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In vitro evaluation of caffeic acid derivatives as efflux pump inhibitor in *Pseudomonas aeruginosa* and *Burkholderia pseudomallei*

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ABSTRACT

Aims: Bacterial pathogens such as *Pseudomonas aeruginosa* and *Burkholderia pseudomallei* are intrinsically resistant to many classes of antibiotics. This is not only due to the poor permeability of their outer membrane but also because of expression of multiple efflux pumps. A promising strategy to minimize the efflux of drugs by these pumps is the use of efflux pump inhibitors (EPIs). In this study, the potential of caffeic acid derivatives as EPIs in *P. aeruginosa* and *B. pseudomallei* were evaluated.
Methodology and results: The potential of caffeic acid and its derivatives, i.e. chlorogenic acid, caffeic acid phenethyl ester (CAPE) and caffeic acid phenethyl amide (CAPA) to act as EPIs in *P. aeruginosa* and *B. pseudomallei* were assessed using the ethidium bromide (EtBr) accumulation and minimum inhibitory concentration (MIC) validation assays. Among the four test compounds, CAPE was found to significantly increased intracellular accumulation of EtBr in both *P. aeruginosa* and *B. pseudomallei.* An increase of 21.4% and 16.8% in cell fluorescence, over a 5-min time frame was observed in *P. aeruginosa* and *B. pseudomallei* respectively. Combination of CAPE with kanamycin significantly reduced MICs of this aminoglycoside by a factor of 8-fold in *P. aeruginosa* and 2-fold in *B. pseudomallei*. Combination of CAPE with gentamicin also led to a reduction of 4-fold MIC value of this antibiotic in *B. pseudomallei*.
Conclusion, significance and impact of study: The *in-vitro* results suggest that CAPE has the potential to act as an EPI in *P. aeruginosa* and *B. pseudomallei*, thus improving the efficacy of aminoglycosides as antimicrobial agents.

Keywords: Gram-negative bacteria, multidrug resistance efflux pump, efflux pump inhibitor, caffeic acid derivatives

INTRODUCTION

The continuous emergence of multidrug-resistant (MDR) bacteria pathogens has made the management and control of infectious diseases a very challenging task. In most of these MDR pathogens, the existence of various efflux mechanisms has been broadly recognized as the major components of resistance to many classes of antibiotics (Lynch and Courvalin, 1997; Piddock, 2006). Most bacteria have the potential to develop MDR traits because many MDR pumps are intrinsically present in bacterial chromosomes. Acquisition of specific resistance determinants such as antibiotic-specific efflux pumps through lateral gene transfer can also happen. Thus, the mechanism of drug efflux in bacteria can be very diverse; some efflux pumps selectively extrude specific antibiotics, while others, such as MDR pumps, expel a variety of structurally and functionally diverse compounds like ethidium bromide (EtBr), acriflavine, triclosan, organic solvents and acylated homoserine lactones (Piddock, 2006). The major antibiotic classes that are known to be effluxed by intrinsic bacterial efflux pumps systems are macrolides, β-lactams, fluoroquinolones, oxazolidinones, and fourth-generation cephalosporins and carbapenems.

Opportunistic pathogens such as *Pseudomonas aeruginosa* and *Burkholderia pseudomallei* are a major cause of nosocomial infections in immune-compromised individuals (Ali *et al*., 2015). Management of infection can be a daunting task because these organisms are intrinsically resistant to wide classes of clinically useful antibiotics, particularly β-lactams, aminoglycosides, macrolides and older-generation cephalosporins (Moore *et al.*, 1999). One of the major contributors to the intrinsic resistance in both *P. aeruginosa* and *B. pseudomallei* is the expression of MDR efflux pumps of the resistance-nodulation-cell-division (RND) superfamily (Chan *et al.*, 2004; Kumar and Schweizer, 2005). For *P. aeruginosa,* the major efflux systems responsible for the intrinsic and acquired MDR in this organism includes both the constitutive MexAB-OprM and MexXY-OprM pumps and the inducible MexEF-OprN and MexCD-OprJ operons (Kumar and Schweizer, 2005). In *B. pseudomallei,*

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resistance to aminoglycosides and macrolides is due to the AmrAB-OprA and BpeAB-OprB efflux pumps, homologues of the *P. aeruginosa* MexXY-OprM and MexAB-OprM pumps (Chan *et al.*, 2004; Mima and Schweizer, 2010). In addition, the BpeEF-OprC pumps are responsible for *B. pseudomallei's* resistance towards trimethoprim and chloramphenicol (Kumar *et al*., 2006).

