Do phenothiazines possess antimicrobial and efflux inhibitory properties?

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Do Phenothiazines Possess Antimicrobial and Efflux Inhibitory Properties?

Summary sentence: The use of phenothiazines as antibiotic adjuvants could be invaluable in the fight against antimicrobial resistance.

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ABSTRACT

Antibiotic resistance is a global health concern; the rise of drug-resistant bacterial infections is compromising the medical advances that resulted from the introduction of antibiotics at the beginning of the 20th century. Considering that the presence of mutations within individuals in a bacterial population may allow a subsection to survive and propagate in response to selective pressure, as long as antibiotics are used in the treatment of bacterial infections, development of resistance is an inevitable evolutionary outcome. This, combined with the lack of novel antibiotics being released to the clinical market, means the need to develop alternative strategies to treat these resistant infections is critical. We discuss how the use of antibiotic adjuvants can minimise the appearance and impact of resistance. To this effect, several phenothiazine-derived drugs have been shown to potentiate the activities of antibiotics used to treat infections caused by Gram-positive and Gram-negative bacteria. Outside of their role as anti-psychotic medications, we review the evidence to suggest that phenothiazines possess inherent antibacterial and efflux inhibitory properties enabling them to potentially combat drug resistance. We also discuss that understanding their mode of action is essential to facilitate the design of new phenothiazine derivatives or novel agents for use as antibiotic adjuvants.

INTRODUCTION

The emergence of antimicrobial resistance (AMR) is seriously compromising the medical advances made possible by the advent of antibiotics in the late 1920s. A review commissioned by the UK government highlighted the burden that AMR places on public health, in terms of morbidity and mortality and estimated that AMR would cause 10 million annual deaths by 2050 (O' Neil 2016). Whilst there is contention regarding the reliability of this estimate (de Kraker *et al.* 2016, Tillotson 2017), this report allowed many outside of the field of AMR to acknowledge antibiotic resistance as a global public health concern.

Previously, antibiotic discovery, research and development (R&D) efforts were able to contain the threat of drug-resistant infections. As of March 2019, there are 42 drugs in the R&D pipeline; of these, only 16 are active against resistant Gram-negative "ESKAPE" pathogens (*Klebsiella pneumoniae, Acinetobacter baumanii, Pseudomonas aeruginosa* and *enterobacter* species) and only 11 possess a novel chemical structure (Tacconelli *et al.* 2017, Trusts 2017). Given the low success rate of clinical drug development, it is likely that only two to three of these agents will be approved for clinical use in the next decade (Ardal *et al.* 2017). Part of the reason behind this reduction in antibiotic R&D is that drug development is extremely time consuming, expensive and the financial rewards for antibiotics are much lower than for drugs that treat chronic illnesses. Therefore, it is important that alternative strategies are developed to prolong the clinical efficacy of currently available antibiotics; one such alternative approach is the use of drug combinations. Combination treatment is invaluable for the treatment of many illnesses including empiric treatment of patients with sepsis (Micek *et al.* 2010), bloodstream infections (Salzberger and Fatkenheuer 2017) as well as those with human immunodeficiency virus (Maenza and Flexner 1998), cancer (Mokhtari *et al.* 2017) and bacterial infections such as *Mycobacterium tuberculosis* (Kerantzas and William R. Jacobs 2017).

USE OF COMBINATION THERAPIES

In terms of multidrug resistance (MDR), both antibiotic-antibiotic or antibiotic-adjuvant combinations are useful for the treatment of drug-resistant infections. An adjuvant is typically a compound that is not antimicrobial when administered alone, but when used in combination potentiates antibiotic activity. Given antibiotic-adjuvant combinations are typically used to target drug-resistance mechanisms (for example, β-lactamase inhibitors), this approach is advantageous as it restores the activity of existing antibiotics. Recently, efforts to produce adjuvants have included synthesising different classes of small molecule inhibitors targeting efflux pumps, β-lactamases or the outer membrane (Lomovskaya *et al.* 2001, Powers *et al.* 2002), or modifying previously known natural chemical products (Choudhury *et al.* 2016). However, drug-drug interactions and the difficulty optimising appropriate dosing regimens accompany the use of drug combinations (Worthington and Melander 2013). An arguably better strategy is to repurpose existing clinically-approved compounds. Considering the pharmacokinetics and toxicology of these compounds are already established, the use of clinically approved drugs would be invaluable in terms of bypassing the costs and time that are associated with drug R&D (Schneider *et al.* 2017).

The utility of this strategy was indicated when systematic screening processes involving previously-approved compounds in combination with clinically used antibiotics revealed that many of these drugs potentiated the activity of a given antibiotic. For example, a study involving the combination of 1,057 FDA approved drugs with the antibiotic minocycline, revealed that 96 compounds including anti-inflammatory, antihistamine, antispasmodic, psychotropic and antihypertensive drugs exhibited synergy with minocycline against *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa* (Ejim et al. 2011). Of these, several phenothiazines were identified to not only synergise with antibiotics, but also possess their own intrinsic antibacterial activity (Melander and Melander 2017).

