TITLE:

Synergistic Interaction Between Phenothiazines And Antimicrobial Agents in

*Burkholderia pseudomallei*

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KEYWORDS:

Prochlorperazine, chlorpromazine, promazine, RND efflux pumps, antimicrobial susceptibility, *Burkholderia pseudomallei*

RUNNING TITLE:

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ABSTRACT

The Gram-negative soil bacillus, *Burkholderia pseudomallei*, is the causative agent of melioidosis, a severe and potentially fatal septicemic disease that is endemic to south-east Asia and northern Australia. Its intrinsic resistance to many antibiotics is attributed mainly to the presence of several drug efflux pumps and therefore inhibitors of such pumps are expected to restore the activities of many clinically important antimicrobial agents that are the substrates of these pumps. The phenothiazine antipsychotic and antihistaminic drugs, prochlorperazine, chlorpromazine and promazine, exert a synergistic interaction with a wide spectrum of antimicrobial agents, thereby enhancing their antimicrobial potency against *B. pseudomallei*. Antimicrobial agents that interacted synergistically with the phenothiazines include streptomycin, erythromycin, oleandomycin, spectinomycin, levofloxacin, azithromycin and amoxicillin-clavulanic acid. The MICs of these antibiotics were reduced by as much as 8000-fold in the presence of the phenothiazines. Antimicrobial agents which did not interact synergistically with the phenothiazines include gentamicin, amoxicillin and ampicillin. Omeprazole, a proton pump inhibitor, provided a similar augmentation of antimicrobial activities as the phenothiazines, suggesting that the phenothiazines might have interfered with the proton gradient at the inner membrane. *B. pseudomallei* cells accumulated more erythromycin in the presence of phenothiazines, similar to the effect of carbonyl cyanide m-chlorophenylhydrazone (CCCP), a proton gradient uncoupler. In the presence of phenothiazines, a much reduced concentration of erythromycin (0.06X MIC) also protected human lung epithelial cells and macrophage cells from *B. pseudomallei* infection and attenuated its cytotoxicity.
INTRODUCTION

Melioidosis is an infectious disease that is caused by the Gram-negative soil bacillus, *Burkholderia pseudomallei*, which is endemic in south-east Asia and northern Australia. Infection may be acquired through direct skin contact with contaminated soil or surface water or by ingestion of such contaminated water or dust. Clinical symptoms depend upon the route of infection, but four clinical forms are generally described: localised infection, pulmonary infection, septicaemia and chronic suppurative infections of the skin. The disease ranges from unsuspected asymptomatic infection to overwhelming and fatal septicaemia. Prostatic, liver and spleen abscesses are common presentations in infected adults whilst acute suppurative parotiditis is observed in almost 40% of pediatric cases in Thailand (7, 24, 25).

*B. pseudomallei* is intrinsically resistant to many common antibiotics including β-lactams, penicillins, narrow-spectrum and expanded-spectrum cephalosporins, most aminoglycosides, macrolides, rifampin and polymyxins (5). Ceftazidime is the drug of choice for the treatment of severe melioidosis, and co-amoxiclav or a combination of co-trimoxazole and doxycycline is used for maintenance therapy, which is typically over 20 weeks. *B. pseudomallei* is also classified as a potential agent for bioterrorism for which co-trimoxazole is recommended for post-exposure prophylaxis in the event of a biological attack as there is no melioidosis vaccine available for humans (2). It has become apparent that efflux-related multidrug resistance (MDR) is a significant complicating factor in the chemotherapy of bacterial infections. The *B. pseudomallei* genome encodes several three-component efflux pumps comprising an integral cytoplasmic membrane drug-proton antiporter of the resistance-nodulation-division (RND) family and a channel-forming outer membrane protein linked together by a periplasmic protein. The majority of these pumps remain uncharacterized, but the BpeAB-OprB and AmrAB-OprA efflux pumps are known to
be responsible for its resistance to aminoglycosides and macrolides through proton gradient-dependent efflux of the drugs (4, 17).

