Chemical Approaches to Tackle the Silent Pandemic of Antibiotic Resistance

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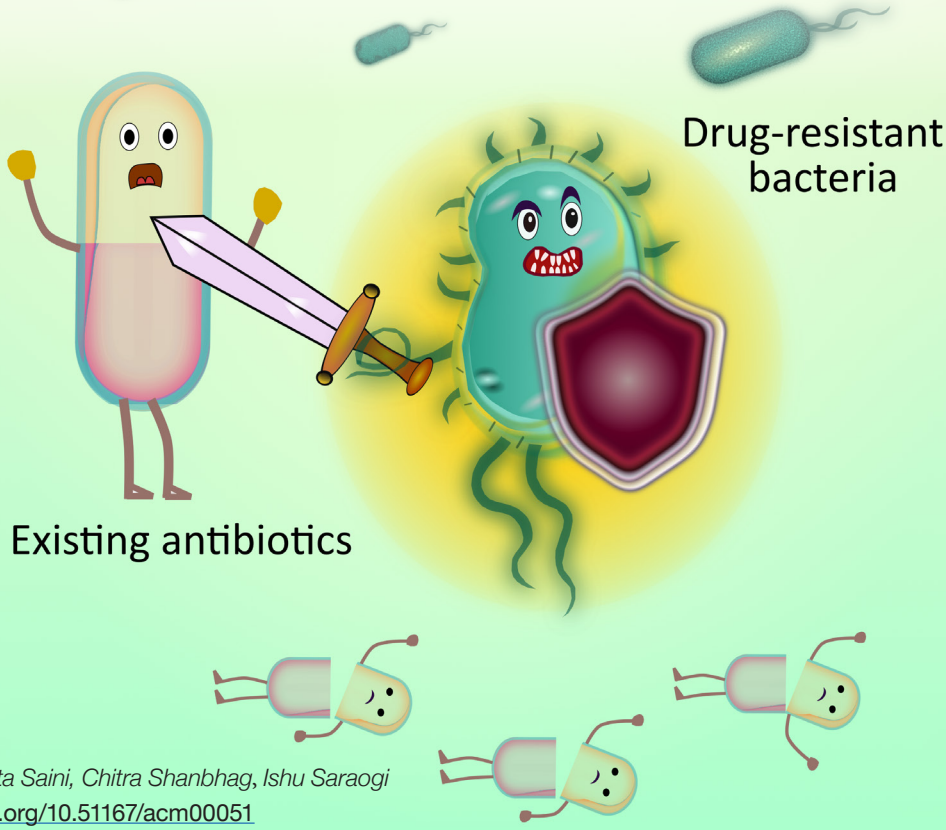

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https://doi.org/10.51167/acm00051

The rapid rise of antibiotic resistance among infectious pathogens is a matter of grave concern. This development of resistance is an evolutionary process, which is intensified due to prolonged and excessive exposure to antibiotics, which will ultimately force us to a situation where well-established lines of treatment will cease to be effective against routine infections. Thus, it is imperative to develop novel strategies to tackle this silent pandemic of antibiotic resistance. Here we briefly summarize the far-reaching implications of antibiotic resistance globally and highlight some key mechanisms by which resistance arises. We also discuss possible approaches to address this problem, including the identification and validation of novel antibacterial pathways that can circumvent existing resistance mechanisms to effectively curb this global threat.

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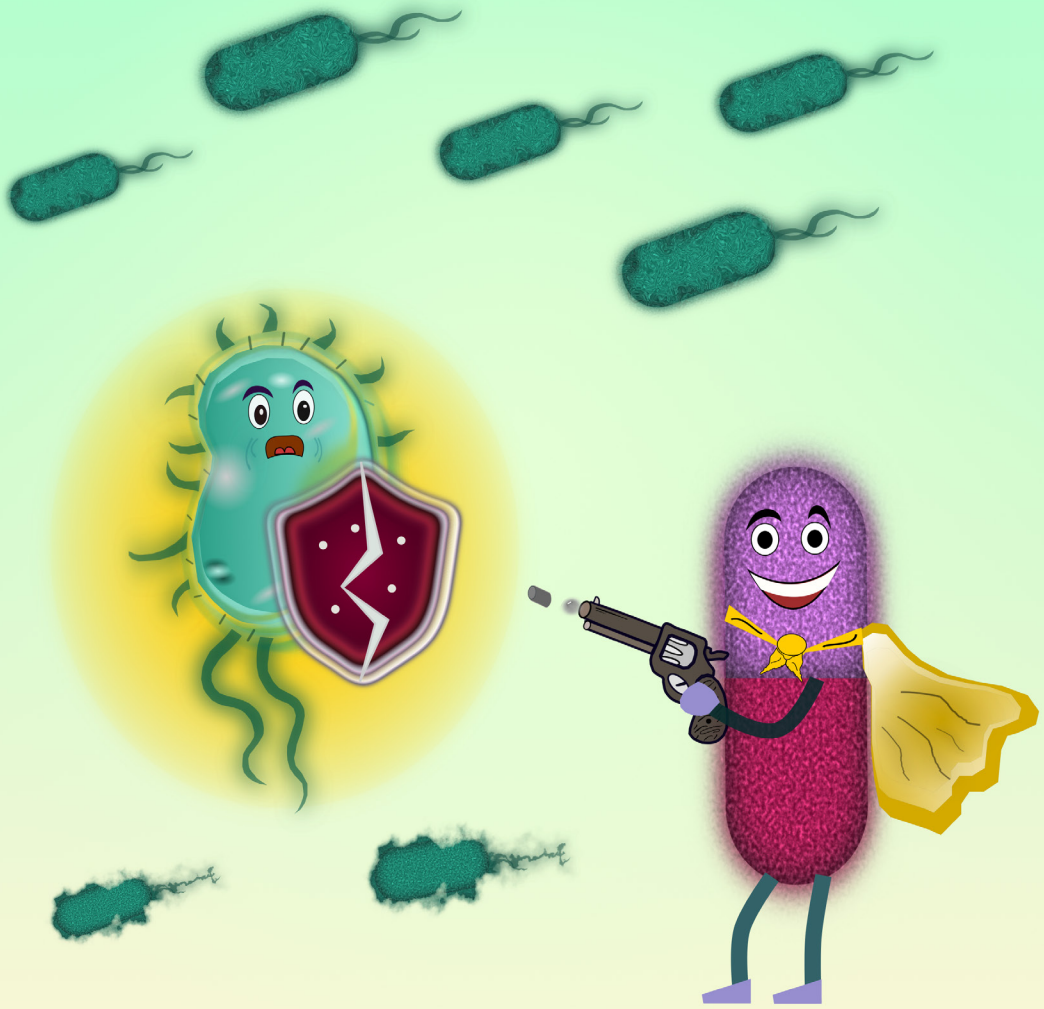

