How antibiotics kill bacteria: from targets to networks

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Abstract | Antibiotic drug–target interactions, and their respective direct effects, are generally well characterized. By contrast, the bacterial responses to antibiotic drug treatments that contribute to cell death are not as well understood and have proven to be complex as they involve many genetic and biochemical pathways. In this Review, we discuss the multilayered effects of drug–target interactions, including the essential cellular processes that are inhibited by bactericidal antibiotics and the associated cellular response mechanisms that contribute to killing. We also discuss new insights into these mechanisms that have been revealed through the study of biological networks, and describe how these insights, together with related developments in synthetic biology, could be exploited to create new antibacterial therapies.

Bactericidal
Antimicrobial exposure that leads to bacterial cell death.

Bacteriostatic
Antimicrobial exposure that inhibits growth with no loss of viability.

Cell envelope
Layers of the cell surrounding the cytoplasm that include lipid membranes and peptidoglycans.

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doi:10.1038/nrmicro2333
Published online 4 May 2010

Our understanding of how antibiotics induce bacterial cell death is centred on the essential bacterial cell function that is inhibited by the primary drug–target interaction. Antibiotics can be classified based on the cellular component or system they affect, in addition to whether they induce cell death (bactericidal drugs) or merely inhibit cell growth (bacteriostatic drugs). Most current bactericidal antimicrobials — which are the focus of this Review — inhibit DNA, RNA, cell wall or protein synthesis[1].

Since the discovery of penicillin in 1929 (REF. 2), other, more effective antimicrobials have been discovered and developed by elucidation of drug–target interactions and by drug molecule modification. These efforts have greatly enhanced our clinical armamentarium. Antibiotic-mediated cell death, however, is a complex process that begins with the physical interaction between a drug molecule and its specific target in bacteria, and involves alterations to the affected bacterium at the biochemical, molecular and ultrastructural levels. The increasing prevalence of drug-resistant bacteria[3], as well as the increased means of gaining resistance, has made it crucial to better understand the multilayered mechanisms by which currently available antibiotics kill bacteria, as well as to explore and find alternative antibacterial therapies.

Antibiotic-induced cell death has been associated with the formation of double-stranded DNA breaks following treatment with inhibitors of topoisomerase II (also known as DNA gyrase)[4], with the arrest of DNA-dependent RNA synthesis following treatment with rifamycins[5], with cell envelope damage and loss of structural integrity following treatment with inhibitors of cell wall synthesis[6], and with cellular energetics, ribosome binding and protein mistranslation following treatment with inhibitors of protein synthesis[7]. In addition, recent evidence points towards a common mechanism of cell death involving disadvantageous cell responses to drug-induced stresses that are shared by all classes of bactericidal antibiotics, which ultimately contributes to killing by these drugs[8]. Specifically, treatment with lethal concentrations of bactericidal antibiotics results in the production of harmful hydroxyl radicals through a common oxidative damage cell death pathway that involves alterations in central metabolism (that is, in the tricarboxylic acid (TCA) cycle) and iron metabolism[8–10].

In this Review we describe our current knowledge of the drug–target interactions and the associated mechanisms by which the main classes of bactericidal antibiotics kill bacteria. We also describe recent efforts in network biology that have yielded new mechanistic insights into how bacteria respond to lethal antibiotic treatments, and discuss how these insights and related developments in synthetic biology could be used to develop new, effective means to combat bacterial infections.

Inhibition of DNA replication by quinolones

DNA synthesis, mRNA transcription and cell division require the modulation of chromosomal supercoiling through topoisomerase-catalysed strand breakage and rejoining reactions[11–13]. These reactions are exploited by the synthetic quinolone class of antimicrobials, including the clinically relevant fluoroquinolones, which target

