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Strategies for the eradication of intracellular bacterial pathogens

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Intracellular pathogens affect a significant portion of world population and cause millions of deaths each year. They can invade and survive inside of host cells and are extremely resistant to immune systems and antibiotics. The current treatments are limited, and new effective therapies are needed to combat this ongoing heath challenge. Active research efforts have developed many new strategies to eradicate these intracellular pathogens. In this review, we focus on the intracellular bacterial pathogens and will first introduce several representative intracellular bacteria and their resultant diseases. We will then discuss the challenges in eradicating these bacteria and summarize the current therapeutics for intracellular bacteria. Finally, recent advances for intracellular bacteria eradication will be highlighted.

1. Introduction

Infectious diseases caused by pathogens, including viruses, bacteria, fungi, and parasites, are ranked as the second death cause by the World Health Organization. Of the 55.4 million deaths reported in 2019, 7.8 million (14%) deaths are due to infectious diseases such as AIDS, influenza, malaria and tuberculosis.[1] Of which, a significant portion of infectious diseases are caused by intracellular bacterial pathogens that are particularly challenging to be treated. A typical example is tuberculosis, caused by the intracellular *Mycobacterium tuberculosis (M. tuberculosis)*, which killed 1.6 million people and affected an estimated 10.6 million people worldwide in 2021.[2] The capability to invade and survive inside of host cells protects these intracellular pathogens from both antibiotics and the host immune systems and makes them extremely recalcitrant to be completely eradicated.[3-6] Even worse, cells infected by intracellular bacteria can act as "Trojan horses", delivering the bacteria to non-infected tissues. The sporadic re-dissemination of bacterial pathogens from these infected cells contributes significantly to treatment failure and recurring infections.

Despite the availability of many highly effective antibiotics against extracellular bacteria, the options for treating intracellular bacterial infections are very limited, due to the poor membrane permeability or dampened intracellular activity of most antibiotics.[7, 8] Therefore, alternative strategies such as new drug delivery system or new biotechnology like vaccines are needed. Currently, various drug delivery approaches and antimicrobial conjugates have been explored as potential alternative strategies to fight against these intracellular bacteria. In this review, we will first introduce several major intracellular bacterial pathogens and the challenges in eradicating them. We will then discuss the current treatment options for the diseases associated and highlight the recent advances in developing new strategies to eradicate intracellular bacteria. Finally, the perspectives of these strategies will also be discussed.

2. Intracellular bacterial pathogens

Various intracellular bacterial pathogens have been reported and they can be classified into either facultative or obligate intracellular bacteria (Table 1).[9] Facultative intracellular bacteria can survive and replicate both inside and outside host cells, with examples including *M. tuberculosis, Salmonella enterica (S. enterica)* and *Listeria monocytogenes (L. monocytogenes).* On the other hand, obligate intracellular bacteria such as *Chlamydia trachomatis (C. trachomatis)*, *Orientia tsutsugamushi* (*O. tsutsugamushi*) and *Coxiella spp* generally require a host cell for replication. In addition to these well-recognized facultative and obligate intracellular bacteria, increasing evidence has shown that some conventionally recognized extracellular bacteria such as *Staphylococcus aureus* (*S. aureus*) and *Escherichia coli* (*E. coli*) are able to invade, survive, and replicate in mammalian cells.[5, 6] The intracellular habitats, diseases associated, lethality, and epidemiology vary among these pathogens (Table 1). While some cause mild infection, others cause deadly diseases such as tuberculosis and listeriosis. Here, we will highlight four representative intracellular bacterial pathogens and their interaction with host cells.

M. tuberculosis. *M. tuberculosis* is a unique bacterium that does not fit into the definition of either Gram-positive or Gram-negative bacteria. Unlike Gram-positive bacteria, the peptidoglycan cell wall of *M. tuberculosis* is further covered by a lipid layer consisting of mycolic acids and trehalose-linked lipids.[10] This exceptional cell wall has permeability characteristics that enable *M. tuberculosis* to evade many antibiotics targeting the cell wall biosynthesis or other intracellular targets, contributing to its extraordinary drug-resistance

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to many antibiotics.[10] *M. tuberculosis* is known to transmit via inhalation of droplets containing the bacteria. Once reaching the pulmonary cavity, *M. tuberculosis* activates phagocytic receptors of alveolar macrophages and gains intracellular entry via phagocytosis.[11] The subsequent survival and replication in macrophages involve preventing the fusion of phagosome with endosome, and thus inhibiting the progression of the phagosome into an acidic, hydrolytically active lysosome.[11] Although phagosomes are the main reservoirs of intracellular *M. tuberculosis,* more recent studies demonstrated that certain *M. tuberculosis* strains can escape into the cytosol.[12, 13] Therefore, a complete eradication of intracellular *M. tuberculosis* requires antimicrobial agents to have sufficient accumulation and activity in both cytosol and phagosome.

S. enterica. *S. enterica* is a Gram-negative bacterium that infects 93.8 million people annually and leads to ~155,000 deaths per year.[14] *Salmonella* infection is contracted through the ingestion of contaminated water or food products. Once ingested, *Salmonella* uses its type three secretion system (T3SS) to breach the intestinal mucosa and infects a variety of intestinal epithelial cells and macrophages via micropinocytosis.[15] *Salmonella* mostly resides in the phagosomal compartment, which is better known as the '*Salmonella*-containing vacuole' (SCV).[16] Similar to *M. tuberculosis, Salmonella* survives by secreting effector proteins to prevent the fusion of SCV with lysosome, thereby avoiding lysosomal activities within macrophages.[15] Moreover, *Salmonella* can modulate the surface proteins of SCV to avoid the surveillance of intracellular inflammasome.[15] Recently, it has also been recognized that a subpopulation of *Salmonella* can escape the SCV and replicate within the host cytosol.[17, 18] However, the escape into cytosol exposes *Salmonella* to the detection of inflammasomes, which have been identified to play a key role in the early host response to *Salmonella*.

***C. trachomatis*.** *C. trachomatis* is a Gram-negative, obligate intracellular bacterium that affects 130-200 million people annually. Incidences are especially common in 20-24-year old male and 16-19-year old female.[19] It is the most common infectious cause of blindness and the most common sexually transmitted bacterium.[20] In women, 70–80% of genital tract infections with *C. trachomatis* are asymptomatic, but 15–40% ascend to the upper genital tract, which can lead to pelvic inflammatory disease, infertility and ectopic pregnancy.[20, 21] The life cycle of *C. trachomatis* is biphasic. Before host cell infection, *C. trachomatis* cells are termed elementary bodies, where the cells have a diameter of 200-400 nm and are encased by a rigid cell wall that allows them to survive outside of a host cell. However, upon invading into host cells, the elementary bodies differentiate into the replicative morphotype known as reticulate bodies and the cell size increases to 600-1,500 nm.[22] Reticulate bodies replicate inside vacuoles, but they eventually differentiate back to the elementary bodies and exit the host cell through extrusion, lysis or possibly other unknown mechanisms.[9, 20]

S. aureus. *S. aureus* is a Gram-positive bacterium that colonizes one-third of world population and is one of the leading causes of bacterial infections globally.[23] In addition to the commonly known skin infections, *S. aureus* also causes many life-threatening diseases such as endocarditis, osteomyelitis, necrotizing pneumonia, sepsis and other deep-seated abscesses in virtually every organ once invaded into bloodstream.[24] Although traditionally regarded as an extracellular bacterium, increasing evidence has shown that *S. aureus* can invade and survive inside of host cells.[25-27] *S. aureus* can either replicate within the acidic phagolysosome by inhibiting the fusion with lysosomes or escape into the cytosol in an α-toxin-dependent manner.[28-30] The intracellular survival of *S. aureus* is highly dependent on the *staphylococcal* genotype, the multiplicity of infection, the growth phase of the bacteria during infection, the susceptibility of host cells to virulence factors and the host cell gene expression.[29]

Table 1. Major intracellular bacterial pathogens and the diseases associated.