Antibiotic resistance: a serious health concern

The discovery of antibiotics in the 1930s has played an imperative role in medical science.[1] Bacterial infections that were otherwise life-threatening could be cured by a short course of antibiotic treatment. In the subsequent decades, several antibiotics were discovered and helped our fight against infectious diseases. Unfortunately, exposure to antibiotics also enabled microbes to develop resistance. This has led to pathogenic bacteria which have rapidly evolved resistance against most antibiotics in clinical use (Figure 1A), and are associated with adverse health and economic consequences.[2] The emergence of resistance against antibiotics is a natural evolutionary process, which occurs when bacterial subpopulations acquire the ability to circumvent the effect of drugs designed to kill them. Genetic mutation or acquisition of genetic material from a resistant bacterium quickly confers resistance to different bacteria against various antibiotic classes through horizontal gene transfer.[3] The consequent emergence of superbugs, which are difficult to treat with existing antibiotics, have led to higher medical costs and increased mortality rates. Increasing pervasiveness of highly pathogenic multidrug-resistant (MDR) and extensively drug-resistant (XDR) bacteria like *Mycobacterium tuberculosis,* Methicillin-resistant *Staphylococcus aureus* (MRSA), and those containing New Delhi metallo-β-lactamase-1 (NDM-1) gene etc. are an alarming threat to society (Figure 1B,C).[4,5]

Antibiotic resistance is a worldwide problem affecting both developing and developed countries. The problem has been accelerated by the overuse and misuse of antibiotics, leading to a dramatic increase in resistance. In Europe, ~25,000 deaths are reported every year owing to antibiotic resistance, which in turn leads to an expenditure of 1.5 billion euros for the European Union economy.[6] Similarly, the US is also severely affected by antibiotic resistance with more than 2 million people infected annually, and an average of 25,000 deaths per year. In Asia and Africa, the situation is much worse (Figure 1D).[7] There are reports that bacteria such as *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* have become resistant to the second and third-generation antibiotics like carbapenem and cephalosporin.[8] A metallo-β-lactamase coding gene (*bla*NDM-1), first detected in New Delhi in 2008,which

encodes a carbapenemase that neutralizes the powerful carbapenem antibiotics has spread globally to more than 70 countries and requires urgent action.[4] The situation has worsened during the COVID-19 pandemic, which has resulted in the broad use of antibiotics due to co-infection or to prevent hospital-acquired infections.[9] Globally, antibiotic resistance is estimated to result in ~10 million deaths by 2050, exceeding that from cancer (Figure 1E).[7]

The silent pandemic of antibiotic resistance warrants collective urgent action to minimize the impact on human life and the economy. Since resistance occurs due to exposure of bacteria to antibiotics, preventing the misuse of antibiotics will partially help control the spread of resistance. Additionally, it is important to discover new antibiotics that the bacteria have not been exposed to, so that existing resistance mechanisms can be circumvented. Unfortunately, the discovery of new antibiotics has not kept up with the appearance of resistance. Over the last few decades, progressively fewer new antibiotics have been brought to the market, due to a combination of various technical and economic challenges (Figure 1F).[10] Thus, there is a pressing need to identify promising antibacterial strategies including targeting key biochemical mechanisms in bacteria which will directly challenge the survival of resistant bacteria.

Mechanisms of antibiotic resistance

Traditionally, antibiotic discovery efforts have focused on a select few biosynthetic pathways in bacteria, which are essential for their survival including the cell wall or cell membrane synthesis, nucleic acid synthesis or protein synthesis.[11] Decades of exposure to these antibacterial agents have led to the development of resistance against them. There are several mechanisms by which resistance appears (Figure 2). These

