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Title: Targeting the hard to reach: Challenges and novel strategies in the treatment of intracellular bacterial infections.

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List of abbreviations

AMP	Antimicrobial peptides
AS-ODN	Antisense oligonucleotide
Azi	Azithromycin
cfu	Colony forming unit
CPP	Cell penetrating peptides
DAPI	4',6-Diamidino-2-Phenylindole
Dox	Doxycycline
EMRSA	Epidemic methicillin resistant *S.aureus*
FITC	Fluorescein isothiocyanate
Gent	Gentamicin
LNA	Locked nucleic acid
MRSP	Methicillin-resistant *Staphylococcus pseudointermedius*
MRSA	Methicillin-sensitive *Staphylococcus aureus*
MSSA	Methicillin-sensitive *Staphylococcus aureus*
NP	Nanoparticle
PAA-+Na	Poly sodium acrylate
PenG	Penicillin G
PEO-b-PAA-+Na	Poly ethylene oxide-b-sodium acrylate
PHMB	Polyhexamethylene biguanide
Pip	piperazine
PLGA	Poly d-L-lactide-co-glycolide
PMO	Phosphorodiamidate morpholino oligomer
PNA	peptide nucleic acid
PS	Phosphorothioate
Rif	Rifampicin
ROS	Reactive oxygen species
Strep	Streptomycin
Tet	Tetracycline
WGA	Wheat germ agglutination
WHO	World Health organization

Abstract

Infectious diseases continue to threaten human and animal health and welfare globally, impacting millions of lives and causing substantial economic loss. The discovery and administration of antibacterials have only been partially successful in reducing disease impact. Bacterial cells are inherently resilient and the therapy challenge is increased by the development of antibacterial resistance, the formation of biofilms and the ability of certain clinically important pathogens to invade and localize within host cells. Invasion into host cells provides protection from both antibacterials and the host immune system. Poor delivery of antibacterial into host cells causes inadequate bacterial clearance, resulting in chronic and unresolved infections. In this review, we discuss the challenges associated with existing antibacterial therapies with a focus on intracellular pathogens. We consider the requirements for successful treatment of intracellular infections and novel platforms currently under development. Finally, we discuss novel strategies that give promise for the treatment of bacteria that present challenges to antibacterial penetration into host cells. As an example, we discuss our recent demonstration that the cell penetrating cationic polymer polyhexamethylene biguanide has antibacterial activity against intracellular *Staphyloccocus aureus.*

Keywords: Infectious diseases, intracellular bacteria, novel therapies, polyhexamethylene biguanide.

Antibacterial resistance and the challenge of infectious disease

Infectious disease remains a major threat to both human and animal populations. In the human population in 2010 approximately 15 million deaths were due to infectious disease, and the World Health Organization (WHO) forecasts that this figure will fall only marginally by 2050 (Dye, 2014). In the animal population, infectious disease continues to affect the health and welfare of livestock, resulting in threats to food security. This situation not only causes huge economic losses but also increases the risk of possible transmission of zoonotic disease to the human population (Tomley and Shirley, 2009).

The discovery of penicillin as an antibacterial in the early 20th century revolutionized treatment for infectious diseases caused by bacteria (Fleming, 1929). Soon after, chloramphenicol, streptomycin and several other antibiotics provided further therapy options. It is without doubt that although the discovery and development of antibacterials improved infectious disease control, the triumph of antibacterial therapy has been short-lived. Increasing consumption of antibacterials to treat illnesses, the use of antibacterials as growth promoters in livestock and their un-controlled released into nature introducing continuous selective pressures, has resulted in the development of resistance (Dye, 2015, Tomley and Shirley, 2009). The World Economic Forum recently concluded that antibacterial resistance is the greatest risk to human health (Howell, 2013). Many infections are now difficult to treat, resulting in high dose administration of antibacterials, in-tolerable toxicity and delays in effective treatment (WHO, 2012). It has been estimated that infections by antibacterial resistant pathogens claim a total of 700,000 lives every year globally, with 10 million projected deaths in the year 2050 (O'Neill, 2014).

The impact of antibacterial resistance is also important within animal health. In livestock a high prevalence of beta-lactam resistant *Staphylococcus aureus*, one of the pathogens responsible for bovine mastitis has made existing therapies less effective, prolonging the disease and increasing the costs of treatment (Barkema *et al*., 2006). Also, in companion animals the emergence of methicillin-resistant *Staphylococcus pseudointermedius* (MRSP), the causative agent of skin, ear and wound infections is a new challenge for veterinary medicine (van Duijkeren *et al*., 2011).