PHENOTHIAZINES

History of Phenothiazines

The history of the phenothiazines began in 1876 upon the synthesis of methylene blue by Henrich Caro (Varga *et al.* 2017). While methylene blue itself is a therapeutically relevant compound, used as an antimalarial drug, its derivatives have amassed significant therapeutic importance due to their wide functional diversity (Taurand *et al.* 2012). The first of these derivatives, phenothiazine, was synthesised in 1883 by Heinrich August Berntthsen (Bernthsen and Laboratorium von A. Bernthsen 1883); its insecticidal (Smith 1937), antihelminthic (Swales 1939, Gordon and Sydney 1945) and antibacterial properties (Deeds *et al.* 1939) were noted in the 1930s and 1940s. However, these were overshadowed when the sedative properties of the phenothiazine derivatives promethazine and chlorpromazine were observed in 1949 by the French army surgeon Henri-Marie Labroit. He noted that previously anxious patients who received a promethazine and chlorpromazine containing 'lytic cocktail' became subdued and indifferent to their surroundings. However, Labroit had difficulty convincing the medical community that phenothiazines would be useful in the field of psychiatry (Ramachandraiah *et al.* 2009, Kunz 2014). Although various researchers tried to prove the effectiveness of phenothiazines, it was not until Elkes and Elkes undertook a successful, randomised and placebo controlled, clinical trial at The University of Birmingham (UK) in 1954 that phenothiazines were universally accepted as a viable clinical option for the treatment of psychological conditions (Elkes and Elkes 1954).

Chemical Structure and use of Phenothiazines in the Treatment of Psychological Disorders

Phenothiazines all have the same three-ring structure containing one sulphur and a nitrogen atom at positions C-9 and C-10 of the tricyclic ring, respectively (Figure 1). The length of the linking alkyl connector, the terminal amine, as well as substituents at the C-2

position, determines the activity of the derivative (Jaszczyszyn *et al.* 2012). Phenothiazines are subdivided into three groups (piperazines, piperadines or aliphatic) dependent on the substituent at the nitrogen atom (Figure 2) (Archer and Hicks 1963).

Considered as classical antipsychotics, phenothiazines are dopamine antagonists. Dopamine over-activity is believed to be a causative factor of several psychological conditions, such as schizophrenia and mania (Seeman and Kapur 2000). Dopamine mediates a variety of biochemical processes in the central and peripheral nervous systems via interactions with dopamine receptors. The primary action of phenothiazines relies on their ability to block post-synaptic D2 dopamine receptors, preventing dopamine binding and further signal transduction (Creese *et al.* 1976). In schizophrenic patients, phenothiazine-mediated inhibition of dopamine results in the reduction of symptoms including: psychosis, hallucinations and delusions. However, phenothiazines will act non-specifically on dopamine receptors affecting other cognitive pathways besides the mesolimbic pathway. In addition to their dopaminergic effects, phenothiazines also have antagonistic effects on histamine, serotonin, glutamine, adrenergic and acetylcholine receptors (Varga *et al.* 2017). As a consequence, this causes significant extrapyramidal side effects.

ANTIBACTERIAL ACTIVITY OF PHENOTHIAZINES

Many of the phenothiazines have a broad range of antibacterial activities (both bacteriostatic and bactericidal) against *Mycobacteria*, some Gram-positive and Gram-negative bacteria (Table 1 and Supplementary Table 1). Both thioridazine and promazine were found to have anti-commensal bacterial activity at 20 μM against 10 of 40 representative species of human gut bacteria (Maier *et al.* 2018).

Table 1: Summary of the antimicrobial activity of chlorpromazine.

Supplementary Table 1: Summary of the phenothiazines that have been shown to possess some antimicrobial activity. The group to which this phenothiazine belongs and its clinical use is described.

The *in vitro* activity of phenothiazines was confirmed in an animal model. Of 60 Swiss albino mice challenged with the median lethal dose of *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium), an 82% mortality rate was observed for an untreated control group. However, upon treatment with trifluoperazine at 15 and 30 µg/20 g mouse (a sub MIC) this mortality rate was reduced to 16% and 13%, respectively. This reduction was accompanied by a decrease in the bacterial load present in the blood, liver and spleen of treated mice (Mazumder *et al.* 2001). Fluphenazine and various other phenothiazine derivatives showed similar protective effects in *S.* Typhimurium and *E. coli* infection (Dastidar *et al.* 1995, Komatsu *et al.* 1997). Considering their large number of mammalian targets, it is unsurprising that the clinical use of phenothiazines as antibiotic adjuvants is limited by their cytotoxicity; chlorpromazine, fluphenazine, thioridazine, trifluoperazine and triflupromazine are toxic to hepatoma tissue culture cells, with EC_{50} values of 45 to 125 µM (de Faria *et al.* 2015). Of the phenothiazines those of the piperidinic class appear to be the most toxic. This cytotoxicity means the average achievable human serum levels (30-100 ng/ml, 0.15-2.5 ng/ml and 0.5-3.0 ng/ml for chlorpromazine, thioridazine and trifluoperazine, respectively) are ~1,000 fold lower than the concentration at which anti-bacterial activity occurs. However, there is a large inter-individual variation in the pharmacokinetics. An issue with the pharmacological data for phenothiazines is that most is derived from steady state dosing as opposed to a C_{max} for a single dose; which may be more appropriate for the use of these drugs as an antibiotic adjuvant. In all cases, the metabolites are often more psychoactive and persistent but little is known about their antibacterial effects.

Interestingly, thioridazine and chlorpromazine are concentrated upon ingestion by macrophages. The MIC of chlorpromazine (>30 mg/l) and thioridazine (18 mg/l) against *S. aureus* occurs at clinically unachievable concentrations. However, when monolayer cultures of human peripheral blood monocyte derived macrophages were pre-treated with chlorpromazine or thioridazine, prior to infection with *S. aureus,* a concentration of 0.1 mg/l of compound completely inhibited the growth of the phagocytosed bacteria. This reduced the MIC of both compounds in this environment to concentrations achievable after routine dosing for the treatment of psychotic disorders (Ordway *et al.* 2002, Ordway *et al.* 2002).