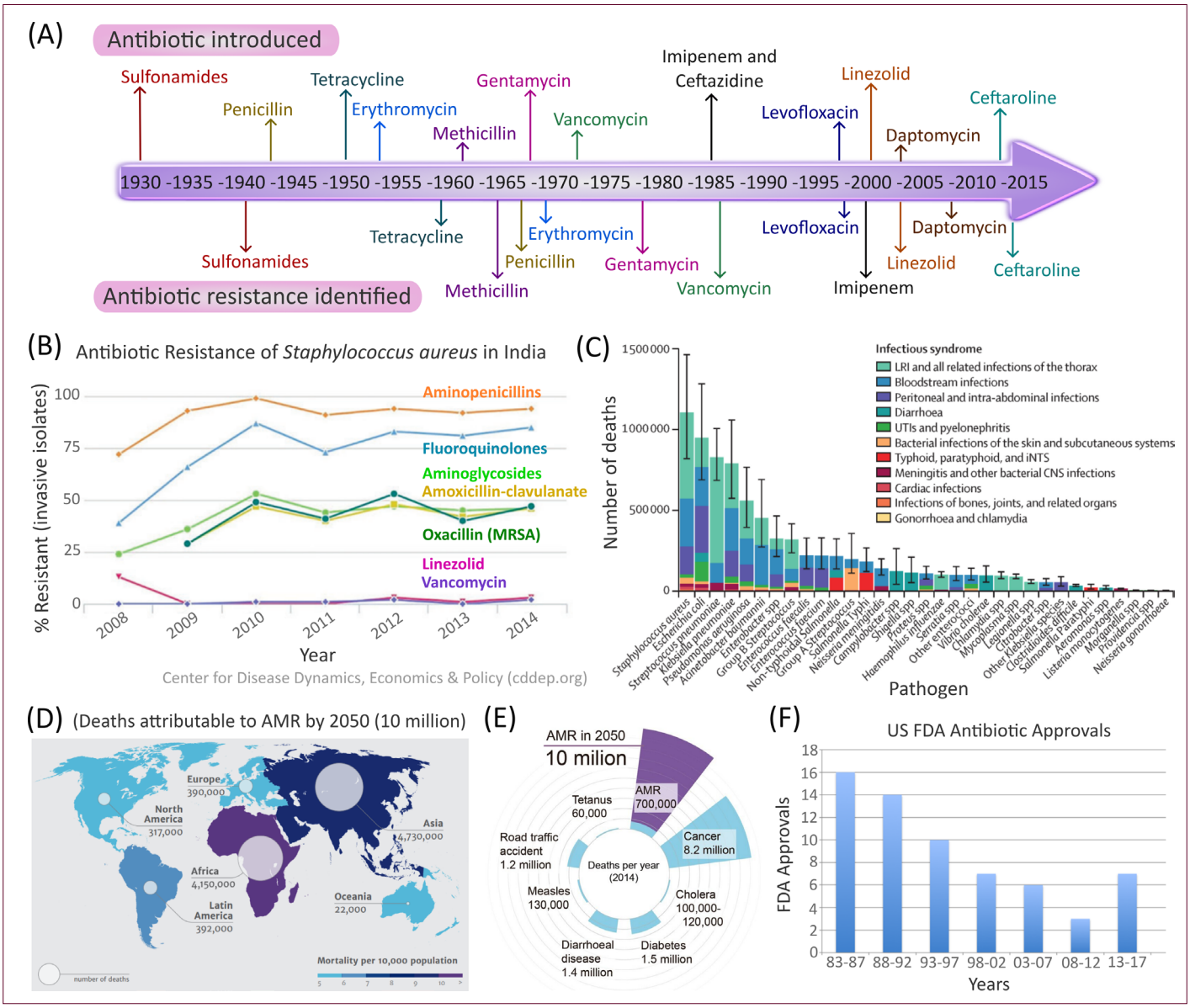

Figure 1. (A) Timeline for antibiotic development and the evolution of antibiotic resistance. The year in which each antibiotic was introduced to the market is depicted above, and the year in which resistance to it was observed is depicted below the timeline (Figure adapted from Ref. 2). (B) Resistance patterns of *Staphylococcus aureus* isolates for different antibiotic classes in India (Figure reproduced from Ref. 4) (C) Global number of deaths by pathogen and infectious syndrome, 2019. Columns show the total number of deaths for each pathogen (reproduced from Ref. 5). (D) Deaths attributable to antimicrobial resistance (AMR) globally by 2050, and (E) compared to other major causes of death by 2050 (Figures reproduced from Ref. 7). (F) A graph depicting the rapid decrease in the number of new antibiotic approvals by US FDA during 1983-2017 (Figure reproduced from Ref. 10).

include the modification of the target so that drug binding is prevented, and reduced uptake, degradation, modification, or efflux of the drug from the bacterial cell.[12] Once resistance has evolved, these genes can be shared between different bacteria through horizontal gene transfer, further accelerating resistance. Below we discuss some mechanisms of microbial resistance.

The bacterial cell wall is made up of peptidoglycan. Damage to its structure causes important metabolites essential for cellular function to leak, leading to cell death. Thus, peptidoglycan is a highly desirable antibacterial target, also because eukaryotic cells do not possess a peptidoglycan cell wall thus minimizing potential toxicity in humans.[13] Examples of this class of antibiotics include β-lactams (e.g. penicillins, carbapenems), glycopeptides (vancomycin), and bacitracin. β-lactams bind to the transpeptidase enzyme important for cell wall synthesis. However, bacteria have developed resistance against these antibiotics through the synthesis of β-lactam-hydrolyzing enzymes such as β-lactamases.[13] For vancomycin, resistance occurs through modification of the target protein via alterations in the peptidoglycan precursors, resulting in weaker interaction of vancomycin with its target.[13]