Conventionally bacteria become resistant to antibiotics through the acquisition of resistance traits. A classic example of this is the acquisition of beta-lactamases, which are hydrolytic

enzymes that break down beta-lactam antibiotics rendering them ineffective. However, the acquisition of resistance traits is only one of the contributing factors in treatment failure. For an antibacterial to be effective in the clinic, it should be able to reach both the bacteria and its molecular targets at effective concentrations that are not toxic to the host. It has been recognized for some time that infections with Gram -ve bacteria can be difficult to treat, because the outer membrane provides a barrier against the diffusion of antibacterials. At the population level, the ability of bacterial communities to form biofilms also provides barriers to drug penetration. These three dimensional multicellular aggregates are inherently resistant to antibacterials. The formation of biofilms by *S. aureus* on medical devices, such as artificial joints or catheters, and *Pseudomonas aeruginosa* on the surfaces of infected sites can bring additional hurdles to existing therapies (McConoughey *et al*., 2014; Winstanley *et al*., 2016). In the case of bovine mastitis, biofilm formation by *S. aureus* on the mammary gland reduces the effectiveness of therapies, creating persistent infections (Melchior, 2011). For detailed knowledge on biofilms and their clinical burden, readers are invited to refer to the following extensive review (Abee *et al*., 2011).

Biofilms present challenges in terms of the access antibacterial to their bacterial targets. In this review we focus on another similar and equally significant challenge to successful therapy; the problem of antibacterial gaining access to bacteria residing within host cells.

Intracellular bacteria represent hard to reach targets

Certain species of bacteria are able to localize inside host cells, followed by multiplication and modulation of the host cell biology. In this way, these bacteria create a niche, from which they can continue the infection cycle (Silva and Silva Pestana, 2013). This group of bacteria, known as intracellular bacteria, can also manipulate the host immune system to permit dissemination to different sites of the body. The classical examples of intracellular bacteria are *Mycobacterium tuberculosis, Salmonella enterica, Chlamydia trachomatis,* and *Listeria monocytogenes* (Armstrong and Hart, 1971; Ibarra and Steele-Mortimer, 2009; Kumar *et al*., 2006; Gaillard *et al*., 1987). Additionally, evidence suggests that some classical extracellular bacteria, such as *S. aureus, Escherichia coli* and *P. aeruginosa* also have the ability to invade and localize inside host cells (Dikshit *et al*., 2015; Garzoni and Kelley, 2009; Angus *et al*., 2008). Table 1 provides a list of intracellular bacteria and their associated disease. Below we

discuss the mechanisms of invasion used by three clinically important pathogens, *S. enterica*, *M. tuberculosis* and *S. aureus*.

Salmonella enterica

Host infections start when *S. enterica* is ingested. On reaching the gastrointestinal tract, *S. enterica* can induce its own uptake into specialized epithelial cells, M cells, that cover Peyer's patches of the intestine (Jensen *et al*., 1998). The bacteria injects effector proteins into the host cell, triggering membrane ruffling and actin rearrangement from inside the cells, leading to bacterial internalization (Patel and Galán, 2005). Internalization into M cells allows the bacteria to cross the intestinal barrier. The bacteria are then engulfed by macrophages and reside in a phagosome called the salmonella containing vacuole. While inside vacuoles, *S. enterica* secrete effector proteins that can prevent fusion of the phagosome with a lysosome, therefore avoiding lysosomal activities within macrophage (Gorvel and Méresse, 2001). Recent evidence also suggests that *S. enterica* can escape into the cytosol (Brumell *et al*., 2002). Migration of infected macrophages can further disseminate bacteria into other organs, such as the liver and spleen (Monack *et al*., 2004).

Mycobacterium tuberculosis

Transmission of *M. tuberculosis* occurs via inhalation of droplets containing the bacilli. Once the pathogen reaches the lung airways, bacteria are phagocytosed by alveolar macrophages. Numerous studies show that *M. tuberculosis* can evade the killing process in macrophages by arresting phagosome fusion with the lysosome, thereby establishing a survival niche within macrophages where replication occurs (Armstrong and Hart, 1971; Rohde *et al*., 2012). However, more recent studies demonstrate that certain *M. tuberculosis* strains can escape into the cytosol, by permeabilizing the phagosome membrane (Watson *et al*., 2012).