Ability of Phenothiazines to Affect Bacterial Cellular Replication and Morphology

The phenothiazines fluphenazine, thioridazine, perphenazine and chlorpromazine have been shown to bind to DNA either by intercalation with, or stacking on, the DNA helix (Ben-Hur *et al.* 1980, de Mol *et al.* 1983, de Mol and Busker 1984, Viola *et al.* 2003). Upon photo-ionisation there is a transfer of electrons between the DNA and the phenothiazine cations; this process is linked to single stranded DNA breaks (Viola *et al.* 2003). Upon intercalation with the DNA helix, the phenothiazine inhibits coiling and uncoiling of the helix as well as all DNA based processes (de Mol *et al.* 1983), most notably cellular replication (Sharma *et al.* 2001, Eisenberg *et al.* 2008). The degree to which phenothiazines can intercalate with DNA is dependent on the guanosine-cytosine content of the DNA helix (de Mol *et al.* 1983). Phenothiazines have also been shown to bind to RNA structural elements with varying binding affinities (Mayer and James 2004). The ability of phentohiazines to act as plasmid curing agents, at sub-inhibitory concentration, (reviewed by Buckner *et al.* 2018) has been speculated to result from the ability of these drugs to intercalate DNA and inhibit plasmid replication and supercoiling (Mandi *et al.* 1975, Molnar and Schneider 1978, Barabas and Molnar 1980, Molnar *et al.* 1980, Molnar *et al.* 1984, Molnar and Nakamura 1988, Molnar *et al.* 1992, Wolfart *et al.* 2006).

In both prokaryotic and eukaryotic cells, phenothiazines have been widely reported as calmodulin antagonists. This ability of phenothiazines to prevent the binding of calcium, to calcium-binding proteins, has been suggested to form the basis of phenothiazine activity against microbial cells (Doroshenko *et al.* 1988, Marshak *et al.* 2002, Martins *et al.* 2011). This hypothesis is based upon data from native PAGE assays that show the phenothiazines chlorpromazine and trifluoperazine prevent the SmCaM1 and SmCaM2 calmodulins from *Schistosoma mansoni* shifting from a compact to an open structure, an essential conformational change to allow interactions with target molecules (Vandonselaar *et al.* 1994, Thomas and Timson 2018). In addition, *Candida albicans* cells grown in the presence of chlorpromazine and trifluoperazine show a reduction in the activity of nuclear calmodulin. This, in addition to phenothiazine-induced DNA damage, is proposed to cause a decrease in cellular replication of *Candida albicans* via delayed entry into, and progression through, the S and G1 phases of the cell cycle (Sharma *et al.* 2001). Thus, it has been suggested that the phenothiazines initiate their pharmacological properties via interactions with the calcium messenger system, inhibiting calcium binding to calmodulin, voltage-gated calcium channels and protein kinase C (Ford *et al.* 1989).

Phenothiazines have been shown, in a species-dependent manner, to directly affect the morphology of bacterial cells at sub-MIC concentrations. For example, at concentrations lower than those which inhibit replication, chlorpromazine causes transient filamentation of *E. coli* (Amaral and Lorian 1991) and an inability of *S. aureus* cells to divide, resulting in large mesosomal-like structures (Kristiansen and Blom 1981). In addition, Amaral *et al.* (2000) noted that exposure of *S.* Typhimurium to sub-MIC concentrations of chlorpromazine resulted in changes in the appearance of the cell wall of a chlorpromazine-resistant mutant. These changes included the loss of an unspecified 55 kDa protein. In the absence of this protein, anti-O antibody was able to bind O-antigen in the presence of chlorpromazine, an

interaction that was initially blocked. The authors suggested that chlorpromazine binds to this absent protein where it can elicit its antimicrobial effects (Amaral *et al.* 2000).

Membrane Damaging Effects

The change in cellular morphology has been suggested to result from the ability of phenothiazines to damage the bacterial membrane of Gram-positive and Gram-negative bacteria (Galeazzi *et al.* 1986). At sub-MIC concentrations, phenothiazines increase outer membrane permeability and fluidity, and depolarise the plasma membrane (Kristiansen 1979, Zilberstein *et al.* 1990, Kaatz *et al.* 2003). At low concentrations this membrane damage causes changes in cell structure and affects the functionality of many inner and outer membrane-bound proteins (Labedan 1988, Rajyaguru and Muszynski 1997, Plenge-Tellechea *et al.* 2018, Wassmann *et al.* 2018). This effect of phenothiazines on the outer and inner membrane may be due to the cationic charge and amphiphilic nature of the compounds. In human erythrocytes and model cell membranes the amphiphilic properties of the phenothiazine, chlorpromazine, has been shown to allow the hydrophobic tricyclic structure to partition into the inner portion of the lipid bilayer and interact with the lipid tails, while the hydrophilic propylamine tail of chlorpromazine is able to interact with the polar headgroups (Jiang *et al.* 2017, Plenge-Tellechea *et al.* 2018). When present in the lipid bilayer, chlorpromazine can assist in lipid translocation and has been shown to cause dissolution of the lipid bilayer at high concentrations (>5 mM) (Jiang *et al.* 2017). Although limited in-depth studies have been performed in bacterial cells, and despite differences in the bacterial cell membrane of eukaryotes and prokaryotes, it has been hypothesised that phenothiazines may affect the bacterial membrane in a similar manner described for mammalian cells (Kristiansen 1979).