Another common mechanism of action for several antibacterial agents is the inhibition of protein synthesis.[14] As proteins are the main functional units of the cell, disruption in their synthesis inhibits normal cellular functioning, leading to bacterial cell death. Several different classes of antibiotics such as aminoglycosides (e.g., gentamicin, amikacin), tetracyclines (e.g., doxycycline, minocycline) and macrolides (e.g., azithromycin, erythromycin) inhibit bacterial protein synthesis by targeting various components of the protein synthesizing factory of the cell called the ribosome.[14] To counter these, bacteria have developed several resistance mechanisms including modification of ribosomal proteins or ribosomal RNA (rRNA).[15] Alterations in the ribosome binding site lead to a decrease in antibiotic binding, thereby reducing or disrupting its ability to inhibit protein synthesis. One such example is the erythromycin ribosome methylase (*erm*) family of genes that methylate the 16S rRNA at the antibiotic binding site thus disrupting binding with macrolides.[15] In a bid to target Erm methyltransferase for combating resistance, Anand and co-workers have recently illustrated the mechanism by which Erm methyltransferase methylates the target base in a complicated RNA scaffold resulting in erythromycin resistance.[16]

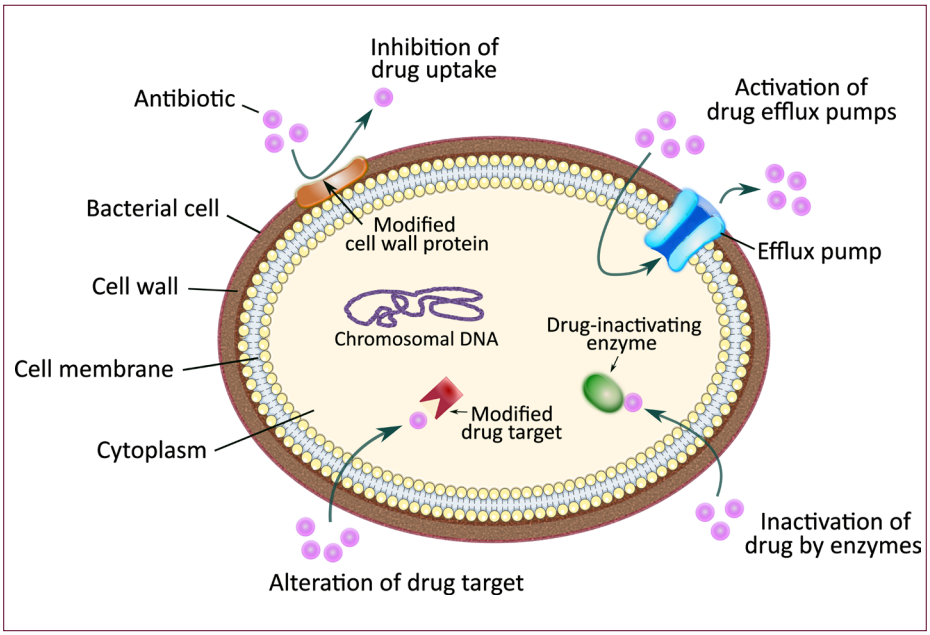

Figure 2. The bacterial cellular structure depicting a few key mechanisms of antibiotic resistance.

Yet another class of antibiotics target nucleic acid biosynthesis by inhibiting the crucial components involved in DNA or RNA synthesis.[17,18] As these two components are fundamental for all cellular processes, inhibition of their biosynthesis leads to bacterial cell death. Examples of this class of antibiotics include fluoroquinolones and rifamycins. However, several pathogenic bacteria have acquired the *qnr* families of quinolone resistance genes which confer resistance against these antibiotics.[19] The *qnr* genes encode proteins which shield topoisomerase IV and DNA gyrase from the inhibitory action of fluoroquinolones.[19]

Any new drugs that target pathways for which resistance mechanisms already exist are not very effective in slowing the emergence of further resistance. This has necessitated the search for novel strategies to tackle antibiotic resistance. New drugs with diverse modes of action will also avoid cross-resistance, i.e., resistance to different antibiotics with a similar mechanism of action.

Strategies to combat antibiotic resistance

Considerable efforts have been made in recent years to address the antibiotic crisis by developing novel therapeutic strategies such as drug repurposing, antibiotic cocktail therapy, phage therapy, photodynamic therapy, and identifying novel drug targets in bacteria.[20–24] Since developing an entirely new drug takes more time, money, and effort; drug repurposing and antibiotic cocktail therapy were favoured by pharmaceutical companies as these rely on utilizing already approved drugs intended for treating other diseases. When tested alone or in combination, some FDA-approved drugs were found to be effective against resistant infectious pathogens.[25] For example, β-lactam (BL) based antibiotics (penicillins, cephalosporins, carbapenems) were used in combination with β-lactamase inhibitors (BLI) to treat multidrug-resistant Gram-negative bacterial pathogens.[26] Examples of successful BL-BLI combinations include ceftolozane-tazobactam, ceftazidime-avibactam, and meropenem-vaborbactam.[26] Unfortunately, resistance to some of these has already been demonstrated. Currently, colistin a membrane-disrupting antibiotic is used to treat drug-resistant hospital-acquired Gram-negative bacterial infections in combination with BL-BLI (Figure 3A).[27] Although the administration of these combinations is promising for a short period, they will eventually lead to cross-resistance. Hence, we must continue to find alternatives to stay ahead in our evolutionary war against bacteria.

