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Targeting bacterial membrane function: an underexploited mechanism for treating persistent infections

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Abstract

Persistent infections involving slow-growing or non-growing bacteria are hard to treat with antibiotics that target biosynthetic processes in growing cells. Consequently, there is a need for antimicrobials that can treat infections containing dormant bacteria. In this Review, we discuss the emerging concept that disrupting the bacterial membrane bilayer or proteins that are integral to membrane function (including membrane potential and energy metabolism) in dormant bacteria is a strategy for treating persistent infections. The clinical applicability of these approaches is exemplified by the efficacy of lipoglycopeptides that damage bacterial membranes and of the diarylquinoline TMC207, which inhibits membrane-bound ATP synthase. Despite some drawbacks, membrane-active agents form an important new means of eradicating recalcitrant, non-growing bacteria.

Antimicrobial chemotherapy seeks to eradicate the infecting pathogen from its host in the shortest possible treatment period, often using antibiotics that effectively kill or inhibit the growth of metabolically active bacteria[1, 2]. Many of these agents were developed during the golden era of antibiotic drug discovery (1940s–1980s), and most target five biosynthetic processes that occur in actively growing bacteria: the biosynthesis of proteins, RNA, DNA, peptidoglycan and folic acid[3]. However, most of these classical antimicrobial strategies are not effective for eradicating persistent infections in which bacteria are quiescent (that is, slow-growing or dormant) (Box 1). Well-known clinical examples of such infections include the staphylococcal biofilms that result in endocarditis and other medical device-related infections, *Pseudomonas aeruginosa* infections of the lungs of patients with cystic fibrosis, streptococcal otitis media[4], ischemic osteomyelitis containing slow-growing microorganisms[5], and tuberculous granulomas that contain latent *Mycobacterium tuberculosis*[6]. These cases require prolonged treatment periods to cure or alleviate the burden of disease. For example, a drug regimen lasting for at least 6 months is required to cure tuberculosis (TB)[7], and for endocarditis and osteomyelitis, a minimum of 4 weeks of treatment is necessary[8, 9]. Alternatively, as is the case for medical device-related infections, surgical removal can often be required. The presence of different subpopulations of persisting bacteria (also known as recalcitrant bacteria) with varying antibiotic susceptibilities further challenges the overall efficacy of an antibiotic, and it may be that no single agent kills all of the subpopulations effectively (although some agents may slowly kill specific subpopulations; pyrazinamide, for example, kills *M. tuberculosis*). Consequently,

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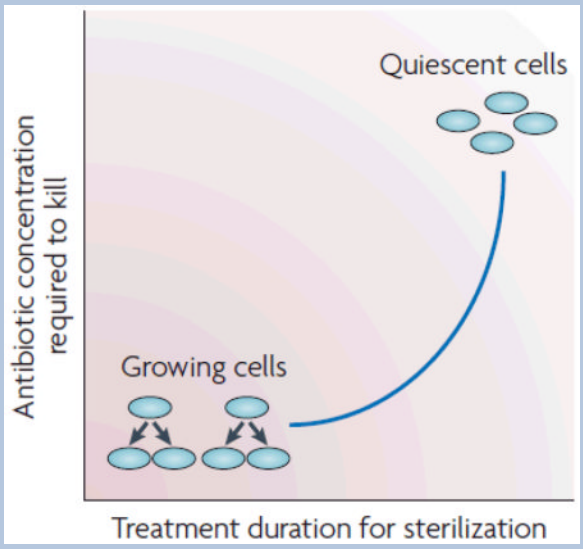
unconventional antimicrobial strategies will be needed to cure infections that contain quiescent bacteria (Box 2), as previously described[1, 6, 10]. Such strategies could shorten the treatment period, reduce disease relapse and limit the emergence of antibiotic resistance arising from surviving bacteria[11]. Therefore, newly approved and emerging therapeutic modalities should be evaluated for their potential to fulfill unmet medical needs such as the treatment of persistent infections. Dormant bacteria have not traditionally been targeted in antibiotic development programmes, in part owing to the difficulty of discovering successful agents. As a result, the types of agents that kill dormant organisms remain poorly defined, although some established drugs, including rifampicin and moxifloxacin, are known to kill some subpopulations of dormant bacteria[12–15]

Box 1

Antibiotic survival and why we need new antimicrobials



Most of the antibiotic classes that are currently in clinical use were discovered during the 'golden era' of antibiotic discovery, which lasted from the 1940s to 1980s. These antibiotics were discovered by screening for the ability to inhibit or kill logarithmically growing bacteria. Therefore, most developed antibiotics target processes required for growth - that is, the biosynthesis of proteins, peptidoglycan, folic acid, DNA and RNA. In response to the bacterial acquisition of antibiotic resistance, chemical modification of the existing drug classes was carried out to obtain analogues with improved activity. Like their precursor drugs, these analogues are efficacious against metabolically active, rapidly growing bacteria (see the figure). However, in several types of infections, bacteria encounter unfavourable conditions that cause cells to enter into a quiescent state of slow growth or even no growth. These metabolically inactive organisms (see the figure) can survive high concentrations of antibiotics, and extended treatment is therefore required for drug efficacy. When the drug concentration falls below levels that kill or inhibit the growing cells, dormant bacteria can reactivate to become growing cells, and the host begins to show symptoms of disease again. Providing that these reactivated cells have not evolved genetic resistance to the drug, they may be killed on subsequent rounds of treatment. The reason why dividing cells become dormant is multifactorial, as this phenomenon occurs in response to environmental factors such as the depletion of nutrients and oxygen, and an acidic pH, all of which decrease and eventually stop bacterial growth. For an extensive review of this phenomenon, see Refs[1,2,10,24].

Biofilm-mediated infections and tuberculosis (TB) provide two major examples of unmet clinical need owing to bacteria that are either slow growing or dormant and therefore hard to treat. Since the mid-1990s, the recognition of biofilm diseases has greatly increased, with estimates by the US CDC suggesting that up to 65% of all infections in developed countries involve biofilms. Biofilm-mediated infections range from those involving medical-device implants, including bloodline catheters and heart implants, to those associated with cystic fibrosis, wounds and superficial skin infections[4]. The unmet medical need for a TB treatment also came to light in the mid-1990s, which saw outbreaks of drug-resistant bacterial strains and the lethal synergy of the disease with HIV. TB is the most devastating bacterial infection of humans, causing ~2 million deaths per year. It is estimated that one-third of the world's population is infected with asymptomatic, dormant M. tuberculosis, from which new cases of active infection arise (~8 million annually)[21].

Now, more than a decade on, and with much intense research effort, it is emerging that antimicrobials that damage the membrane structure to perturb its function may provide a novel avenue for treating persisting infections. Similarly, organisms that can become dormant, such as M. tuberculosis and biofilm bacteria (for example, Staphylococcus aureus and Pseudomonas aeruginosa) may use anaerobic metabolism (for example, substrate level phosphorylation and anaerobic respiration) to sustain viability in the absence of growth. Because of this, enzymes involved in these processes are also being explored as targets for drug development.