Effects on Energy Generation

Phenothiazines have been widely reported to affect the flux of ions across the bacterial membrane. An increase in calcium influx and potassium efflux has been noted at sub-MIC concentrations of chlorpromazine and thioridazine in a variety of bacterial and fungal species including *S. aureus* and *Saccharomyces cerevisiae* (Kristiansen 1979, Kristiansen *et al.* 1982, Eilam 1983, Eilam 1984, Zilberstein *et al.* 1990). The addition of high concentrations of each cation partially reverses this effect, with higher concentrations of phenothiazine being required to elicit the same response. The effect of phenothiazines on ion flux has been shown to be dependent on the presence of metabolic energy with the removal of glucose causing reversion of the cell to a pre-exposure phenotype (Eilam 1983). This suggests that the phenothiazine does not affect ion flux simply through increased membrane permeability. The hypothesis that phenothiazines require energy for uptake into the cell was not supported as chlorpromazine was able to cross the bacterial membrane in the absence of glucose (Eilam 1984). This effect of phenothiazines on ion flux has been suggested to occur by one, or both, of two mechanisms. The first is a result of inhibition of calcium-dependent processes, and the second is a result of disruption of cation-dependent ATPases (Eilam 1983, Zilberstein *et al.* 1990).

The changes in ion flux induced by phenothiazines results in disruption of the bacterial membrane potential and proton motive force (PMF) (Zilberstein *et al.* 1990, Kaatz *et al.* 2003). Membrane potential is a difference in the electrical charge between the inside and outside of the cell, with most bacterial cells having a resting membrane potential of -40 mV to -70 mV with respect to the outside of the cell. Any change in the flux of ions across the membrane can alter the electrical potential and result in hyperpolarisation or depolarisation of the membrane. The PMF is one of the ways by which cellular energy is created and is dependent on both the electrical potential and pH gradients. In short, redox

reactions occurring as a result of electron transfer between electron carriers in the cell membrane cause protons to be transported across the inner membrane, forming a concentration gradient. The PMF drives protons to flow back across the inner membrane along their concentration gradient. The F_0F_1 ATP synthase complex couples the energy released by the PMF-driven flux of protons with the synthesis of ATP (Lodish *et al.* 2000, Krulwich *et al.* 2011). Though a change in a single component of the PMF is usually buffered by a counteracting increase in the other, a change in either the membrane potential or pH gradient can cause a disturbance in the maintenance of the PMF, which can have detrimental impacts in terms of metabolism and energy-dependent cellular process (Farha *et al.* 2013). Such processes include ATP synthesis, cell division (Strahl and Hamoen 2010), efflux of toxic substances (Paulsen *et al.* 1996), flagellar motility (Manson *et al.* 1977) and nutrient uptake (Tanaka *et al.* 2018).

Phenothiazines have been shown to inhibit many ATPases found in eukaryotic and microbial cells, including: F_1F_0-ATPase, Na^+/K^+-ATPase, Ca^{2+}/Mg^{2+}-ATPase and Ca^{2+}-ATPase (Bullough *et al.* 1985, Dabbeni-Sala and Palatini 1990, Bhattacharyya and Sen 1999). Inhibition of ATPases is hypothesised to result from the ability of certain phenothiazines to change the conformation of these membrane-associated protein complexes. For example, photoactivated chlorpromazine and trifluoperazine are able to covalently bond with different locations of F_1 and F_0 of F_1F_0-ATPase causing irreversible inhibition (Dabbeni-Sala and Palatini 1990). Plenge-Tellechea *et al.* (2018) revealed that chlorpromazine at 0.1-1 mM inhibits hydrolytic activity of the erythrocyte Ca^{2+}-ATPase. This inhibition was suggested to result from the ability of chlorpromazine to disturb the lipid bilayer and thus interfere with the functionality of the membrane-associated proteins (Plenge-Tellechea *et al.* 2018). However, at this concentration chlorpromazine does not irreversibly modify the membrane environment or affect the lipid content. Effects of chlorpromazine on calmodulin

were dismissed upon the observation that increasing concentrations of calcium restores hydrolytic activity of Ca^{2+}-ATPase. These results indicate that the drug interacts directly with the enzyme; computational modelling supports this by showing the presence of two potential chlorpromazine binding sites within Ca^{2+}-ATPase (Plenge-Tellechea *et al.* 2018). At high concentrations (>100 μM) chlorpromazine is also able to significantly change the conformation of Na^{+}/K^{+}-ATPase and Ca^{2+}/Mg^{2+}-ATPase (Bhattacharyya and Sen 1999). Detailed experiments of the interaction of phenothiazines with ATPases have been primarily conducted using those from mitochondrion or erythrocytes. However, ATPases from bacterial sources possess very similar structural and functional features and may be expected to respond to phenothiazines in a similar manner. Given that ATP synthases play the primary role in energy generation, inhibition of these proteins by phenothiazines may lead to significant detrimental cellular effects.

Phenothiazines are able to interfere with the respiratory chain in *M. tuberculosis*. Weinstein *et al.* (2005) showed that oxygen is rapidly consumed upon the addition of nicotinamide adenine dinucleotide (NADH) to *M. tuberculosis* membrane particles, a consumption which is inhibited by 1 mM of trifluoperazine (Weinstein *et al.* 2005). The restoration of respiration by the addition of ascorbic acid and 3,3,5,5-tetramethylphenylenediamine, indicated this inhibition occurred upstream of the cytochrome *c* complex. Subsequent titration experiments of NADH: quinone 2 oxidoreductase activity revealed that inhibition of oxygen consumption may be related to the inhibition of Type-2 NADH dehydrogenase (NDH-2) homologues by chlorpromazine and thioridazine with IC_{50} values of ~10 μM (Weinstein *et al.* 2005). NDH-2 plays an important role in oxidative phosphorylation, which is involved in the generation of respiratory ATP and consists of two processes; chemiosmosis and the electron transport chain. Electrons are able to enter the electron transport chain through NDH-2 and the subsequent redox reactions that occur result