A non-antibiotic strategy for killing infectious bacteria is phage therapy which leaves human cells unharmed.[21] Phages are bacterial parasites (a form of virus) that specifically recognize bacterial cell surface receptors and inject their genome within the bacterial host (Figure 3B). The phage genome integrates within the bacterial genome, hijacking bacterial replication

machinery to produce the next generation of phage progeny, leading to lysis of the bacterial cell. Many countries have extensively used phage therapies to treat infections caused by *S. aureus*, *P. aeruginosa* and *K. pneumoniae*.[21] Recently, early-stage clinical trials in adults with cystic fibrosis who carry *P. aeruginosa* in their lungs have been initiated to evaluate the safety of phage therapy.[28]

Another novel approach is antibacterial photodynamic therapy (PDT) which relies on utilizing non-toxic photosensitizers, which upon exposure to light generate reactive oxygen species (ROS) such as superoxide, peroxide, and hydroxyl radicals that act as deadly weapons to kill bacteria.[22] Thus, PDT has broad-spectrum activity against a wide variety of pathogens to treat localized infections. The most frequently used photosensitizers (PS) in clinical applications include phenothiazinium dyes, 5-aminolevulinic acid methyl ester, methylene blue etc. (Figure 3C).[22] There are prominent examples of the utilization of PDT in clinical treatment of dental plaques, chronic leg ulcers, acne vulgaris. Further, nanoparticle-mediated delivery was utilized for enhanced PS uptake in bacteria to substantiate the therapeutic index of PDT.[29] Small molecules harbouring redox functionalities that can generate ROS to kill drug-resistant *Mycobacterium tuberculosis* have also been explored.[30]

Novel antibacterial targets

Besides the development of non-antibiotic approaches described above, several labs have focused on validating cellular pathways with pivotal roles in bacterial survival and virulence as novel antibacterial targets. Ideally, the chosen cellular pathway should be conserved across bacterial species, but exhibit minimal eukaryotic homology to prevent toxicity to humans. Identifying new inhibitors to such essential pathways would expand the antibiotics toolbox to disarm pathogenic bacteria. Several essential cellular pathways have exhibited potential or are upcoming targets in the fight against antimicrobial resistance. In this review, we discuss a few representative examples related to proteins involved in cellular signal transduction pathways such as bacterial histidine kinases, GTPases, and bacterial protein transport and secretion systems with reference to their reported inhibitors.[31–33]

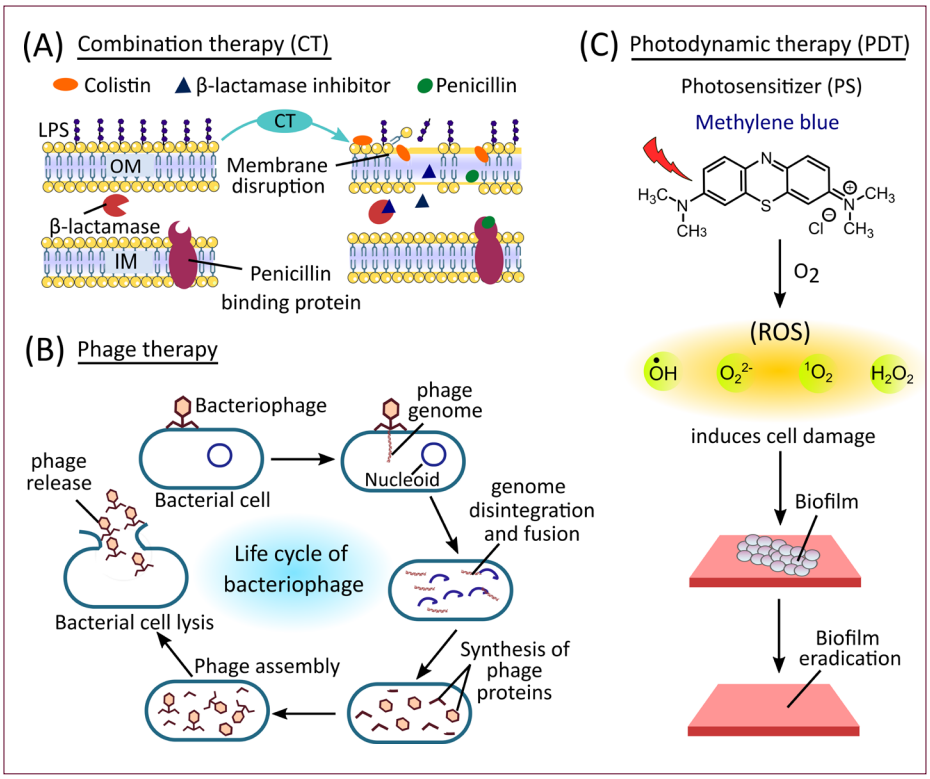

Figure 3. (A) A schematic depiction of combination therapy (CT) using three drugs. Colistin disrupts lipopolysaccharide (LPS) and outer membrane (OM), thus facilitating cellular uptake of penicillin (which binds to penicillin binding protein) and a β-lactamase inhibitor (BLI). (B) Schematic representation of bacteriophage life cycle and its use in phage therapy. Bacteriophage specifically infects bacteria to reproduce, and lyse the bacterial cell. (C) Schematic depiction of the principle behind antibiotic photodynamic therapy in which an excited photosensitive molecule reacts with molecular oxygen to produce reactive oxygen species (ROS) that induces bacterial cell damage resulting in eradication of the biofilm.