Staphylococcus aureus

S. aureus is a Gram-positive pathogen that can cause various disease conditions including complicated skin infections (Dryden, 2010) and bloodstream infections in hospitalized patients (hospital acquired infections) (Burton *et al*., 2009). In animals, *S. aureus* is one of the main pathogens that causes mastitis, a disease manifested by inflammation of the udder (Jamali *et*

al., 2014). *S. aureus* was historically known as an extracellular bacterium until recently. However, accumulating evidence suggests that this bacteria can invade and survive in various host cells including keratinocytes, endothelial cells, epithelial cells, fibroblast, osteoblasts and bovine mammary epithelial cells (Mempel *et al*., 2002; Garzoni *et al*., 2007; Sinha and Hermann 2005; Hanses *et al*., 2011; Reott *et al*., 2008; Hébert *et al*., 2000).

S. aureus invades host cells through a zipper uptake mechanism involving adhesion to the host cell surface (Fraunholz and Sinha, 2012). Attachment leads to signal transmission that results in cytoskeletal rearrangement, allowing movement of *S. aureus* into host cells (Sinha *et al*., 1999; Ahmed *et al*., 2001; Edwards *et al*., 2011). Once inside the host cell, *S. aureus* can either survive and replicate within the acidic phagolysosome (Brouillette *et al*., 2003) or escape from the phagosome into the cytosol (Fraunholz and Sinha, 2012). *S. aureus* invasion can induce cell death, allowing the bacteria to escape and start a new cycle of infection, subsequently entering the blood stream to cause septicemia (Soong *et al*., 2012).

The problem of antibacterial delivery to bacteria residing within host cells is of paramount importance. Some of these bacteria are responsible for devastating diseases. For example, *M. tuberculosis*, the causative agent of tuberculosis, causes approximately 1.5 million deaths per year and is the second leading cause of death due to a single infectious agent (Lewandowski *et al*., 2015). Species belonging to the *Salmonella* group are important foodborne pathogens responsible for enteric diseases and cause over one billion infections annually (Buckle *et al*., 2012). Moreover, methicillin-resistant *Staphylococcus aureus* (MRSA) is responsible for 20% of mortality due to the blood stream infections in the hospital-acquired setting (Thomer *et al*., 2016). Therefore, the effective delivery of antibacterial into host cells containing these pathogens is a critical goal of novel antibacterial therapies.

Current challenges in the treatment of intracellular bacterial infections

The delivery of antibacterials into desired locations in the body is one of the main challenges for successful therapeutics. Depending on the routes of uptake and the location of the infections, antibacterials may need to cross the epithelial cells of the gastrointestinal tract to reach the bloodstream (oral antibacterial), the thick stratum corneum for skin infections (topical antibacterial), and the mucosa for respiratory tract infections (pulmonary antibacterial).

For infections that are caused by pathogens that reside extracellularly, antibacterials can exert activities rapidly. However, if bacteria reside intracellularly, antibacterials face another challenge; they need to cross the host cell membrane either through diffusion or endocytosis. Localization of bacteria inside host cells provides protection and, although there are multiple antibacterials options available for treatment, more than two thirds are ineffective against intracellular pathogens (Abed and Couvreur, 2014).

The plasma membrane of mammalian cells is composed of a lipid bilayer embedded with peripheral and integral proteins, and is impermeable to most polar or charged solutes. Small (< 700 Da in size) lipophilic antibacterials such as beta lactams, macrolides and quinolones enter mammalian cells via diffusion across the lipid bilayer (Tulkens, 1991). Uptake via endocytosis occurs when a compound is large or does not readily diffuse across the membrane. Endocytosis involves internalization of molecules bound within vesicles from the membrane, followed by invagination of the vesicles into the host cells. Once taken up by the host cells, the vesicles are directed to the endosomal route, where acidification takes place. Certain compounds can trigger membrane destabilization, therefore are released into the cytosol of the host cell.

Aminoglycoside antibiotics are known to enter host cells via endocytosis. They bind to megalin, the endocytic receptor abundantly expressed in the renal proximal tubule that promotes uptake into the host cells (Nagai and Takano, 2004). Because of the specificity of aminoglycoside towards megalin, accumulation of the antibiotics in the kidney can cause nephrotoxicity in patients (Nagai and Takano, 2004).