in proton translocation, allowing for the establishment of the PMF, which in turn, activates ATPsynthase to generate cellular ATP. Inhibition of NDH-2 may potentially collapse the PMF and lead to a reduction in the generation of ATP. However, outside of *M. tuberculosis*, in many bacteria NDH-2 is not essential for survival and the production of ATP by oxidative phosphorylation can be compensated for by the utilisation of fermentable carbon sources using substrate level phosphorylation (Hunt *et al.* 2010). While important in the context of aerobic bacteria, the observation that inhibition of NDH-2 by phenothiazines may not be relevant when considering the action of these inhibitors against facultative anaerobic organisms including Staphylococci, *E. coli* and *Salmonella*.

EFFLUX INHIBITORY PROPERTIES OF PHENOTHIAZINES

Efflux pumps

Located in the cell membrane of Gram-positive and Gram-negative bacteria, efflux pumps recognise toxic substances that have breached the bacterial cell wall entering the cytoplasm or the periplasm. Once recognised, efflux pumps extrude them to the external environment. Efflux pumps may be specific for a single substrate, or they may export a wide variety of structurally unrelated compounds. Substrates include: antibiotics, dyes, biocides and degradation products from cellular metabolism .

Efflux pumps are classified by their structure, the number of transmembrane domains they contain and their substrates. In prokaryotes there are six major super-families; the major facilitator superfamily (MFS), the resistance nodulation division (RND) family, the small multidrug resistance (SMR) family, the ATP binding cassette superfamily (ABC), the proteobacterial antimicrobial compound efflux (PACE) family and the multidrug and toxic compound extrusion (MATE) family. These six families are grouped into two classes dependent on their energetic requirements; primary transporters that utilise the energy of ATP

hydrolysis (ABC) and secondary transporters (RND, MATE, MFS, PACE and SMR) that rely on the energy produced from transmembrane electrochemical gradients (Li and Nikaido 2009).

Inhibition of efflux may possibly occur via a number of mechanisms: competitive inhibition, non-competitive inhibition, interference with the outer membrane channel, collapse of the mechanism required for the production of the energy, interference with expression of a component of the tripartite pump, disruption of the assembly of the tripartite structure and changes in the structure of pump substrates (Opperman and Nguyen 2015).

Chlorpromazine as an Efflux Inhibitor

While the antimicrobial activities of phenothiazines generally occur at concentrations greater than those clinically achievable, the ability of phenothiazines to prevent selection of antibiotic-resistant bacteria at sub-inhibitory concentrations was noted as early as 1969. Manion *et al.* (1969) observed that isoniazid resistance in *Mycobacterium* can be delayed or prevented when combined with sub-inhibitory concentrations of phenothiazine (Manion *et al.* 1969, Kristiansen *et al.* 2007). The mechanism was not reported.

Chlorpromazine and other phenothiazines display synergy with and potentiate the activity of efflux pump substrates for many bacteria including *S. aureus* (Kaatz *et al.* 2003), *Salmonella* (Bailey *et al.* 2008) and *E. coli* (Amaral *et al.* 2011) (Table 2 and Supplementary Table 2). However, the mode of efflux inhibition is poorly understood. The nature of their interaction with efflux proteins or substrates, or even if such an interaction exists, is currently unknown. In terms of this review, efflux inhibition by phenothiazines is summarised for the RND transporter AcrAB-TolC as most research has been carried out in this context.

Table 2. Summary of the published antibiotic potentiation by chlorpromazine. Where N/D is written no information on fold decrease in MIC is available.

Supplementary Table 2. Summary of the published antibiotic potentiation by phenothiazines. Where N/D is written no information on fold decrease in MIC is available.

AcrAB-TolC is a tripartite system involving an inner membrane transporter (AcrB) complexed with a periplasmic adaptor protein (AcrA) and an outer membrane protein (TolC) (Du *et al.* 2018). In *E. coli,* AcrAB-TolC is regulated via global and local transcriptional regulation; locally by AcrR which represses *acrAB* transcription and globally by the AraC/XylS transcriptional activators MarA, SoxS and Rob (Weston *et al.* 2017). Although not present in *E. coli,* in many other Enterobacteriales, such as *S.* Typhimurium, *Klebsiella pneumoniae* and *Enterobacter cloacae*, RamA, a homologue of MarA, is also involved (Schneiders *et al.* 2003, Bailey *et al.* 2008, Blair *et al.* 2015, Raczkowska *et al.* 2015).

Efflux inhibition may be competitive or non-competitive. Non-competitive in that the compound prevents the protein from functioning (either by preventing the conformational changes that are essential for extrusion, preventing pump assembly or blocking the exit channel), or competitive where the inhibitor is a preferential substrate and is extruded into the extracellular environment instead of, or before, the antibiotic. In both situations, the antibiotic remains within the cell where it can interact with its intracellular target. Bailey *et al* (2008) revealed that chlorpromazine had a poor antimicrobial effect against wild-type *S.* Typhimurium, with MIC values of 512-1,024 µg/ml (Bailey *et al.* 2008). However, hypersusceptibility to chlorpromazine, and other phenothiazines, was seen in strains with deletions in efflux pump genes (*acrB, acrD, acrF* and, *tolC*) or regulatory genes (*marA* and *ramA*). The greatest extent of hyper-susceptibility occurred in strains with mutations in *acrB* or *tolC,* suggesting that chlorpromazine may be a substrate of the AcrAB-TolC efflux pump. This hypersusceptibility to phenothiazines (chlorpromazine and thioridazine) for *S.* Typhimurium strains lacking *acrB* or *tolC* was confirmed by Yamasaki *et al.* (2016) (Yamasaki *et al.* 2016). In addition, overexpression of *acrAB* or *acrEF* conferred resistance

to chlorpromazine and thioridazine for a *ΔacrB S.* Typhimurium strain (Yamasaki *et al.* 2016). This, combined with the synergy that occurs when chlorpromazine is combined with a range of antibiotics, provides data to suggest that chlorpromazine may interact with the AcrAB-TolC system and behave as an efflux inhibitor, binding preferentially to antibiotic binding sites giving rise to intracellular accumulation of the substrate.

