotetrazolium
KBr	Potassium bromide
*K*i	Inhibition constant
LB	Luria Bertani

MHB	Mueller Hinton Broth
MDR	Multidrug resistant
MIC	Minimum inhibitory concentration
MS	Mass spectrometry
Na_2HPO_4	Sodium phosphate dibasic
$NaCO_3$	Sodium carbonate
NaH_2PO_4	Sodium phosphate monobasic
NaOH	Sodium hydroxide
$NaSO_4$	Sodium sulphate
NMR	Nuclear Magnetic Resonance
OD	Optical density
P. aeruginosa	*Pseudomonas aeruginosa*
PDB	Protein databank
pH	Potential of hydrogen
RMSD	Root mean square deviation
RNA	Ribonucleic acid
RND	Resistance-nodulation-cell-division
S. aureus	*Staphylococcus aureus*
TLC	Thin Layer Chromatography
TDZ	Thioridazine
TMS	Tetramethylsilane
UV	Ultraviolet
UV-Vis	Ultraviolet-visible

LIST OF UNITS AND SYMBOLS

°C	Degree celcius
CFU/mL	Colony-forming units per militer
g	Gram
L	Litre
H	Hertz
Kcal/mol	Kilocalorie per mol
M	Molar
mg	Milligram
mg/mL	Milligram/mililitre
mL	Mililitre
mM	Milimolar
nm	Nanometer
µg/mL	Microgram/microlitre
µL	Microliter
µM	Micrometer
ppm	part per million
R_f	Retardation factor
%	Percentage
x *g*	Times gravity

RAMALAN *In Siliko* DAN PENILAIAN *In Vitro* TERHADAP KAFEIK ASID DAN TERBITANNYA SEBAGAI PERENCAT PAM EFLUKS DALAM *Pseudomonas aeruginosa*

ABSTRAK

Pseudomonas aeruginosa adalah sejenis bakteria pathogen yang secara intrinsiknya rintang terhadap pelbagai jenis antibiotik. Faktor utama yang menyumbang ke arah kerintangan intrinsik ini adalah disebabkan oleh lapisan membran yang kurang telap dan pengekspresian pelbagai pam efluks. Salah satu strategi yang menarik untuk mengatasi masalah kerintangan dalam bakteria adalah dengan penggunaan perencat pam efluks (EPI). Dalam kajian ini, potensi asid kafeik dan terbitannya untuk bertindak sebagai EPI diramal dengan menggunakan kaedah *in siliko* pendokan molekul dan dinilai secara *in vitro* menggunakan asai pengumpulan etidium bromida (EtBr). Dua protein yang memainkan peranan utama dalam pengangkutan keluar antibiotik dalam *P. aeruginosa* (MexB dan MexY) telah digunakan sebagai protein sasaran. Berdasarkan kajian interaksi protein-ligand, ester feniletil asid kafeik (CAPE) dan amida feniletil asid kafeik (CAPA) yang memperolehi tenaga bebas pengikatan yang terendah dalam kedua-dua protein telah dikenalpasti berpotensi sebagai calon EPI. Potensi asid kaffeik, CAPE dan CAPA untuk bertindak sebagai EPI dalam *P. aeruginosa* telah dinilai melalui asai pengumpulan EtBr. Antara tiga sebatian yang diuji, CAPE didapati sangat berkesan dalam menyebabkan peningkatan pengumpulan EtBr intraselular dalam *P. aeruginosa*. Untuk tempoh jangka waktu 5 minit, peningkatan sebanyak 21.4% dalam pendafluor telah diperhatikan dalam *P. aeruginosa*. Ini menyarankan bahawa CAPE berupaya mengganggu gugat sistem pam efflux, dan membawa kepada pengumpulan EtBr dalam

sel bakteria ini. Kombinasi setiap satu sebatian yang diuji (CAPE, CAPA dan asid kafeik) dengan suatu sebatian aminoglikosida, kanamisin juga telah menyebabkan penurunan kepekatan merencat minima (MIC) pada faktor 4-kali ganda sehingga 8-kali ganda. Dengan itu, berdasarkan interaksi ikatan yang baik ditunjukkan oleh CAPE melalui kaedah *in siliko* pendokan molekul dan keupayaannya untuk mengumpulkan lebih banyak EtBr dalam *P. aeruginosa* daripada asid kafeik dan CAPA melalui asai pengumpulan EtBr, hasil kajian ini mencadangkan CAPE berpotensi sebagai perencat pam efluks dalam *P. aeruginosa*. Keupayaan CAPE dan terbitan asid kafeik lain yang dikaji untuk mengurangkan MIC kanamisin dalam *P. aeruginosa* juga turut meningkatkan potensi terbitan asid kafeik ini sebagai EPI, justeru dapat memperbaiki keberkesanan aminoglikosida ini sebagai ajen antimikrob.

In Silico PREDICTION AND *In Vitro* EVALUATION OF CAFFEIC ACID AND ITS DERIVATIVES AS POTENTIAL EFFLUX PUMP INHIBITOR(S) IN *Pseudomonas aeruginosa*

ABSTRACT

Pseudomonas aeruginosa is a bacterial pathogen that is intrinsically resistant towards various antibiotics. The main factors that contribute to this intrinsic resistance are due to the lower outer membrane permeability and expression of multiple efflux pumps. One of the promising strategies to circumvent the problem of bacterial resistance is the use of efflux pump inhibitor (EPI). In this study, the efflux pump inhibitory potential of caffeic acid and its derivatives were predicted using *in silico* molecular docking, and accessed using *in vitro* ethidium bromide (EtBr) accumulation assay. Two proteins (MexB and MexY) that play important roles in the effluxing of antibiotics in *P. aeruginosa* were used as the target proteins. Based on the protein-ligand interaction studies, caffeic acid phenethyl ester (CAPE) and caffeic acid phenethyl amide (CAPA) that scored the lowest free energy of binding in both proteins were identified as potential EPI candidates. The potential of caffeic acid, CAPE and CAPA to act as EPIs in *P. aeruginosa* was evaluated using ethidium bromide (EtBr) accumulation assay. Among the three compounds tested, CAPE was found to significantly increase the intracellular accumulation of EtBr in *P. aeruginosa*. An increase of 21.4% in fluorescence over 5-min time frame was observed in *P. aeruginosa*. This suggests that CAPE was able to disrupt or compromise the efflux pumps, thus leading to the accumulation of EtBr in these bacterial cells. Combination of each test compound (CAPE, CAPA and caffeic acid) with an aminoglycoside, kanamycin has reduced the minimum inhibitory

concentration (MIC) by 4-folds to 8-folds. Thus, based on the good binding interaction showed by CAPE from the *in silico* molecular docking study and its ability to accumulate higher EtBr in *P. aeruginosa* than caffeic acid and CAPA in the *in vitro* EtBr accumulation assay, these suggest that CAPE has the potential to act as an EPI in *P. aeruginosa*. The ability of CAPE and the other caffeic acid derivatives tested to reduce the MIC of kanamycin in *P. aeruginosa* further promote the potency of of these caffeic acid derivatives as EPIs, thus improving the efficacy of this aminoglycoside as an antimicrobial agent.