Inhibitors of MDR efflux pumps would be expected to restore the activities of antimicrobial agents that are substrates of these pumps. In *B. pseudomallei*, inhibition of BpeAB-OprB would also provide an additional benefit of attenuating virulence because of the involvement of the pump in the efflux of quorum sensing autoinducers (3). The search for candidate efflux pump inhibitors can be costly and time consuming, so it makes reasonable sense to examine selected drugs that are already in clinical use as potential inhibitors. Phenothiazines are dopamine receptor antagonists, which are used clinically as antihistaminic agents or neuroleptics for the management of psychosis and have been shown to have modest but antimicrobial activities against a wide array of microorganisms (13). When combined at one-fourth their MICs with common antimicrobial substrates of MDR pumps of *Staphylococcus aureus*, phenothiazines augmented the antimicrobial activities of these substrates (11). Phenothiazines also potentiated the activity of some anti-tubercular drugs against multidrug-resistant *M. tuberculosis* strains (26). In this regard, phenothiazines could be used as adjuncts to antibiotics where resistance is noted. Phenothiazines are hypothesized to inhibit the proton-motive force-dependent pumps, possibly via their direct interaction with the pump and, to a lesser extent, a reduction in the transmembrane potential (11).

In this study, we addressed the effect of phenothiazines, such as prochlorperazine (PCPZ), chlorpromazine (CPZ) and promazine (PMZ), on the augmentation of antimicrobial activities of several antibiotics, especially the aminoglycosides and macrolides, which are the substrates of the *B. pseudomallei* BpeAB-OprB and AmrAB-OprB efflux pumps. We showed that phenothiazines produced a similar synergistic interaction with the antibiotics as omeprazole (OPZ), a proton pump inhibitor, thus suggesting that phenothiazines might
disrupt the proton gradient required by the \textit{B. pseudomallei} RND efflux pumps. This was verified by an impaired efflux of erythromycin by \textit{B. pseudomallei} in the presence of the phenothiazines, PCPZ and CPZ. Additionally, we showed that the inclusion of a phenothiazine as adjunct treatment, together with a sub-inhibitory concentration of erythromycin, protected human lung epithelial cells and macrophage cells from \textit{B. pseudomallei} infection and cytotoxicity.

\textbf{METHODS}

\textbf{Bacterial strains and culture conditions}

\textit{Burkholderia pseudomallei} KHW is a virulent clinical isolate which we have described and used previously in our studies (6). For routine maintenance cultures and erythromycin accumulation assay, \textit{B. pseudomallei} KHW was cultured on Luria-Bertani (LB) agar or LB broth (Becton Dickinson, Cockeysville, Md.). For determination of antimicrobial susceptibility and checkerboard titration assay, \textit{B. pseudomallei} KHW was cultured in Muller-Hinton broth (Becton Dickinson).

\textbf{Determination of antimicrobial susceptibilities}

Minimal inhibitory concentration (MIC) was assayed using 96-well microtiter plates by the standard broth microdilution method as described previously (4, 18). All antibiotics used were purchased from Sigma (St Louis, Mo.), except amoxicillin-clavulanic acid (AMC), levofloxacin (LVX) and azithromycin (AZM), which were purchased from Beecham Pharmaceuticals (Brentford, England), Aventis Pharma Deutschland GmbH (Frankfurt, Germany) and Pfizer S.A. (New York, USA), respectively. The checkerboard titration assay used for assessing interaction between either prochlorperazine (PCPZ), chlorpromazine (CPZ), promazine (PMZ) or omeprazole (OPZ) and an antibiotic was performed in 96-well
microtiter plates according to the protocol described by Lomovskaya et al (2001). Antibiotics tested included streptomycin (STR), gentamicin (GEN), erythromycin (ERY), oleandomycin (OLE), amoxicillin (AMX), ampicillin (AMP), spectinomycin (SPT), amoxicillin-clavulanic acid (AMC), levofloxacin (LVX) and azithromycin (AZM). MICs of the antimicrobial agents were determined either alone or together with phenothiazines or omeprazole at concentrations ranging from 0.98 to 1000 µM. Prochlorperazine dimaleate salt, chlorpromazine hydrochloride, promazine hydrochloride and omeprazole were purchased from Sigma.