Once inside the host cells, antibacterials have to be retained and accumulate at sufficient concentrations for a period that is sufficient to exert their effects. Although macrolide and quinolone can enter the host cells via diffusion, they are subjected to P-glycoprotein efflux pumps, leading to reductions in antibiotic accumulation inside the host cells (Seral *et al*., 2003). For compounds entering the host cell via endocytosis, if the compound remains in the endosome, it will be exported out from the host cell via the exocytosis route.
In addition to penetration and retention inside host cells, to be effective, antibacterials must also reside within the same sub-cellular compartment as their bacterial target. As discussed previously, intracellular bacteria can reside within intracellular compartments or the cytosol. Therefore, antibacterial must penetrate the specific compartment where bacteria are residing.

The choice of intracellular location (vesicle or cytosol) brings additional challenges to treatment. Certain bacteria such as *Salmonella* localize and replicate in acidified phagosomes where the pH is in between 4-5.5. Therefore to be active against intracellular bacteria selected antibacterials must also resist pH insult (Lemaire *et al*., 2011).

To adapt to the stress of the host cell environment, intracellular bacteria transform their physiological condition to a non-replicating or slowly replicating state (Grant and Hung, 2014). *S. enterica* can change into a state of non-replicating persistence inside macrophages and, *S. aureus* can change into small colony variants inside epithelial cells (Helaine *et al*., 2014; Vesga *et al*., 1996). Changes in their physiology make these variants less susceptible to antibacterials that are often only active against the variants with normal growth rates (Nguyen *et al*., 2009). *M. tuberculosis* enters a non-replicating state within the host to cause latent infections that are resistant to conventional treatment (Wayne and Sohaskey, 2001). Therefore, to be able to clear intracellular infections by non-replicating bacteria, antibacterial must be effective against both replicating and non-replicating states.

The potency of existing therapies for intracellular infections

Quinolones are often considered to be the best choice for treatment of intracellular infections. They have potent activities against a range of Gram +ve and Gram -ve bacteria and mycobacteria (Hartley 2011; Jacobs, R, 1999). They enter and accumulate in mammalian cells (Tulkens, 1991) and diffuse across subcellular compartments (Carlier *et al*., 1990). Although quinolones have been shown to be more effective against intracellular bacteria, compared to other classes of antibacterials (Carryn *et al*., 2003), their potency against bacteria that are located intracellularly is still much lower relative to their potency against extracellular bacteria (Seral *et al*., 2005).

Derivatives of tetracycline, such as tigecycline, have also shown efficacy against intracellular bacteria. This antibiotic has potent activities against a range of Gram +ve and Gram -ve bacteria (Peterson, 2008). Tang *et al*., 2011 demonstrated bactericidal activities of tigecycline at 0.5 mg/L against intracellular *S. typhimurium* in peripheral blood mononuclear cells (Hung-Jen Tang *et al*., 2011). In contrast, another study found that tigecycline at 1 mg/L, demonstrated only bacteriostatic activities against intracellular *S. aureus* in polymorphonuclear neutrophils

(Ong *et al*., 2005). These observations provide examples of how intracellular localization influences the activity of antibacterials.

Existing antibiotics can be improved by increasing their ability to penetrate host cells. Barcia-Macay *et al*., 2006 made a comparison between vancomycin and televancin (a hydrophobic derivative of vancomycin), against intracellular *S. aureus* in macrophages. Televancin displayed bactericidal activities against intracellular *S. aureus* within six hours of treatment, while vancomycin required 24 hours to demonstrate the same efficacy (Barcia-Macay *et al*., 2006). The reduced efficacy of existing antibiotics has driven the need to improve existing therapies. Some of the many platforms that can potentially improve the outcome and provide a better solution for intracellular infections are discussed below.

Novel promising therapies in the treatment of intracellular infections

Antimicrobial peptides

Antimicrobial peptides (AMP) are chains of amino acids produced by living organisms as part of the hosts innate immunity (Zasloff, 2002). They are expressed on the primary barriers of organisms such as the skin or the mucosal epithelial cells (Guan-Guerra *et al*., 2010). These peptides show potent antimicrobial activities against bacteria, viruses and fungi. AMPs have antibacterial activity through membrane disruption and pore formation, causing leakage of the cellular contents. Additionally, AMPs may enter and interact with intracellular molecules within bacteria, thereby inhibiting DNA, RNA and protein synthesis (Peters *et al*., 2010).