Pathogens	Obligate/ Facultative	Sub-cellular compartment	Disease associated	Epidemiology	Reference
M. tuberculosis	Facultative	Phagosome, cytosol	Tuberculosis	10 million incident cases and 1.2-1.5 million death each year	10-13
S. enterica	Facultative	Phagosome, cytosol	Typhoid and paratyphoid	93.8 million foodborne illnesses and 155,000 deaths per year	14-18
L. monocytogenes	Facultative	Cytosol	Listeriosis	0.1 to 10 cases per 1 million people per year and 15-20% mortality rate	9
C. trachomatis	Obligate	Vacuole	Genital infection and trachoma	130 million new genital infections annually and 40 million people with active trachoma	19-21
O. tsutsugamushi	Obligate	Cytosol	Scrub typhus	1 million infections per year	9
Coxiella spp	Obligate	Phagosome	Q fever	Ubiquitous in animals; potential for outbreaks among agricultural workers	9
S. aureus	Facultative	Endosome, cytosol	Skin infections, mastitis, osteomyelitis	Ubiquitous	22-28
E. coli	Facultative	Vacuole	Urinary tract infections, mastitis	Ubiquitous	5, 6

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3. Challenges in the eradication of intracellular pathogens

Precise and effective delivery of adequate quantity of antimicrobial agents into infected host cells is critical for the elimination of intracellular pathogens. To date, many antibiotics have been used clinically to treat infections caused by intracellular bacteria; however, complete eradication of intracellular bacteria still faces numerous challenges.[5, 31-33] Here, we discuss several major challenges in eradicating intracellular bacteria (Figure 1).

Insufficient intracellular accumulation. Poor membrane permeability and low intracellular accumulation of some antibiotics is one of the major reasons for the insufficient activity against intracellular pathogens, especially in the case of hydrophilic antibiotics such as aminoglycosides and glycopeptide antibiotics.[34-36] Intracellular bacteria reside in the phagosome and/or cytosol, and they are encased by at least one membrane barrier. To kill these intracellular bacteria, effective permeation through the membrane barriers and sufficient intracellular accumulation of antibiotics are essential. However, this is not always achievable for many antibiotics. Eukaryotic cell membranes, though showing good permeability to small lipophilic molecules, have poor permeability for hydrophilic molecules, especially for ionic molecules or those with molecular weight over 500 Da.[37] Unlike small lipophilic antibiotics (< 500 Da), such as β-lactams, macrolides and quinolones, which enter mammalian cell lipid bilayer via diffusion,[38] endocytosis or pinocytosis could be the major pathway for some hydrophilic or large antibiotics.[39] But this pathway requires antibiotics to escape from endosome before lysosomal degradation or exocytosis/transcytosis. Moreover, even if a portion of antibiotics can enter the cell, it is still difficult to eliminate intracellular bacteria. On the one hand, the phagosome or vacuole membrane will set another barrier; On the other hand, the short residence time and low intracellular accumulation due to the efflux pumps or rapid exocytosis will allow the intracellular bacteria to survive and grow back quickly. For example, some antibiotics like macrolides and quinolones can be quickly depleted by host P-glycoprotein efflux pumps before they can reach their minimum effective concentration.[40] Excessive doses of antibiotics may increase their intracellular accumulation, but various side effects and toxicities are inevitable. Insufficient intracellular accumulation results in incomplete clearance of bacteria, leading to chronic and unresolved infection.

Inactivation of antibiotic. Antibiotic activity can be affected by various intracellular factors such as pH, redox status, and enzymes. The structural integrity of antibiotics is critical to their antimicrobial activity; however, some antibiotics, such as penicillins and cephalosporins, have a β-lactam ring that can be cleaved by β-lactamase and lose their activity.[41, 42] Moreover, the relatively low phagosomal pH can also deactivate antibiotics. Despite most intracellular bacteria survive by inhibiting the fusion of phagosome with lysosome, the phagosomal pH is still relatively acidic. For example, *M. tuberculosis* phagosomes have a pH of 6.3-6.5.[43] Some intracellular bacteria, like *Salmonella*, reside in acidified phagosomes with pH of 4.0-5.0.[44] This low pH may negatively impact the structure and activity of antibiotics that have eventually gained access to intracellular bacteria. In fact, it has been reported that some antibiotics including ampicillin, cefalothin, cefamandole, cefazolin and cefotaxime, have significantly compromised antimicrobial activity against *Salmonella* at pH 5.2 compared to pH 7.2.[45]

Low susceptibility to dormant intracellular bacteria

Many antibiotics kill or inhibit bacteria by disrupting their normal metabolism pathways such as the synthesis of proteins, nucleic acids, and cell walls.[46] However, due to the unamiable intracellular environment, some intracellular bacteria may transform into a dormant state with low metabolism activity.[4, 47] For example, *M. tuberculosis* changes into a non-replicating state within the host cells and causes latent infection that is resistant to conventional treatment.[4, 48] Such physiological change significantly reduces their susceptibility to antibiotics. However, dormant bacteria can be activated and replicate rapidly within the cell under a favorable intracellular environment, leading to recurrence of infection.

Ineffective subcellular antibiotic localization. Effectively delivering antibiotics to the location where bacteria reside within host cells is of paramount importance in eradicating intracellular bacteria. However, different bacteria inhabit and survive in distinct subcellular compartments (such as vacuoles for *S. aureus* and cytosol for *S. typhimurium*).[49-51] Tulkens, Skold and Zon et al. investigated the cellular uptake and subcellular localization of a series of antibiotics.[52-56] Their reported that the aminoglycoside antibiotics exclusively localized in lysosomes but almost absent in other subcellular compartments, including the bacteria containing phagosomes. However, the enzymatic and acidic environment of lysosomes deactivates antibiotics, resulting in low antibacterial effect.[53] Recently, Gutierrez and coworkers studied the subcellular distribution of antibiotics in *M. tuberculosis*-infected human primary macrophages.[57, 58] They observed heterogeneous accumulation of pyrazinamide in intracellular compartments and the maximum accumulation was achieved in acidified phagosomes. However, *M. tuberculosis has developed mechanisms to* escape from phagosomes into neutral cytosol where pyrazinamide is inactive.[59, 60] Precise subcellular antibiotic localization remains challenging.

Antibiotic resistance. Due to the abuse of antibiotics, bacteria have developed resistance to antibiotic via different mechanisms, including reduced antibiotics uptake by changing the membrane permeability, inhibition of the interaction of antibiotics with targets by modifying the antibiotic targets, inactivating of antibiotics by

enzymatic modification or destruction, and efflux of antibiotics from bacterial cells through efflux pumps.[61, 62] Of which, efflux pumps are particular important in antibiotic resistance. Bacterial efflux pumps are membrane proteins that allows the microorganisms to remove toxic substances, including antimicrobial agents, metabolites and quorum sensing signal molecules. There are many different efflux pumps, including the ATP-binding cassette (ABC) superfamily, the major facilitator superfamily (MFS), the multidrug and toxic compound extrusion (MATE), the small multidrug resistance (SMR) family and the drug metabolite transporter (DMT) superfamily.[63] The up-regulated expression of efflux pumps then counteract the threat from antibiotics by effectively pumping them out.[61, 63]

In addition, the difficulty in antimicrobials discovery and development is also a real fact. To successfully and completely eliminate intracellular bacteria, new antimicrobials with enhanced intracellular accumulation, acidic stability and the capability to locate and kill bacteria in any state are needed.