\[ ^{14}\text{C}} \text{-erythromycin accumulation assay} \]

Efflux of erythromycin was monitored by measuring the amount of \[^{14}\text{C}}\text{-erythromycin} in intact \textit{B. pseudomallei} KHW cells according to the procedure described previously (4). Briefly, 5 ml of LB medium was inoculated (1:50) with an overnight culture of \textit{B. pseudomallei} KHW and incubated for 2 h at 37°C, shaken, until OD\text{600} ∼0.5. PCPZ, CPZ, or carbonyl cyanide \textit{m}-chlorophenylhydrazone (CCCP, Sigma) was added to a final concentration of 250 µM, 500 µM and 20 µM, respectively. After 10 min, \[^{14}\text{C}}\text{-erythromycin} was added to a final concentration of 0.1 µg/ml. One ml aliquots were removed at the beginning of the assay and 4 h later and the amount of \[^{14}\text{C}}\text{-erythromycin} in the cells measured using an LS6500 multipurpose liquid scintillation counter (Beckman Instruments Inc., Fullerton, Ca.).

\textbf{Cell invasion and cytotoxicity assays} 

Invasion of A549 human lung epithelial cells and THP-1 human macrophage cells with wild-type \textit{B. pseudomallei} KHW were performed as described previously, with the following modifications (4). The bacteria were added to the mammalian cells in 1.5 ml of
culture medium containing one of the following and incubated for 4 h: PCPZ (250 µM), CPZ (500 µM), PCPZ (250 µM) and erythromycin (8 µg/ml), CPZ (500 µM) and erythromycin (8 µg/ml), erythromycin (8 µg/ml) only, or erythromycin (128 µg/ml) only. No erythromycin or phenothiazine was added to the positive control. Human lung epithelial cells (A549) and macrophage cells (THP-1) were cultured using DMEM and RPMI 1640 media, respectively, which were supplemented with 10% (v/v) fetal bovine serum (Sigma). The assay was performed in triplicate.

Cytotoxicity of *B. pseudomallei* on A549 and THP-1 cells was determined by measuring the release of lactate dehydrogenase enzyme using a Cytotoxicity Detection kit (Roche, Mannheim, Germany) as described previously (3). Briefly, mid-log phase *B. pseudomallei* KHW cells were added at a MOI of 100 to the wells of a 24-well microtitre plate containing A549 and THP-1 cells (10^5 cells/well), respectively in culture medium comprising of either PCPZ (250 µM), CPZ (500 µM), PCPZ (250 µM) and erythromycin (8 µg/ml), CPZ (500 µM) and erythromycin (8 µg/ml), erythromycin (8 µg/ml) alone, or erythromycin (128 µg/ml). The cells were incubated for 4 h. Only PCPZ (250 µM) or CPZ (500 µM), but no *B. pseudomallei*, was added to the cells in the negative controls. The culture plate was then centrifuged and 100 µl of the culture supernatant from each well was used for the LDH assay. The assay was performed in triplicate.