The extrusion of toxic compounds such as antibiotics by these efflux pumps limits the accumulation of the antibiotics inside the cell. Hence, inevitably, the bacteria become insensitive to antibiotics. The usage of efflux pump inhibitors (EPI) offers a promising strategy to circumvent the efflux of drugs. Co-administration of EPI with antibiotics has not only led to the lowering of the antibiotic minimal inhibitory concentration (MIC) but also successfully reduced the invasiveness of *P. aeruginosa* (Lamers *et al*., 2013). Therefore, the ability to inhibit these drug efflux mechanisms will not only restore the clinical usefulness to some older compounds but also improve drug potency as well as minimize the potential development of resistant bacteria strains (Zechini and Versace, 2009).

EPI is usually a small molecule that can bind to the efflux pump either competitively or non-competitively and block the pump from binding to the antibiotics. To date, a number of bacterial efflux pump inhibitors of significant potentiating activity have been discovered. Phenyl-arginine beta-naphthylamide (Paβn) was the first established EPI that could inhibit all four clinically relevant efflux systems in *P. aeruginosa* and in other Gram-negative bacteria such as *Escherichia coli* and *B. pseudomallei*. However, this EPI has not reached the clinical use for several reasons. One of the important reasons is that this compound can affect the membrane integrity of bacteria that can lead to lipopolysaccharide modification and consequently the induction of undesirable resistance profiles by reducing drug penetration (Sun *et al.*, 2014).

Since ancient times, plants have been extensively used as traditional medicine to control bacterial, viral and fungal infections (Dupont *et al*., 2006; Temrangsee *et al*., 2011). The repertoire of phytochemicals is bountiful with extensive functional group chemistry, chirality and ultimately chemical diversity. One ubiquitous phytochemical in herbs and plants is caffeic acid. It is found in all plants since it is a key intermediate in the biosynthesis of lignin, one of the principal sources of biomass (Boerjan *et al*., 2003). Caffeic acid and its derivatives such as chlorogenic acid (3-O-Caffeoylquinic acid) and caffeic acid phenethyl ester (CAPE) have been gaining increasing attention for their various pharmaceutical properties and health-promoting benefits (Celik *et al*., 2009; Ikeda *et al*., 2011). Derivatives of caffeic acid, such as dicaffeoylquinic acid and N-caffeoylphenalkylamide have been shown to be active against a broad range of Gram-negative and Gram-positive bacteria. For example, the co-administration of N-caffeoylphenalkylamide derivatives with norfloxacin resulted in an 8-fold reduction of the norfloxacin MIC at 292 µM (100 µg/mL) in MDR *Staphylococcus aureus* (Michalet *et al*., 2007). In another finding, the dicaffeoylquinic acid derivatives from *Artemisia absinthium* demonstrated very weak antimicrobial activity against the Gram-positive bacteria, *B. cereus* and *E. faecalis*, MIC (64 µg/mL) but resulted in a 4-fold difference between MICs in *S. aureus* 8325-4 and the *S. aureus* NorA multi-efflux pump, knock-out mutant (Fiamegos *et al.*, 2011). This observation suggests that the caffeic acid derivatives are potential candidates as EPIs.

This study was initiated to evaluate the potential of caffeic acid and its derivatives, chlorogenic acid, caffeic acid phenethyl ester (CAPE) and caffeic acid phenethyl amide (CAPA) to inhibit efflux pump activities in *P. aeruginosa* and *B. pseudomallei*, two opportunistic pathogens known to possess multiple number of efflux pump systems. A few methods are available for monitoring the efflux pump activity in bacterial cells (Hasdemir *et al*., 2004; Lechner *et al*., 2008; Viveiros *et al*., 2008; Daugelavicius *et al*., 2010). One of the methods, ethidium bromide (EtBr) accumulation assay utilizes EtBr as the fluorescent probe (Kamicker *et al*., 2008; Paixão *et al*., 2009). The principle of this assay is the transport of EtBr across the cytoplasmic membrane and its subsequent intracellular accumulation inside the bacterial cell. In Gram-negative bacteria, EtBr traverses the bacterial cell wall via porin channels and it can be concentrated inside the cell to a point where it fluoresces when excited by ultraviolet light (Borges-walmsley *et al*., 2003; Amaral *et al*., 2011). MDR efflux pumps will recognize EtBr as a substrate and bacteria with active efflux pumps can efficiently efflux EtBr out of their systems. However, in the presence of an EPI, accumulation of EtBr in the cell will occur. Putative EPI candidates as determined through the EtBr accumulation, were further characterized to investigate if any of these EPI candidates could cause a reduction in the MIC values of selected antibiotics in *P. aeruginosa* and *B. pseudomallei*.