Certain AMPs are amphiphilic (contain hydrophilic and hydrophobic regions). This property facilitates AMP penetration into mammalian cells. A number of studies have shown their promise as a potential therapy for intracellular infections. One example of an AMP that can enter mammalian cells is Cathelicidin LL-37, which is naturally expressed by the human skin. Noore *et al.*, demonstrated that LL-37 was effective against intracellular *S. aureus* in osteoblasts (Noore *et al*., 2013). Temporin, an AMP isolated from frog skin was found to be bactericidal against intracellular methicillin-sensitive *Staphylococcus aureus* (MSSA) and MRSA in keratinocytes, and promoted wound healing by stimulating keratinocyte migration (Di Grazia *et al*., 2014). Another study found that equine alpha-helical antimicrobial peptide eCATH1 killed *Rhodococcus equi* in macrophages (Schlusselhuber *et al*., 2013). Brinch *et al*.,

2010 demonstrated that Plectasin, an AMP derived from the pezizalean fungus *Pseudoplactenia nigrella* was effective against intracellular *S. aureus* in human and mouse monocytes (Brinch *et al*., 2010).

Antisense oligonucleotide based technologies

Antisense oligonucleotide (AS-ODN) based technology is a platform designed to control gene expression at the RNA level. AS-ODNs are short oligomers of nucleic acids or nucleic acid mimics; consisting of 10-30 residues that are complimentary to the target mRNA of interest. Hybridization of AS-ODN to the target mRNA can inhibit the translation process, resulting in repression of gene expression (Sahu *et al*., 2007). Phosphorothioate (PS), peptide nucleic acid (PNA), locked nucleic acid (LNA), and phosphorodiamidate morpholino oligomer (PMO), are among the most studied AS-ODNs (Chan *et al*., 2006). To improve delivery into bacterial or mammalian cells, AS-ODNs are often attached to cell penetrating peptides (CPP) (Nekhotiaeva *et al*., 2003).

For antibacterial purposes, AS-ODNs are designed to target genes essential to the survival of the bacteria (Good, 2002). In this way, antisense technology serves to silence or completely knock-out the function of selected genes. Antisense PNAs and PMOs have been shown to effectively inhibit bacterial growth *in vitro* and *in vivo*. Good *et al.,* 2001 demonstrated bactericidal activity of a peptide-PNA conjugate targeted to the acyl-carrier protein (*acp*), an essential gene involved in fatty acid biosynthesis in *E. coli* (Good *et al*., 2001). Similarly, Tilley *et al*., 2007 showed that CPP conjugated PMO targeted to the *acp* gene reduced bacteremia and promoted survival of mice infected with *E. coli* (Tilley *et al*., 2007).

For the potential of AS-ODNs to be fully realized, the challenge of delivery across both bacterial and mammalian membrane must be overcome. Ma *et al.,* 2014 showed that electroporation improved the delivery of a peptide-PNA targeting bacterial RNA polymerase and killed intracellular *S. typhimurium* (Ma *et al*., 2014). Also, Mitev *et al.,* 2009 introduced piperazine (Pip) linkages between bases of PMO, to introduce cationic charges to the peptide-PMO, to further enhance its delivery into mammalian cells. The Pip-peptide-PMO showed potent efficacy against intracellular *S. typhimurium* and killed > 99% of the bacteria inside

macrophages (Mitev *et al*., 2009). These studies suggest that antisense technology, with further improvement on delivery issues; represents a promising strategy against intracellular bacteria.

Nanoparticles

Nanoparticles are nano-scale materials derived from metallic, metal oxide, semiconductors, polymers or carbon-based materials (Hajipour *et al*., 2012). Nanoparticles have long been applied in the material sciences field, but recent evidence suggests that they have potential applications in the medicinal field. Certain nanoparticles demonstrate potent antibacterial activities and may help to potentiate small molecule antibiotics. For example, Azam *et al*., 2012 demonstrated antibacterial activities of Zinc oxide, Cuprum oxide and Ferum oxide nanoparticles against Gram +ve and Gram -ve pathogens (Azam *et al*., 2012).