RESULTS

Effect of phenothiazines on antimicrobial properties of antibiotics

For the checkerboard titration assay, none of the phenothiazines alone exhibited antimicrobial effect on *B. pseudomallei*, even at concentrations up to 1000 µM (data not shown). PCPZ, CPZ and PMZ, potentiated the antimicrobial properties of a wide spectrum of antibiotics against *B. pseudomallei*, ranging from aminoglycosides, macrolides, β-lactams
and a fluoroquinolone. Each of the phenothiazines interacted synergistically with streptomycin, spectinomycin, erythromycin, oleandomycin, azithromycin, amoxicillin-clavulanic acid and levofloxacin, but the most pronounced synergistic interaction was with the aminoglycoside and macrolide antibiotics (Tables 1). PCPZ (500 µM) produced the greatest synergistic interaction with spectinomycin, resulting in more than 1000-fold reduction in its MIC for *B. pseudomallei*, but it had very little synergistic interaction with spectinomycin when added at concentrations lower than 500 µM (Table 1). In comparison, CPZ and PMZ were both more potent than PCPZ in their synergistic interactions with the aminoglycoside and macrolide antibiotics. For example, CPZ and PMZ reduced the MICs of streptomycin for *B. pseudomallei* KHW by >4000-fold and >2000-fold, respectively, as compared to a reduction of only 4-fold by PCPZ. Similarly, CPZ and PMZ reduced the MICs of oleandomycin by >8000-fold and 4000-fold, respectively, compared to >30-fold reduction by PCPZ. CPZ and PMZ were also more effective in the reduction of the MIC of erythromycin to >500-fold compared to only 16-fold for PCPZ (Tables 1). There was also a significant difference in the synergistic interaction between each of the phenothiazines and erythromycin. Both CPZ and PMZ reduced the MICs of erythromycin by greater than 500-fold but a much smaller reduction in MIC of 16-fold was achieved with PCPZ (Tables 1). The checkerboard titration assays showed that significant synergistic interaction between a phenothiazine and an antibiotic was achievable mostly with phenothiazine concentrations above 250 µM and there was a distinct concentration threshold below which such synergistic interaction was not evident.

There was no synergistic interaction between phenothiazines and the β-lactam antibiotics, amoxicillin and ampicillin, probably because these antibiotics are not actively effluxed by the *B. pseudomallei* RND pumps (data not shown). However, each of the phenothiazines tested increased the susceptibility of *B. pseudomallei* to amoxicillin-
clavulanic acid by at least 32-fold, with PCPZ (250 µM) being the most effective by reducing
the MIC of amoxicillin-clavulanic acid >64-fold from 16 µg/ml to less than 0.25 µg/ml
(Table 1). There was also no synergistic interaction between the phenothiazines and
gentamicin (data not shown). This was unexpected as the aminoglycoside is also a substrate
of both the BpeAB-OprB and AmrAB-OprB RND efflux pumps.

Omeprazole provided similar synergistic interaction with antibiotics as phenothiazines
The proton pump inhibitor, omeprazole (OPZ), provided similar reductions on the
MIC values of the antibiotics as PCPZ, CPZ and PMZ. Addition of 1000 µM OPZ reduced
the MIC of streptomycin, oleandomycin and erythromycin by >2000-fold, >8000-fold and
>500-fold, respectively (Table 1). This suggests that the phenothiazines probably interfered
with the membrane proton-gradient that is required for active efflux of the susceptible
antibiotics. In contrast to the phenothiazines, OPZ produced a slightly different effect in that
it interacted only minimally with spectinomycin and azithromycin and showed no interaction
at all with amoxicillin-clavulanic acid (Table 1).

Prochlorperazine and chlorpromazine inhibits efflux of [14C]-erythromycin
Erythromycin is a substrate of the RND efflux pumps BpeAB-OprB and AmrAB-
OprA and hence only low levels of [14C]-erythromycin were detected in the B. pseudomallei
KHW cells when these were exposed to a sub-inhibitory concentration (0.1 µg/ml) of
erthromycin (Fig. 1)(4, 17). Almost 4-fold more [14C]-erythromycin accumulated in the B. pseudomallei
KHW cells in the presence of either 250 µM PCPZ or 500 µM CPZ as a result
of reduced erythromycin efflux (Fig. 1). PCPZ and CPZ yielded the same effects on the
intracellular accumulation of erythromycin as that obtained when B. pseudomallei was
exposed to a sub-inhibitory concentration (20 µM) of carbonyl cyanide m-
chlorophenylhydrazone (CCCP), a proton-gradient uncoupler, thus suggesting that the
phenothiazines might have either disrupted membrane proton gradient or directly interacted
with the pump to inhibit the efflux of erythromycin, or both.