CHAPTER 1: INTRODUCTION

1.1 Background of study and problem statement

The alarming rate of antimicrobial resistance and continuous emergence of multidrug resistant (MDR) bacteria pathogens in various environments have made the control and management of infectious diseases a daunting task. Recently, the World Health Organization (WHO) and Centers for Disease Control and Prevention (CDC) highlighted this issue as a serious threat to public health that requires action from the government sector and society all around the world (CDC, 2016; WHO, 2018). This is due to the fact that the patients who are infected by MDR pathogens are prone to experience increased risk of adverse clinical outcomes than the patients infected with non-resistant bacteria (WHO, 2018). Consequently, the patients will consume more healthcare resources and require higher dose of antimicrobial agents that can be harmful.

Apart from the extensive use of antimicrobial agents in various clinical settings, the existence of various efflux mechanisms in most of the MDR pathogens has been broadly recognized as the major resistance component (Poole, 2001; Piddock, 2006; Anes *et al.*, 2015). The mechanisms of drug efflux in bacteria are intricate, where some can be resistant for specific antibiotics while others, such as MDR efflux pumps, can confer resistance to variety of structurally and functionally unrelated compounds. These compounds include ethidium bromide (EtBr), acriflavine, triclosan, organic solvents and acylated homoserine lactones (Piddock, 2006). Examples of major antibiotic classes that are known to be effluxed by the intrinsic bacterial efflux pumps systems include

macrolides, β-lactams, fluoroquinolones, oxazolidinones, fourth-generation cephalosporins and carbapenems (Kumar and Schweizer, 2005).

Pseudomonas aeruginosa is an opportunistic human pathogen, recognized as ubiquitous organism due to its ability to survive and adapt in a wide range of environment (Matthew *et al*., 2003). *P. aeruginosa* is also one of the leading causes of nosocomial infections (Weinstein *et al*., 2005). Immuno-compromised individuals with underlying diseases such as cystic fibrosis, cancer and diabetes, and in patients who suffered burns or other severe trauma are particularly susceptible to *P. aeruginosa* infections (Botzenhart and Döring, 1993; Kominos *et al*., 1972). For example, in the United States of America, the threat level by MDR *P. aeruginosa* has been characterized as very serious, with 6700 MDR *Pseudomonas* infection and 440 death in 2014 (CDC, 2014).

Over the years, *P. aeruginosa* has become increasingly resistant towards many antimicrobials, leading them to be known as a 'superbug' (Breidenstein *et al*., 2011). The intrinsic resistance in *P. aeruginosa* towards multiple classes of antibiotics is most probably contributed by the expression of MDR efflux pumps of the resistance-nodulation-cell-division (RND) superfamily (Li *et al*., 1994; Lynch *et al*.,, 1997; Piddock, 2006; Strateva and Yordanov, 2009). These efflux pumps include the constitutively expressed MexAB-OprM and MexXY-OprM, and the inducible MexCD-OprF and MexEF-OprN operons (Kumar and Schweizer, 2005; Piddock, 2006). The ability of these efflux pumps to facilitate the extrusion of antibiotics has limited their accumulation inside the cell; thus making the antibiotics less effective.

The usage of efflux pump inhibitor(s) (EPI) offers a very promising approach to minimize the effluxing of drugs. EPI is a small molecule that acts as an adjuvant to enhance the activity of conventional and/or older-generation antibiotics. This molecule usually binds to the efflux pump either competitively or non-competitively and thwarts the extrusion of antibiotics. In addition, the application of EPI in the treatment of bacterial infections can also elevate drug potency and reduce the development of new bacterial resistant strain (Zechini and Versace, 2009). To date, many EPIs have been discovered, including the established EPI, phenyl-arginine beta-naphthylamide (PAβN). PAβN was the first broad-spectrum RND pump inhibitor that could inhibit all the four clinically significant efflux systems in *P. aeruginosa* and as well as other Gram-Negative bacteria (Lomovskaya *et al*., 2001; Mamelli *et al*., 2003; Hasdemir *et al*., 2004). However, to date, this EPI has not reached the clinical usage, probably due to its unfavourable pharmacokinetics and toxicological profiles (Lomovskaya and Bostian, 2006) and its effect on the membrane integrity of bacteria (Lomovskaya *et al*., 2001; Lamers *et al*. 2013).

For centuries, plants have been widely used as traditional medicine for the treatment of diseases related to bacterial, fungal and viral infections (Dupont *et al*., 2006; Temrangsee *et al*., 2011). The abundance of phytochemicals with diverse functional groups and chirality in plants also contributes towards many health-promoting benefits. Hence, taking advantage of the therapeutic potential of plant resources as antimicrobials, this research is conducted to evaluate the use of caffeic acid and its derivatives as potential EPI(s) for *P. aeruginosa*. Caffeic acid is a phytochemical ubiquitiously found in plants and herbs. Caffeic acid and its derivatives, such as

chlorogenic acid and caffeic acid phenethyl ester (CAPE) have been shown to demonstrate various pharmaceutical properties such as antibacterial, anticancer, antifungal, antioxidant and antiviral (Mirzoeva *et al*., 1993; Chen *et al*., 1996; Tsuchiya *et al*., 1996; Tamura *et al*., 2006; Fatoni *et al*., 2008; Lou *et al*., 2011). Therefore, by assessing the efflux pump inhibitory activities of the caffeic acid and its derivatives, it could give an insight as to how effective these phytochemicals could reduce the MDR problem in *P. aeruginosa,* thus enhancing the antimicrobial efficacy.

1.2 Objectives of study

The general objective of this study is to identify and evaluate new candidate(s) of EPI from caffeic acid and its derivatives as an approach to reduce MDR-related problem in *P. aeruginosa* using *in silico* and *in vitro* approaches. The specific objectives of this study include:

1. To predict the binding interaction of caffeic acid and its selected derivatives with the multidrug resistance efflux pumps MexB and MexY proteins in *P. aeruginosa,* using *in silico* molecular docking technique.

2. To synthesize and characterize potential caffeic acid derivative candidate(s) based on the molecular docking results.

3. To evaluate the efflux pump inhibitory activities of the selected caffeic acid derivatives from their ability to accumulate ethidium bromide (EtBr) and ability to reduce the minimum inhibitory concentration (MIC) of test antibiotics in *P. aeruginosa*, using *in vitro* assay.

CHAPTER 2: LITERATURE REVIEW

2.1 Multidrug resistance of *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is a Gram-Negative rod bacterium that belongs to the family of *Pseudomonadaceae*. It is an opportunistic pathogen and has been recognized as the leading cause of life-threatening nosocomial infections in patients with impaired defenses (Weinstein *et al*., 2005). These include the patients suffering from burns and wounds, which can lead to bacteraemia during complication (Sadikot *et al*., 2005). In addition, it can also cause urinary tract infection, nosocomial pneumonia and chronic lung infections in cystic fibrosis (CF) patients (Hare *et al*., 2012). However, this bacterium rarely causes infection in healthy people.

The infections caused by *P. aeruginosa* is due to its ability to survive in a wide range of environment such as in soil, water and plants (Matthew *et al*., 2003). In hospitals, this bacterium can spread through the cleaning solution, non-sterilized medical equipment or even *via* the hands of the healthcare workers (Kominos *et al*., 1972). Hence, this is why most hospitalized patients with weakened immune system are always at a higher risk of being infected by this organism (CDC, 2016). The risk of infection caused by *P. aeruginosa* in the Intensive Care Unit (ICU) also remains high with mortality rates as high as 30 % to 60 % in bacteraemia and up to 70 % in patients with nosocomial pneumonia (Aliaga *et al*., 2002; Alp *et al*., 2004).