Box 2

Targeting persistence

Targeting of proteins involved in the formation of quiescent cells is being considered as a co-therapy approach to treat persistent infections[152]. Although this concept is plausible, genetic mechanisms that c confer bacterial persistence are currently not well understood and may involve multiple genes and redundant pathways[46], which would complicate target selection for drug discovery. Indeed, a screen of a well-defined transposon library in *Escherichia coli* (the Keio collection) failed to identify a single mutant unable form persisters, supporting the hypothesis that bacteria use multiple pathways to reduce growth and form persister cells[46, 153, 154]. Hence, an alternative strategy could be to target processes that are required to maintain bacterial viability in already formed quiescent cells. These cell types are often present in an infection before therapy is administered[1, 10].

In our opinion, antimicrobials that target either the organization of the bacterial membrane bilayer (in bacterial biofilms) or the functions of membrane-associated respiratory enzymes (in latent TB) are promising therapeutic approaches for treating slow-growing or dormant bacterial infections. The general actions of these types of agents are shown in Fig. 1. Inhibitors of energy metabolism bind directly to target enzymes in the membrane that are involved in energy generation and the redox balancing of NAD^+/NADH (Fig. 1a). By contrast, membrane-damaging antimicrobials are generally lipophilic in nature and directly interact with the bacterial membrane bilayer, disrupting its function (or functions) and its physical integrity (Fig. 1b). Although some membrane-damaging agents may also disrupt the function of membrane-bound proteins, such as the enzymes involved in energy production, this action is indirect. The clinical effectiveness of damaging the bacterial membrane is demonstrated by the recently approved agents daptomycin and telavancin, which disrupt the membrane bilayer and are in clinical use for treating *Staphylococcus*

aureus infections (Fig. 2; Table 1). Furthermore, the drug TMC207 disrupts energy production in *M. tuberculosis* by targeting its ATP synthase, and it is presently in Phase II clinical trials for TB treatment (Fig. 3; Table 1). Several other membrane-active agents and inhibitors of energy metabolism are at various stages of pre-clinical and clinical development (Table 1). The potential therapeutic benefits of such agents arises from the fact that the membrane is vital to both active and metabolically inactive pathogens, and quiescent bacteria, like all living cells, require some form of cellular energy and redox homeostasis to maintain cell viability, even in the absence of growth[16]. Although these emerging paradigms have arisen from seemingly separate research areas, they represent a change from traditional discovery efforts. In this Review, we discuss whether antimicrobial strategies can be devised to treat infections of dormant and slow-growing bacteria, and whether lessons learned from the development of new therapeutics for one indication can be applied to another. We focus in particular on studies concerning the potential use of membrane-active antibiotics against biofilms and of agents that attack the membrane-associated anaerobic respiratory system of dormant *M. tuberculosis*. Accordingly, we stress that the concept of using membrane disruption to counter bacterial biofilms should be applicable to killing dormant *M. tuberculosis* to treat chronic TB; similarly, as membrane-mediated anaerobic metabolism is increasingly being recognized as essential for the maintenance of biofilms[17, 18, 19, 20], corrupting this function may also counter biofilm diseases. As the clinical relevance of biofilm formation by *M. tuberculosis* is unknown[21, 22], this Review does not address the action of antimicrobials against *M. tuberculosis* biofilms.

The problem of antibiotic recalcitrance

Resistance to antibiotics is widely associated with treatment failure. In addition to genetic resistance, pathogenic bacteria commonly undergo physiological adaptations that render cells slow-growing or non-growing and the bacteria thereby become recalcitrant to killing by bactericidal antibiotics (Box 1) without becoming genetically resistant; they regain sensitivity to antibiotics on the resumption of growth. This recalcitrance is due to two phenomena: 'antibiotic persistence'[6,23], in which a fraction of non-growing specialized persister cells arises during stationary phase and can survive antibiotic exposure, and 'antibiotic indifference'[2,24] in which a bacterial subpopulation becomes slow-growing or non-growing in response to unfavourable conditions (such as host responses, low pH, nutrient deprivation and oxygen deprivation) and is therefore not susceptible to killing by antibiotics. 'Antibiotic survival', a more comprehensive term encompassing both phenomena, indicates that bacteria have multiple ways of avoiding being killed by bactericidal antibiotics[11]. As a result of several studies, a consensus is emerging that persisting bacteria can evade antibiotic killing by substantially downregulating the biosynthetic processes that are targeted by most antibiotics without affecting bacterial survival in themetabolically inactive state[1,2,6,10,11,24] (Box 1). For example, β-lactam antibiotics kill by activating autolysins and this requires active peptidoglycan synthesis in cells undergoing division. Below, we explain why agents that disorganize the structure of the membrane or that inhibit respiratory enzymes involved in energy production and establishing the membrane's proton motive force are valid approaches for treating infections containing quiescent bacteria.

A case for targeting the cell membrane

Antimicrobial properties that are applicable to the discovery and therapeutic use of membrane-active agents include the essentiality of the bacterial membrane target, as well as the ability of some agents to kill target pathogens selectively or to disrupt multiple parts of the cell; together, these are potent bactericidal properties that result in low prospects for the emergence of resistance.

Essentiality and selectivity

The bacterial membrane is essential, irrespective of the metabolic status of the cell, as it provides selective permeability for cellular homeostasis and metabolic energy-transduction. In addition, the membrane contains about one third of the proteins in a cell and is the site for crucial processes, such as active transport of nutrients and wastes, bacterial respiration, establishment of the proton motive force in association with respiratory enzymes (Fig. 1a), ATP generation and cell-cell communication in biofilms[25]. Antimicrobial peptides (AMPs) made by the host and several bioactive molecules that act on the membrane[26,27] validate its significance as an antibacterial target site. In spite of this, traditional discovery efforts have not focused on the membrane as a target for antibiotic development. Several synthetic and natural-product chemotypes that damage the bacterial membrane, and could possibly kill dormant bacteria, are under-explored as chemotherapeutics, possibly owing to concern over the potential for these agents to disrupt the mammalian plasma membrane as well[27] and the lack of knowledge regarding chemical optimization of such molecules to attain pathogen selectivity (Box 3). However, the successful medical use of the membrane-active antibiotics daptomycin (a cyclic lipopeptide) and telavancin, oritavancin and dalbavancin (lipoglycopeptides) indicate that bacterial specificity is achievable. These agents preferentially bind to bacterial membranes owing to the predominance of negatively charged phospholipids (phosphatidylglycerol and cardiolipin) and zwitterionic phosphatidylethanolamine, which are rare in the outer leaflets of mammalian cells[28], and the absence of cholesterol. For example, daptomycin oligomerizes in the presence of calcium ions (Ca^{2+}) to form a micelle-like amphipathic structure, with its hydrophobic decanoyl side chain facing inwards[29] and this provides the drug with a pseudo-positively charged surface that increases its affinity for the negatively charged bacterial membranes[29]. The micelle is disrupted by interaction with the membrane, allowing the insertion of the hydrophobic tail of daptomycin, similar to the action of cationic antimicrobial peptides[29]. Similarly, oritavancin carries a net positive charge and destabilizes model membranes that contain large amounts of bacterial phosphatidylglycerol and cardiolipin[30,31]. In addition, binding to membrane-located peptidoglycan precursors and bacterial membrane proteins may contribute to the specificity of daptomycin and lipoglycopeptides.

Box 3

Challenges and opportunities for membrane-active agents

Disruption of the membrane may offer a superior approach for treating dormant infections, but several obstacles need to be considered during the discovery and assessment of these agents. The most obvious challenges and opportunities are outlined below.