Synergistic activities of phenothiazines and erythromycin protects A549 and THP-1
cells from *B. pseudomallei*

We have showed previously that any impairment to the BpeAB-OprB efflux pump
function would result in an attenuation of cell invasion and cytotoxicity by *B. pseudomallei*
KHW (3). The similar properties of the phenothiazines and CCCP on antibiotic accumulation
in *B. pseudomallei* suggests that synergistic interaction between phenothiazines and
erythromycin could also protect human lung epithelial and macrophage cells from invasion
and cytotoxic effects of *B. pseudomallei*. Invasion of both human lung epithelial cells (A549)
and human macrophage cells (THP-1) by *B. pseudomallei* KHW were significantly
attenuated by adding a sub-inhibitory concentration of erythromycin (0.06 x MIC or 8
µg/ml) to the culture medium together with either PCPZ (250 µM) or CPZ (500 µM) (Fig.
2A, B). Without phenothiazines, invasion of A549 and THP-1 cells by *B. pseudomallei*
KHW was attenuated only by adding erythromycin at its MIC (128 µg/ml), but not at the sub-
inhibitory concentration of 8 µg/ml. Similarly, 250 µM PCPZ and 500 µM CPZ alone
afforded no protection against cell invasion by *B. pseudomallei* KHW (Figs. 2A, B).

The cytotoxicity of *B. pseudomallei* KHW on A549 and THP-1 cells was attenuated
by about 4-fold and 3-fold, respectively, in the presence of erythromycin at its MIC value of
128 µg/ml, but not in the presence of 250 µM PCPZ or 500 µM CPZ alone. However, in the
presence of either 250 µM PCPZ or 500 µM CPZ, the sub-inhibitory concentration of
erthyromycin (0.06 MIC or 8 µg/ml) was now equally effective in attenuating the
cytotoxicity of *B. pseudomallei* KHW towards A549 and THP-1 cells, thus affirming the synergistic interaction between the phenothiazines and erythromycin (Figs. 3A, B).

**DISCUSSION**

The intrinsic resistance of *B. pseudomallei* to many antibiotics, especially aminoglycosides and macrolides, is largely attributed to the activities of the RND efflux pumps, BpeAB-OprB and AmrAB-OprA (4, 17). The efflux is an active process which is dependent on the membrane proton gradient (19). Efflux pumps can be considered as potentially effective antibacterial targets and the resultant efflux pump inhibitor (EPI)-antibiotic combination drug should exhibit increased potency, enhanced spectrum of antimicrobial activity and reduced propensity for acquired resistance. EPIs that have been identified function either as competitive or non-competitive substrate inhibitors, or prevent ATP-binding or disturb the proton gradient. These include the synthetic dipeptide amide, L-Phe-L-Arg-β-napthylamide (MC-207,110) which has been shown to significantly decrease the level of intrinsic resistance of *Pseudomonas aeruginosa* to fluoroquinolones, reversed the acquired resistance due to the overexpression of efflux pumps, and reduced the emergence of *P. aeruginosa* strains that are highly resistant to fluoroquinolones. Such EPIs can cause increased accumulation of substrates without disrupting the proton gradient (16).