MATERIALS AND METHODS

Chemicals

Ethidium bromide and antibiotics (kanamycin sulfate and erythromycin) were purchased from Amresco, USA, chloramphenicol and gentamicin sulfate was purchased from Sigma-Aldrich, USA. Standard EPIs thioridazine (TDZ) and carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) were purchased from MP Biomedicals, USA. Caffeic acid and chlorogenic acid were purchased from Cayman Chemical, USA, and CAPE was purchased from Sigma, USA. CAPA was synthesized and purified in our laboratory.

Bacterial strains and culture conditions

The stock cultures for test strains, *Pseudomonas aeruginosa* ATCC 27853 and *Burkholderia pseudomallei* K96243 were maintained on Luria-Bertani agar (HiMedia Laboratories, India) at 4 °C with regular passage. Mueller-

Hinton broth II (Sigma-Aldrich, USA) was used in the broth microdilution technique for determination of MIC.

Ethidium bromide accumulation assay

The potential of caffeic acid and its derivatives, chlorogenic acid, CAPE and CAPA to function as efflux pump inhibitors in *P. aeruginosa* ATCC 27853 and *B. pseudomallei* K96243 cells were assessed using the EtBr accumulation assay, according to the adapted protocols as described by Kamicker *et al.* (2008) and Coldham *et al*. (2010). All experiments were performed in triplicates and error bars reported as standard deviation. For this assay, the stock solutions of test compounds (caffeic acid, chlorogenic acid, CAPE, CAPA, thioridazine (TDZ) and carbonyl cyanide 3-chlorophenylhydrazone (CCCP) were dissolved in 100% DMSO to a final concentration of 10 mM. Then, 20 µL of the prepared solution of each compound was aliquot into fresh Eppendorf tube and diluted with 80 µL of 100% DMSO.

Bacteria culture was inoculated on a fresh Luria-Bertani (LB) agar plate, 24 h prior to use. To prepare the inoculums, four to five well-isolated colonies of each bacterium were selected from the overnight plate culture. Each colony was transferred with a sterile streaking loop into a test tube containing about 10 mL of sterile LB broth and incubated overnight at 37 °C with agitation. On the following day, 5 mL of the overnight culture was diluted with 45 mL of fresh medium and incubated at 37 °C with agitation for 4 h. The culture was then transferred to a 50 mL centrifuge tube and centrifuged at 4000 × *g* for 10 min. The supernatant was discarded and the cell pellet resuspended in 50 mL of sodium phosphate buffer (SPB), pH 7.0. The cells were then recentrifuged at 4000 × *g* for 10 min and the SPB discarded. Subsequently, the cells were resuspended in SPB. The cell turbidity was then adjusted to an OD_{600} = 0.5 (Hitachi U-2000 Double-Beam UV/Vis Spectrophotometer). For every 47.5 mL of cell inoculum, 0.5 mL of glucose (1 M) was added to give a final concentration of about 0.01 M.

For the EtBr accumulation assay, the bacterial inoculum was cultured in 96-well black tissue culture plate (SPL Life Sciences), in triplicates. A volume of 175 µL bacterial cells supplemented with glucose, was added to each well containing 20 µL of 100 µM EtBr and incubated for 1 h. Then, 5 µL of each test compound (2 mM) was added to each well of the daughter plate, in triplicates. CCCP and TDZ were used as the positive controls while DMSO, as the negative control. The plate was then placed into the Promega Glomax Fluorescence plate reader and the fluorescence intensity of the accumulated EtBr was read for 5 min at excitation = 530 nm and emission = 600 nm with a 1-min time interval. The loss of EtBr from the cells was indicated by the reduction in the fluorescence intensity as compared to the positive and negative control. The raw data obtained was statistically analyzed and a graph of the EtBr accumulation in the bacterial cell against time was plotted using Graphpad Prism 5.0 and the percentage increase in fluorescence was calculated as below:

$$[(Ft_5 – Ft_0)/ Ft_0 × 100]$$

Where, Ft_5 is the EtBr fluorescence at time 5-min and Ft_0 is fluorescence at time 0 min (Hong and James, 1999).

Antibiotic/compound susceptibility test and minimal inhibitory concentration determination

Before conducting the EPI/antibiotic combination studies, the susceptibility of *P. aeruginosa* ATCC 27853 and *B. pseudomallei* K96243, to the selected antibiotics and test compound were determined. For *P. aeruginosa*, the MIC values for kanamycin and chloramphenicol were determined, while for *B. pseudomallei*, its susceptibility to kanamycin, gentamicin and erythromycin were tested. Both the organisms' susceptibility to all test compounds (EPI candidates) was also determined.