- Selective toxicity should, ideally, be achieved against the target pathogen. There is a wealth of potential compounds in chemical-product and natural-product screening libraries, many of which have been shown to kill pathogens, but these candidates often also perturb mammalian membranes[27]. Therefore, structure–activity relationships need to be established to understand how to create species-specific molecules. A starting point would be to apply the design strategies from antimicrobial peptides and peptidomimetics to small molecules.
- During antibacterial whole-cell screening, the red blood cell haemolysis assay provides a simple approach for eliminating molecules that damage mammalian membranes. Rather than discarding these molecules, counter-selective screening in bacterial membrane-damage assays[155] should be carried out to catalogue molecules with some selectivity for bacteria. These compounds can then be subjected to structure–activity relationship medicinal chemistry efforts.

- The physicochemical properties of membrane-active agents may not be best suited for oral administration. Therefore, these parameters need to be considered during optimization and may require suitable formulations for oral and parenteral delivery, including specialized dosing regimens and formulations. This is exemplified by the antifungal amphothericin B, which damages the membrane of Candida albicans: its toxicity can be mitigated by specialized liposomal formulations, allowing this drug to be used effectively at higher doses to treat systemic mycoses[156].
- Not all antibacterial drugs kill in the same manner. The determination of bactericidal kinetics for membrane-active agents, to determine whether they are concentration dependent or time dependent, may be used to develop optimum dosing regimens that limit the development of resistance and maximize efficacy and safety. Furthermore, not all membrane-active agents will be bactericidal against all forms of persisting infections. Hence, the spectrum of diseases that can be covered by agents should be defined early during the discovery stages. This will enable further clinical development or chemical optimization to expand the potential for activity against bacteria in different quiescent states.
- The complex lipid-rich cell wall of *Mycobacterium tuberculosis* contributes to its resilience against chemical challenges and may restrict molecules from reaching the plasma membrane target. The outer-membrane of Gram-negative bacteria will impose similar effects. Therefore, the amphipathic properties of molecules must be taken into account during the development of agents for use against these organisms.
- As is the case for pyrazinamide, agents showing high minimum inhibitory concentration values in vitro might still eradicate dormant cells from an infection by synergizing with the host immune system. But the discovery of such molecules would require suitable animal models or in vitro systems that can reproduce the complex conditions found in the host.
- In addition, minimum inhibitory concentration values are not indicative of antimicrobial potency against non-growing bacteria; therefore, more representative parameters such the minimum stationary-cidal concentration[1, 37], minimum dormicidal concentration[1] or minimum biofilm eradication concentration[50] should be determined during discovery of compounds for treating persistent infections.
- As membrane-active agents do not rely on intracellular targets for their activities, they may be immobilized to the surface of medical devices as part of a ‘smart surface approach’ to prevent bacterial contamination and biofilm formation. This is demonstrated by the agent chitosan[157] and by a new class of antimicrobial peptidomimics known as ceragenins[158].

A complex multitarget mode of action

Several membrane-damaging agents interfere with multiple targets[29, 31, 32] through the interaction of a lipophilic moiety with the bacterial membrane (causing disruption of membrane architecture and functional integrity), through steric inhibition of membrane-embedded proteins and/or through alteration of the proton motive force, which may lead to leakage of cytosolic content and eventual cell death (Fig. 1). Agents causing damage to the membrane may lead to lethal pleiotropic effects in quiescent bacteria, but dissipation of the proton motive force alone is not bactericidal in all species (although this property may constrain energy supply in already metabolically inactive cells). By contrast, *M.*

tuberculosis, unlike other pathogens[33, 34], seems to require a fully energized membrane for survival under both aerobic and hypoxic conditions[16, 35], and therefore dissipation of the proton motive force by the ionophores nigericin or valinomycin is highly bactericidal to active and dormant *M. tuberculosis*[35]. Furthermore, increasing the proton permeability of the membrane makes mycobacteria more sensitive to the effect of reactive free radicals (nitric oxide and superoxide) in macrophages, as this mechanism is enhanced at acidic pHs[36].

A low potential for the development of resistance

As membrane-active agents may interact with multiple targets in the membrane, the ability of bacteria to acquire resistance to these agents is limited. In vitro studies have been carried out with lipopeptides[22], lipoglycopeptides (I.C. and A.J.O., unpublished observations), a range of cationic antimicrobial peptides[13] and HT61, a recently described quinolone-derived membrane-active compound[37], and these studies suggest that de novo mutations conferring resistance to membrane-active antibiotics do not readily arise. The induction of lipopolysaccharide modification systems (such as those regulated by the PmrA–PmrB two-component system; also known as the BasR–BasS system) and the expression of efflux pumps (including MexAB–OprM) allow Gram-negative organisms such as *P. aeruginosa* to avert killing by cationic peptides[38, 39]. However, the rapid elimination of pathogens that are inherently susceptible to membrane-active drugs should reduce the likelihood of resistance emerging, as long as drug concentrations remain bactericidal at the site of infection.

Membrane-damaging agents against biofilms

Several agents that disrupt the bacterial cytoplasmic membrane possess activity against biofilms in vitro (Fig. 2; Table 1), and one of these, daptomycin, has been used clinically since 2003. Telavancin was approved for use in the United States and Canada in 2009, and other lipoglycopeptides are in late-stage development[40]. Daptomycin kills staphylococcal cells quicker than most other antibacterials and can, in some cases, completely eradicate *S. aureus* biofilms[41, 42]. Indeed, clinical observations indicate that it is useful for treating biofilm-mediated staphylococcal and enterococcal endocarditis[43]. However, daptomycin may not be able to eradicate all types of staphylococcal biofilm infections, as in a mouse model it cleared less than 7% of catheter-associated biofilms after 7 days of treatment[44]. Thus, even a dormant bacterial population may contain different subpopulations of persistent cells, depending on the existing environmental or host conditions[6, 17, 45, 46]; similarly, the composition of membrane lipids may vary between different subpopulations of persistent cells[47, 48], thereby affecting the interactions of the drug with the membrane[11]. Combination therapies may be required to eradicate particular biofilm diseases, as the clinical efficacy of daptomycin, like that of all other antimicrobials, will depend on the pharmacokinetics and pharmacodynamics of the drug at the site of infection. Furthermore, the finding that daptomycin is less effective against stationary phase bacteria than against cells in logarithmic phase exemplifies the fact that not all membrane-damaging agents will be effective in certain slow-growing or dormant infections[49]. This does not mean, however, that other, structurally distinct membrane-disrupting agents will lose their bactericidal activities against the different forms of slow-growing or dormant bacteria[50, 51]. For example, telavancin can eradicate staphylococcal and enterococcal biofilms at concentrations close to those that inhibit the growth of planktonic cells[52]. Similarly, the related lipoglycopeptide oritavancin is equally potent for killing staphylococcal biofilms and stationary phase cells[51]. Although telavancin and oritavancin have multiple modes of action, including inhibition of peptidoglycan biosynthesis, their anti-biofilm activity probably results primarily from their ability to permeabilize the bacterial membrane[51, 52]. Corroborating this idea, the prototypical glycopeptide vancomycin, which inhibits peptidoglycan crossbridge formation but lacks activity against the staphylococcal membrane, is inactive against staphylococcal

biofilms[51]. These membrane-active lipoglycopeptides may have an important role in combating osteomyelitis, endocarditis and catheter-related infections, as suggested by experimental animal models, as long as they can reach the site of infection[40]. Dalbavancin, the lipophilic derivative of the glycopeptide teicoplanin, may also have membrane-damaging properties, but its mechanism of action and potential activity against quiescent bacteria, including *S. aureus* biofilms, is unknown; however, a study examining its efficacy in preventing *S. aureus* colonization of catheters in rabbits indicates some improvement over the related agent vancomycin[53].