Nanoparticles display antibacterial activities through various mechanisms. For example, the cationic charges of titanium and aluminium oxide nanoparticles promote their adsorption onto bacterial surfaces, resulting in destabilization of the membrane, leading to cellular leakage (Ruparelia *et al*., 2008; Pal *et al*., 2015). Silver nanoparticles can produce free radicals that can cause lipid peroxidation of the membrane, resulting in loss of the normal functions, such as bacterial respiratory activities (Allahverdiyev *et al*., 2011). Zinc nanoparticles internalized by the bacteria can induce production of reactive oxygen species (ROS), resulting in ROS-mediated cell damage (Patra *et al*., 2015; Zhao and Drlica, 2014).

Although nanoparticles are very large structures relative to drug molecules, they are able to improve cell entry properties to access intracellular targets. This effect is being exploited in several therapeutic areas aiming to improve the intracellular delivery and cell type targeting of biomolecules or drugs. Nanoparticles are thought to enter mammalian cells through phagocytosis or the endocytosis pathway (Oh and Ji-Ho, 2014). This ability makes nanoparticles useful weapons in the fight against intracellular bacteria. Pati *et al.,* 2014 demonstrated zinc oxide uptake by macrophages. Zinc oxide induced ROS and nitric oxide production in the cells and subsequently killed intracellular *Mycobacterium smegmatis* (Pati *et al*., 2014).

Certain nanoparticles such as liposomes, polymeric nanoparticles, solid lipid nanoparticles and dendrimers can be tailored to display desired charge or composition for combination with other biomolecules; for example, drugs, antibodies, proteins and oligonucleotides. The nanoparticle surfaces can also be decorated with material that is responsive to certain stimuli (e.g pH or temperature) allowing for controllable drug release in a specific place, for example in the acidified endosome (Xu *et al*., 2006). Therefore, together with the ability to enter mammalian cells, the nanoparticle platform can also be utilized to improve the delivery of existing antibiotics into host cells (Zhang *et al*., 2010). A number of studies have investigated the ability of nanoparticles to potentiate antibiotic activities against intracellular bacteria and these are listed in Table 2. The efficacies of penicillin, gentamicin and tetracycline against intracellular *S. aureus* (Meo *et al*., 2012; Ranjan *et al*., 2009; Maya *et al*., 2012), rifampicin and isoniazid against intracellular *M. tuberculosis* (Clemens *et al*., 2012), streptomycin and doxycycline against intracellular of *Brucella melitensis* (Seleem *et al*., 2009) and rifampicin and azithromycin against intracellular *Chlamydial trachomatis* (Toti *et al*., 2011), have all been markedly improved over the free drug by delivering them as nanoparticle conjugates.

Recent advances with a polymeric biocide

Polyhexamethylene biguanide (PHMB) is a cationic polymer composed of repeating biguanide groups linked by hexamethylene chains (Figure 1). PHMB alone is a potent topical antimicrobial against Gram +ve and Gram -ve bacteria (Gilbert and Moore, 2005), fungi(Messick *et al*., 1999; Hiti 2002), parasites and viruses (Romanowski *et al*., 2013). It has been widely used as antiseptic in medicine, food industries and domestic applications (Gilbert and Moore, 2005). PHMB applications include impregnation in wound dressing (Moore and Gray, 2007), water treatment (Kusnetsov *et al*., 1997), mouthwash and disinfection in contact lenses (Hiti, 2002). Although PHMB has been used for over 40 years, there are no reports of bacterial resistance towards this compound (Gilbert and Moore, 2005).

PHMB antibacterial activities involve the interaction of biguanide groups with the cytoplasmic membrane, lipopolysaccharide and peptidoglycan of the bacterial cell wall. This binding is believed to displace the divalent cation Ca^{2+} causing membrane destabilization and cellular leakages (Gilbert and Moore, 2005). Simultaneously, the hexamethylene segment can interact with phospholipids on the membrane, causing a phase separation that disturbs random

distribution of lipids, further destabilizing the membrane structure (Broxton *et al*., 1984). Furthermore, recent findings in our laboratory demonstrated that PHMB enter bacteria cells and this leads to chromosome condensation (Chindera *et al*., 2016). Therefore, PHMB may have at least two mechanisms of action, and this may help to explain why acquired antibacterial resistance to PHMB has not yet been reported.