The treatment of infections caused by *P. aeruginosa* usually requires higher end antibiotics and higher healthcare cost (Carmeli *et al*., 1999; Slama, 2008). The common classes of antibiotics used to treat *P. aeruginosa* infections include aminoglycoside,

carbapenem, cephalosporin, penicillin, polymyxin and quinolone (Hancock and Speert, 2000). The treatment of *P. aeruginosa* infection has become more challenging as the organism is becoming increasingly resistant towards these antibiotics. Thus, this bacterium has been categorized as multidrug resistant (MDR) organism due to its ability to resist more than one class of antibiotics (CDC, 2014).

Among factors that can lead to the multidrug resistance in *P. aeruginosa* is due to the over reliance and inappropriate use of antibiotics (CDC, 2014). Besides this factor, *P. aeruginosa* is also associated with its high intrinsic resistance towards various antibiotics (Breidenstein *et al*., 2011). The low outer membrane permeability of *P. aeruginosa* has contributed to its high intrinsic resistance (Navon-Venezia *et al*., 2005; Hirsch and Tam, 2011). In addition, the ability of *P. aeruginosa* to survive in a wide range of environments also enables them to develop other intrinsic and adaptive secondary mechanisms of resistance such as the over-expression of multidrug efflux pumps (Li *et al*., 1995; Li *et al*., 2000) and β-lactamase production (Nakae *et al*., 1999). Table 2.1 summarizes the overview of the different types of resistance exhibited by *P. aeruginosa*.

Table 2.1: Overview of the different types of resistance exhibited by *P. aeruginosa*.

Type of resistance	Mechanisms	Examples of genes involved
Intrinsic	Low outer membrane permeability, β-lactamase production and efflux pump overexpression	*crc, lon, psrA*
Acquired	Horizontal transfer, mutations leading to reduced drug/substrate uptake and efflux pump overexpression	*ampD, gyrA, nalA, nfxB, cbrA, MBLs*
Adaptive	Gene expression changes including β-lactam and efflux pump overexpression due to the factors triggering expression of regulatory genes	*ampC, mexZ, phoQ*

From Breidenstein *et al*.(2011).

Considering all the factors that lead to the antibiotic resistance, it is not surprising that *P. aeruginosa* has reached to the level of 'superbug' that is resistant to most of the currently available anti-pseudomonal antibiotics and actively developing MDR strain (Breidenstein *et al*., 2011). This problem is contributed by various mechanisms of resistance exhibited by *P. aeruginosa*.

2.2 Mechanisms of bacterial antibiotic resistance

Over the years, bacteria have evolved and developed sophisticated mechanisms of drug resistance in order to survive the effect of antibiotics. A single bacterium can exhibit various mechanisms of resistance at the same time, therefore, rendering them more difficult to be eradicated. Three fundamental mechanisms of antibiotic resistance in bacteria include the alteration of the bacterial target site; degradation or modification of the antibiotic and the reduction of the intracellular concentration of the antibiotic, by

decreased permeability of the cell wall or by the efflux of the antibiotic from the cell (Munita *et al*., 2016). Figure 2.1 describes the image for the different mechanisms of bacterial antibiotic resistance.

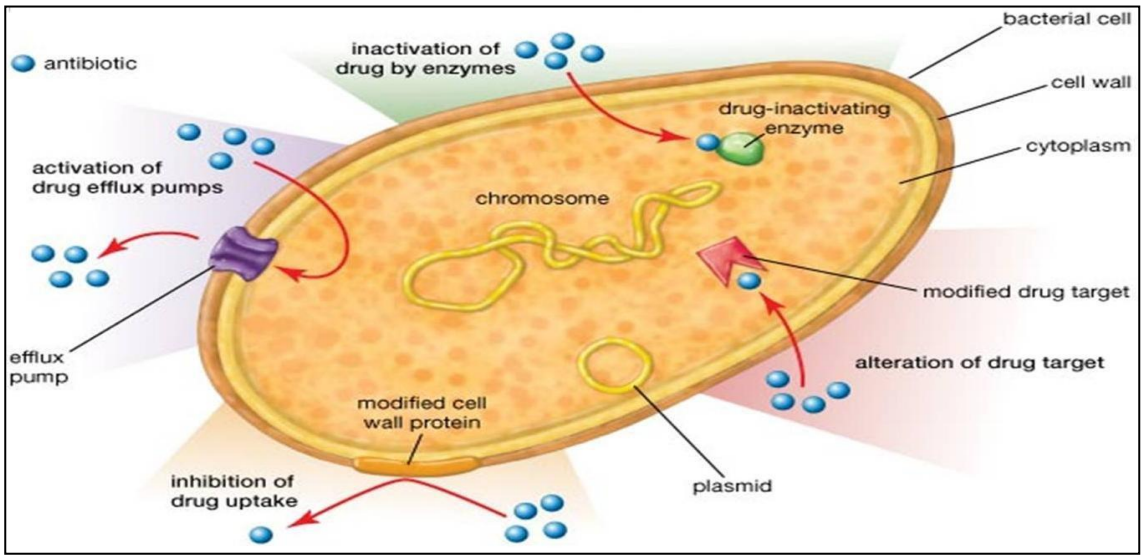


Figure 2.1: Diagram of different mechanisms of bacterial antibiotic resistance. This picture was adapted from Encyclopedia-Britannica, (2009).

2.2.1 Alteration of the bacterial target site

In bacteria, alteration of the antibiotics target site can be achieved by:

A. the target protection that prevent the antibiotics from reaching its target (e.g. the development of tetracycline resistance by the expression of tetracycline resistance determinants Tet(M) and Tet(O) in *Steptococcus spp.* and *Campylobacter jejuni*, respectively. These resistance determinants prevent the tetracycline from reaching their binding sites by interacting with the ribosome) (Connell *et al*., 2003), and/or by

B. the modifications of the target site, which involve a few strategies, such as:

i. the point mutations in the genes encoding the target site (e.g. the evolution of rifampin (RIF) resistance *via* the inhibition of the DNA-dependent RNA polymerase binding site, encoded by *rpoB* gene that prevent bacterial transcription) (Campbell *et al*., 2001),

ii. the enzymatic modifications of the binding site (e.g. the development of macrolide resistance through the methylation of the ribosome, which is catalyzed by an enzyme encoded erythromycin ribosomal methylation, *erm* genes) (Leclercq, 2002), and/or

iii. the replacement or bypass of the original target (e.g. the development of methicillin resistance in *Staphylococcus aureus* caused by the acquisition of foreign gene, *mecA* that encodes penicillin-binding protein, PBP (PBP2a), which has low likeness towards all β-lactams) (Hiramatsu *et al*., 2013).

2.2.2 Degradation or modification of the antibiotic

Degradation or modification of antibiotics by the production of enzyme can be achieved by two main strategies:

A. the chemical alterations of the antibiotic (e.g. the development of aminoglycosides resistance *via* the activity of aminoglycoside modifying enzymes (AMEs) that can covalently alter the amino or hydroxyl groups of the aminoglycoside molecule) (Ramirez and Tolmasky, 2010), and/or by

B. the degradation of the antibiotic molecule (e.g. the evolution of β-lactam resistance by the production of β-lactamase enzyme that is able to destroy the amide bond of the β-lactam ring) (Abraham and Chain, 1940).