Several pre-clinical or experimental membrane-active agents also display potent activity against biofilms (Fig. 2; Table 1). The novel anti-staphylococcal porphyrin agents XF70 and XF73 perturb the bacterial membrane[54] and eradicate both *S. aureus* biofilms and stationary phase *S. aureus* cells at concentrations close to the minimum inhibitory concentration (MIC) for planktonic cultures, similarly to lipoglycopeptides[50]. Anti-biofilm activity has also been noted for derivatives of the tetramic acid natural product reutericyclin (Fig. 2) (obtained from *Lactobacillus reuteri*[55]), which depolarize staphylococcal cells and therefore have potential for treating persistent topical infections[56]. Interestingly, reutericyclin derivatives also kill stationary phase Clostridium difficile cells that are insensitive to vancomycin and metronidazole (J.G.H. and R.E.L., unpublished observations). Antimicrobial peptides such as LL-37 (Ref. 57) and synthetic peptidomimetics[58] (Fig. 2), which cause permeabilization of the inner and outer membranes in Gram-negative bacteria, can kill bacterial biofilms.

The small quinolone-derived compound HT61 was discovered in a screen for novel antimicrobials that kill non-growing bacteria[37]. In this unorthodox but highly commendable approach, compounds were prioritized according to their killing of non-growing cells rather than on conventional MIC testing against growing bacteria (Box 3). HT61 rapidly and preferentially kills non-growing staphylococci through multiple mechanisms involving rapid permeabilization of the bacterial membrane (Table 1), and the drug is currently in clinical trials as a topical agent for nasal decolonization of methicillin-resistant *S. aureus*. No anti-biofilm activity has been reported for HT61, but its method of discovery could prove useful for guiding other approaches to obtain a new generation of antimicrobials that kill persisting cells[37].

Challenges for membrane-active agents

Despite these encouraging examples, there are problems associated with the current anti-biofilm agents and their evaluation.

Test methods

A standardized methodology for evaluating anti-biofilm activity is lacking, and there is substantial variation in the experimental approaches that are used by different laboratories[1]. Existing methods include the use of static biofilm devices (such as minimum biofilm eradication concentration (MBEC) assays and colorimetric microtitre systems), in which biofilms are cultured without a continuous flow of fresh nutrients, and flow biofilm models (including the CDC biofilm reactor and the BioFlux system), in which biofilms are cultured with a continuous supply of fresh nutrients[59]. Consequently, the activity of agents tested in unrelated studies cannot be directly compared, and the relative activities of these agents against biofilms is therefore unknown.

The spectrum of activity

Most approaches do not consider that medical biofilms often have a polymicrobial composition[60] and that an effective anti-biofilm strategy must therefore eliminate multiple bacterial species or at least synergize with other antibiotics to achieve complete

sterilization[11]. Furthermore, the use of membrane-active antimicrobials that also target Gram-negative biofilms needs to be expanded, but this requires agents that either damage the outer membrane (as has been shown for the anti-biofilm action of polymyxin[38]) or traverse this layer and damage the cytoplasmic membrane. Furthermore, little is known about the structure–activity relationships that are required for small membrane-active molecules to display specificity for bacterial membranes[11, 61]. However, we believe that this gap can be filled by applying concepts from antimicrobial peptides or peptidomimetics to the design of amphipathic molecules that selectively target bacterial membranes (Box 3).

Pharmacology

Commonly associated toxicities (such as nephrotoxicity) and factors that affect drug disposition and efficacy (such as serum binding and tissue penetration) are also problems intrinsic to the design and development of membrane-active agents. For instance, the key to the clinical development of daptomycin was the choice of dosing regimen — it is dosed once per day at a high concentration — to maximize its concentration-dependent killing mechanism and to avoid an associated time-dependent myotoxicity[43]. Other membrane-active drugs will probably have similar physiochemical properties and will thus perhaps also exhibit pharmacological liabilities. Such drugs will tend to be partly lipophilic in nature, and they will therefore become bound to protein and may not distribute well into all tissues. For example, daptomycin is inactive against respiratory infections, and this is thought to be due to high levels of protein binding, as occurs in epithelial lining fluid (ELF) obtained by bronchoalveolar lavage[62]. Telavancin is also highly protein bound (90–93%) compared with vancomycin (10–55%), but its efficacy is not affected by ELF, as the concentration of unbound telavancin remains above the MIC against methicillin-resistant *S. aureus*[63, 64]. Dalbavancin and oritavancin are also highly protein bound (93–98% and 86–90%, respectively), with prolonged terminal half-lives (195 hours and 257 hours, respectively) and high retention times in mammalian cells and tissues[48], which could extend the duration of potential side effects[40]. In culture models, accumulation of oritavancin, a drug that is important for killing intracellular *S. aureus*, induces lysosomal lipid storage disorder, but its corresponding toxic effects in vivo are unknown[65, 66]. These factors, along with a narrower safety margin, have discouraged the development of membrane-active agents in the past. However, owing to the urgent need for antibiotics that decrease the time required to effectively treat biofilm infections and other persistent infections, such agents may become important parts of future antibacterial treatment strategies.

Resistance

Laboratory studies indicate that the development of resistance is less frequent for several membrane-active agents than for other drugs; nonetheless, resistance (including cross-resistance between different structural classes of membrane-active agents with similar mechanisms of action) could emerge following widespread clinical use of these agents. For example, resistance to daptomycin was detected shortly after its clinical introduction[67, 68], but the resulting daptomycin-resistant strains are exceedingly rare. This resistance is multifactorial and partially mediated by overproduction of phosphatidylglycerol lysyltransferase (MprF)[69, 70], which adds L-lysine to phosphatidylglycerol, thereby increasing the net positive charge of the bacterial surface and decreasing the binding of daptomycin and some cationic antimicrobial peptides[70, 71]. However, telavancin is still active against daptomycin-resistant mutants of *S. aureus* containing mutations in MprF[72], suggesting that MprF does not affect telavancin activity. Expression of the *dltABCD* operon, which encodes the enzymes that mediate the addition of alanine to lipoteichoic acid, also confers daptomycin non-susceptibility by increasing the net positive charge of the bacterial surface[73], but its effects on other membrane-active agents is unknown. Lipoglycopeptide resistance can also result from expression of the *vanA* gene clusters; this expression leads to

the production of peptidoglycan precursors ending in D-Ala-D-lactate instead of D-Ala-D-Ala, reducing the binding of glycopeptides to peptidoglycan by up to 1,000-fold[74]. Vancomycin-resistant *S. aureus* and vancomycin-resistant enterococci expressing *vanA* are resistant to dalbavancin (MIC > 32 μg ml ^{1}) and only partially susceptible to telavancin (MIC = 2–16 μg ml ^{1}) compared with wild-type strains (MIC ≤4 μg ml ^{1} for both drugs)[63, 72]. However, these resistant strains remain sensitive to oritavancin owing to an enhanced ability of this drug to form homodimers to improve its interaction with peptidoglycan precursors; this interaction is facilitated by the chlorobiphenyl side chain of oritavancin, which ensures that the drug is anchored to the membrane[75, 76]. Despite this, constitutive expression of *vanA* can lead to decreased susceptibility to oritavancin[77]. The clinical prevalence of *vanA*-mediated resistance in *S. aureus* is low, as expression of the *vanA* cluster is associated with a fitness cost[74, 78]; this may enable long-term and widespread use of lipoglycopeptides to treat staphylococcal infections. As current and future membrane-active antimicrobials progress, examination of the associated mechanisms of resistance, the ease with which resistance can occur and the potential for cross-resistance will be important parameters for the clinical development of different chemical classes of these agents.