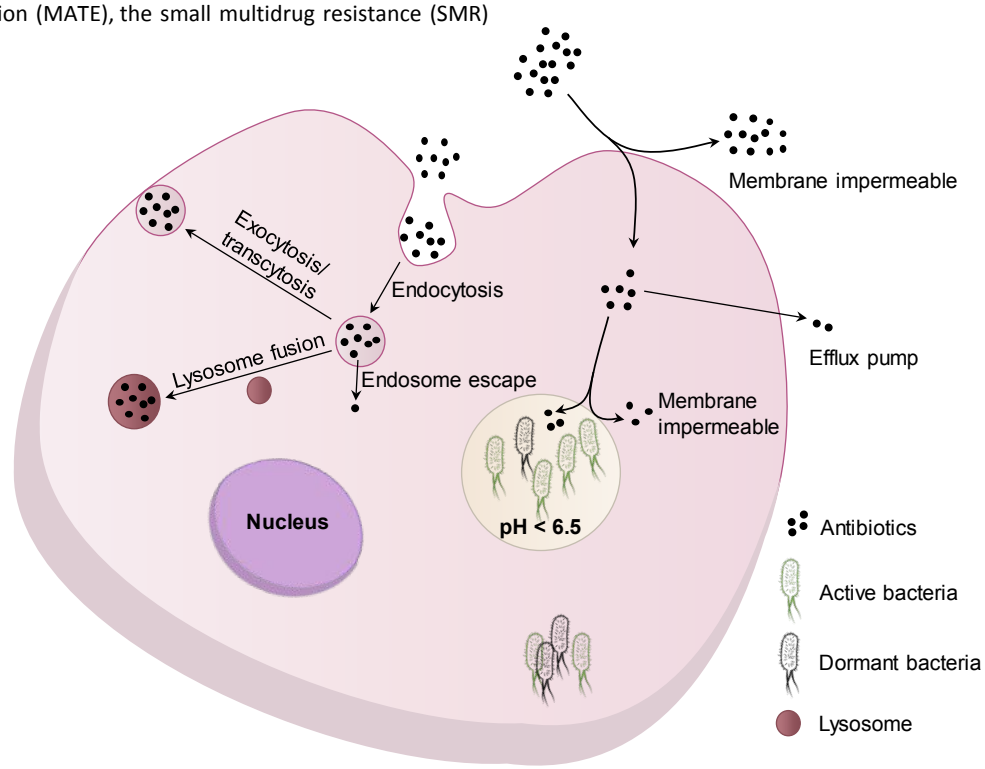

Figure 1. Membrane barriers, low metabolism of dormant bacteria and low phagosomal pH negatively impact the intracellular antibacterial activity of antibiotics.

4. Current antibiotic therapies

Currently, conventional antibiotics are still the first choice for intracellular bacterial infection treatment. A list of such antibiotics is shown in Figure 2a. It should be noted that the necessity of treatment and the types of antibiotics selected vary case-by-case. For example, the treatment of latent tuberculosis needs to choose one or two antibiotics from isoniazid, pyrazinamide, ethambutol and rifampin. However, for active tuberculosis, particularly if it is caused by a drug-resistant strain, a combination of several antibiotics is usually required and the inclusion of fluoroquinolones is recommended.[64, 65] The situation for *Salmonella* is even more complicated, as some studies suggested that improper choice of antibiotics can exacerbate the infection.[66, 67] Woodman and colleagues reported that children with salmonellosis are more likely to show prolonged excretion and clinical relapse if treated with ampicillin or amoxicillin, compared to those who were given placebo.[68] Therefore, antimicrobial therapies should be carefully selected and only be given to patients with severe illness or patients with risk factors for extraintestinal spread of infection.[66] Therefore, a case-by-case selection of antibiotics is also recommended for diseases caused by intracellular bacterial pathogens.

A closer structure analysis revealed that the majority of antibiotics that have been proven to be effective against intracellular bacteria are either small molecular antibiotics (100-300 Da), such as isoniazid, pyrazinamide, ethambutol, sulfamethoxazole, chloramphenicol and beta-lactams, or antibiotics with relative lipophilic structures, such as fluoroquinolones, tetracyclines, macrolides, and rifampin (Figure 2b). These small molecular or lipophilic antibiotics usually have good membrane permeability and can accumulate intracellularly. However, though these antibiotics have relatively higher intracellular antibacterial activity than other antibiotics, their intracellular activity is significantly decreased compared with their activity extracellularly. Increasing the hydrophobicity of antibiotics has been proven to be an effective strategy to optimize their intracellular accumulation. By rendering antibiotics more hydrophobic, the enhanced membrane permeability allows them to diffuse across the lipid bilayer more efficiently. The hydrophobic nature facilitates antibiotics to partition into the lipid bilayer, thereby elevating their local concentration proximal to the cell membrane and accelerating their diffusion. Recently, various hydrophobic derivatives of commercially available antibiotics have been developed. For example, telavancin, a hydrophobic derivative of vancomycin, showed enhanced activity against intracellular *S. aureus* compared to vancomycin.[69] Similarly, the intracellular antibacterial activity of rifalogue, a lipophilic derivative of rifampicin developed by Genetech, was improved by over 1000-fold.[25] To note, the intracellular accumulation rifalogue is over 100-fold higher than rifampicin.[25] However, it's also important to note that excessive hydrophobicity can lead to some problems, such as poor solubility, reduced bioavailability, and potential toxicity. The optimization of hydrophobicity should be carefully considered.

Though the above hydrophobic antibiotics offer numerous short-term benefits, intracellular bacteria can soon gain resistance. Besides, hydrophobic antibiotics did not show superior antimicrobial effects for dormant bacteria. Therefore, developing new therapies for intracellular bacteria is still intriguing.

5. Antibiotic delivery systems

Challenges for the treatment of intracellular pathogens partially lies on the poor membrane permeability of antibiotics and the emergence of multidrug resistance (MDR).[5, 70] Further challenge is the severe side effects associated with an overly high dose required for therapeutic efficacy and recurrent infection.[71, 72] The development of efficient intracellular drug delivery systems is then emerged as a promising approach.[73] Significant amount of research has designed various antibiotic delivery systems and some of them have demonstrated good therapeutic efficacy for clinical translation.