Bacterial signal transduction components

Signal transduction systems are composed of complex protein networks involved in regulating cellular signalling cascades that respond to molecular or physical cues from the environment.[31,32] These proteins utilize nucleotide triphosphates such as ATP or GTP as a currency to mediate cell signalling, often accompanied by phosphate transfer or release. Examples of such protein families include ATPases, GTPases, and kinases etc. which are evolutionarily conserved but relatively simple and well-studied in prokaryotes. There are several instances where components of signal transduction have been found to be essential in bacterial cell survival or the production of virulence factors.[31,32] Hence, identifying and validating these components as viable antibiotic targets will open avenues to tackle antibiotic resistance. Below, we briefly discuss bacterial kinases and GTPases, components of signal transduction that have been targeted with small molecules as antibacterial agents.

Bacteria can readily adapt to diverse environmental conditions and often utilize signal transduction pathways to respond to external environmental stimuli. These stimuli might include variations in pH, osmotic pressure, redox state, antibiotics, etc. The capability of the bacterial cell to deal with such environmental cues relies on phosphotransfer-based systems, such as the two-component regulatory system (TCS).[31] The bacterial TCS system, which is relatively simple compared to eukaryotes, is comprised of a membrane-associated histidine kinase (HK), a homodimeric protein that senses external stimuli. Its cognate receptor, called response regulator (RR), facilitates cellular response by altering gene expression. Signal transduction occurs via recognition of the extracellular stimulus by the sensor domain of HK, resulting in ATP-mediated autophosphorylation of a specific histidine residue within the cytoplasmic domain of histidine kinase. The transfer of phosphate from HK to the cognate RR generates phosphorylated RR, which binds to DNA and orchestrates differential gene expression in response to the external stimulus (Figure 4A). The involvement of TCS in mediating bacterial virulence and activating resistance to vancomycin has been reported.[34] Recently, a mycobacterium-specific cyclic-di-GMP responsive TCS that controls transcriptional modulation for sustenance during nutrient deprivation was also reported.[35] Thus, TCS function is important to generate appropriate bacterial response under stress.

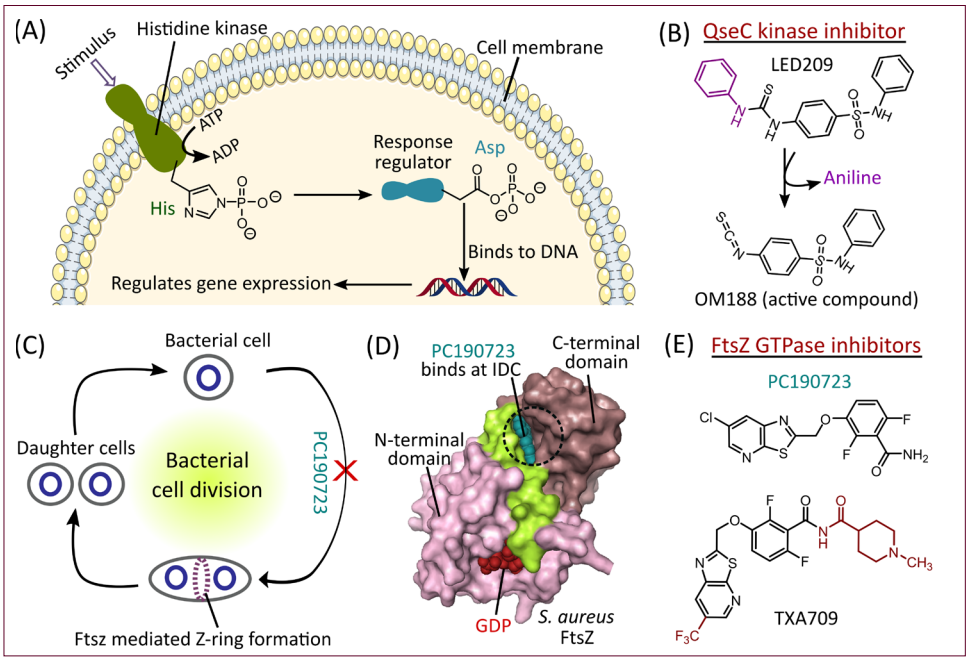

Figure 4. (A) Schematic depiction of the function of two component system (TCS) in bacteria. Upon stimulation, membrane bound histidine kinase (HK) autophosphorylates and catalyzes the phosphorylation of a conserved Asp residue of the response regulator (RR). RR in turn binds to DNA to regulate differential gene expression in response to environmental cues. (B) The QseC histidine kinase inhibitor LED209 gets cleaved to the active compound OM188, while aniline is released as the side product. (C) Schematic representation of the functional role of the GTPase FtsZ in bacterial cell division, and its inhibition by PC190723. (D) Surface view of the crystal structure of *S. aureus* FtsZ (PDB: 4DXD) showing binding of the inhibitor PC190723 at an interdomain cleft (IDC) (dotted circle), which is situated between the N and C-terminal domains separated by a H7-helix (lemon green). (E) Chemical structures of the FtsZ inhibitor PC190723 and its improved prodrug version TXA709 (modifications are highlighted in red).