Membrane-damaging agents against tuberculosis

The concept of damaging the structure of the mycobacterial membrane bilayer as a therapeutic strategy has received little attention. The development of membrane-active drugs with activity against *M. tuberculosis* could prove valuable in the endeavour to shorten the treatment period for TB, as is evident from the anti-TB activities of ionophores and the third-line anti-TB drug clofazimine. The mode of action of clofazimine is not well defined, but it may affect membrane architecture[79, 80], with subsequent accumulation of lysophospholipids and depletion of K^{+} (Ref. 81). These effects are associated with membrane depolarization. Not surprisingly, clofazimine shows potent in vitro bactericidal activity against dormant *M. tuberculosis* under hypoxia[82] and near-sterilizing activity in mice[83]. Although clofazimine causes reversible skin discoloration, this molecule is a chemotype for the development of less toxic derivatives[84].

Several host-derived peptides are known to be active against many important bacterial pathogens, but only recently have these antimicrobials been reported to kill *M. tuberculosis*[85]. For example, ubiquitin-derived peptides kill persister cells that are tolerant to rifampicin (G. E. Purdy, personal communication). Although the therapeutic development of antimicrobial peptides is challenged by several factors, including the high costs of production, peptidomimetics that also cause membrane damage[86] may provide another way forward for obtaining novel anti-TB drugs.

Damaging membranes in atypical bacteria

Most bacterial pathogens can form persistent infections that thwart antibiotic efficacy[87, 88]. Therefore, as our understanding of various types of bacterial persistent infections improves, it will become possible to define other bacterial pathogens to which the concepts about membrane-damaging agents may be applied. In particular, studies will be required to determine whether this approach is applicable to atypical microorganisms that have both extracellular and intracellular niches during dormant infections; such microorganisms include *Mycoplasma* spp.[89, 90] and *Chlamydia* spp.[91]. Several studies report that antimicrobial peptides[92–95] and analogues of host-derived membrane-active antimicrobial lipids[96] kill metabolically inactive extracellular elementary bodies of *Chlamydia trachomatis*[91] effectively. By contrast, few of these studies describe the killing of the metabolically active intracellular reticulate bodies of *Chlamydia* spp., which may indicate

that poor cell penetration by these peptides is an issue[97]. Indeed, the intracellular expression of the peptide melittin on a tetracycline-inducible plasmid in mammalian cells achieved a 75% reduction in reticulate bodies[98]. Similarly, Pep-1, a synthetic transport carrier peptide in eukaryotes, is active against reticulate bodies, although it lacks activity against elementary bodies[97]. If cell penetration can be achieved by antimicrobial peptides, their peptidomimetics or other membrane-active agents, activity against reticulate bodies may be achieved to provide a comprehensive therapeutic strategy. Cell culture and animal models also indicate that some membrane-active peptides are bactericidal against persistent infections of *Mycoplasma* spp.[99–101]. These examples suggest that the future could see the application of membrane-damaging agents to atypical pathogens, although variations in membrane lipid composition (stemming from biphasic lifestyles and the ability of these pathogens to display eukaryote-like lipids in their membrane[102–104]) may alter drug–target interactions and the activities of membrane-active agents.

Targeting energy metabolism

Persistent colonization of the host requires that bacteria adopt various mechanisms of energy production to survive the scarcity of nutrients and oxygen that may occur in these infections. Dormant *M. tuberculosis* and organisms in biofilms survive these conditions and maintain redox homeostasis by using fermentation and anaerobic respiration to fulfil the lower energy demands of these persisting bacteria[16, 19, 105]. Anaerobic respiration is driven by the proton motive force, yielding a lower, but adequate, amount of ATP energy that sustains the viability of dormant cells. As dormant bacteria require ATP and redox balancing to survive, the inhibition of membrane-bound proteins that facilitate anaerobic metabolism is increasingly being proposed as a strategy to limit the survival of persisting bacteria[16, 105–107]. Although this strategy is a new direction for antibiotic discovery, there are several challenges. First, bacteria possess multiple ways of recycling or generating energy to compensate for the loss of their favoured energy-producing mechanisms. For example, staphylococci can forgo their respiratory chain to form small-colony variants that survive solely by fermentation and that persist in the host for prolonged periods[108, 109]. Second, as persisting infections consist of metabolically heterogeneous populations, a failure to kill all populations will necessitate the use of additional agents to sterilize the infection. Nevertheless, as our understanding of bacterial metabolism in dormant bacteria increases, novel targets will be identified that may be suitable for therapeutic intervention. Notable examples illustrating the potential value of this strategy are described here for *P. aeruginosa* and *M. tuberculosis*, and these examples reveal that targeting energy metabolism is likely to be a pathogen-specific approach.

Inactivation of cytochrome cbb_3 oxidase 1, cytochrome cbb_3 oxidase 2 and the cyanide-insensitive oxidase in *P. aeruginosa* results in impaired growth and poor biofilm formation under aerobic and microaerophilic conditions[110]. Similarly, deletion of the rhlRI quorum sensing system (which acts as a transcriptional regulator of anaerobic respiration) kills *P. aeruginosa* in biofilms owing to intoxication by intracellular nitric oxide[18], which is generated as a by-product of denitrification. Biofilm formation in staphylococci is also disrupted by nitric oxide[111]. Therefore, using prodrug nitroheterocyclic antibiotics to generate nitric oxide in bacteria, a strategy currently in development for the treatment of latent TB, may be a viable strategy for the treatment of other diseases.

Dormant *M. tuberculosis* is exceptionally susceptible to agents that inhibit its respiratory chain (Figs 1, 3) and therefore cause membrane depolarization, ATP depletion and changes in the cellular redox state of the bacterium[35, 112]. This high sensitivity seems to correlate with an unusual need for *M. tuberculosis* to maintain a fully energized membrane under both aerobic and anaerobic conditions, which could reflect the inability of the species to survive

by fermentation alone. Therefore, ATP synthase, which couples the proton motive force to energy transduction, is essential for the survival of active and dormant *M. tuberculosis* cells, even though this enzyme is downregulated during dormancy[113]. ATP synthase is also important in *Streptococcus pneumoniae*[27], but it can be deleted in other bacteria, including *Escherichia coli* and *Salmonella enterica*, although this leads to virulence attentuation[114]. Thus, inhibition of ATP synthase may be only an adjunctive approach in some organisms.