For MIC determination, stock solutions of gentamicin (10 mg/mL), kanamycin (10 mg/mL) and TDZ (10 mg/mL) were dissolved in distilled water and filter-sterilized. Stock solutions for erythromycin (10 mg/mL), chloramphenicol (10 mg/mL) and CCCP (10 mg/mL) were dissolved in ethanol. Stock solutions for caffeic acid (5 mg/mL), chlorogenic acid (10 mg/mL) and CAPE (10 mg/mL) were prepared in DMSO. All stock solutions were kept at -20 °C. For preparation of working solutions, stock solutions of antibiotics were diluted in sterile distilled water, stock solution of CCCP was diluted in sterile MHB II broth, and stock solutions of caffeic acid derivatives were dissolved in normal saline to obtain the desired working concentrations. For susceptibility testing of antimicrobials alone and EPI alone, working solutions at 4× the final desired concentrations were prepared. For MIC testing of antibiotic/EPI combination, working solutions at 8× the final desired concentrations for antibiotics and 4× the final desired concentrations for EPI were prepared.

For MIC determination, the standard two-fold broth microdilution technique by tetrazolium microplate assay (TEMA) was performed, as described by Eloff J. N. (1998). Five well-isolated single colonies from a pure overnight culture on LB agar were inoculated in 5 mL MHB II broth and incubated for 4 h at 37 °C with shaking, until its turbidity reaches 0.5 McFarland standard (approximately 1-2 × 10^8 CFU/mL). The inoculum was further diluted to a ratio of 1:100 in MHB II broth. The diluted inoculum was used within 15 min to minimize changes in organism density.

Briefly, 100 µL of Mueller Hinton II broth was dispensed into rows A to G, of the 96-well microtiter plate (SPL Life Sciences, South Korea) while row H, which served as negative control well was filled with 200 µL of the broth. Next, 100 µL of each antibiotic or EPI working solution (1024 µg/mL) was added to the wells in the first column in triplicates. The drugs were serially diluted in a ratio of 1:2 through six dilutions, and 100 µL of excess medium from the final dilution was discarded. Then, 100 µL of bacterial inoculum was added to wells in rows A to G, with a final organism density of 5 × 10^5 CFU/mL. The wells in row G served as a positive growth control (inoculum only) and wells in row H served as a sterility control (without inoculum). Each well contained a total

volume of 200 µL. The plates were sealed with Parafilm, wrapped in aluminium foils and incubated for 18 - 20 h at 37 °C. On the following day, 50 µL of 1.0 mg/mL freshly-prepared p-iodonitrotetrazolium violet, INT (Sigma-Aldrich, USA) was added to all wells. The plates were reincubated for 30 min to 1 h. MIC was defined as the lowest concentration of drug required whereby, no visible color change is observed in the inoculums. A colorless solution denoted no bacterial growth, and a purple color indicated growth.

The MIC interpretations of susceptible (S) or resistant (R) against antibiotics gentamicin (S ≤ 4, R > 4), kanamycin, and erythromycin were based on EUCAST clinical breakpoints for *Pseudomonas* spp., and interpretation for chloramphenicol (S ≤ 8, R > 8) was based on *Enterobacteriaceae*.

Antibiotic-EPI combination test in the presence of EPI or test compounds

MIC determination of antibiotics in combination with standard EPI or test compounds was carried out as previously described, with minor modifications. A constant concentration of EPIs or test compounds at a sub-inhibitory concentration of 100 µg/mL or lower was employed. Briefly, 50 µL antibiotic at 8× final desired concentration was serially diluted in 50 µL MHB II broth, and 50 µL of excess media in the final dilution was discarded. Next, 50 µL of EPI or test compound at 4× the final desired concentration was added to all wells containing serially diluted antibiotics. Then, 100 µL of 1:100 diluted inoculum with turbidity set to 0.5 McFarland standard, was added to all wells (except the sterility control wells) for a final cell density of 5×10^5 CFU/mL. Each well would achieve a total volume of 200 µL. The plates were sealed, covered, and incubated overnight at 37 °C prior to the addition of 50 µL of 0.6 - 1.0 mg/mL INT. The plates were then reincubated for 30 min to 1 h and MICs were recorded based on color changes.

RESULTS AND DISCUSSION

Over dependence and inappropriate use of antibiotics have led to the rapid emergence of multi-drug resistant bacteria, worldwide. Although antimicrobial resistance can be due to various factors, such as modification of drug targets and enzymatic inactivation or modification of drugs, the active efflux of drugs by intrinsic bacterial efflux pumps is also a cause of major concern. The problems of active drug efflux in bacteria is especially eminent because the expression of a single type of multi drug efflux pump, such as those of the RND family, can lead to simultaneous resistance to a number of drugs, yielding a MDR phenotype. Hence, the application of an EPI that could counter the active expression of efflux pumps offers an attractive strategy to manage and overcome microbial infections.