2.2.3 Decreased permeability of the cell wall or by efflux of antibiotics

Two important mechanisms of resistance that prevent antibiotics from reaching its intracellular or periplasmic target include:

A. the decrease uptake of the antibiotics due to the changes in the permeability of the outer membrane (e.g. resistance to β-lactams, tetracyclines and some fluoroquinolones) and/or due to the porin-mediated antibiotic resistance (e.g. the development of resistance to imipenem by the unusual production of OprD porin in *P. aeruginosa*) (Quinn *et al*., 1986), and/or by

B. the efflux of antibiotics from within the cell or membrane by utilizing membrane bound protein transporter known as the efflux pump (e.g. resistance to various antibiotics due to the activity of the MexAB-OprM efflux pump in *P. aeruginosa* and AcrAB-TolC efflux pump in *Escherichia coli* (Piddock, 2006).

Among the mechanisms of antibiotic resistance described, efflux appears be an important mechanism that contributes to the multidrug resistances in bacteria. Besides actively extruding antibiotics, efflux is also involved in the low intrinsic susceptibility, cross-resistance to chemically unrelated classes of molecules, and selection/acquisition of additional mechanisms of resistance (Mahamoud *et al*., 2007).

2.3 Major classes of bacterial efflux pumps

The genes encoding the efflux pumps can be either chromosomally or non-chromosomally (plasmid) encoded. They are often regulated at the level of transcription in response to the presence of high concentrations of drugs (Piddock, 2006). Two major

efflux pump transporters usually encoded in bacteria include primary transporter such as ATP-binding cassette (ABC) transporter, and secondary transporter, which includes major facilitator superfamily (MFS), small multidrug resistance (SMR), resistance-nodulation-cell division (RND), and multidrug and toxic compound extrusion (MATE). In general, primary transporter utilizes ATP hydrolysis as their energy source while secondary transporter derives energy from the trans-membrane proton or sodium ion gradient (Piddock, 2006). Figure 2.2 shows the five major super families of bacterial efflux pumps.

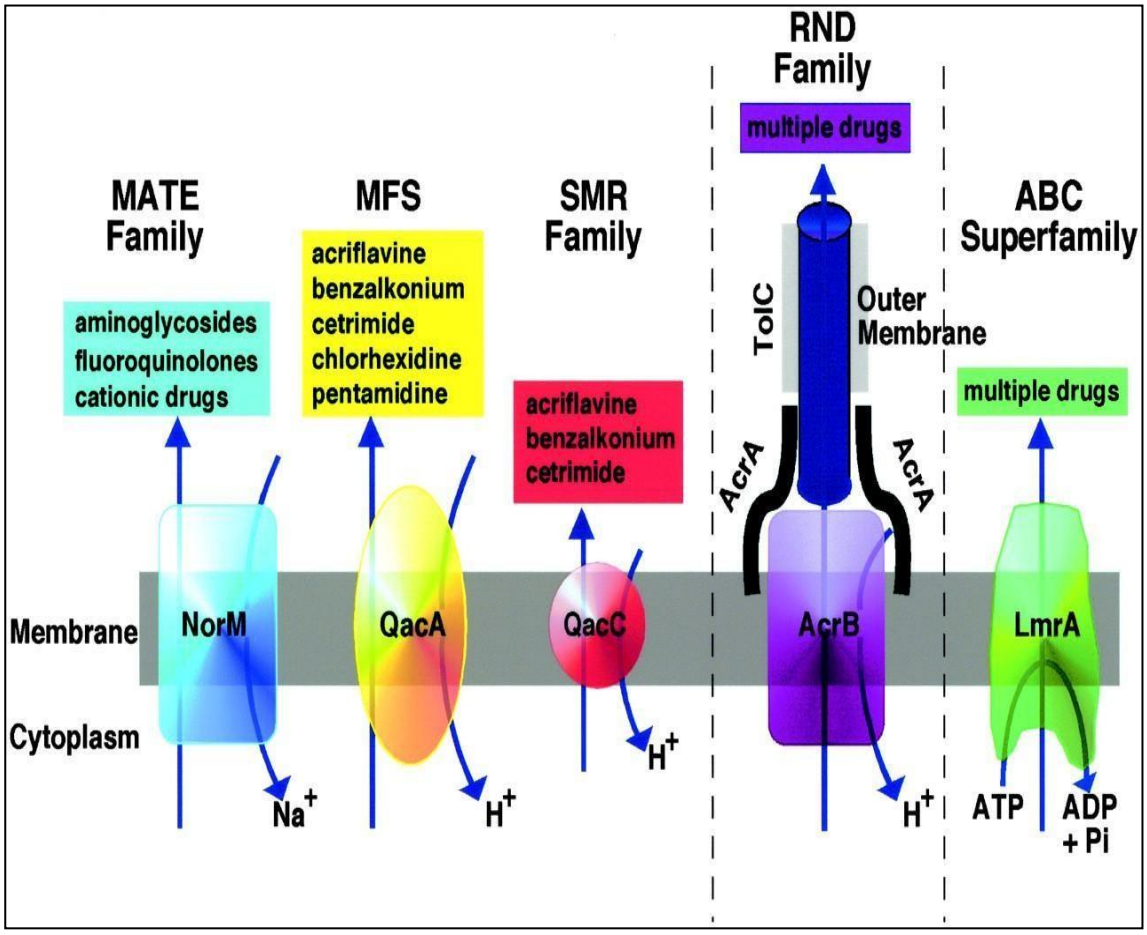


Figure 2.2: Diagram of different major super families of bacterial efflux pump with their common substrates. This picture was adapted from (Piddock, 2006).

Of these transporters, RND pumps are primarily found in Gram-Negative bacteria. These pump not only play a major role in both their intrinsic and acquired resistance to a diverse clinically significant antibiotics, but can also efflux biocides, dyes, detergents and organic solvents (Kumar and Schweizer, 2005; Bhardwaj and Mohanty, 2012). Apart from that, there are also other type of efflux transporters present in Gram-Negative bacteria but are less prominent in mediating resistance to clinically relevant antibiotics (Nilsen *et al*., 1996; Edgar and Bibi, 1997; Miyamae *et al*., 1998).

2.4 Resistance-nodulation-cell division efflux pumps encoded in *P. aeruginosa*

The common efflux pumps that contribute to the multidrug resistance in *P. aeruginosa* mostly come from the RND super family (Kumar and Schweizer, 2005; Piddock, 2006). To date, 7 out of 12 RND pump-encoding operons have been characterized in this organism, which include: MexAB-OprM (Poole *et al*., 1993), MexXY-OprM (Mine *et al*., 1999), MexEF-OprN (Köhler *et al*., 1997), MexCD-OprJ (Poole *et al*., 1996), MexJK-OprM (Chuanchuen *et al*., 2002), MexGHI-OpmD (Aendekerk *et al*., 2002) and MexVW-OprM (Li *et al*., 2003). The major efflux systems responsible for the intrinsic and acquired resistance in this bacterium include MexAB-OprM, MexXY-OprM, MexEF-OprN and MexCD-OprJ (Kumar and Schweizer, 2005). The operons, MexAB-OprM and MexXY-OprM efflux systems, are constitutively expressed in *P. aeruginosa*, thus rendering this bacterium the ability to extrude multiple unrelated compounds (Bambeke *et al*., 2013). The list of substrates and regulators for each characterized RND efflux systems in this organism are summarized in Table 2.2.