Phenothiazines belong to a class of non-antibiotic drugs that could inhibit efflux pumps that confer resistance to fluoroquinolones in *S. aureus* (11, 12). We showed that the phenothiazines, PCPZ, CPZ and PMZ, did not exhibit any antimicrobial activities on *B. pseudomallei* KHW, at concentrations up to 1 mM. However, when used together with antibiotics, these phenothiazines interacted synergistically with streptomycin, erythromycin, oleandomycin, spectinomycin, levofloxacin, azithromycin and amoxicillin-clavulanic acid, albeit to varying degrees, to enhance their antimicrobial potency against *B. pseudomallei*. 
The synergistic interaction between phenothiazines and antibiotics was most pronounced for the aminoglycosides (streptomycin and spectinomycin) and macrolides (erythromycin, oleandomycin and azithromycin), which are also substrates of two *B. pseudomallei* RND efflux pumps, BpeAB-OprB and AmrAB-OprA (4, 17). Inhibitory effects of phenothiazines on multidrug efflux pumps have previously been reported, including the augmentation of potency of common efflux pump substrates against *S. aureus* strains possessing different MDR efflux-related resistance mechanisms, and the inhibition of NorA pump function and non-NorA-related efflux phenotypes in a concentration-dependent manner. The mechanism of inhibition of efflux pumps by phenothiazines and thioxanthenes appears more likely to involve direct interaction of these compounds to the pump, and disruption of the proton gradient is involved to somewhat lesser extent (11). The lack of significant cytotoxicity effects despite the relatively high concentrations of drugs used in our experiments is also an argument against their role as proton gradient inhibitors. It is also generally acknowledged that proton motive force inhibitors would not make good EPIs because they would exhibit significant cytotoxicity effects. Phenothiazines have also been shown to inhibit the function of eukaryotic MDR efflux pumps (10). CPZ and PMZ were the most potent of the phenothiazines tested, achieving at least 500-fold more augmentation of the antimicrobial activity of streptomycin against *B. pseudomallei* than a similar concentration of PCPZ. Similar variations in efficacy were observed for the interaction between the different phenothiazines and oleandomycin. We attribute these variations in potency of the phenothiazines to their different charges at neutral pH. CPZ, PMZ and PCPZ each contain a cationic –N-CH3 group, which could potentially interact with the membrane to alter its permeability (22). The pKa values of CPZ, PMZ and PCPZ are 9.3, 9.4 and 8.1, respectively, suggesting that CPZ and PMZ would be more positively charged than PCPZ at pH 7 and
thus, were expected to be more effective in altering the outer membrane permeability to
dissipate the proton gradient (8).

One of the targets which give rise to the synergistic interaction between the
phenothiazines and the susceptible antibiotics seems to be the proton gradient. CPZ has been
shown to affect ion flux across the membrane in *S. aureus* and *Sacchromyces cerevisiae*, and
alter the transmembrane potential in *Leishmania donovani* (9, 13, 27). Thus, we expect that
*B. pseudomallei* RND efflux pumps, which depend on the membrane proton gradient to
energize substrate translocation of antibiotics, to be affected by phenothiazines. Such pumps
would include the *B. pseudomallei* BpeAB-OprB and AmrAB-OprB pumps which actively
efflux aminoglycosides and macrolides (4, 17). Indeed, our data showed that the addition of
phenothiazines not only rendered *B. pseudomallei* more susceptible to the aminoglycosides
and macrolides but also to levofloxacin and the β-lactam, amoxicillin-clavulanic acid. It is
interesting to note that phenothiazines had no effect on amoxicillin alone but a synergistic
effect was observed between phenothiazines and amoxicillin-clavulanic acid, suggesting that
clavulanic acid could be a substrate of a yet uncharacterized RND efflux pump in *B.
pseudomallei*. In *P. aeruginosa*, β-lactamase inhibitors are substrates of the MexAB-OprM
and MexEF-OprN efflux pumps (15). Phenothiazines have also rendered methicillin-resistant
*S. aureus* (MRSA) more susceptible to oxacillin, probably also due to their effect on a similar
efflux pump (14). The ability to reproduce similar synergistic interaction between the same
antibiotics and omeprazole, a proton pump inhibitor, also supports the notion that
phenothiazines might affect the functions of the *B. pseudomallei* RND efflux pumps through
changes in the proton gradient. Omeprazole also inhibited the activity of the *S. aureus* NorA
pump, but as in *B. pseudomallei*, the concentration of omeprazole required was above what is
clinically attainable (1). In this respect, however, we are unable to explain why gentamicin,
which is also a substrate of BpeAB-OprB multidrug efflux pump in *B. pseudomallei*, was
unaffected by the phenothiazines (4). Gentamicin, like streptomycin, spectinomycin and azithromycin, are all cationic and could disrupt an otherwise intact and impermeable LPS layer by cationic binding, and streptomycin, with two extra amine groups is more cationic and hence is more disruptive on the LPS than gentamicin (23). However, if the mechanism of inhibition of efflux pumps by phenothiazines could be attributed principally to their direct interaction with the pump, then a plausible explanation would be that the phenothiazines interfered with the binding of streptomycin and spectinomycin to the efflux pumps, but not gentamicin. Moreover, as the relatively large concentrations of phenothiazines used in this study did not exhibit any significant cytotoxicity effects, it is plausible that these drugs do not function primarily as proton gradient inhibitors (Fig. 3). We have ascertained that there was no change in the MIC of gentamicin in the presence or absence of omeprazole (data not shown). Thus, we believe that the mechanism of synergistic interaction between phenothiazines and antibiotics is likely to involve direct interference with the antibiotic-pump interaction, as well as by a limited disruption of the proton motive force.