Plants have always been recognized to be a reservoir for bioactive metabolites and lead compounds. Quite a number of EPI candidates have been derived out from plant sources. For instance, the plant alkaloid reserpine that inhibits multidrug transporter NorA in *Staphylococcus aureus* (Neyfakh *et al.*, 1993) and verapamil that inhibits LmrA, an ATP-dependent transporter pump in *Lactococcus lactis* (Poelarends *et al.*, 2002). Unfortunately, these compounds have been shown to be toxic for human consumption since the effective dose needed to inhibit bacterial efflux pumps was high (Schmitz *et al.*, 1998; Poelarends *et al.*, 2002). Thus, although quite a number of natural and synthetic EPIs have been identified, none of them has been approved for management of bacterial infections due to their adverse effects and unreliable clinical efficiency (Rana *et al.*, 2014). Hence, this illustrates the call for search of more potent but non-toxic efflux pump inhibitors that can be safely used in clinical setting.

Table 1: Minimum inhibitory concentrations (MIC) of ethidium bromide, standard efflux pump inhibitors and caffeic acid derivatives against *P. aeruginosa* ATCC 27853 and *B. pseudomallei* K96243.

	MIC (µg/mL) of test compound	
Compound	*Pseudomonas aeruginosa* ATCC 27853	*Burkholderia pseudomallei* K96243
EtBr	256	64
CCCP	256	64
TDZ	> 256	> 256
Caffeic acid	> 256	> 256
Chlorogenic acid	64	> 256
CAPE	> 256	> 256
CAPA	256	256

(EtBr, ethidium bromide; CPZ, chlorpromazine; TDZ, thioridazine; CCCP, carbonyl cyanide m-chlorophenyl hydrazone; CAPE, caffeic acid phenethyl ester; CAPA, caffeic acid phenethyl amide)

Our search for EPI candidates that could effectively be applied in pathogens with well-established efflux pump systems such as *P. aeruginosa* and *B. pseudomallei*, were focused on compounds that are known to be abundant in plants, i.e., caffeic acid and its derivatives chlorogenic acid, CAPE and CAPA. For initial screening, the Ethidium bromide (EtBr) accumulation assay (Kamicker *et al.,* 2008; Coldham *et al.,* 2010) was conducted to evaluate the potential of the selected caffeic acid derivatives as EPI(s) in *P. aeruginosa* ATCC 27853 and *B. pseudomallei* K96243. Prior to evaluation of selected test compounds as EPI(s) using the EtBr

accumulation assay, the tolerance/susceptibility of *P. aeruginosa* ATCC 27853 and *B. pseudomallei* K96243 cells towards EtBr were assessed (Table 1). The high MIC of EtBr (256 µg/mL) obtained for *P. aeruginosa* ATCC 27853 indicates that this organism is not susceptible towards this substrate. However, the MIC of EtBr in *B. pseudomallei* ATCC 27853 was lower (64 µg/mL), which can suggest a lower tolerance of this bacterium towards EtBr.

The susceptibility of both *P. aeruginosa* ATCC 27853 and *B. pseudomallei* K96243 cells towards caffeic acid, chlorogenic acid, CAPE and CAPA were also evaluated. Established EPIs, thioridazine (TDZ) and carbonyl cyanide m-chlorophenyl hydrazone (CCCP) were used as positive controls. As shown in Table 1, the standard EPIs; TDZ and CCCP have high MICs (≥ 256 µg/mL) against *P. aeruginosa* ATCC 27853, which indicated that they possess minimal antibacterial activity against the bacteria. Similarly, the MIC value of TDZ for *B. pseudomallei* K96243 was also high, i.e., ≥ 256 µg/mL, indicating that this bacterium is not susceptible to this compound. However, the MIC for CCCP in *B. pseudomallei* was lower, with a value of 64 µg/mL, suggesting that this compound might exhibit some bacteriostatic/bactericidal activity in this bacterium. With the exception for the MIC value of chlorogenic acid in *P. aeruginosa* ATCC 27853 (64 µg/mL), the MIC values for the test compounds were also high, i.e., ≥ 256 µg/mL, indicating that both bacterial cells were also not susceptible to the test compounds.

Generally, various phytochemicals have been shown to have some antimicrobial activities against a broad spectrum of bacteria, albeit, at a lower potency than conventional antibiotics (Kyaw *et al*., 2012). However, with the exception of chlorogenic acid *in P. aeruginosa* ATCC 27853*,* the test compounds demonstrated very minimal antibacterial activities in both *P. aeruginosa* ATCC 27853 *and B. pseudomallei* K96243*,* based on their high MIC values. This attribute therefore, qualifies the test compounds as potential EPI candidates whereby; to be categorized as an ideal EPI, the said compound should not demonstrate intrinsic antimicrobial activity (Kamicker *et al*., 2008).