Table 2.2: Resistance-nodulation-cell division-type multidrug efflux systems in *P. aeruginosa*

Efflux components[a]			Regulator (s)	Substrates [b]	References
MFP	**RND**	**OMP**			
MexA	MexB	OprM	MexR	AC, AG, BL, CM, CV, EB, ML, NO, SDS, TC, TM, TR	Poole *et al*., 1993; Li *et al*., 1995; Poole *et al*., 1996
MexC	MexD	OprJ	NfxB	CM, CP, FQ, TC, TR	Poole *et al*., 1996
MexE	MexF	OprN	MexT	CM, FQ	Köhler *et al*., 1997
MexH	MexI	OpmD	?	AC, EB, HL, NO, RD, VD	Aendekerk *et al*., 2002; Sekiya *et al*., 2003
MexJ	MexK	OprM/ OpmH	MexL	EM, TC, TR	Chuanchuen *et al*., 2002
MexV	MexW	OprM	?	AC, CM, EB, EM, FQ, TC	Li *et al*., 2003
MexX	MexY	OprM	MexZ	AG, ML, TC	Mine *et al*., 1999; Nikaido *et al*., 1999

[a]MFP: membrane fusion protein; RND: resistance-nodulation-cell-division; OMP: outer membrane protein.
[b]AC, acriflavine; AG, aminoglycosides; AP, ampicillin; BL, β-lactams, CM, chloramphenicol; CP, cephalosporins; CV, crystal violet; EB, ethidium bromide, EM, erythromycin; FQ, fluoroquinolones; HL, homoserine-lactones; ML, macrolides; NO, novobiocin; RD, rhodamine; SDS, sodium dodecyl sulfate; TC, tetracycline; TM, trimethoprim; TR, triclosan; VD, vanadium; ?, unknown.
From Kumar and Schweizer, (2005), modified.

MexAB-OprM was the first RND efflux pump reported in *P. aeruginosa* and has the broadest substrate range of all characterized *P. aeruginosa*'s efflux pumps. Deletion of components of this efflux system in a wild-type strain of *P. aeruginosa* has made the strain hypersusceptible to many drugs such as chloramphenicol, fluoroquinolones, nalidixic acid and tetracycline (Poole *et al*., 1993; Li *et al*., 1995). Like MexAB-OprM, MexXY-OprM efflux system also contributes to the intrinsic resistance of *P. aeruginosa* towards several antibiotics including aminoglycosides, erythromycin and fluoroquinolones (Aires *et al*., 1999).

2.4.1 Structure of MexAB-OprM and MexXY-OprM efflux pumps

The main RND-type efflux pumps encoded in wild-type strain of *P. aeruginosa* (i.e. MexAB-OprM and MexXY-OprM) are comprised of a periplasmic membrane fusion protein (MFP) component, the inner-membrane drug/H^+ antiporter or the RND component (IMP), and the outer-membrane channel (OMP) component. Figure 2.3 shows the structure of the RND-type efflux pump based on the individual structures of MexAB-OprM efflux system in *P. aeruginosa*.

Based on the tripartite RND efflux complex, the MFP (e.g. MexA or MexX) component has been identified to function as a membrane bridge protein that connects the IMP and the OMP components (Akama *et al*., 2004). This component is essential for the pump assembly and function, whereas the OMP (e.g. OprM) functions in facilitating the exit of the substrates out of the cell (Akama *et al*., 2004). Meanwhile, the inner membrane RND transporter, IMP (e.g. MexB or MexY) component plays the central role of the efflux pump that recognizes the molecule to be effluxed and catalyzes the pH

dependent drug transport (Sennhauser *et al*., 2009). This protein is involved in the extrusion of the substrates with the aid of the proton motive force (Venter *et al*., 2015). The tripartite assembly of this efflux system allows the direct extrusion of the substrates from the inner membrane to the extracellular medium (Bambeke *et al*., 2013).

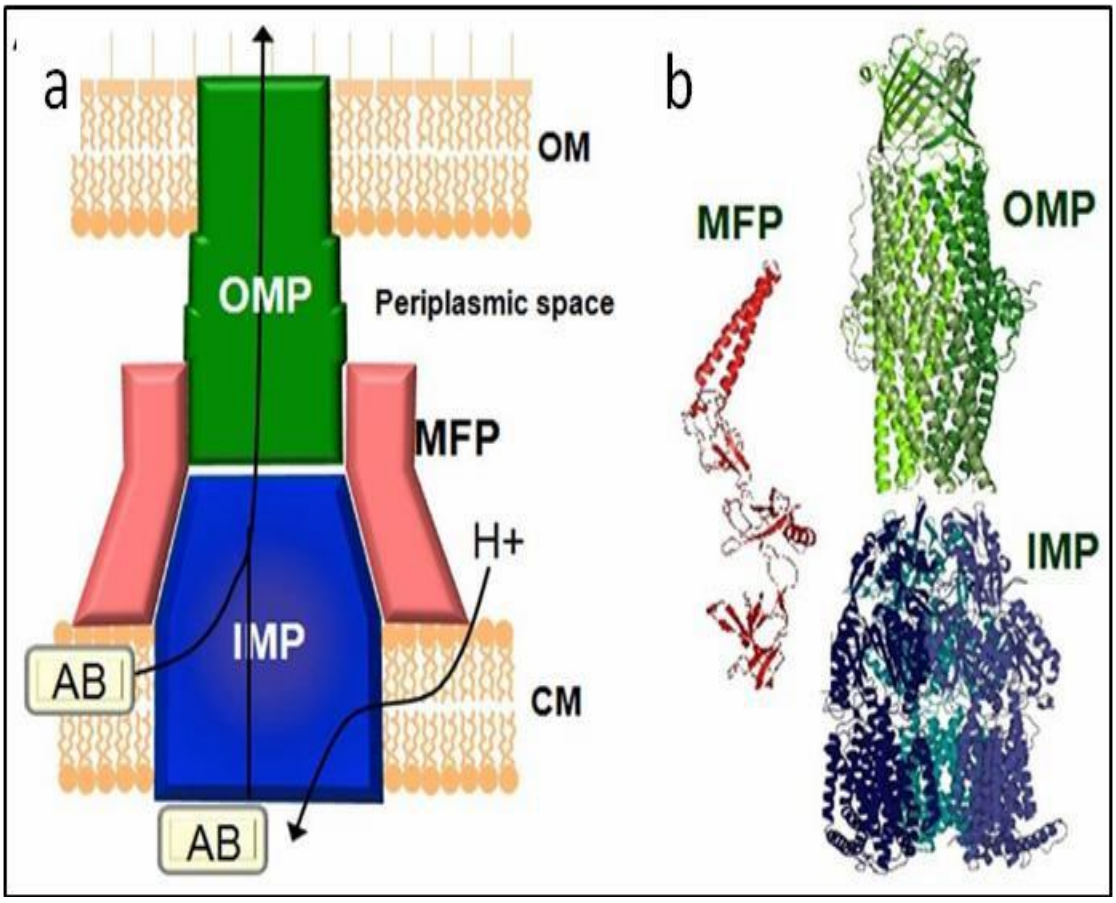


Figure 2.3: Schematic representation of a Resistance-Nodulation Division-type efflux pump. a) The pump consists of three proteins that span the inner membrane (CM), the periplasmic space and the outer membrane (OM). The proteins include the inner-membrane protein (IMP) that is connected to the outer-membrane protein (OMP) by the membrane fusion protein (MFP). The picture illustrated the process of drug (AB) extrusion by the efflux pump, which requires energy (H+). b) Protein structures of the individual components of the RND efflux pump; MFP (MexA; pdb: 2V4D), IMP (MexB; pdb: 2V50) and OMP (OprM; pdb: 1WP1) in *P. aeruginosa*. Picture was obtained from (Venter *et al*., 2015).