In mycobacteria, enzymes that initiate the transfer of electrons into the respiratory chain, enzymes that are involved in menaquinone biosynthesis, and enzymes that maintain NAD^+/NADH redox pools are further points of vulnerability. For example, the inhibition of menaquinone production kills *M. tuberculosis*[115], and the NADH type II dehydrogenase (NDH2; present in two copies encoded by ndh and ndhA), a nonproton-pumping enzyme that transfers electrons to the respiratory chain, is essential in active and dormant *M. tuberculosis*[35, 107] and is important for recycling NAD^+, the main cellular oxidant and an important cofactor in cells[35, 112]. Hence, the inhibition of NDH2 deprives *M. tuberculosis* of both energy production and maintenance of its redox pools, which leads to cell death. As NAD^+ recycling is also important in biofilms[116, 117] and under anaerobic conditions[118, 119], this enzyme could be a promising target for drugs. This will require studies to probe the antimicrobial potential of inhibiting NADH dehydrogenases and other enzymes that affect redox homeostasis in biofilms, as the effects are still, for the most part, unknown[120].

On the basis of studies in *E. coli*, it has been proposed that several key bactericidal agents (that is, β-lactams, aminoglycosides and fluoroquinolones) cause cell death through a common mechanism of disturbing bacterial metabolism and the respiratory chain, leading to the formation of lethal reactive oxygen species[121]. However, it remains to be seen whether this concept is applicable to other classes of bactericidal antibiotics and other bacteria. Nevertheless, by directly perturbing the membrane and essential respiratory components, it may be possible to generate reactive oxygen species to impair numerous cellular processes and, eventually, cause cell death. Other respiratory and metabolic enzymes have been proposed as targets for anti-infective approaches in *P. aeruginosa*[105, 106], *S. aureus*[120, 122] and *M. tuberculosis*[107, 123], but this is beyond the scope of this article.

Inhibiting energy metabolism in tuberculosis

With the renewed interest in drug discovery for TB treatment, energy metabolism in *M. tuberculosis* has re-emerged as an important drug target. Pyrazinamide (PZA) provided the first indication that energy metabolism is vulnerable in dormant TB, as this drug reduced the period of TB treatment from 9 months to 6 months. PZA disrupts the intracellular pH, an important component of the proton motive force, and only kills metabolically dormant cells at acidic pH and under oxygen-limiting conditions[124, 125]. Paradoxically, PZA has poor in vitro activity (MIC ≈ 60 mg l^{-1}), even under acidic conditions, which is not predictive of its potent sterilizing properties. It is possible that by reducing the intracellular pH, PZA synergizes with host-derived reactive oxygen and nitrogen species[36], thus reducing the MIC in vivo. Agents possessing this activity would thus be expected to kill more efficiently in acidified phagosomes in the host. This demonstrates the potential technical challenges when developing agents that specifically target latent or slow-growing subpopulations: namely, that such agents will probably have poor activity in standard MIC assays against dividing bacteria, and that the in vivo conditions that favour their synergistic activity are difficult to reproduce in vitro.

Among the anti-TB agents discovered in the past decade, the diarylquinoline TMC207, which is active against metabolizing and non-growing cells, seems to be the leading candidate[126]. TMC207 specifically binds to subunit C of the mycobacterial F_0F_1-ATPase,

sterically blocking the enzyme's rotational motor and thereby inhibiting proton translocation and coupled ATP biosynthesis[127]. Mutations in subunit C confer resistance to TMC207 (Ref. 128). Combinations of PZA and TMC207 are highly synergistic in mice (compared with other anti-TB combinations)[129], due to their inhibition of sequential processes: the uncoupling of the proton gradient by PZA and specific inhibition of the ATP synthase by TMC207. Further studies to explore this observation are needed, as synergy with PZA was not reported in clinical trials with TMC207 (Ref. 130). Like PZA, TMC207 displays slow-onset killing[131], which might point to the partial use of alternative energy systems by the bacteria or the presence of a pool of ATP in the bacteria that needs to be depleted before the bactericidal effects of inhibiting ATP synthase can be detected. However, in one study using a guinea pig model of TB infection, TMC207 was unable to kill a fraction of *M. tuberculosis*[132]. These findings illustrate the complexity of using a single antibiotic to sterilize the different physiological states of persisting bacteria, and they also highlight the need for standardized test models as well as for models that evaluate the effects of antibiotics on dormant cells exposed to multiple stresses (for example, simultaneous nutrient and oxygen starvation)[133], which may be more representative of in vivo conditions. Efforts are already underway to find other ATP synthase inhibitors using structure-aided technologies[134].

Although there are currently no drugs approved for TB treatment that target mycobacterial NDH2, experimental observations with the NDH2 inhibitors phenothiazines, chlorpromazine and thioridazine (which are antipsychotic drugs) provide a case for targeting NDH2 (Refs 135, 136). Chlorpromazine and thioridazine are both bactericidal and reduce the pulmonary mycobacterial load in mice[137]. However, phenothiazines are present at subinhibitory levels at the site of infection, and toxic side effects occur following routine administration, two factors that slow their clinical development[138]. Nevertheless, these agents demonstrate that NDH2 inhibition kills both nutrient-starved and hypoxic *M. tuberculosis* at concentrations similar to those that kill cells in the logarithmic phase[35, 139]. The lethality of NDH2 inhibition, resulting from disruption of both ATP synthesis and NAD^+/NADH recycling, demonstrates that antibiotics acting at different points of the *M. tuberculosis* respiratory chain may display enhanced sterilizing activity by disrupting redox homeostasis. As the development of non-phenothiazine inhibitors of NDH2 progresses[140, 141], attention must be paid to potential cross-resistance with isoniazid-resistant mutants that contain mutations in NDH2 (Ref. 142).

Prodrug nitroheterocyclic antibiotics are used to treat infections with parasites and anaerobic bacteria, and derivatives of these drugs with the potential to sterilize the lungs have emerged as good candidates for TB therapy. These agents include two nitroimidazoles (PA-824 and OPC-67683) that are in current clinical trials[143], nitazoxanide[144], and nitrofurans that are still in the early discovery stage[145]. These agents are activated in the bacterium by reduction of the precursor, resulting in the formation of reactive oxygen or nitrogen intermediates that damage multiple targets, including cell envelope lipids, DNA and proteins. Furthermore, the nitric oxide that is generated is predicted to poison the respiratory chain of dormant cells by inhibiting cytochrome oxidases[112], thereby changing the redox status of cells and dramatically reducing the amount of intracellular ATP[146]. Cross-resistance between nitroheterocyclic antibiotics is a concern, particularly if they require the same nitroreductase or biochemical pathway for activation. However, the potential for cross-resistance can be reduced by optimizing the electrochemical potential of agents such that different nitroreductases and pathways are used to activate the prodrugs[147]. Indeed, changing the head group of OPC-67683 to a nitrofuran (Fig. 3) maintained the efficacy of the drug against *M. tuberculosis* mutants resistant to PA-824 and OPC-67683 (J.G.H., R. B. Lee, R.E.L., M. S. Scherman and M. R. McNeil, unpublished observations). Nitroheterocyclic antibiotics could potentially also be used as anti-biofilm agents, given their multitarget mode of action and

good chemical tractability, and indeed nitazoxanide inhibits the formation of biofilms for *E. coli* and *S. aureus*[148, 149]. Similarly, ranbezolid, which is active against *M. tuberculosis* and contains a nitrofuran ring fused to an oxazolidone scaffold, exhibits good anti-biofilm properties against staphylococci compared with linezolid[150]. Ranbezolid is also reported to damage the membrane of *Staphylcoccus epidermidis*, probably owing to nitroheterocyclic action[151]. However, the use of nitroheterocyclic antibiotics for anti-biofilm control will require the presence of a nitroreductase that can activate the prodrug, and for that nitroreductase to be expressed in active and quiescent subpopulations. In addition, these molecules would have to be non-mutagenic, so they would have to avoid reduction by the host redox enzymes.