The potential of caffeic acid, chlorogenic acid, CAPE and CAPA to act, as EPI(s) in either *P. aeruginosa* or *B. pseudomallei* were further assessed using the EtBr accumulation assay. The levels of EtBr accumulation in *P. aeruginosa* ATCC 27853 and *B. pseudomallei* K96243 cells treated with the standard EPIs (TDZ and CCCP), DMSO (negative control) and test compounds (caffeic acid, chlorogenic acid, CAPE and CAPA) as monitored within a 5-min time frame using the fluorescence microplate reader are depicted in Figures 1(a) and 1(b). Intracellular accumulation of EtBr, expressed as percentage increase in fluorescence over the 5-min time frame for both *P. aeruginosa* ATCC 27853 and *B. pseudomallei* K96243 cells are summarized in Table 2.

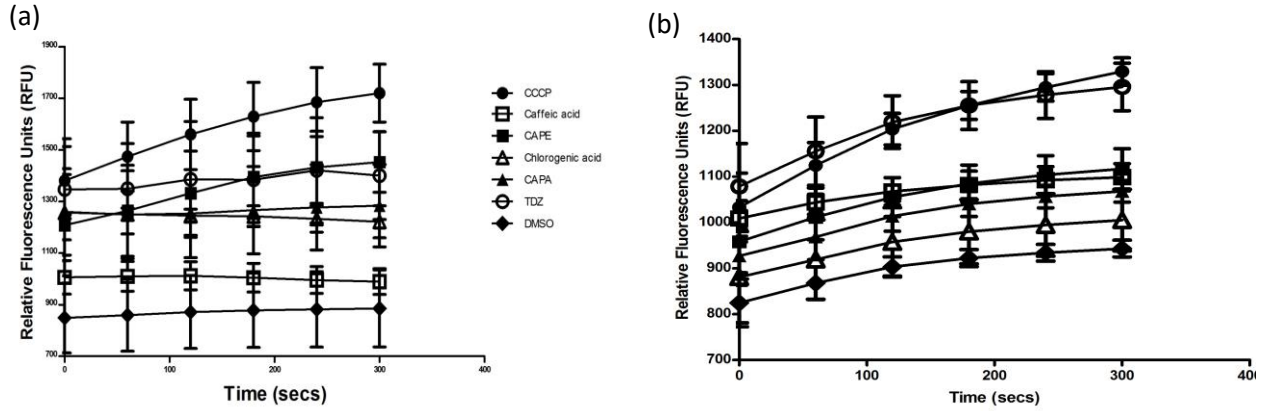

Figure 1: Effect of EPIs (CCCP and TDZ), caffeic acid derivatives (caffeic acid, chlorogenic acid, CAPE, CAPA) and DMSO on the accumulation of EtBr in (a) *P. aeruginosa* ATCC 27853 and (b) *B. pseudomallei* K96243.

Table 2: Percentage increase in fluorescence over a 5-min time frame, indicative of EtBr accumulation in *P. aeruginosa* ATCC 27853 and *B. pseudomallei* K96243.

	Percentage (%) increase in fluoresence during 5-min time frame	
Compound	*Pseudomonas pseudomallei* ATCC 27853	*Burkholderia pseudomallei* K96243
CCCP	24.8	29.0
TDZ	4.2	20.5
Caffeic Acid	Insignificant increase	8.9
Chlorogenic Acid	Insignificant increase	14.5
CAPE	21.4	16.8
CAPA	2.0	15.4

Accumulation of EtBr was lowest for *P. aeruginosa* ATCC 27853 and *B. pseudomallei* cells treated with DMSO and highest for cells treated with CCCP, followed by cells treated with TDZ. The slow rate of EtBr accumulation in cells treated with DMSO (negative control) indicated that both *P. aeruginosa* and *B. pseudomallei* cells can efficiently efflux out EtBr molecules. As expected, cells treated with CCCP and TDZ displayed very high accumulation of EtBr. CCCP-treated *P. aeruginosa* ATCC 27853 cells experienced 24.8% increase in fluorescence over a 5-min time frame while CCCP-treated *B. pseudomallei* K96243 cells had 29.0% increment. However, intracellular accumulation of EtBr was notably less efficient for TDZ-treated *P. aeruginosa* ATCC 27853 cells as compared to TDZ-treated *B. pseudomallei* K96243 cells. After 5 min, TDZ-treated *P. aeruginosa* ATCC 27853 cells only showed 4.2% fluorescence increase while *B. pseudomallei* K96243 cells experienced 20.5% increase.