Recent discoveries in our laboratory also demonstrate that PHMB can enter a range of mammalian cells and co-localize with intracellular MRSA (Chindera *et al*., 2016; Firdessa *et al*., 2015; Kamaruzzaman, NF; Firdessa, R; Good 2016). Furthermore, we demonstrated that PHMB causes a marked reduction in survival of intracellular MRSA inside the host cells (Figure 2). This finding shows, for the first time, that PHMB has potential value to be further developed as a novel therapy for intracellular infections.

Summary and outlook

The discovery of penicillin is considered to be one of the most significant advances in medicine, and the subsequent development and use of conventional antibacterials has saved large numbers of lives. However, the current problem of antibacterial resistance threatens our ability to treat and control infections. Intracellular bacteria, which are generally harder to reach than extracellular bacteria, may not be resistant to antibacterials in the conventional sense, yet nevertheless represent a population of bacteria that are difficult to treat, resulting in frequent treatment failures and limited treatment options. The difficulty of reaching intracellular bacteria was recognised over 50 years ago (Holmes et al., 1966) and in this review we have discussed the challenges of antibacterial therapy for intracellular infections.

The outlook for the treatment of intracellular infections is positive. There are a number of new technologies that offer promise over conventional treatments. AMPs and AS-ONDs have the potential to increase the choice of treatments and offer the advantage that acquired resistance to these compounds may be infrequent (Guilhelmelli *et al*., 2013); however, such technologies still face challenges in gaining entry into host cells. The most promising advances in this area may be found in the nanoparticle arena, where several studies have shown antibacterials to have successful access to and efficacy against bacteria residing within host cells. In this review, we have focused on targeting the bacteria however there are also several strategies that target the host. One example is the use of statins to improve the outcome of infection with *M.*

tuberculosis. Statins lower host cholesterol by the inhibition of HMG-CoA reductase, an enzyme in the cholesterol biosynthetic pathway. The ability to utilise host cholesterol as a carbon source is required for *M. tuberculosis* persistence. Lowering cholesterol levels in the host by the use of statins improves clearance of the bacteria by autophagy (Parihar *et al*., 2014). Therefore, interference with the host cellular pathways provides an additional alternative strategy in the battle against intracellular bacterial pathogens (Hawn *et al*., 2015).

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Box 1 Glossary of biological terms.

Biofilm - a structured community of bacterial cells enclosed in a self-produced polymeric matrix and adherent to an inert or living surface.

M-cells - highly specialised cells present within the epithelium of the small and large intestine. They play a central role in the initiation of mucosal immune responses by transporting antigens and microorganisms to the underlying lymphoid tissue.

Peyer's patches - are small masses of lymphatic tissue found in the small intestine. They monitor intestinal bacteria populations and preventing the growth of pathogenic bacteria in the intestines.

Actin - a protein that forms filaments and provides a structural scaffold for cells. It is also a component of muscle fibres.

Macrophages – white blood cells that engulf invading pathogens or cells that are not recognised as self.

Phagosome - a vacuole in the cytoplasm of a cell, containing an engulfed particle enclosed within a part of the cell membrane.

Lysosome - an organelle in the cytoplasm of eukaryotic cells containing degradative enzymes enclosed in a membrane.

Cytosol - the aqueous component of the cytoplasm of a cell within which various organelles and particles are suspended.

Phagolysosome - the name of the vacuole formed when the phagosome is fused with the lysosome and the contents of the lysosome gains access to the bacteria residing within.

Exocytosis/endocytosis - Endocytosis is the process of capturing a substance or particle from outside the cell by engulfing it with the cell membrane, and bringing it into the cell. Exocytosis describes the process of vesicles fusing with the plasma membrane and releasing their contents to the outside of the cell

Table 1 Summary of diseases associated with intracellular bacteria

Bacteria	Associated disease	Host cells	Localisation inside host cells	Reference
Salmonella enterica	Typhoid and paratyphoid	Macrophages	Modified phagosome*	Gorvel and Méresse, 2001, Brumell *et al*., 2002
Mycobacterium tuberculosis	Tuberculosis	Macrophages	Phagosome, cytosol	Armstrong and Hart, 1971; Rohde *et al*., 2012, Watson *et al*., 2012
Chlamydia species	Ocular and genital infections	Conjunctiva and genital epithelial cells	Vacuole**	Kumar *et al*., 2006
Listeria monocytogenes	Listeriosis	Epithelial cells	Cytosol	Gaillard *et al*.,1987
Staphyloccocus aureus	Skin infections, mastitis, osteomyelitis	Keratinocytes, bovine mammary epithelial cells, osteoblast	Endosome, cytosol	Brouillette *et al.*, 2003 Fraunholz and Sinha, 2012
Escherichia coli	Urinary tract infections, mastitis.	Bladder epithelial cells, mammary epithelial cells	Vacuole	Dikshit *et al.*, 2015