Concluding remarks

Owing to the paucity of treatment options for infections involving quiescent bacteria, we have begun to explore the premise that the bacterial membrane and enzymes involved in anaerobic respiration might be potential drug targets for controlling such infections, as exemplified by TB and biofilm diseases. Although these strategies are potentially advantageous, the main challenge will be obtaining molecules that are selective for bacteria. Although the fortuitous discovery of TMC207 reveals that this can be achieved for respiratory enzymes, an understanding of which genetic determinants are essential for anaerobic metabolism is needed for several bacterial pathogens. If these targets are sufficiently structurally distinct from human counterparts, this could then allow established drug discovery methods to be used. These efforts will probably result in pathogen-specific molecules owing to there being different mechanisms for energy production in bacteria. However, we believe that membrane-active agents could emerge as a superior therapeutic approach, with the advantages that they have multitarget effects, a potentially rapid bactericidal action, activity against growing and dormant subpopulations and low resistance prospects — providing that such agents can be developed with an acceptable selectivity verses human membranes. Moreover, disruption of membrane structure is also likely to affect membrane-embedded enzymes that carry out anaerobic metabolism and redox reactions. As most of the recently described membrane-active agents act on Gram-positive pathogens, there is a real need to extend this paradigm to Gram-negative biofilms and dormant *M. tuberculosis*. We anticipate that different forms of persistent bacterial infections, not covered in detail here, would be susceptible to killing by agents that can disrupt the membrane integrity of the pathogen. As this exciting field progresses, an understanding of the structure–activity relationships needed to obtain pathogen-selective molecules will prove crucial for guiding the discovery and development of novel agents that could shorten treatment periods and improve clinical outcomes for persistent infections.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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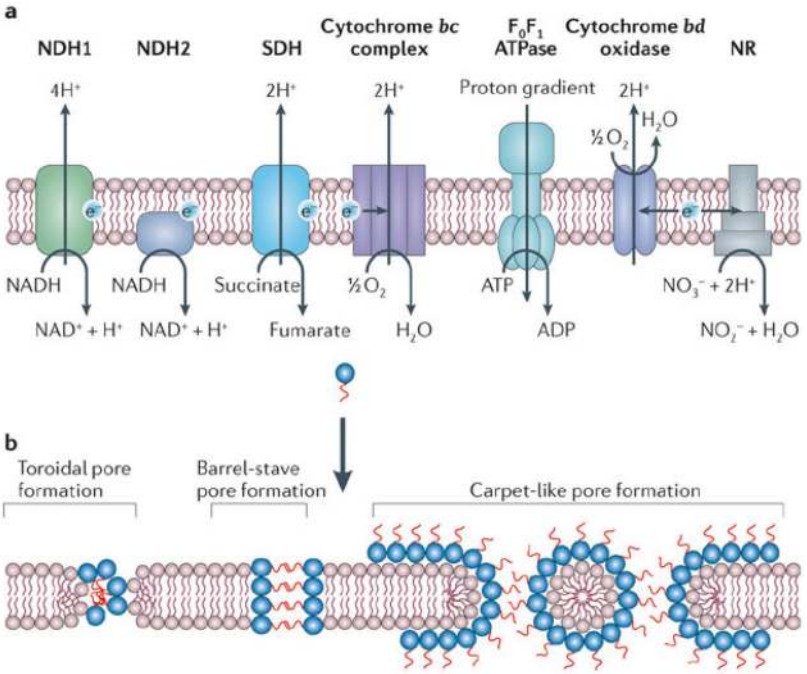

Figure 1.

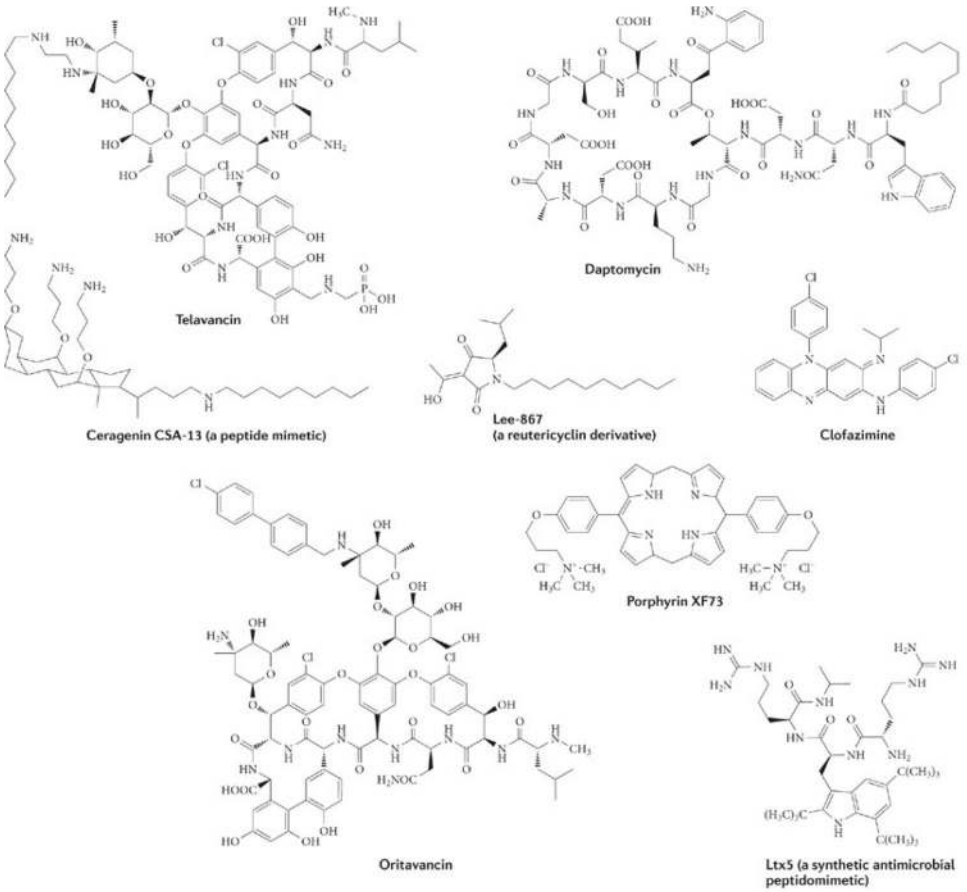

Figure 2.