While changes in the MIC of efflux pump substrates in the presence and absence of efflux inhibitors provides valuable information, this approach has limited sensitivity to detect antibiotic potentiation, largely because subtle differences are often difficult to determine. The degree of efflux inhibition can be determined by measuring efflux directly, or by measuring substrate accumulation in the presence of a putative inhibitor. The most commonly used of these methods rely on measuring the fluorescence of an efflux pump substrate that is able to intercalate DNA; usually either ethidium bromide or Hoescht H33342. The greater the extent of efflux inhibition, the higher the level of substrate fluorescence due to intracellular accumulation (Blair and Piddock 2016).

Phenothiazines, at sub-inhibitory concentrations, give rise to increased accumulation of ethidium bromide and antibiotics such as norfloxacin and ciprofloxacin (Kaatz *et al.* 2003, Bailey *et al.* 2008, Amaral *et al.* 2011). This occurs in wild-type, efflux-deficient (Δ*tolC S.* Typhimurium) and MDR strains (e.g. *norA* over-expressing *S. aureus* strains) (Kaatz *et al.* 2003). Bailey *et al.* (2008) showed that chlorpromazine exerted no inhibitory effects when used against an *acrB*-deficient strain; perhaps because chlorpromazine is no longer able to interact with its binding site (Bailey *et al.* 2008, Yamasaki *et al.* 2016). This supports the hypothesis that certain phenothiazines (e.g. chlorpromazine) may directly interact with individual components of efflux pumps and behave as competitive inhibitors. Considering AcrB is a major contributor to efflux of many compounds, when it is no longer present, inhibition of (usually) minor efflux systems has a minimal effect. Yamasaki *et al.* (2016)

confirmed that exposure to chlorpromazine or thioridazine does not increase ethidium bromide accumulation in Δ*acrB S.* Typhimurium. However, the authors do not discuss that the data shows both phenothiazines cause a concentration-dependent increase in the initial accumulation of ethidium bromide (Yamasaki *et al.* 2016). Given that ethidium bromide accumulation may be affected by factors including changes in cell permeability, the initial increase in ethidium bromide accumulation may be due to the ability of chlorpromazine to permeabilise the membrane allowing a greater initial influx of this compound (Coldham *et al.* 2010).

Effect of Chlorpromazine on AcrAB-TolC Gene Expression

Bailey *et al.* (2008) determined the effects of chlorpromazine on expression of the *ramA* and *acrB* genes of *S.* Typhimurium. Chlorpromazine caused an increase in the expression of *ramA*, whilst simultaneously causing a reduction in the expression of *acrB.* This reduction in expression correlated with an increase in the susceptibility of *S.* Typhimurium for a variety of AcrAB-TolC substrates (Lawler *et al.* 2013). Furthermore, chlorpromazine and other phenothiazines increased the expression of *ramA* to levels greater than those observed in response to inactivation of *acrB.* Although inactivation of the transcriptional activator *ramA* conferred increased susceptibility to chlorpromazine (Bailey *et al.* 2008), data indicates that chlorpromazine does not directly induce the expression of *ramA.* It was proposed that the bacterium compensates for lack of AcrB via a positive feedback mechanism on *ramA.* This may occur from increased intracellular accumulation of metabolites that may bind to the transcriptional repressor RamR, increasing *ramA* transcription (Lawler *et al.* 2013). Upon removal of chlorpromazine, the amount of the RamA protein decreases to pre-exposure levels. *Salmonella* strains with a non-functional Lon protease are unable to degrade RamA and thus the abundance of this protein is not reduced post-chlorpromazine exposure (Ricci *et al.* 2014). Lon protease mediated degradation of

462 transcriptional activators is dependent on the energy of ATP hydrolysis. If chlorpromazine
463 interferes with the ability of the bacterial cell to produce ATP, the Lon protease will be
464 rendered non-functional and unable to degrade RamA, accounting for the increased
465 expression of this activator seen in the presence of chlorpromazine (Ricci *et al.* 2014).

466 **Non-selectivity of phenothiazines as efflux inhibitors**

467 Data suggests that phenothiazines have multiple modes of action including effects on the
468 bacterial membrane, cellular replication and energy generation, as well as several effects on
469 mammalian cells. Therefore, while many of these compounds may be substrates of AcrAB-
470 TolC and directly interact with this protein complex, it is unlikely that their efflux inhibitory
471 effects are selective. Indeed, their non-specific effects may contribute to their ability to
472 inhibit efflux.