As histidine kinases are initiators of TCS function, they are viable antibacterial targets. Here, we discuss the example of QseC HK inhibition to reduce pathogenicity in enterohemorrhagic *Escherichia coli* (EHEC), an infective agent of the human colon that activates virulent gene expression. Rasko *et al.* reported a small molecule called LED209 that prevented QseC histidine kinase receptor phosphorylation leading to the inhibition of QseC-mediated virulence gene expression in EHEC, including *Salmonella typhimurium, and Francisella tularensis*.[36] Later, Curtis *et al.* showed that LED209 is a prodrug that gets cleaved in the bacterial environment to the active compound OM188 (Figure 4B).[37] The isothiocyanate moiety of OM188 covalently modifies the lysine residues of QseC HK, thus impairing autophosphorylation. LED209, which has desirable pharmacokinetic properties and was shown to be safe in rodents, is a promising clinical candidate for drug-resistant infections.[36]

Another family of viable antibacterial targets include the GTPases, which are hydrolase enzymes that bind to GTP and hydrolyze its γ-phosphate to produce guanosine diphosphate (GDP) and inorganic phosphate (Pi). Bacterial GTPases that control major cellular signalling events including those involved in cell division, ribosome assembly, tRNA modification, protein synthesis and secretion are attractive antibacterial targets.[32] Some inhibitors for GTPases involved during protein synthesis are currently in antibiotic use, e.g., fusidic acid.[38] The appearance of resistance to fusidic acid took ~40 years, leading to the expectation that the development of resistance to antibiotics against complex systems like GTPases will be slower than in the case of classic antibiotic targets.[38]

Efforts towards novel GTPase inhibitors have led to a potent inhibitor of the GTPase FtsZ, which has recently moved to clinical trials.[39] FtsZ, which is essential for bacterial cell division, functions by polymerizing itself in a GTP-dependent manner to provide a ring structure. The ring (called Z-ring) acts as a site of constriction for bacterial cell division to produce daughter cells (Figure 4C).[39] The GTPase activity of FtsZ was potently inhibited by PC190723, which binds to an inter-domain cleft on *S. aureus* FtsZ monomer with nanomolar affinity (Figure 4D). This resulted in the disruption of FtsZ-mediated Z-ring formation, thereby hampering bacterial cell division.[40] However, PC190723 was found to be metabolically unstable as it was susceptible to the action of cytochrome P450.[41] To bypass this problem, a prodrug TXA709 which was a derivative of PC190723 was developed (Figure 4E) that displayed improved metabolic stability and pharmacokinetic properties. TXA709 is currently under clinical trials to treat multidrug-resistant *S. aureus* infections.[41]

Bacterial protein transport and secretion systems

The synthesis of proteins takes place primarily in the cytoplasm. However, a large fraction of these proteins must be delivered to various cellular destinations, where they can carry out their designated function. Many bacterial pathogens also secrete proteins involved in promoting pathogenicity (e.g., streptolysin O from *Streptococcus species*) or in acquiring antibiotic resistance (e.g., β-lactamase in Gram-negative bacteria).[33] To transport these proteins from their site of origin (cytoplasm) to their destination (periplasmic space or plasma membrane), bacteria utilize dedicated protein transport pathways (Figure 5A). Thus, inhibiting these pathways will disrupt proper protein localization within bacterial cells, eventually killing them.[42]

As hydrophobic proteins might aggregate or fold prematurely in the cytoplasm, their transport which begins at the ribosome during translation is referred to as co-translational transport. In bacteria, co-translational protein transport is carried out by the GTPases of the Signal Recognition Particle (SRP) pathway, which bring ribosomes translating membrane and secretory proteins to the plasma membrane (Figure 5A).[43] Bacterial SRP is a ribonucleoprotein complex composed of a non-coding RNA called SRP RNA (4.5S RNA in *E. coli*) and a protein called Ffh. The translating ribosome-SRP complex is brought to the SecYEG translocon by the interaction of SRP with its receptor (SR), in a process that requires GTP hydrolysis from both SRP and SR. Studies have shown that the SRP pathway is not only essential for bacterial cell survival, but is also important in the secretion of virulence factors.[44] Hence, the bacterial SRP pathway has been proposed as a potential antibacterial target. In a recent report, an antisense peptide nucleic acid (PNA) was reported to inhibit the interaction of *E. coli* SRP RNA with the Ffh protein.[45] This led to the inhibition of SRP-mediated

GTP hydrolysis and *E. coli* cell growth in a dose-dependent manner, thereby validating bacterial SRP as a potential antibacterial target.[45]

The post-translational transport of various periplasmic, membrane, and secretory proteins in bacteria is mediated by the secretion (Sec) pathway after the termination of protein synthesis.[33] Several Gram-negative bacterial pathogens use the Sec pathway to transport virulence factors through the plasma membrane. The Sec pathway utilizes two protein components namely SecA and SecB that facilitate the translocation of synthesized proteins in their premature, unfolded state (Figure 5A). SecB binds to signal sequences on presecretory proteins, prevents their folding, and delivers them to SecA. The ATPase domain of SecA provides energy for protein translocation and guides the protein to the SecYEG translocation channel. The secreted proteins are then folded upon delivery to the periplasm.[46] As SecA is well-conserved among bacteria, is essential for bacterial viability, and is absent in eukaryotes, it is a promising antibacterial target.[47] Among several reported inhibitors, SCA-107 was the most active allosteric inhibitor of SecA ATPase activity and showed antibacterial activity against several Gram-negative (e.g., *E. coli*, *P. aeruginosa*, and *A. baumannii*) and Gram-positive (e.g., *S. aureus*) bacteria.[47] *In vivo* studies have also shown that SCA-107 protects mice against lethal infection caused by *S. aureus*.[47] These studies validate SecA as a promising antibacterial target.