Table 1 | **Antibiotic targets and pathways**

Drug type	Drug	Derivation	Species range	Primary target	Pathways affected
Fluoroquinolones*					
DNA synthesis inhibitor	Nalidixic acid, ciprofloxacin, levofloxacin and gemifloxacin	Synthetic	Aerobic Gram-positive and Gram-negative species, some anaerobic Gram-negative species (*C. perfringes*) and *M. tuberculosis*	Topoisomerase II (DNA gyrase), topoisomerase IV	DNA replication, SOS response, cell division, ATP generation, TCA cycle, Fe–S cluster synthesis, ROS formation, and envelope and redox-responsive two-component systems
Trimethoprim–sulfamethoxazole					
DNA synthesis inhibitor	Co-trimoxazole (a combination of trimethoprim and sulfamethoxazole in a 1:5 ratio)	Synthetic	Aerobic Gram-positive and Gram-negative species	Tetrahydrofolic acid synthesis inhibitors	Nucleotide biosynthesis and DNA replication
Rifamycins					
RNA synthesis inhibitor	Rifamycins, rifampin and rifapentine	Natural and semi-synthetic forms of ansamycins (derived from *S. mediterranei*)	Gram-positive and Gram-negative species, and *M. tuberculosis*	DNA-dependent RNA polymerase	RNA transcription, DNA replication and SOS response
β-lactams*					
Cell wall synthesis inhibitors	Penicillins (penicillin, ampicillin, oxacillin), cephalosporins (cefazolin, cefoxitin ceftriaxone, cefepime) and carbapenems (imipenem)	Natural and semi-synthetic forms of carbonyl lactam ring-containing azetidinone molecules (from *P. notatum*, *C. acremonium* and *S. cattleya*)	Aerobic and anaerobic Gram-positive and Gram-negative species	Penicillin-binding proteins	Cell wall synthesis, cell division, autolysin activity (regulated by LytSR–VncRS two-component system), SOS response, TCA cycle, Fe–S cluster synthesis, ROS formation, and envelope and redox-responsive two-component systems
Glycopeptides and glycolipopeptides					
Cell wall synthesis inhibitor	Vancomycin; teicoplanin	Natural and semi-synthetic forms of amino sugar-linked peptide chains (for glycopeptides) or of fatty acid-bearing, amino sugar-linked peptide chains (for glycolipopetides) derived from actinobacteria	Gram-positive species	Peptidoglycan units (terminal D-Ala-D-Ala dipeptide)	Cell wall synthesis, transglycosylation, transpeptidation and autolysin activation (VncRS two-component system)
Lipopeptides					
Cell wall synthesis inhibitors	Daptomycin and polymixin B	Natural and semi-synthetic forms of fatty acid-linked peptide chains (from *S. roseosporus* and *B. polymyxa*)	Gram-positive species (daptomycin), Gram-negative species (polymixins)	Cell membrane	Cell wall synthesis and envelope two-component systems
Aminoglycosides					
Protein synthesis inhibitors	Gentamicin, tobramycin, streptomycin and kanamycin	Natural and semi-synthetic forms of amino sugars (-mycins from *Streptomyces* spp. and -micins from *Micromonospora* spp.)	Aerobic Gram-positive and Gram-negative species, and *M. tuberculosis*	30S ribosome	Protein translation (mistranslation by tRNA mismatching), ETC, SOS response, TCA cycle, Fe–S cluster synthesis, ROS formation, and envelope and redox-responsive two-component systems
Tetracyclines					
Protein synthesis inhibitors	Tetracycline and doxycycline	Natural and semi-synthetic forms of four-ringed polyketides (from *S. aureofaciens* and *S. rimosus*)	Aerobic Gram-positive and Gram-negative species	30S ribosome	Protein translation (through inhibition of aminoacyl tRNA binding to ribosome)
Macrolides					
Protein synthesis inhibitors	Erythromycin and azythromycin	Natural and semi-synthetic forms of 14- and 16-membered lactone rings (from *S. erythraea* and *S. ambofaciens*)	Aerobic and anaerobic Gram-positive and Gram-negative species	50S ribosome	Protein translation (through inhibition of elongation and translocation steps) and free tRNA depletion

Table 1 (cont.) | **Antibiotic targets and pathways**

Drug type	Drug	Derivation	Species range	Primary target	Pathways affected
Streptogramins					
Protein synthesis inhibitors	Pristinamycin, dalfopristin and quinupristin	Natural and semi-synthetic forms of pristinamycin I (group B, macrolactone ringed-peptides) and pristinamycin II (group A, endolactone oxazole nucleus-bearing depsipeptides) (from *Streptomyces* spp.)	Aerobic and anaerobic Gram-positive and Gram-negative species‡	50S ribosome	Protein translation (through inhibition of initiation, elongation and translocation steps) and free tRNA depletion
Phenicols					
Protein synthesis inhibitor	Chloramphenicol	Natural and semi-synthetic forms of dichloroacetic acid with an aromatic nucleus and aminopropanediol chain (from *S. venezuelae*)	Some Gram-positive and Gram-negative species, including *B. fragilis*, *N. meningitidis*, *H. influenzae* and *S. pneumoniae*	50S ribosome	Protein translation (through inhibition of elongation step)