Both of the intrinsic RND pumps, MexB and MexY from MexAB-OprM and MexXY-OprM efflux systems, respectively, play major roles for the substrate specificity and energy of the transport process (Zgurskaya and Nikaido 1999; Elkins and Nikaido 2002). These proteins are closely related to the AcrB pump in *E. coli* with sequence identity of 69.8% (MexB) and 51.0% (MexY), and sequence similarity of 83.2% (MexB) and 19.0% (MexY) (Sennhauser *et al*., 2009; Lau *et al*., 2014). The presence of these three-dimensional structures has given rise to a better understanding of the mechanism of efflux in Gram-Negative bacteria (Pagès *et al*., 2005).

2.4.2 Mechanism of RND efflux pump in Gram-Negative bacteria

The presence of crystal structures from the RND efflux pump and antibiotic/inhibitor-bound RND efflux pump has shed light towards a better understanding of the mechanism of drug efflux. For instance, Murakami *et al*. in 2006 had revealed the functionally rotating mechanism of the RND efflux pump using the crystal structure of doxorubicin and minocycline-bound AcrB (homolog of MexB) protein from *E. coli*. From this study, it shows that the RND components exist as asymmetric trimer with individual monomers, in a rotating fashion, adapting one of the three conformations that represent the different steps of the drug export process. These conformations include access/loose (L) conformation, binding/tight (T) conformation and extrusion/open (O) conformation. The efflux process through this rotating monomers requires energy from the proton translocation across the membrane (Murakami *et al*., 2006; Seeger *et al*., 2006). Studies have also shown that, deactivation of any one of the three monomers can lead to the dysfunction of the entire trimer

(Takatsuka and Nikaido, 2009). Figure 2.4 shows the schematic illustrations of the proposed mechanism for RND efflux pump (i.e. AcrB) in the process of drug extrusion.

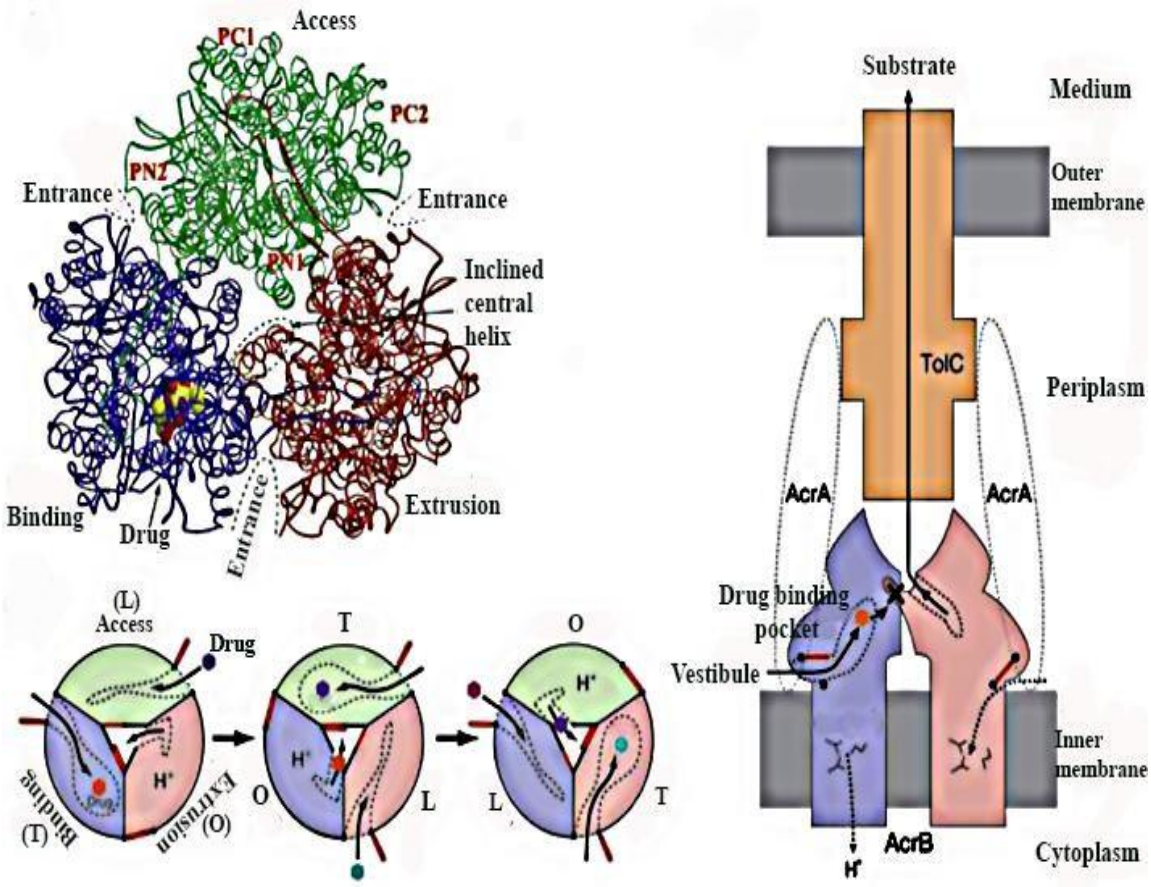


Figure 2.4: Schematic illustrations of substrate and proton pathways in the functionally rotation mechanism of AcrB (homolog of MexB). a) Top view of a ribbon representation of the AcrB trimer with the antibiotics (in yellow and red colour) locates in the binding protomer. The three protomers are colored as green, blue and red, according to their conformation: access, binding and extrusion, respectively. b) Top view from the distal side of the cell showing rotating motion of the protomers during the efflux of drugs. c) The side view of the AcrAB-TolC efflux system parallel to the membrane plane. Drugs are illustrated as hexagons. The red lines represent the entrance and exit of each protomer. The drug-binding pocket and translocation pathway are described as dotted lines. Picture was adapted from (Murakami *et al*., 2006).

Based on Figure 2.4, Murakami *et al*. (2006) postulated that the individual monomer cycles sequentially through these conformational states L, T, O and back to L, by three-step functionally rotating mechanism. Initially, the drug first enters the pump in the access pocket of the L monomer, and then is the subsequent accommodation of the drug in the binding pocket during the L to T transition to the T monomer. Next, the drug is extruded in the O monomer into the outer membrane (OM) channel-forming constituent through an opening at the top of the RND component as shown in Figure 2.4(b) and Figure 2.4(c).