Lipid nanoparticles. Lipids have been extensively studied and used as drug-carriers, due to their low toxic and non-immunogenic membrane originality, and the adaptivity to encapsulate various drugs of different properties.[74-76] Lipid vesicles for antibiotics encapsulation have been used for the treatment of intracellular infections.[77, 78] Among lipid nano-carriers, liposomes have been widely used because they can easily fuse with bacterial cell membranes, thereby releasing high doses of antibacterial drugs directly inside the bacteria.[79] Some liposomal products are currently in clinical research, such as AmBiosome® and MiKasome®.[80] Lehr, Loretz and co-workers developed colistin-loaded liposomes whose surface is functionalized with extracellular adherence protein (Eap), an invasive moiety derived from *S. aureus*.[81] These liposomes enhance the intracellular delivery of colistin and significantly reduce the intracellular bacterial burden in both HEp-2 and Caco-2 cells that are infected with *S. enterica*. Targeted delivery of antibiotics is important for the intracellular infection treatments because it can efficiently internalize encapsulated antibiotics.[82] For instance, gentamicin-loaded liposomes with mannose decoration has been proved to be more effective in killing intracellular bacteria.[83] Similarly, Yang and co-workers developed a type of active-targeting lipid nanoparticles (NP-Antibiotic@EV) for antibiotic delivery to eliminate the intracellular *S. aureus* (Figure 3a).[84] In this work, the antibiotic-preloaded PLGA nanoparticles were coated with the membrane of extracellular vesicles (EVs) secreted by *S. aureus*, which contain the *S. aureus* antigens. These nanoparticles can be internalized at higher efficiency by *S. aureus*-infected macrophages. They found that these nanoparticles, when administrated intravenously into a *S. aureus*-infected mouse model, exhibit considerable accumulation in the infected organs and can significantly reduce the bacterial load. More interestingly, by switching the coating membrane to the outer membrane vesicle (OMV) secreted by *E. coli*, the resulting NP@OMV nanoparticles can actively target *E. coli*-infected macrophages, but not *S. aureus* infected-ones, suggesting the selectively of the designed nano- for specific intracellular pathogens. In addition, a novel gentamicin-coated phosphatidylcholine-chitosan nanoparticle delivery system (GPC NPs) also showed good treatment effects. It not only inhibited the biofilm formation of Gram-positive and Gram-negative microorganisms with different maturity, but also effectively eliminated intracellular bacteria in infected RAW264.7 cells with a 20 μg/mL gentamicin.[85] In addition to liposome and nanostructured lipid carriers, solid lipid nanoparticles (SLNs) are also promising for antibiotic delivery.[86, 87] For instance, doxycycline-encapsulated SLNs showed improved efficacy to clear *B. melitensis* infection. More importantly, this SLN enhanced the antibacterial efficacy of

doxycycline in the treatment of both acute and chronic brucellosis infections and prevented its recurrence *in vivo* simultaneously.[86, 88]

(a)

Intracellular bacteria	Antibiotic treatment options
M. tuberculosis	Isoniazid, pyrazinamide, ethambutol, rifampicin, and/or fluoroquinolones
Salmonella	Ampicillin, amoxicillin, and/or trimethoprim-sulfamethoxazole
Listeria	Ampicillin, penicillin G, and/or gentamicin
C. trachomatis	Azithromycin, doxycycline, and/or amoxicillin
O. tsutsugamushi	Tetracyclines, chloramphenicol, macrolides, and/or rifampicin

(b)

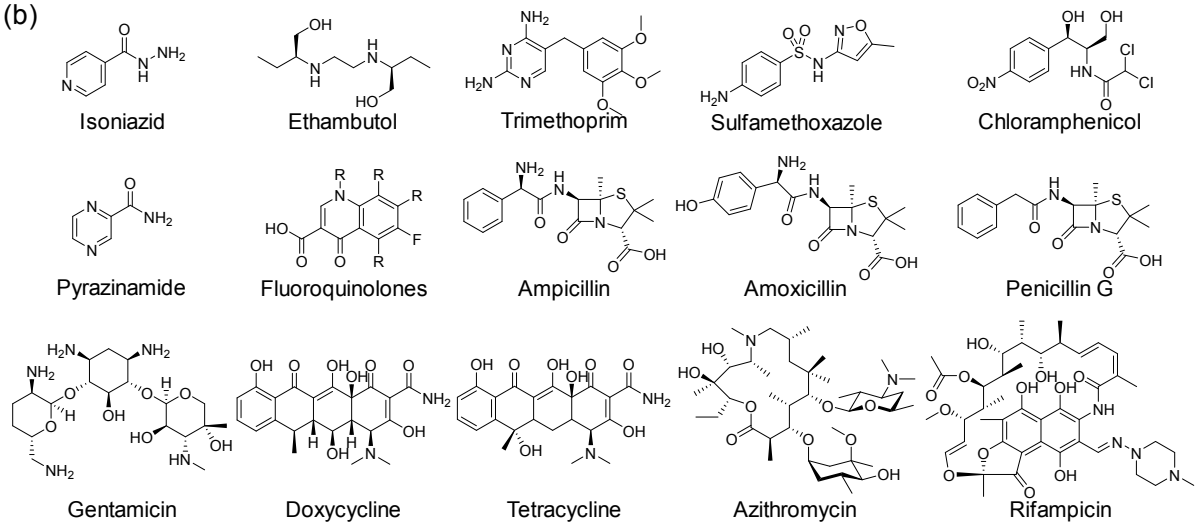

Figure 2. (a) Table for intracellular bacterial pathogens and their corresponding antibiotic treatment options. (b) Structure of antibiotics used in intracellular bacterial eradication therapy.

Polymeric nanoparticles (PNPs). Polymeric nanoparticles are considered as a promising candidate for antibiotic delivery because of their biocompatibility, structural diversity, and potential biomimetic properties, etc.[89] Rationally designed PNPs can efficiently deliver drugs to location of interest and can controllably release drug cargo at patients' demand, which may provide an effective treatment to the recalcitrant intracellular bacterial infection.[90, 91] Scott et al., developed a series of gentamicin-loaded PLGA nanoparticles (GNPs) with high drug-loading efficiency (13.5% w/w) using a water-in-oil-in-water formulation strategy.[92] They demonstrated that these GNPs, after phagocytosed by *K. pneumoniae*-infected macrophages and transported to the intracellular bacteria reservoir, dramatically reduce the viability of intracellular bacteria without concomitant stimulation of pro-inflammatory or pro-apoptotic pathways. In fact, PLGA nanoparticles have also been previously used by Panyam, Whittum-Hudson and co-workers for the delivery of antibiotics to eradicate intracellular *chlamydial*.[93] In addition, a GRAS-approved (Generally Recognized as Safe by the United States Food and Drug Administration) natural antimicrobial polymer, chitosan and its derivatives has been widely used in antibacterial.[94] Hollow chitosan-dextran sulphate (CD) nanocapsules, prepared by layer-by-layer (LbL) deposition on a sacrificial silica nano-template, were also explored as an antibiotic delivery system to treat intraphagosomal pathogens. For example, when loaded with ciprofloxacin, CD nanocapsules can efficiently target and clear *Salmonella* infection.[95] Recently, a novel acid-transforming chitosan (ATC), soluble in neutral conditions but insoluble in the mildly acidic intracellular compartment, was designed and proved to treat *S. Typhimurium* infection. More interestingly, when ATC was complexed with fragmented DNA (fDNA), the resulting nano-sized spherical polyplexes can effectively eradicate intracellular *S. Typhimurium* in RAW264.7 macrophages.[94] More recently, Li et al. reported a type of traceable and enzyme-responsive nanoparticles for intracellular antibiotic delivery and tracking (Figure 3b).[96] In their work, mannose-grafted polymers containing enzymes-responsive moieties and tetraphenylethylene segments (mPET) were assembled into nanoparticles and loaded with the conjugate of deferoxamine-ciprofloxacin-Fe^{3+} ($D_{Fe}C$). Before entering the cell, the aggregation-induced emission (AIE) of mPET is quenched by $D_{Fe}C$ in the nanoparticle. After mannose-mediated endocytosis, the nanoparticles are degraded by lipase and phospholipase to release mPET and $D_{Fe}C$. The intensity of AIE can be used to monitor the antibiotic release profile. *S. aureus*-infected mice showed 100% survival rate after the treatment with the designed system.