* Modified phagosome also known as salmonella containing vacuole

** Vacuole also known as inclusion

Table 2 Summary of studies showing the promise of nanoparticles in the improvement of therapies against intracellular infections

Host cell/ organs	**Bacteria**	**Nanoparticle platform**	**Antibiotic**	**Outcome**	**Reference**
Macrophages	*Staphylococcus aureus*	Squanelene	Penicillin G (PenG)	NP-Pen G killed 87% and free PenG killed 56%	Semiramoth *et al*., 2012
THP-1 and HEK- 293 cells	*Staphylococcus aureus*	Chitosan	Tetracycline (Tet)	NP-Tet killed ~97% in THP-1 and ~95% in HEK 293 Free Tet killed ~83% in THP-1 and ~85% in HEK 293	Maya *et al*., 2012
Macrophages	*Mycobacterium tuberculosis*	Polyethylenimine coating mesoporous siica	Rifampicin (Rif)	NP-Rif reduced 3.3 log Free Rif reduced 1.6 log	Clemens *et al*., 2012
Lung epithelial Hep2 cells	*Chlamydia trachomatis*	Poly d-L-lactide-co-glycolide (PLGA) polymer	Rifampicin (Rif) or azithromycin (Azi)	NP- Rif reduced 40% and free Rif reduced 20% NP-Azi reduced 40% and free Azi showed no reduction	Toti *et al*., 2011
Murine Spleen and liver	*Brucella melitensis*	Poly ethylene oxide-b-sodium acrylate (PEO-b-PAA-+Na) and poly sodium acrylate (PAA-+Na) co polymers	Streptomycin (Strep) and doxycycline (Dox)	NP-Strep-Dox reduced 0.72 log in spleen and 0.79 in liver Free Strep-Dox reduced 0.51 log in spleen and 0.42 log in liver	Seleem *et al*., 2008

Murine Spleen and liver	*S. typhimurium*	$PAA^{-}\ ^{+}Na$-*b*-PEO-*b*-PPO-b-PEO-b-$PAA^{-}\ ^{+}Na$ block copolymers	Gentamicin (Gent)	NP-Gent reduced 0.29 log in spleen and 1.07 log in liver Gent only reduced 0.23 log in liver but not in spleen (0.34 increase)	Ranjan *et al*., 2009

Figure 1 The structure of PHMB. PHMB is a cationic polymer with a repeating 20-30 units of hexamethylene biguanide with average n=10-12.

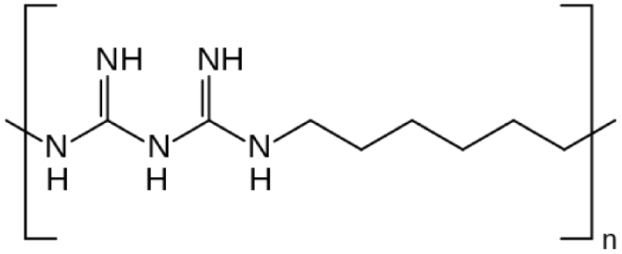

Figure 2 Intracellular location and bactericidal activities of nadifloxacin and PHMB against intracellular MRSA. (a) Colocalization of PHMB-FITC with EMRSA-15 in keratinocytes. Keratinocytes were infected with EMRSA-15 followed by treatment with PHMB-FITC (green). Keratinocytes were labeled with DAPI (blue) and WGA (red). Upper panels are images of infected cells and merged images. Lower panels are enlarged images that clearly show colocalisation between PHMB-FITC (green) and EMRSA-15 (blue). White scale bar is 25 μm **(b) Survival of EMRSA-15 within keratinocytes after treatment with nadifloxacin and PHMB**. Keratinocytes infected with strains EMRSA-15 were either untreated or treated with increasing concentrations of nadifloxacin or PHMB. Untreated cultures were used to establish cfu values corresponding to 100% survival.