To evade the host immune system during infection, bacterial cells use specific machineries to secrete effector proteins crucial for pathogenesis including toxins, anti-host factors, etc. into the host cell.[33] Although these systems are not essential for bacterial viability, they have an impact on virulence. Hence, the inactivation of bacterial secretion systems would lead to attenuation of pathogenesis, without impacting bacterial survival, thus slowing down the emergence of resistance. So far seven types of bacterial secretion systems (Type I-VII) have been identified based on their ability to secrete proteins across single, double, or triple phospholipid membranes. Among them, Type (I-VI) are prevalent in Gram-negative bacteria whereas Type VII secretion system is found in Gram-positive bacteria.[33] The Type III secretion system (T3SS) is an attractive drug target due to its importance in delivering effector proteins into the cytoplasm of the host eukaryotic cell across three membranes.[23] Among the reported small molecules, salicylidene acylhydrazides effectively inhibited T3SS in *Y. pseudotuberculosis* and reduced its pathogenicity.[49] However, these molecules are reported to have multiple targets or act indirectly on T3SS by impairing bacterial physiology.[23] To specifically target T3SS, antibodies against several tip proteins have been reported. An antibody MEDI3902 against the T3SS tip protein PcrV, was effective against *P. aeruginosa* and a wide range of clinical isolates and is currently under phase II clinical trials.[50] These studies suggest that T3SS can be used for anti-virulent therapy.

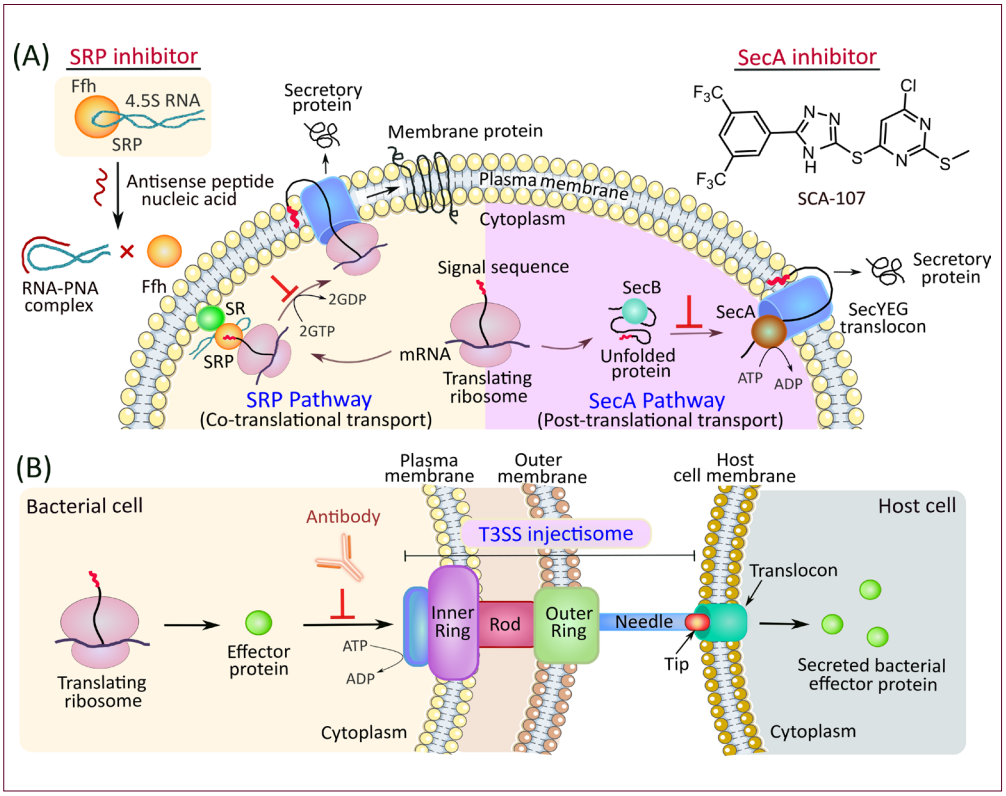

Figure 5. (A) The SRP pathway mediates co-translational transport of membrane and secretory proteins to the bacterial plasma membrane (left). SRP, which is a ribonucleoprotein complex (4.5S RNA and Ffh protein) recognizes the signal sequence (red) of the nascent proteins on translating ribosomes and delivers them to the plasma membrane with the help of its receptor SR. Antisense peptide nucleic acid (PNA) inhibits the formation of functional SRP by forming an RNA-PNA complex and disrupts the SRP pathway. The post-translational mode of protein transport is mediated by the Sec pathway which involves the ATPase SecA and the SecB chaperone (right). The small molecule SCA-107 inhibits the ATPase activity of SecA and prevents SecA mediated protein transport in bacteria. (B) A schematic illustration of Type 3 secretion system (T3SS) bearing the injectisome that invades the host cell by injecting effector proteins (e.g., toxins and anti-host factors) during host-pathogen interaction. Inhibition of T3SS with specific antibodies against different components of the T3SS injectisome inhibits secretion of effector proteins into host cells.

Conclusion

Antibiotic resistance in pathogenic bacteria is a relatively neglected global pandemic. The upsurge in the morbidity and mortality rates among patients infected with pathogenic bacteria demands the development of effective antibiotics and new strategies that can eradicate or minimize antibiotic resistance. Drug repurposing and antibiotic combination therapies are quick and reliable, but in the long run, will result in cross-resistance and generate superbugs. Thus, non-antibiotic-based strategies such as phage therapy and photodynamic therapy might be explored to circumvent antibiotic resistance problems. In addition, identifying and targeting either the molecular mechanisms of resistance or other essential biochemical pathways in bacteria will be important to tackle antimicrobial resistance.

Acknowledgments

Work on antibiotic resistance in our laboratory is generously supported by Science and Engineering Research Board and IISER Bhopal.

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