Mesoporous silica nanoparticles (MSNs). Mesoporous silica nanoparticles possess honeycomb-like porous structure and high surface area. They can encapsulate relatively larger amounts of molecules compared to the solid nanoparticles. Moreover, if a stimuli-responsive gate is incorporated, MSNs can achieve trigger-responsive drug release.[97] Consequently, MSNs have received significant attention as an efficient nanocarrier for drug delivery.[98] The use of MSNs for intracellular antibiotic delivery was also explored.[99, 100] For example, when the ciprofloxacin is encapsulated into arginine-decorated nanoparticles (Cip Arg-MSN), the nanoparticles exhibited two-fold higher intracellular antibacterial activity than the ciprofloxacin alone in both macrophage and epithelial cell models infected with *Salmonella*.[101] Tang et al.

designed gentamicin-loaded MSNs coated with infected microenvironment-responsive lipid bilayers and a bacteria-targeting peptide $UBI_{29–41}$ (Gen@MSN-LU) (Figure 3c). The nanomaterial can significantly inhibit the growth of planktonic and intracellular *S. aureus*.[102] Another study found that the rifampicin-loaded MSNs, compared to the free rifampicin, have superior uptake by macrophages that were infected by small colony variants (SCV) of *S. aureus* and can kill intracellular SCV efficiently.[103]

Inorganic solid nanoparticles. Inorganic solid nanoparticles, with a high specific area that can be used for drug conjugation, have also been studied for intracellular bacteria eradication. Moreover, some oxides and metals, like titanium dioxide, copper and silver, have intrinsic antimicrobial activity and are particular attractive to be used as antimicrobial agents for intracellular bacteria treatment.[104-108] Ruoslahti et al. developed vancomycin-loaded silver nanoparticles conjugated with a cyclic 9-amino-acid peptide CARGGLKSC (CARG) that can specifically bind to *S. aureus*. The vancomycin-AgNPs-CARG selectively accumulates in *S. aureus*-infected tissues and cells, and remarkably improves the survival of *S. aureus*-infected mice, but not the survival rate of *Pseudomonas*-infected mice.[109] Compared with silver, gold nanoparticles do not have intrinsic antibacterial activity, but it can be used in combination with other antimicrobials or through reasonable surface modification to obtain antibacterial activity.[110] For instance, a C-terminally hexahistidine-tagged antimicrobial peptide, A3-APO^{His}, was loaded onto gold nanoparticles conjugated with His-tag DNA aptamer (AuNP-Apt^{His}) (Figure 3d). It was demonstrated that this type of nanoparticles can completely inhibit the colonization of *S. Typhimurium* in the infected mice organs and results in 100% survival rate.[111] Bhunia et al. found that the conjugate of gold nanoparticle and an antimicrobial peptide VG16KRKP (VARGWKRKCPLFGKGG) can efficiently kill intracellular *S. Typhi* in both epithelial and macrophage cells.[112] In addition to being used alone, the hybrid silver-gold nanoparticles has also been proved to not only improved the dispersion stability and activities of silver but also showed the combinatorial effect. Niidome et al. prepared one-layer gold atoms-coated silver nanoplates. They showed strong antibacterial activity against intracellular *S. typhimurium residing in RAW264.7*.[113] In addition, Sliver-coated gold hybrid nanoparticles also showed great potential for ROS mediated killing of a broad range of drug resistant bacterial strains. They are also potential antimicrobial agents to combat biofilm formation and eliminate intracellular infections.[105]

Nano-MOFs. Nanoscale metal organic frameworks (nanoMOFs) have emerged as a class of versatile, biodegradable, and nontoxic drug nanocarriers due to their high porosity, drug loading capacity, good biocompatibility, and tunable functionality.[114, 115] The antimicrobial activity of various MOF systems has been investigated.[116] Wang et al. designed a pH-responsive MOF/antibiotic three-in-one delivery system, tetracycline (Tet)@ZIF-8@hyaluronic acid (HA), for the efficient and targeted elimination of intracellular bacteria. HA can specifically bind to the cell-surface CD44 antigen receptors and promote cellular uptake. A clearance rate of the intracellular *S. aureus* was reported to be over 98% after treatment with these nanocomposites.[117] Co-encapsulation of multiple drugs into one MOF nanoparticle remains to be challenging, due to the complex preparation process and the mutual inhibition. It was found that when some drugs are co-encapsulated, they would impede each other and dramatically reduce the loading efficiency. To address this challenge, nanoMOFs with two distinct “compartments” or mesoporous cages were prepared based on porous iron (III) trimesate (Figure 3e).[118] The mesoporous iron carboxylate nanoMOF can efficiently co-encapsulate amoxicillin and potassium into different compartments, whose diameters are 24 and 27 Å, respectively.[119] Notably, the nanoMOFs alone show some antibacterial properties. Together with drugs, the drug-loaded nanoMOFs can significantly reduce the intracellular bacteria.[119] More recently, Haag et al. designed a group of MOF-derived 2D carbon nanosheets (2D-CNs) modified with phase transformable thermally responsive brushes (TRB) to fabricate the TRB-ZnO@G.[120-123] This system combined the extraordinary photothermal conversion capability of 2D graphene and the chemically tunability of MOF nanomaterials to achieve local multiple therapeutic modalities to fight pathogenic bacteria. Notably, TRB-ZnO@G can form 2D-CNs-bacteria aggregations upon near-infrared irradiation, which can enhance the Zn^{2+} ion penetration, physical cutting and thermal effects. Destruction of bacterial membranes and intracellular substances was thus synergistically improved while not causing normal skin tissues damages and accumulative toxicities.

In addition, dendrimers were also explored to be used for targeted antibiotic delivery.[124, 125] Govender et al. designed pH-responsive lipid–dendrimer hybrid nanoparticles (LDH-NPs) that can delivery vancomycin to the site of infection and reach significant clearance of intracellular bacteria (Figure 3f).[126] Cationic antimicrobial peptides and cell penetrating peptides, when administrated as an antibiotic adjuvant, have also demonstrated improved antibiotic delivery efficacy.[127, 128]

Although nanoparticles have the potential to be used as antibacterial agents, their potential toxicity cannot be ignored. Many factors, such as nanoparticle size, shape, agglomeration state, surface functionalization, and exposure duration can influence their toxicity.[129, 130] The chitosan coated nanoparticles have been suggested to be toxic, and its toxicity is associated with the molecular weight (MW) and acetylation degree of surface-coated chitosan.[131, 132] Silver-nanoparticles have been shown to cause mitochondrial dysfunction and cell death by affecting the metabolic activity and generating ROS, which mainly due to the unleashed silver ions.[133] Moreover, nanoparticles’ toxicity mechanisms can also be affected by various factors, such as size and shape, as exemplified by cell death caused by 1-nm gold nanoparticles through necrosis and apoptosis, although gold nanoparticles with larger sizes have commonly been considered as inert and safe.[134, 135] Therefore, the comprehensive evaluation of nanoparticle’s toxicity and the balance between therapeutic potential and adverse effects are crucial for further development.

6. Antimicrobial conjugates

In addition to the antibiotic delivery systems, various antimicrobial conjugates, such as antibody-antibiotic conjugates and cell-penetrating peptide (CPP)-antibiotic conjugates, have also been developed and evaluated, with the aim of improving the membrane permeability, intracellular antibacterial efficacy, pharmacokinetics

(PK) and pharmacodynamics (PD) of free antibiotics. The significant advantage of antimicrobial conjugates over the drug delivery systems is the simplified composition, which allows the easier and more accurate control of PK/PD profile. Moreover, the conjugate strategy usually combines two different functional components into one entity and allows them to function synergistically.