Both CCCP and TDZ are well-established EPIs and are known to disrupt efflux pump efficiency. For instance, CCCP is an energy uncoupler that could alter the transmembrane electrochemical gradient of proton (H^+)-driven efflux pumps, leading to inhibition of the efflux pump (Zhang *et al.*, 2010). The phenothiazine, TDZ, has also been proven to be a potent inhibitor of efflux pumps of multi-drug resistant bacteria and yeast (Kaatz *et al.,* 2003; Kolaczkowski *et al.*, 2003; Amaral *et al.*, 2011).

Among the four compounds tested, the level of EtBr accumulation induced by CAPE treatment was comparatively higher for both *P. aeruginosa* ATCC 27853 *and B. pseudomallei* K96243 cells. Within 5 min, CAPE-treated *P. aeruginosa* ATCC 27853 cells exhibited 21.4% increase in fluorescence while *B. pseudomallei* K96243 cells showed 16.8% increase. Meanwhile, for *P. aeruginosa* ATCC 27853 cells treated with, either CAPA, chlorogenic acid or caffeic acid, the percentage increase in fluorescence was minimal or insignificant (Table 2). The efflux pump inhibitory activities of CAPA, chlorogenic acid or caffeic acid were, however, more pronounced in *B. pseudomallei* K96243, as observed in the level of fluorescence increase over the 5-min time frame (Table 2).

With the exception for CAPE, the efflux pump inhibitory activities of caffeic acid, chlorogenic acid and CAPA on the EtBr accumulation appeared to be comparatively more efficient in *B. pseudomallei* K96243 than *P. aeruginosa* ATCC 27853. Among the factors that could have contributed to this observation include, differences in cell-wall permeability (Amaral and Lorian, 1991), efflux pump substrate affinity, substrate specificity, and substrate promiscuity (Mao *et al*., 2002; Nikaido and Pages, 2012).

In both *P. aeruginosa* and *B. pseudomallei*, efflux pumps of the resistance nodulation cell division (RND) family are the clinically most significant efflux systems (Poole, 2005; Podnecky *et al*., 2015). The expression of these pump have rendered these organisms resistance to to clinically significant antibiotics, including aminoglycosides, chloramphenicol, fluoroquinolones, and tetracyclines (Moore *et al*., 1999; Chan *et al.*, 2004; Chuanchuen *et al.*, 2008; Lau *et al.*, 2014). Our results showed that *B. pseudomallei* has relatively high MICs for gentamicin (32 µg/mL), kanamycin (8 µg/mL), and erythromycin (64 µg/mL). As defined by EUCAST guidelines, these MIC values fall in the resistant range. Meanwhile, *P. aeruginosa* ATCC 27853 exhibited high MICs for kanamycin (64 µg/mL) and chloramphenicol (128 µg/mL). This is not unexpected, as *P. aeruginosa* has been known to express both the MexXY-OprM and MexAB-OprM respectively. MexXY-OprM is usually known to be the *P. aeruginosa* efflux pump that extrudes aminoglycosides (Chuanchuen *et al.*, 2008; Lau *et al.*, 2014) while MexAB-OprM can efflux widely different substrates including chloramphenicol (Kumar and Schweizer, 2005). Thus, co-administration of efflux pump inhibitors would certainly be beneficial as this could reduce the amount of antibiotics required for improved efficiency in management of microbial infections.

Initial assessment of caffeic acid and its derivatives, chlorogenic acid, CAPE and CAPA using the EtBr accumulation assay implied that these compounds have the potential to disrupt the efflux pump systems in *P. aeruginosa* ATCC 27853 and *B. pseudomallei* K96243. Co-administration of these putative EPI(s) with selected antibiotics revealed some interesting observations for both *P. aeruginosa* ATCC 27853 (Table 3) and *B. pseudomallei* K96243 (Table 4).

Table 3: Value of MIC (µg/mL) of antibiotics when used in combination with standard EPIs or caffeic derivatives, against *Pseudomonas aeruginosa* ATCC 27853.

Compound (Sub inhibitory concentration, µg/mL)	MIC values of antibiotics (µg/mL)	
	Kanamycin	Chloramphenicol
No addition of EPI candidate	64	128
CCCP (64 µg/mL)	16*	32*
TDZ (100 µg/mL)	16*	16*
Caffeic acid (100 µg/mL)	≤ 8*	128 N.R.
Chlorogenic acid (16 µg/mL)	16*	128 N.R.
CAPE (100 µg/mL)	8*	128 N.R.
CAPA (100 µg/mL)	8*	128 N.R.