2.5 Strategies to circumvent drug resistance *via* efflux mechanisms

The dissemination of MDR pathogen in hospital and in the community has resulted in challenging treatment of infectious diseases. This problem thus highlights the urgency for the development of new compounds that would be able to circumvent the drug resistance in bacteria and improve the usefulness/efficacy of both old and new generations antibiotics. As described earlier, efflux pumps play a major role in contributing to the development of drug resistance, especially in Gram-Negative bacteria. Alternatively, the development of efflux pump inhibitor (EPI) appear to be a promising strategy to increase the intracellular drug concentration level, restore the activity of the drug in the MDR strain, as well as capable to prevent further development of resistant strain (Askoura *et al*., 2011). In addition, the inhibition of the efflux pump can be achieved through different mechanisms, such as:

i. The design of new antibiotics or change the design of the existing antibiotic (Chopra, 2002; Vaara, 2010)

ii. Interfere with the assembly of the efflux pump (for tripartite RND pump) (Zgurskaya and Nikaido, 2000; Malléa *et al*., 2002)

iii. Block the energy required by the efflux pumps to operate (Mallea *et al*., 1998)

iv. Competitive or non-competitive inhibition of efflux pumps (Lomovskaya *et al*., 2001; Thorarensen *et al*., 2001)

v. Interfere with the regulatory steps in the expression of the efflux pump genes (Bornet *et al*., 2003; Yoshihara and Inoko, 2011), and

vi. Blocking the efflux pump protein/gene (Oethinger and Levy, 2002)

To date, many EPIs have been tested in various drug-resistant bacteria including *E. coli, Enterobacter aerogenes, Klebsiella pneumoniae, Campylobacter jejuni, P. aeruginosa and Salmonella enterica* serovar Typhimurium (Lomovskaya *et al*., 2001; Thorarensen *et al*. 2001; Malléa *et al*., 2002; Chevalier *et al*., 2004; Bohnert and Kern 2005). Besides that, several companies such as Microcide, Pfizer, Paratek and several academic laboratories have also invested in the search for bacterial EPIs from synthetic, natural products and peptidomimetics (Lomovskaya and Bostian, 2006; Bhardwaj and Mohanty, 2012).

An example of established EPI is the carbonyl cyanide m-chlorophenylhydrazone (CCCP). This compound which was known as the proton uncoupler, could affect the energy required for the efflux pump to operate and the cell viability by leading to the dissipation of the proton motive force of the membrane (Mahamoud *et al*., 2007). The efflux pump inhibitory activity of CCCP have been reported in *Mycobacterium smegmatis* (Choudhuri *et al*., 1999), but to date, CCCP has not reached the clinical use due to the high toxicity and adverse effect of this compound

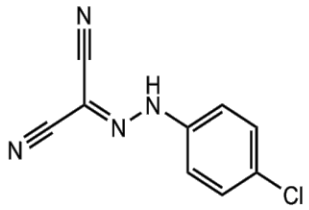
on the cell (Mahamoud *et al*., 2007). Another established EPI, 1-(1-*n*aphthylmethyl)-piperazine (NMP) from the members of arylpiperazine family was able to reverse the bacterial resistance towards several antibiotics such as chloramphenicol, fluoroquinolones and linezolid (Bohnert and Kern, 2005). However, the obscure mechanism of action of NMP and its "serotonin agonist" properties have made this compound too toxic for clinical usage and unsuitable to act as an EPI (Pagès and Amaral, 2009).

In addition, one of the broad-spectrum and prominent competitive inhibitor of the efflux pump being studied in *P. aeruginosa* is the dipeptide amide, phenylalanine arginyl β-napthylamide (PAβN or MC-207, 110). This molecule competitively binds to the active site of the efflux pump, thus allowing the entrance of antibiotics into the cell. The co-administration of PAβN with levofloxacin has resulted in 8-fold and 32- to 64-fold reductions of the levofloxacin MIC in a wild-type strain and efflux pump overexpressing strain of *P. aeruginosa*, respectively (Lomovskaya *et al*., 2001). However, to date, this EPI has not reached the clinical settings probably due to the unfavourable pharmacokinetics and toxicological profiles of this compound, and its effects on the membrane integrity (Lomovskaya *et al*., 2001; Lomovskaya and Bostian, 2006).

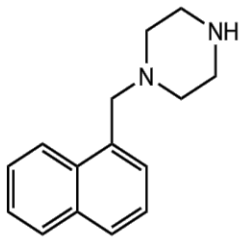
Another group of EPI, pyridopyrimidines, is the other broad-spectrum EPIs that have been extensively characterized to date. Its derivative, [{2-[({[(3R)-1-{8-[(4-tert-butyl-1,3-thiazol-2-yl)carbamoyl]-4-oxo-3-[(E)-2-(1H-tetrazol-5-yl)ethenyl]-4H-pyrido[1,2-a]pyrimidin-2-yl}piperidin-3-yl)oxy}carbonyl)amino]ethyl} (dimethyl)ammonio] acetate (ABI-PP) is the AcrAB/MexAB specific EPI. ABI-PP has

been identified as a potential EPI candidate due to its high solubility, good safety profile in an acute toxicity assay and excellent activity *in vivo* tested on a rat pneumonia model of *P. aeruginosa* (Yoshida *et al*., 2007). However, its limitation was that it could only inhibit the efflux of all substrates of AcrAB and MexAB efflux systems, but have little effect on the other efflux systems (Lomovskaya and Bostian, 2006). Other established EPI are the tricyclic neuroleptic phenothiazines such as thioridazine and chlorpromazine. The strategy of efflux pump inhibition being hypothesized for these EPIs is such that they could disrupt the electron transport chain and the processing of metabolic energy that is required by the efflux system (Weinstein *et al*., 2005; Martins *et al*., 2011). These EPIs have shown their efflux pump inhibitory activity against *B. pseudomallei, P. aeruginosa, E. coli, S.* Typhimurium and *S. aureus* (Amaral and Lorian, 1991; Kristiansen *et al*., 2003; Chan and Chua, 2005; Spengler *et al*., 2012). However, the direct use of phenothiazines as EPI was not clinically applicable due to the high concentration needed to inhibit the bacterial growth *in vitro* (Zechini and Versace, 2009). Figure 2.5 depicts the chemical structure of the aforementioned EPIs.

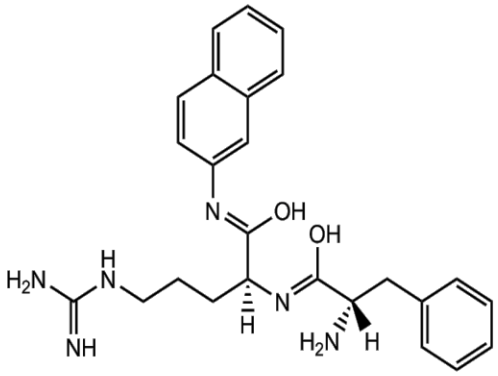
To date, many putative EPIs have been discovered, unfortunately, very few of them has reached the clinical settings due to the problem of toxicity and their adverse clinical outcomes. One EPI candidate that has successful advance to the human clinical trial stage is a compound known as MC-601, 205 (Zechini and Versace, 2009). The co-administration of an aerosolized formulation of MC-601, 205 with ciprofloxacin has reached the phase II clinical trial in the treatment of pulmonary exacerbations of cystic fibrosis patient conducted by Mpex Pharmaceuticals. However, to date, the structure and specific mode of action of this compound is still unclear (Zechini and Versace, 2009).