We have previously shown that the disruption of BpeAB-OprB function could attenuate *B. pseudomallei* virulence in cell invasion and cytotoxicity assays (3). In this study, we showed that the use of a sub-inhibitory concentration (0.06 MIC) of erythromycin, together with a phenothiazine as adjunct treatment, not only inhibited *B. pseudomallei* growth but also protected the mammalian cells from infection *B. pseudomallei* and its cytotoxicity. Erythromycin is an effective therapy against infection by intracellular pathogens as it is able to enter eukaryotic cells and was shown to be markedly concentrated within polymorphonuclear leukocytes (21). CPZ is also concentrated by human macrophages where it exerted bacteriocidal properties against *S. aureus* phagocytosed by human monocyte-derived macrophages (20). The successful attenuation of cell invasion and killing by *B. pseudomallei* using a combination of phenothiazine and a sub-inhibitory concentration of
erythromycin might be the result of an accumulation or concentration of erythromycin and
the phenothiazine within the mammalian cells, thus enhancing their bacteriostatic and
bacteriocidal properties. Alternatively, the combination of phenothiazine and erythromycin
could also exert an inhibitory effect on the efflux pump, and consequentially reduced its
virulence by inhibiting the efflux of a key metabolite(s) that is required for expression of
virulence in B. pseudomallei.

The data supports the potential role of phenothiazines as efflux pump inhibitors (EPIs) in B.
pseudomallei. Although we acknowledge that the concentrations (250 µM to 1 mM) at which
phenothiazines provide such beneficial effects on the antimicrobial agents are not clinically
achievable, it nevertheless provide a basis for future development of compounds that can
either inhibit the multidrug efflux pumps or dissipate the proton motive force required for
active efflux of antimicrobial agents. It is also possible that maximal bacterial pump
inhibition may not be required to achieve a beneficial effect in vivo, but this would require
verification using animal studies. It is envisaged that chemical modification of the
phenothiazines could result in compounds with less central nervous toxicity but improved
inhibitory activity towards the bacterial efflux pumps. The wide spectrum of antibiotics
which interact synergistically with the phenothiazines further lends support to the
development of these drugs into EPIs as they could augment the antimicrobial activities of
nearly all clinically-used antibiotics. Although B. pseudomallei KHW is a virulent clinical
isolate that has been well-characterized in our laboratory, we are cognizant that the extent of
the synergistic interaction between phenothiazines and antibiotics could vary from strain to
strain and recommend further studies to include more B. pseudomallei isolates.
ACKNOWLEDGEMENTS

This work was supported by the National Medical Research Council of Singapore (Grant NMRC 1012/2005).