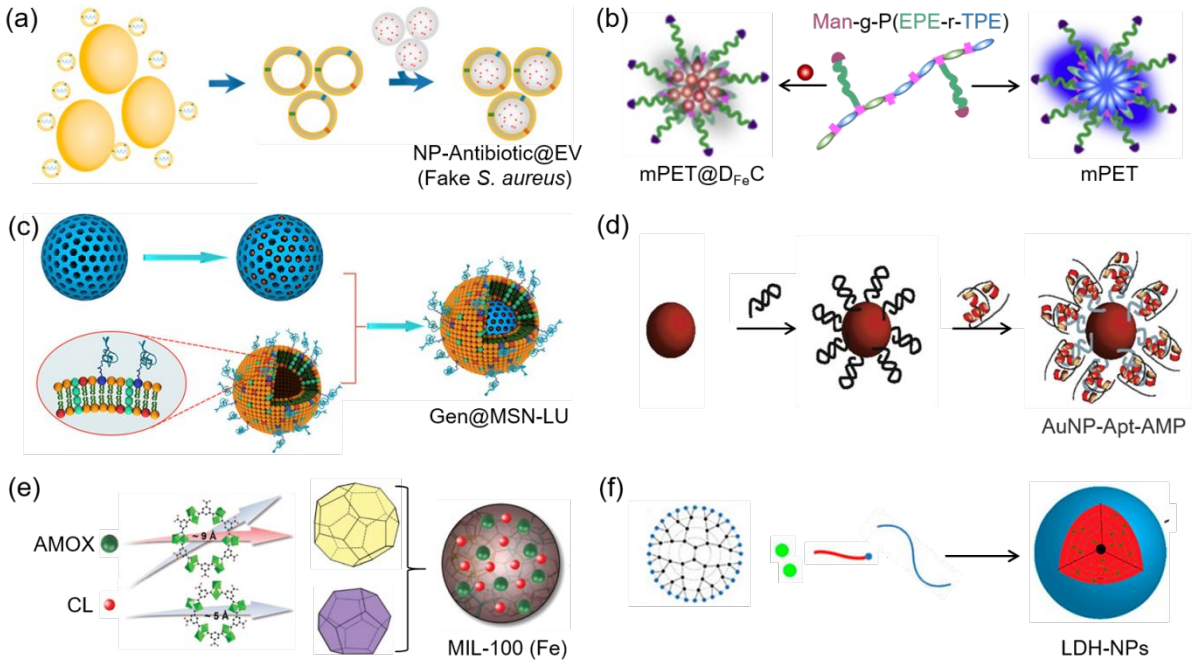

Figure 3. Antibiotic delivery systems. (a) Extracellular vesicle (EV)-coated nanoparticles (NP-antibiotics@EV) for the intracellular delivery of antibiotics to selectively eradicate intracellular *S.aureus*.[84] Adapted with permission from ref. 84. Copyright 2019, American Chemical Society. (b) Enzyme responsive polymer nanoparticles (mPET@D_{Fe}C) with deferoxamine-ciprofloxacin-Fe^{3+} (D_{Fe}C) and tetraphenylethylene molecules for traceable intracellular delivery of antibiotics.[96] Adapted with permission from ref. 96. Copyright 2020, Elsevier. (c) Gentamycin-loaded mesoporous silica nanoparticles (MSN) coated with a lipid bilayer containing a bacteria-targeting peptide (Gen@MSN-LU) were used for intracellular antibiotic delivery.[102] Adapted with permission from ref. 102. Copyright 2018, American Chemical Society. (d) Gold nanoparticles functionalized with DNA aptamer and antimicrobial peptide for the eradication of intracellular bacteria.[111] Adapted with permission from ref. 111. Copyright 2016, Elsevier. (e) Nano MOFs with two distinct compartments for the intracellular delivery of antibiotics.[119] Adapted with permission from ref. 119. Copyright 2019, John Wiley and Sons. (f) pH-responsive lipid–dendrimer hybrid nanoparticles (LDH-NPs) for the intracellular delivery of vancomycin.[126] Adapted with permission from ref. 126. Copyright 2019, American Chemical Society.

6.1 Antibody-antibiotic conjugates

Inspired by the recent success in the development of antibody-drug conjugate (ADC) for cancer treatment,[136] one attractive therapeutic approach with the potential to treat bacterial infections is the development of antibody–antibiotic conjugate (AAC),[25, 137-142] which combines the key attributes of both antibody and antibiotic in one single molecule. Specifically, AACs possess the antibacterial activity of antibiotics, and the specificity and high affinity of antibodies. They also have improved absorption, distribution, metabolism, and elimination (ADME) properties and longer *in vivo* circulation half-life. Recently, Lehar, Mariathasan and coworkers designed a type of AAC where the antibiotic rifalogue (dmDNA31) is conjugated to a monoclonal THIOMAB™ antibody that can specifically bind to cell wall teichoic acid of *S. aureus* (Figure 4). The antibody and antibiotic are connected by a linker that is responsive to the phagolysosomal protease. The resulting AAC does not diffuse into mammalian cells by itself and has no direct antibacterial activity when bound to planktonic *S. aureus*.[25] However, when AAC-opsonized bacteria are internalized by host cells, the intracellular proteases cleave the linker and release the activated antibiotic. Because many AACs are capable of binding to a single bacterium and the high antibacterial activity of rifalogue, the intracellular antibiotic concentration is high enough to completely eradicate intracellular *S. aureus*. Excellent *in vitro* and *in vivo* intracellular antibacterial activities were demonstrated, and good efficacy in rescuing mice intravenously infected with *S. aureus* was achieved. Later, Mariathasan and Tan demonstrated this AAC was able to effectively reduce the pathogen loads compared with two conventional antibiotics currently used to treat refractory S. aureus infection in mouse infection model.[137] Kamath et al. focused on the PK and PD of this kind of THIOMAB™ antibody-antibiotic conjugate (DSTA4637S, developed by Roche/Genentech) and found that DSTA4637A (a liquid formulation of DSTA4637S) has typical monoclonal antibody PK behavior in both non-infected and *S. aureus*-infected mice, with improved PK and PD profiles compared to free antibiotic rifalogue.[138] Recently, they not only observed same monoclonal antibody-based therapeutic in complicated rats and monkeys models, but also developed an integrated PK model. This model effectively elucidated the PK behavior of DSTA4637A in mice, rats and monkeys, and displayed a reasonable capability to predict PK in human.[143] Excitingly, DSTA4637S was investigated in two phase I trials and completed in 2020.[144]

6.2 Cell penetrating peptide-antibiotic conjugates

As poor membrane permeability is one of the major players that limit the intracellular accessibility of antibiotics, increasing the membrane permeability has been regarded as a promising approach to increase their intracellular antibacterial activity. Therefore, there has been a growing interest in developing CPP-antibiotic conjugates, based on the rationale that the membrane permeable CPPs can bring the conjugated antibiotics into the host cells, either by direct membrane penetration or by enhanced endocytosis, to kill the intracellular pathogens. Currently, a variety of CPP-antibiotic conjugates have been reported. They are either based on natural CPPs,[145-147] such as oligoarginines and TAT, or synthetic CPPs,[146, 148-155] such as P14LRR and mitochondria targeting peptides. In early work, Wender, Mcleod and coworkers developed several CPP-antibiotic conjugates by ligating triclosan to octaarginine via a hydrolyzable glutaric anhydride linker and evaluated their activity against intracellular parasite *Toxoplasma gondii* bradyzoites (Figure 5a).[146] The conjugate Tr8 is significantly more active than triclosan alone in killing *T. gondii in vivo*, and it can kill ~80% of *T. gondii* at 12.5 µM. Moreover, they also demonstrated that conjugates with a hydrolysable linker are more active than those with a non-releasable linker.