(*, showed reduction in MIC; NR, no reduction)

In *P. aeruginosa* ATCC 27853, co-administration of the standard EPIs, CCCP and TDZ, caused 4-fold to 8-fold reduction in the MIC of kanamycin and chloramphenicol respectively (Table 3). The aminoglycoside, kanamycin, when used in combination with the caffeic acid–based compounds showed between 4-fold and 8-fold reduction in MIC values (Table 3). CAPE and CAPA at their sub-inhibitory concentrations (100 µg/mL) were shown to reduce the MIC of kanamycin by 8-fold each. Chlorogenic acid (16 µg/mL) reduced the MIC of kanamycin by 4-fold while caffeic acid, caused more than 8-fold reduction in the MIC of kanamycin. However, none of the four compounds had any effect on the MIC value of chloramphenicol in *P. aeruginosa* ATCC 27853.

In *B. pseudomallei* K96243, the effect of EPI/antibiotics combinations on the MIC values showed a more erratic trend (Table 4). For instance, the standard EPI CCCP, was only suitable for use in combination with gentamicin (2-fold reduction in MIC) while TDZ caused a 4-fold reduction of erythromycin MIC in *B. pseudomallei* K96243. For the test compounds, CAPE reduced the MIC of gentamicin by 4-fold, kanamycin by 2-fold, but showed no reduction in the MIC of erythromycin in *B. pseudomallei* K96243. The actions of caffeic acid and chlorogenic acid were, however, either non-effective or antagonistic when used in combinations with gentamicin, kanamycin, and erythromycin. Caffeic acid caused a 2-fold increase in the MIC of gentamicin, kanamycin, and erythromycin in *B. pseudomallei* K96243. Chlorogenic acid also increased the MIC of gentamicin by 2-fold but showed no changes in the MIC of kanamycin and erythromycin (Table 4).

Table 4: Value of MIC (µg/mL) of antibiotics when used in combination with standard EPIs or caffeic acid derivatives, *against Burkholderia pseudomallei* K96243.

Compound (Sub inhibitory concentration, µg/mL)	MIC values of antibiotics (µg/mL)		
	Kanamycin	Gentamicin	Erythromycin
No addition of EPI candidate	8	32	64
CCCP (16 µg/mL)	8 NR	16*	64 NR
TDZ (100 µg/mL)	8 NR	32 NR	16*
Caffeic acid (100 µg/mL)	16 NE	64 NE	128 NE
Chlorogenic acid (100 µg/mL)	8 NR	64 NE	64 NR
CAPE (100 µg/mL)	4*	8*	64 NR

(*, showed reduction in MIC; NR, no reduction; NE, negative effect/caused increase in MIC)

In all these antibiotic-EPI combination tests, a sub-inhibitory concentration of EPIs at 100 µg/mL or lower was used in combination with multiple dilutions of antibiotics. This was to ensure that any observed reduction in MIC was primarily due to efflux inhibition by the EPI, and not by the effects of partial killing activity of the compounds.

Among the four caffeic acid derivatives tested, only CAPE demonstrated potential efflux pump inhibitory activities against the extrusion of the aminoglycosides, kanamycin and gentamicin in both *P. aeruginosa* ATCC 27853 and *B. pseudomallei* K96243 cells. This is based on the observation that CAPE-treated *P. aeruginosa* ATCC 27853 and CAPE-treated *B. pseudomallei* K96243 cells exhibited a significant increase in fluorescence due to EtBr accumulation in their cells. The possibility of CAPE as potential EPI candidate is further enhanced by its ability to cause reduction in the MIC values of kanamycin in both *P. aeruginosa* ATCC 27853 and *B. pseudomallei* K96243 cells. Combination of CAPE-gentamicin also led to a very significant reduction in MIC value of this aminoglycoside in *B. pseudomallei* K96243. In both organisms, it has been well established that the extrusion of aminoglycosides from the cells are facilitated by the efflux pumps of the resistance nodulation cell division (RND) family (Poole, 2005). However, the underlying mechanisms of actions of CAPE still require further study in order to validate whether the compound acts directly or indirectly on RND efflux pumps.

CONCLUSION

To summarize, CAPE is a promising compound that can be further developed into an effective EPI. However, CAPE is sparingly soluble in aqueous solution, thus it did not fully dissolved when diluted into its working concentration. Due to this insolubility problem, it is assumed that the actual concentration of CAPE tested was lower than what was intentionally prepared. Even at low solubility, CAPE managed to reduce the MIC of some of the antibiotics by several folds lower against both bacteria. Further studies should be done to evaluate its clinical potential as an efflux pump inhibitor. The safety profile of this compound also needs to be established in order to implement the use of the combinations in the clinical setting.

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