REFERENCES


TABLE LEGENDS

Table 1.

Synergistic *in vitro* activity of prochlorperazine (PCPZ), chlorpromazine (CPZ), promazine (PMZ) and omeprazole (OPZ) combined with antibiotics against *B. pseudomallei* KHW
FIGURE LEGENDS

Figure 1
Effect of PCPZ, CPZ and CCCP on the intracellular accumulation of [14C]-erythromycin in B. pseudomallei KHW. The amount of [14C]-erythromycin retained in intact B. pseudomallei KHW cells 4 h after exposure to exogenous [14C]-erythromycin was determined as described in Materials and Methods. B. pseudomallei KHW cells were treated with either 500 µM chlorpromazine (+ CPZ), 250 µM prochlorperazine (+ PCPZ) or 20 µM carbonyl cyanide m-chlorophenylhydrazone (+ CCCP) for 4 h. The control experiment contained untreated B. pseudomallei KHW cells. The assay was performed in triplicate.

Figure 2
Synergistic and protective effects of phenothiazines and erythromycin against invasion of human lung epithelial cells and macrophages by B. pseudomallei KHW. Invasion of A549 human lung epithelial cells (A) and THP-1 human macrophage cells (B) by B. pseudomallei KHW were performed as described under Materials and Methods. PCPZ500 and CPZ250 refer to the final concentrations of 500 µM PCPZ and 250 µM CPZ, respectively, whilst the ERY8 and ERY128 refer to the concentrations of erythromycin at 0.06X MIC (8 µg/ml) or MIC (128 µg/ml), where appropriate. (+) and (-) indicate presence or absence of the compounds and B. pseudomallei KHW culture.

Figure 3
Synergistic and protective effects of phenothiazines and erythromycin against cytotoxicity of B. pseudomallei KHW. Cytotoxicity of B. pseudomallei KHW on A549 (A) and THP-1 cells (B) were determined by the release of lactate dehydrogenase from the cells as described in Materials and Methods. PCPZ500, CPZ250, ERY8 and ERY128 refer to their final
concentrations of 500 µM, 250 µM, 8 µg/ml and 128 µg/ml, respectively, while KHW refer to *B. pseudomallei* KHW. (+) and (-) indicate presence or absence of the compounds or bacteria.
TABLE 1.  Synergistic *in vitro* activity of phenothiazines and omeprazole with antibiotics against *B. pseudomallei* KHW

<table>
<thead>
<tr>
<th>Antibiotics</th>
<th>MIC of antibiotics (µg/ml) in the presence of phenothiazines and omeprazole (µM)</th>
<th>Max. fold decrease</th>
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<sup>a</sup> denotes the lowest concentration of antibiotic used in this assay.

Values in the shaded boxes denote synergistic interaction between each respective phenothiazine, or omeprazole, and antibiotic.
Intracellular levels of $[^{14}C]$-erythromycin (ng/OD$_{600}$)
Figure 2

A

No. of intracellular bacteria

KHW  +  +  +  +  +  +  +  +
PCPZ_{500}  -  +  -  +  -  -  -  -
CPZ_{250}  -  -  +  -  +  -  -  -
ERY_{8}  -  -  -  +  +  +  -  -
ERY_{128}  -  -  -  -  -  -  -  +
Figure 2

B

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<th>CPZ&lt;sub&gt;250&lt;/sub&gt;</th>
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Figure 3
Figure 3

B

% Cytotoxicity

KHW  +  +  +  +  +  +  +  -  -
PCPZ$_{500}$  -  +  -  -  +  -  -  +  -
CPZ$_{250}$  -  -  +  -  -  +  -  -  +
ERY$_8$  -  -  -  +  +  +  -  -  -
ERY$_{128}$  -  -  -  -  -  -  +  -  -