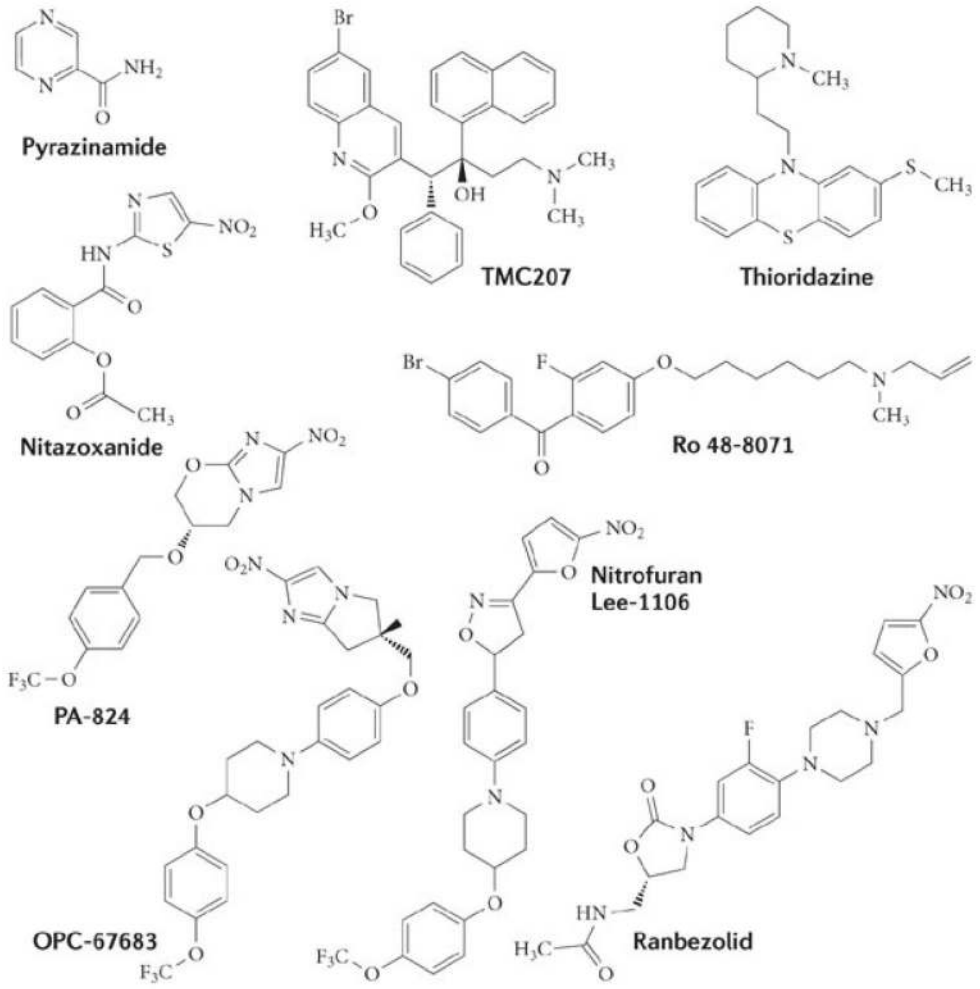

Figure 3.

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Table 1

Antibiotic	Active against	Mode of action	Antibiotic status	Refs
Daptomycin	Gram-positive bacteria (for example, *Staphylococcus aureus* and *Enterococcus* spp.); active against biofilms	Membrane permeabilization and depolarization; disruption of multiple cellular processes	Approved (2003) (or cSSSI; *S. aureus* bacteremia and right-side endocarditis	160
Telavancin	Gram-positive bacteria (for example, *S. aureus*, including VISA); active against biofilms	Inhibition of peptidoglycan biosynthesis by binding to the D-Ala-D-Ala termini; membrane permeabilization and depolarization; disruption of multiple cellular processes	Approved (2009) for cSSSI; completed Phase III for pneumonia	63
Oritavancin	Gram-positive bacteria (*S. aureus*, including VISA, and Enterococcus spp.); active against biofilms and stationary phase cells	Inhibition of peptidoglycan biosynthesis by binding to the D-Ala–D-Ala termini; membrane permeabilization and depolarization; disruption of multiple cellular processes	Completed Phase III trials for cSSSI and Phase II trials for *S. aureus* bacteremia	63
Dalbavancin	Gram-positive bacteria (for example, *S. aureus*, including VISA); prevents biofilm formation *in vivo*	Inhibition of peptidoglycan biosynthesis by binding to the D-Ala-D-Ala termini; alternative modes of action are presumed to involve membrane disruption	Completed Phase III trials for cSSSI	53.63
Reutericyclin (Lee-867)	Gram-positive bacteria (for example, *S. aureus*); active against biofilms	Membrane depolarization; disruption of multiple cellular processes	Discovery stage for topical use against *S. aureus*	56
XF-73	Gram-positive bacteria (for example, *S. aureus*)	Membrane permeabilization and depolarization; disruption of multiple cellular processes	Discovery stage for topical use against *S. aureus*	50.54
CSA-13	Broad spectrum drug, mainly active against Gram-positive bacteria (for example, *S. aureus*); active against biofilms	Membrane permeabilization and depolarization: disruption of multiple cellular processes	Pre-clinical stage for topical use and biofilm prevention on medical devices	158.161.162
HT61	Gram-positive bacteria (for example, *S. aureus*)	Membrane permeabilization and depolarization; disruption of multiple cellular processes	Clinical trials for nasal decolonization of *S. aureus*	163
Clofazimine derivatives	*Mycobacterium tuberculosis* and *S. aureus*; active against NRP cells	Complex action, including membrane depolarization	Discovery optimization stage	TB Alliance *
TMC207	*M. tuberculosis*; active against NRP cells	Inhibition of mycobacterial ATP synthetase	Phase II trials for TB disease	TB Alliance *
Nitroimidazoles (PA-824 and OPC67683)	*M. tuberculosis*; active against NRP cells	Activated by the Rv3547–Fgd1 pathway to Form reactive free radical antimicrobial species	Phase II trials for TB disease	TB Alliance * and Otsuka pharmaceuticals‡
Nitrofurans (such as Lee-1106)	*M. tuberculosis*; active against NRP cells	Activated by an unknown mechanism to form reactive free antimicrobial species	Discovery optimization stage	164.165
Nitazoxanide	Broad spectrum drug, active against *Escherichia coli* and *S. aureus* biofilms and NRP *M. tuberculosis*	Inhibition of PFOR; undergoes reduction by other redox enzymes to form reactive free radical antimicrobial species	Approved for anaerobic intenstinal parasites (for example, *Giadria lamblia* and *Cryptosporidium parvum*)	147.166

Antibiotic	Active against	Mode of action	Antibiotic status	Refs
Ro48-8071	*M. tuberculosis*	Inhibition of menaquinone biosynthesis, leading to respiratory chain arrest	Discovery optimization stage	115 and TB Alliance *

cSSSL, complicated skin and skin structure infection; NRPcells, non-replicating persistent cells; PFOR, pyruvate–ferredoxin oxidoreductase; Fgd1.F420-dependent glucose-6-phosphate dehydrogenase; TB, tuberculosis; VISA, vancomycin-intermediate *S. aureus*.

* TB Alliance (The Global Alliance for TB Drug Development) can be Found at www.tballiance.org

‡ Otsuka Pharmaceuticals can be found at http://www.otsuka-us.com.