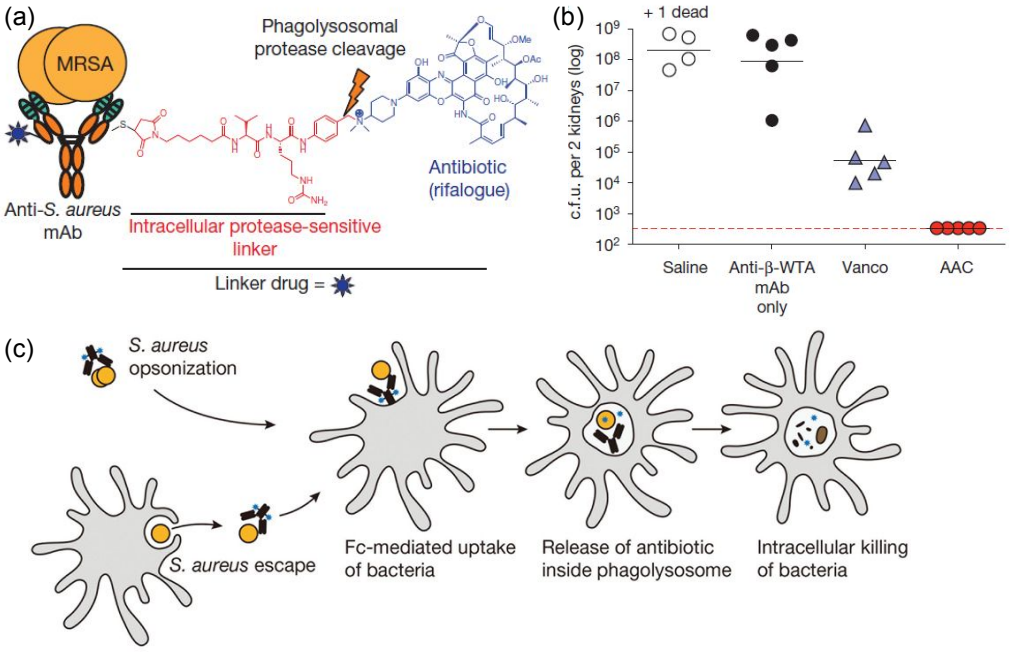

Figure 4. Antibody-antibiotic conjugate (AAC) for effective eradication of intracellular *S. aureus*. (a) Model of AAC. (b) *In vivo* activity of AAC in a mouse intravenous infection model. Wild-type mice were treated with saline, anti-β-WTA antibody used in the AAC (monoclonal antibody (mAb)), vancomycin, or anti-MRSA AAC. (c) Mechanism of AAC action. [25] Reprinted with permission from ref. 25. Copyright 2015, Springer Nature.

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Kelley and coworkers conjugated methotrexate (Mtx), an inhibitor of bacterial dihydrofolate reductase (DHFR), to a series of synthetic CPPs with alternating hydrophobic (cyclohexylalanine and/or phenylalanine) and cationic residues ($_D$-arginine) for the eradication of *L. monocytogenes* (Figure 5b).[148] It was demonstrated that these conjugates could penetrate into the Hela cells and specifically co-localize with the intracellular *L. monocytogenes.* By optimizing the structure of mitochondria-targeting CPPs, they designed a conjugate that is 10-fold more active than their initial candidate. This optimized conjugate could kill ~80% of intracellular *L. monocytogenes* at 10 μM. Moreover, the conjugate could also act as a prodrug to reduce the non-specific cytotoxicity of Mtx. Later, the same group designed another conjugate that was responsive to the β-lactamase secreted by intracellular *mycobacteria* for targeted eradication of intracellular mycobacteria.[150] This conjugate displayed low cytotoxicity and good activity against intracellular *M. smegmatis*. It could kill ~95% of intracellular *M. smegmatis* at 2 μM.

Chmielewski and coworkers reported another type of CPP-antibiotic conjugates in which kanamycin is attached to a synthetic cationic polyproline helix P14LRR via a disulfide linker (Figure 5c).[151] The disulfide linker was responsive to intracellular reducing environment and allowed the releases of free kanamycin upon getting into the cell. Meanwhile, the P14LRR by itself also had some antimicrobial activity and ad been previously reported to be active against *Salmonella typhimurium* and *Brucella abortus* (60-90% of killing at 15 μM).[152] Decent intracellular antimicrobial activity (95% of killing at 10 μM) was demonstrated for conjugate P14KanS, and it was more active than P14LRR, kanamycin or 1:1 mixture of P14LRR and kanamycin. Interestingly, the releasable P14KanS was more active than the non-releasable conjugate P14KanC, underscoring the importance of maintaining the free form of some drugs. *In vivo* efficacy was demonstrated in a *Caenorhabditis elegans* model infected with *Salmonella enteritidis*. It was reported to achieve 90% of killing at 60 μM.