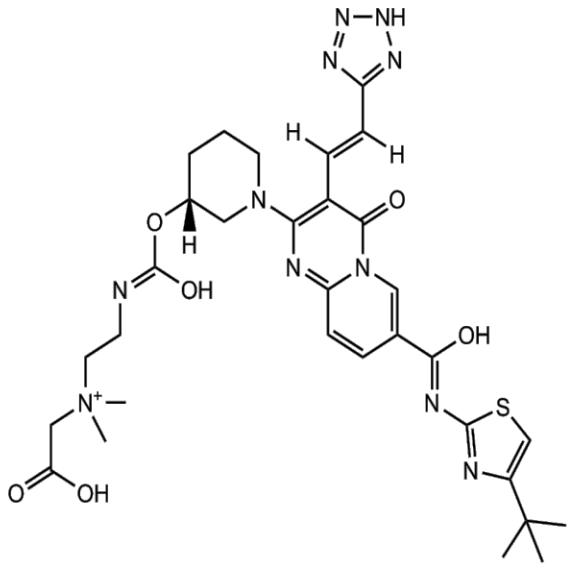
Carbonyl cyanide m-chlorophenyl hydrazine



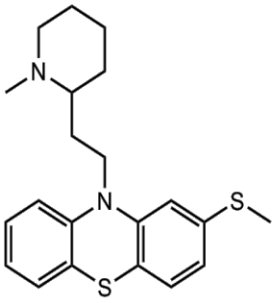
1-(1-Naphthylmethyl)-piperazine



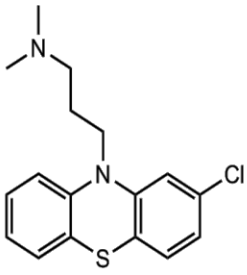
Phenylalanine arginine beta-naphthylamide



Pyridopyrimidine derivative



Thioridazine



Chlorpromazine

Figure 2.5: Chemical structure of efflux pump inhibitors

Besides that, several other EPIs were only reported on their partial efficacy. This is probably due to their limitations on bioavailability, selectivity, stability and toxicity (Pagès and Amaral, 2009). Hence, in addressing this matter, the search for natural sources or plant-based EPIs offers a potentially safer alternative (Rana *et al*., 2014; Prasch and Bucar, 2015).

2.6 Efflux pump inhibitor from natural products

For centuries, natural products have already been known as potential source of drugs. In general, natural products are naturally occurring substances produced by living organisms and also refers to secondary metabolites that are not involved in the main life processes (Newman and Cragg, 2012) In fact, plants, which are the main sources of natural products, are able to produce many cytotoxic compounds that can protect themselves from pathogenic microbes. Thus, this is why less infective diseases are seen in wild plants (Stavri *et al*., 2007). Besides that, the chemical diversity and specific action on targets have also made natural products in favor of approaches in drug discovery (Prasch and Bucar, 2015).

The repertoire of phytochemicals in herbs and plants has also contributed to the discovery of many plant-based EPIs, recently. One of the ubiquitous phytochemical in plants is the caffeic acid derivatives. Caffeic acid is a natural compound that consists of both phenolic and acrylic functional groups. It is the major representative of hydroxycinnamic acids and the predominant phenolic acids. Caffeic acid is abundant in most plants as it is the key intermediate in the biosynthesis of lignin, which is the main source of biomass. Besides that, caffeic acid can also be found in our daily foodstuffs

and beverages such as asparagus, cabbage, coffee, olives, olive oil, spinach, white grapes and wine (Rice-Evans *et al*., 1996). Figure 2.6 shows the structures of caffeic acid and some of its important derivatives.

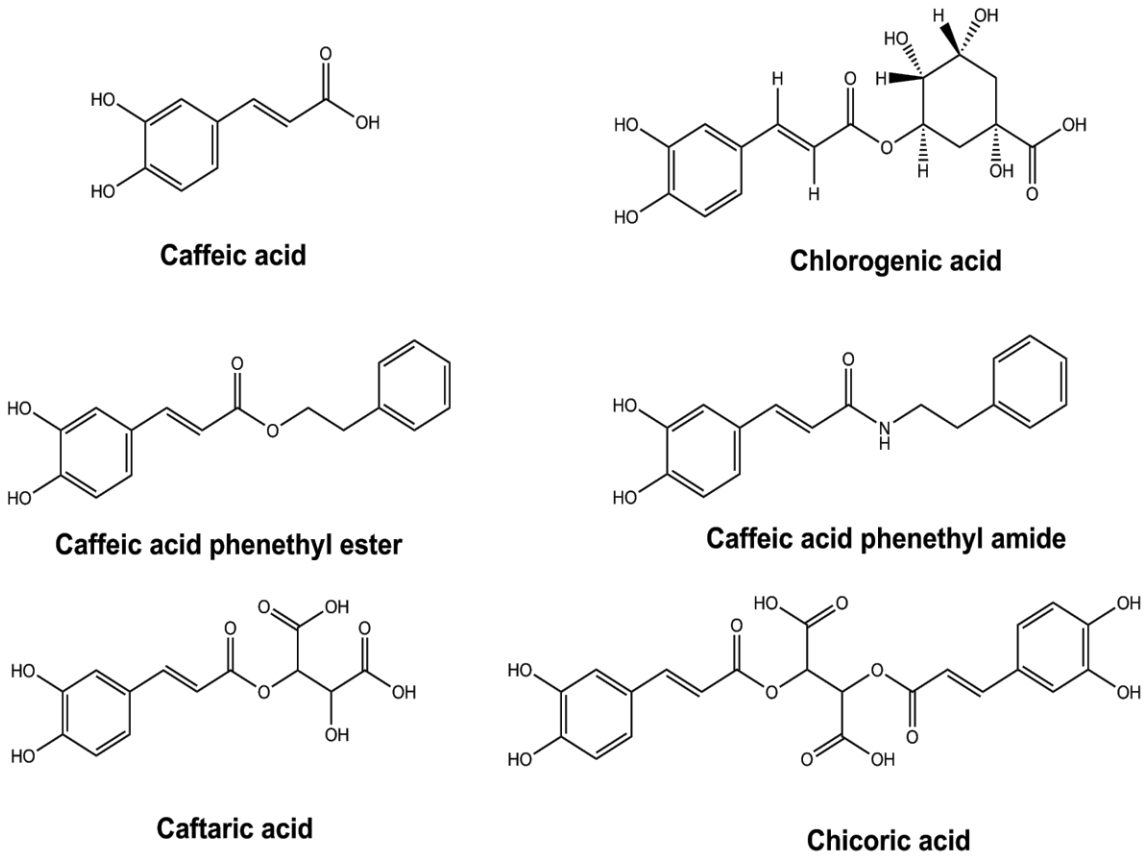


Figure 2.6: Structure of caffeic acid and some of its derivatives

Caffeic acid usually exists in plants as various simple derivatives such as glycosides, amides, esters and sugar esters (Cowan, 1999; Touaibia and Doiron, 2011). Some of the important caffeic acid derivatives include chlorogenic acid (3-*O*-caffeoylquinic acid) and caffeic acid phenethyl ester (CAPE). Chlorogenic acids are formed from the esterification of hydroxycinnamic acids (e.g. caffeic acid, ferulic, p-coumaric acid) and quinic acid. Chlorogenic acid is one of the main components in