473 Considering that many efflux pumps, including AcrAB-TolC, are proton/substrate
474 antiporters driven by the PMF (Blair *et al.* 2015), efflux is inhibited upon interference with
475 the ability of the bacterial cell to generate or maintain an energised cell membrane. Amaral *et*
476 *al.* (2011) and Rodrigues *et al.* (2009) revealed that the increased accumulation of ethidium
477 bromide caused by *N*-hydroxylalkyl-2-aminophenothiazines at pH 7.4 is significantly reduced
478 in the presence of glucose (Rodrigues *et al.* 2008, Amaral *et al.* 2011). This suggests that the
479 addition of a source of metabolic energy is able to reverse the inhibitory effects of
480 phenothiazines and demonstrates the role that such energy plays in the activity of the *E. coli*
481 AcrAB-TolC efflux pump.

482 As stated above, studies have suggested that chlorpromazine interferes with calcium
483 binding to calcium-binding proteins (Molnar *et al.* 1997). Martins *et al.* (2011) noted that at
484 pH 8.0, the chlorpromazine-induced accumulation of ethidium bromide was decreased by the
485 addition of calcium chloride (Martins *et al.* 2011). The author speculated that chlorpromazine

interferes with the binding of calcium to calcium-dependent ATPases, thus inhibiting the hydrolysis of ATP. The consequent lack of protons then collapses the PMF, inhibiting efflux. Upon addition of calcium chloride, the excess calcium ions out-compete chlorpromazine and bind to calcium-binding proteins which reverses the efflux inhibition and allows the efflux of ethidium bromide.

However, the generation and hydrolysis of ATP by calcium-dependent ATPases is only one avenue by which ATP can be produced and hydrolysed, and does not take into consideration calcium-independent generation of ATP. For example, the F_0F_1 ATPase (and many other ATPases) is not calcium dependent and ATP will continue to be produced and hydrolysed in the absence of this ion. Therefore, it is unlikely that any net loss of ATP generation as a result of inhibition of the calcium dependent ATPases is sufficiently large enough that it cannot be ameliorated by the activity of other enzymes.

Apart from chlorpromazine, little work has been done regarding the mode of action of phenothiazines as efflux inhibitors. However, recently Wassmann *et al.* (2018) selected for *S. aureus* mutants resistant to thioridazine. These mutants contained mutations in *cls*, important for the synthesis of membrane cardiolipin. Given that thioridazine interacts with negatively charged phospholipids, the authors proposed that thioridazine may bind to cardiolipin allowing it to pass into, and accumulate within, the cytoplasmic membrane. This disturbance of the membrane in turn damages the electrochemical gradient giving rise to inhibition of a variety of energy-dependent processes. Interestingly, growth kinetic experiments revealed that while deletion of *cls* results in resistance to thioridazine, the strain shows a growth kinetic profile similar to the wild type when thioridazine was used in combination with dicloxacillin. Therefore, while cardiolipin was suggested to be important for the bactericidal activity of thioridazine it is not essential when considering the ability of thioridazine to potentiate the activity of antibiotics.

EFFECT OF PHENOTHIAZINES ON BIOFILM FORMATION

Many persistent and chronic bacterial infections are linked to the formation of biofilms (Flemming *et al.* 2016). Given that changes in the expression of genes encoding efflux and transporter proteins occurs during the establishment of a biofilm, efflux pumps have been suggested to be involved in their formation and maintenance. Up-regulation of genes encoding efflux and transporter proteins is a common feature of many biofilms. the transcriptional profiles of the *E. coli* UTI strains 83972 and VR50 showed that 128 of the 600 genes upregulated during biofilm growth encoded efflux pumps and other transporters (Kvist *et al.* 2008). In addition, transposon mutagenesis of *E. coli* revealed that the efflux genes *emrY, fsr* and *emrE* were essential for biofilm growth. Further studies have also shown that *E. coli* and *Salmonella* strains lacking *acrB, acrD, acrE, mdtE* and *emrE* grew poorly in a biofilm when compared to the wild-type strain (Han *et al.* 2010, Baugh *et al.* 2012). Alav *et al* have recently reviewed the interplay between biofilm formation and efflux pumps (Alav *et al.* 2018)**.**

It is unclear whether inhibition of efflux pumps will inhibit the formation or maintenance of a biofilm. At sub-MIC concentrations, thioridazine and chlorpromazine have been shown to inhibit the formation of biofilms in the following organisms: *Francisella novicida* (Dean and van Hoek 2015), *E.coli* MG1655 (Baugh *et al.* 2014), *E. coli* F18, *E. coli* UTI strains 83972 and VR50, *S. aureus* NCTC 8532 (Baugh *et al.* 2014), *P. aeruginosa* PAO1 (Baugh *et al.* 2014), *Proteus mirabilis* (Nzakizwanayo *et al.* 2017), *S.* Typhimurium (Baugh *et al.* 2012) and *K. pneumoniae* I222-86 (Nzakizwanayo *et al.* 2017), as well as clinical isolates of *Proteus mirabilis, E. coli* and *P. aeruginosa* (Nzakizwanayo *et al.* 2017). *P. mirabilis* possesses the Bcr/CflA efflux system that is essential for the development of biofilms by this species. Thioridazine at half-MIC reduced the rate of biofilm formation by *P. mirabilis* on catheters, In silico modelling predicted an interaction between thioridazine and

536 the hydrophobic binding pocket of the Bcr/CflA efflux system (Nzakizwanayo et al. 2017).
537 This suggests that part of the mode of action may be as a competitive inhibitor of efflux.