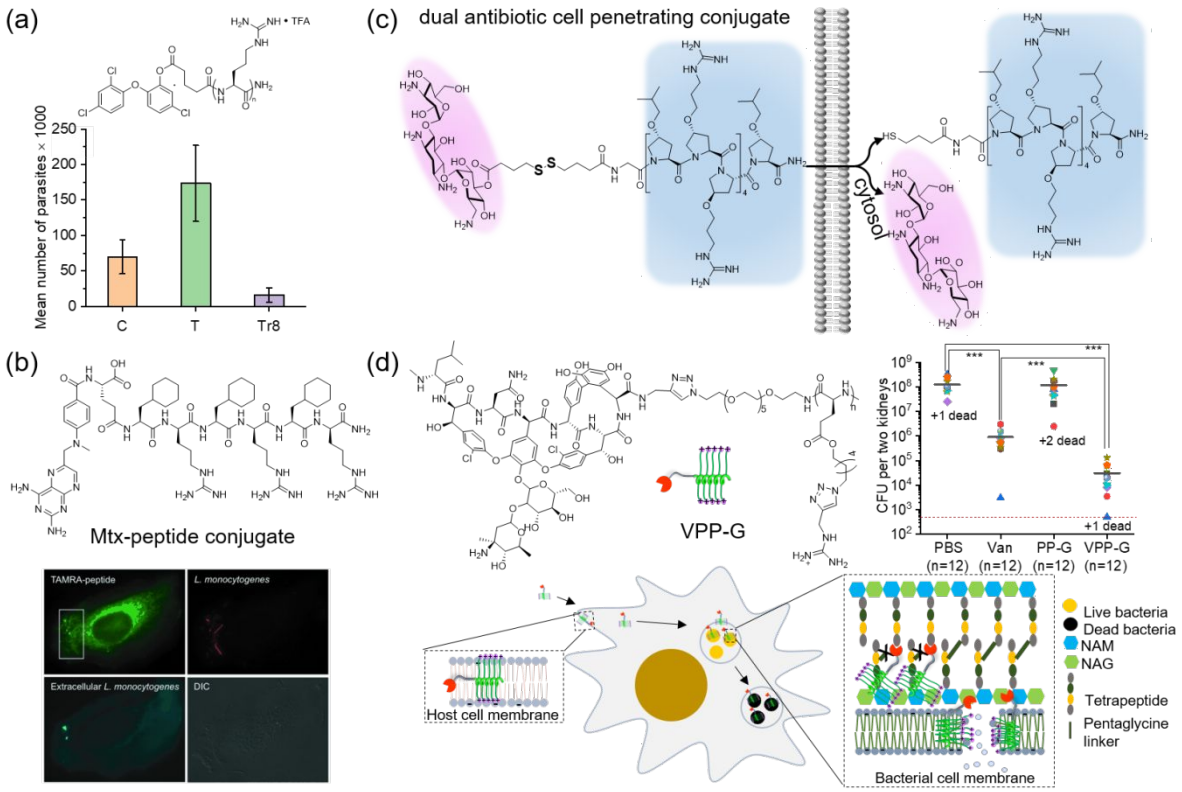

Figure 5. Representative CPP-antibiotic conjugates for the eradication of intracellular pathogens. (a) Octaarginine-triclosan conjugate (Tr8) significantly reduce intracellular parasite *T.gondii* in a mouse intraperitoneal infection model at 12.5 μM.[146] T: triclosan (12.5 μM); C: PBS. Adapted with permission from ref. 146. Copyright 2003, National Academy of Sciences. (b) Mtx-peptide conjugate for eradication of intracellular *L. monocytogenes*.[148] Fluorescence images show that the fluorescently labelled peptides (shown in green) co-localize specifically to mitochondria and *L. monocytogenes* (shown in red) in HeLa cells. Adapted with permission from ref. 148. Copyright 2013, John Wiley and Sons. (c) Cleavable cell penetrating peptide P14LRR-kanamycin conjugate for eradication of intracellular bacteria.[151] Adapted with permission from ref. 151. Copyright 2018, American Chemical Society. (d) Metaphilic cell penetrating peptide-vancomycin conjugate, VPP-G, efficiently eradicates intracellular *S. aureus* both *in vitro* and *in vivo* via a dual antimicrobial mechanism. The dual antimicrobial mechanism, structure and in *vivo* efficacy of VPP-G are shown.[156] Adapted with permission from ref. 156. Copyright 2020, American Chemical Society.

More recently, Cheng, Luijten and co-workers reported another class of CPP-antibiotic conjugate, VPP-G, that has high membrane permeability and intracellular antimicrobial activity.[156] Unlike most CPP-antibiotic conjugates reported so far, which are usually based on arginine-rich CPPs with relatively low membrane permeability, the conjugate reported by Cheng is based on a "metaphilic" CPP that has long flexible side chains and very high membrane permeability (up to 20 times more membrane penetrative than TAT).[157-159] The conjugate penetrates the host cell membrane directly via a unique "metaphilic" membrane penetrating process, which is enabled by the capability of these long flexible side chains to adapt to different microenvironment (hydrophilic, amphiphilic and hydrophobic) by being metaphilic, rather than static amphiphilic.[160] The conjugate exhibits excellent *in vitro* antimicrobial activity against intracellular *S. aureus* (99.9% of killing at 9 μM). More interestingly, this conjugate was proved to have a dual antimicrobial mechanism: disruption of bacterial membrane and inhibition of cell wall biosynthesis. This dual mechanism prevented bacteria from developing drug resistance and which assisted the eradication of dormant bacteria. Significantly, this conjugate demonstrated excellent *in vivo* activity against intracellular *S. aureus* in a mouse intravenous infection model.

Recent studies suggested that CPP-antibiotic conjugates have enhanced intracellular antibacterial activity compared to either CPP or antibiotics alone, or the mixture of CPP and antibiotics. While some of them demonstrated decent intracellular antimicrobial activity, the majority of them have sub-optimal intracellular activity requiring further improvement for potential clinical translation. Moreover, the *in vivo* efficacy to treat infectious diseases caused by intracellular bacteria has not been sufficiently evaluated. Further understanding and success in clinical translation remain to be achieved so far.

6.3 Antimicrobial peptides and their conjugates

Antimicrobial peptides (AMPs), also known as host defense peptides (HDPs), have emerged as promising alternatives to conventional antibiotics and garnered significant attention due to their broad-spectrum antibacterial activity and the potential to avoid antibiotic resistance.[161-164] Compared with conventional antibiotics, which usually have highly specific targets, AMPs and their mimics act on the plasma membrane or multiple intracellular targets of pathogenic bacteria, exhibiting potent activity against both extracellular and intracellular drug-resistant bacteria.[165-167] Recently, Feng, Bai and co-workers designed an oligoguanidine-based peptidomimetic that can precisely target and eliminate intracellular *S. aureus* located in the phagolysosome lumen, and is active to its dormant state.[168] Moreover, synergistic antibacterial effects can be achieved by conjugating AMP with other compounds, including CPPs or antibiotics.[169-172] Wang and coworkers constructed two CPP-AMP conjugates (B6N2 and T11N2) and found that these conjugates mainly locate in endosomes of RAW264.7 macrophage cells. Moreover, these conjugates exhibited enhanced activity against intracellular *S. typhimurium* as compared to AMP alone or non-conjugated mixtures.[173, 174] AMP-based therapies provide a promising platform to eradicate the intracellular bacteria; however, their therapeutic efficacy and clinic development are limited by high cost, rapid degradation, systemic toxicity and other side effects. Therefore, the design and optimization of AMP and mimics still require ongoing efforts.

7. Summary and Perspective

Infectious diseases caused by intracellular pathogens pose a major threat to human health. Alarmingly, in addition to these well-recognized facultative or obligate intracellular pathogens, some conventional extracellular bacteria have also been found to be capable of invading and surviving inside of host cells. More members of this group of bacteria could be revealed through active research. Invasion into the host cells protects these pathogens from the attack of both immune systems and conventional antibiotics, making them particularly recalcitrant to be eradicated. New, effective therapies are needed to eradicate these pathogens.

To address such need, extensive research efforts have developed various strategies to eradicate intracellular bacteria, examples including the development of lipophilic derivatives of conventional antibiotics, antibiotic delivery systems, antibody-antibiotic conjugates, and CPP-antibiotic conjugates. However, some major challenges remain to be solved before these new strategies can be considered for further clinical study. For conventional antibiotics, the rapid development of drug resistance and the poor efficiency over dormant intracellular bacteria are the key barriers. Drug delivery systems based on lipid and polymer nanoparticles also suffer from instability in body fluid, premature drug release, and difficulty in drug loading. Nanoparticles such as metal nanoparticles and mesoporous silica nanoparticles with conjugated or encapsulated antibiotics potentially have high stability. However, their long-term toxicity and biodegradability could be new concerns. Nano-MOFs represent a new class of drug delivery vesicles, but the applicability and biocompatibility remain to be verified in clinical settings. Similarly, the antimicrobial conjugates are also facing some key challenges limiting their actual application. The majority of CPP-antibiotic conjugates reported so far have low-to-moderate intracellular activity, and their *in vivo* efficacy is largely underexplored. Moreover, these CPP-antibiotic conjugates usually have membrane activity-associated toxicity, which substantially limits their therapeutic window. Therefore, to facilitate the clinical translation of these new therapies, creative drug/vehicle design, systemic *in vivo* activity and toxicity evaluation, and histological studies are required. Overall, despite these obstacles, the various strategies have been developed and offer promising pipelines to address the infectious diseases caused by intracellular bacteria.

Author Contribution

The manuscript was written through the contributions of all authors. All authors have given approval to the final version of the manuscript.

Conflicts of interest

There are no conflicts to declare.

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