538 The establishment and maintenance of a biofilm is regulated by quorum sensing (cell-
539 to-cell signalling). In *F. novicida* the observed biofilm inhibition by phenothiazines was
540 dependent on the virulence factor QseC, a quorum sensing histidine kinase that forms part of
541 the QseBC two-component system (Dean and van Hoek 2015). QseBC is also found in *E.*
542 *coli* and shares homology with PmrAB of *S.* Typhimurium suggesting that phenothiazines
543 may inhibit quorum sensing. In turn, this will have downstream impacts on virulence factor
544 production, motility and biofilm formation. Some bacterial species containing deletions in
545 efflux pump genes are unable to secrete quorum sensing signals and thus form a biofilm.
546 Similarly, compounds that are known to inhibit efflux via disturbance of the PMF (CCCP)
547 have also been shown to inhibit quorum sensing in *E. coli* by preventing extrusion of toxic
548 quorum sensing signals (Varga *et al.* 2012). Given this, the inhibition of biofilms by
549 phenothiazines may indirectly result from inhibition of efflux pump activity by disturbances
550 to the PMF.

551 In enterohemorrhagic *E. coli*, QseBC acts as a virulence factor responsible for
552 activating transcription of motility genes (Clarke *et al.* 2006). This implies that
553 phenothiazines decrease biofilm formation by inducing a response that increases motility,
554 allowing the bacterium to move away from the toxic inhibitor. The ability of phenothiazines
555 at sub-inhibitory concentrations to inhibit motility and swarming has also been shown in *P.*
556 *vulgaris* (Molnar *et al.* 1992). Considering the flagellum is energised by transmembrane ion
557 gradients, it was postulated that the ability of phenothiazines to inhibit motility results from
558 inhibition of the bacterial proton gradient. Type IV pilli are another key virulence factor that
559 contribute to both motility and the ability of the bacterium undergo homologous
560 recombination (Craig *et al.* 2004). Recently, Denis *et al* (2019) reported that trifluoperazine

(at sub-inhibitory concentrations) is able to affect the functionality of Type IV pilli as seen by a reduction in pilli-dependent twitching motility and subsequent dispersal of aggregates produced by *Neisseria meningitides* and *Neisseria gonorrhoeae.* This was not observed in a retraction-defective Δ*pilT* mutant or a mutant overexpressing the outer membrane protein PilC. Of note, all piperazine and piperidine classes of phenothiazines, but not the aliphatic class (with the exception of promazine), induced aggregate dispersal of meningococcal aggregates. *N. meningitidis* mutants resistant to trifluoperazine and thioridazine were found to contain mutations in the Na^+ pumping NADH: ubiquinone oxidoreductase complex (Na^+-NQR). This respiratory chain enzyme is essential in the maintenance of an inner membrane Na^+ gradient. The accompanying mutations in *lgtE* or *galE* may compensate for the resulting osmotic stress by altering lipopolysaccharide structure. This, in combination with the observation that the addition of NaCl inhibits the aggregate dispersal activity of the phenothiazines, suggests these compounds are able to affect bacterial motility via alterations to the inner membrane Na^+ gradients . Given that the Na^+ gradient allows electrons to enter the electron transport chain disruption of Na^+-NQR may have downstream impacts on the ability of the cell to generate the energy required for other crucial biosynthetic pathways .

CAN PHENOTHIAZINES BE USED CLINICALLY AS ANTIBIOTIC ADJUVANTS?

Several questions arise from the use of phenothiazines in psychiatry that could be useful for determining their clinical impact as antibiotic adjuvants. For instance, are patients who receive phenothiazines less likely to have a bacterial infection or does phenothiazine administration improve the clinical outcome of patients with bacterial infections treated with antibiotics? In addition, given that the usefulness of efflux inhibitors will be limited if bacteria develop resistance to the adjuvant, does bacterial resistance to phenothiazines occur

in commensal organisms in patients administered this drug for its neuroleptic properties? Unfortunately, there are currently no published studies addressing these questions.

Another question often raised about the use of these drug combinations is whether the drug-drug interactions of antibiotics and phenothiazines limit their use in combination? Phenothiazines and many antibiotics share a similar organ distribution and very few antibiotics interact negatively with phenothiazines . However, there are no published studies showing that phenothiazines synergise with antibiotics *in vivo.* Drug interactions are highly complex and mechanistically relevant models will need to be built to determine whether phenothiazines synergise or enhance the activity of antibiotics in a clinically useful manner. In addition, the concentrations at which phenothiazines can be administered therapeutically without cytotoxicity is ~1,000 fold lower than the concentration at which antibiotic-adjuvant activity is observed. Therefore, the current clinical usefulness of these compounds may be limited. However, understanding the mode of action of phenothiazines as efflux-adjuvants may allow for the design of phenothiazine derivatives or novel compounds as efflux inhibitors without the accompanying cytotoxicity.

CONCLUDING REMARKS

Phenothiazines have been very useful clinical agents within the field of psychiatry and many of their additional biological properties, although largely overlooked, have been known for many years. Over the last decade, researchers have begun to study the diverse activities of the phenothiazines for use as antibiotic adjuvants. Phenothiazines have been shown to interfere with cellular replication, affect cellular energy generation, possess plasmid curing properties and inhibit biofilm formation. Of particular interest is the evidence to suggest that phenothiazines are efflux inhibitors, capable of potentiating the antimicrobial activity of

existing antibiotic and increase the intracellular concentration of antibiotics. Considering that the pharmacokinetics and toxicology of phenothiazines are well-described, these compounds could be useful as antibiotic-adjuvants. Unfortunately, the cytotoxicity of these compounds will limit their clinical use. The rational design of more active and less cytotoxic efflux inhibitors, either novel compounds or phenothiazine derivatives will be achieved through understanding of the mechanisms of phenothiazine activity against bacteria. Irrespective of their clinical use, the use of phenothiazines in academic research has greatly enhanced the understanding of many biological systems including plasmid conjugation, biofilm formation and efflux pumps.

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