*Drug efficacy can vary across species range based on drug generation. ‡When used as a combination of pristinamycin I and pristinamycin II. *B. fragilis*, *Bacillus fragilis*; *B. polymyxa*, *Bacillus polymyxa*; *C. acremonium*, *Cephalosporium acremonium*; ETC: electron transport chain; *H. influenzae*, *Haemophilus influenzae*; *M. tuberculosis*, *Mycobacterium tuberculosis*; *N. meningitidis*, *Neisseria meningitidis*; *P. notatum*, *Penicillum notatum*; ROS, reactive oxygen species; *S. ambofaciens*, *Streptomyces ambofaciens*; *S. aureofaciens*, *Streptomyces aureofaciens*; *S. cattleya*, *Streptomyces cattleya*; *S. erythraea*, *Saccharopolyspora erythraea*; *S. mediterranei*, *Streptomyces mediterranei*; *S. pneumoniae*, *Streptococcus pneumoniae*; *S. rimosus*, *Streptomyces rimosus*; *S. roseosporus*, *Streptomyces roseosporus*; *S. venezuelae*, *Streptomyces venezuelae*; TCA, tricarboxylic acid.

DNA–topoisomerase complexes[4,14,15]. Quinolones are derivatives of nalidixic acid, which was discovered as a byproduct of the synthesis of chloroquine (a quinine) and was introduced in the 1960s to treat urinary tract infections[16]. Nalidixic acid and other first generation quinolones (for example, oxolinic acid) are rarely used today owing to their toxicity[17]. Second (ciprofloxacin), third (levofloxacin) and fourth (gemifloxacin) generation quinolone antibiotics (TABLE 1) can be classified on the basis of their chemical structure and of qualitative differences between the killing mechanisms they use[16,18].

The quinolone class of antimicrobials interferes with the maintenance of chromosomal topology by targeting topoisomerase II and topoisomerase IV, trapping these enzymes at the DNA cleavage stage and preventing strand rejoining[4,19,20] (FIG. 1). Despite the general functional similarities between topoisomerase II and topoisomerase IV, their susceptibility to quinolones varies across bacterial species[20] (TABLE 1). For example, several studies have shown that topoisomerase IV is the primary target of quinolones in Gram-positive bacteria (for example, *Streptococcus pneumoniae*[21]), whereas in Gram-negative bacteria (for example, *Escherichia coli*[13] and *Neisseria gonorrhoea*[22]) their primary target is topoisomerase II (and topoisomerase IV is the secondary target).

Introduction of DNA breaks and replication fork arrest. The ability of quinolone antibiotics to kill bacteria is a function of the stable interaction complex that is formed between drug-bound topoisomerases and cleaved DNA[4]. On the basis of studies using DNA cleavage mutants of topoisomerase II[23] and topoisomerase IV[24] that do not prevent quinolone binding, and studies that have shown that strand breakage can occur in the presence of quinolones[25], it is accepted that DNA strand breakage occurs after the drug has bound to the enzyme. Therefore, the net effect of quinolone treatment is to generate double-stranded DNA breaks that are trapped by covalently (but reversibly) linked topoisomerases, the function of which is compromised[26–28]. As a result of quinolone–topoisomerase–DNA complex formation, the DNA replication machinery becomes arrested at blocked replication forks, leading to inhibition of DNA synthesis, which immediately leads to bacteriostasis and eventually cell death[4] (FIG. 1). It should be noted that the effects on DNA replication that correlate with bacteriostatic concentrations of quinolones are thought to be reversible[4,29]. Nonetheless, considering that topoisomerase II has been found to be distributed approximately every 100 kb along the chromosome[30], inhibition of topoisomerase function by quinolone antibiotics and the resulting formation of stable complexes with DNA have substantial negative consequences for the cell in terms of its ability to deal with drug-induced DNA damage[31].

The role of protein expression in quinolone-mediated cell death. The introduction of double-stranded DNA breaks following topoisomerase inhibition by quinolones induces the DNA stress response (SOS response), in which RecA is activated by DNA damage and promotes self-cleavage of the LexA repressor protein, inducing the expression of SOS response genes such as DNA repair enzymes[32]. Notably, several studies have shown that preventing the induction of the SOS response enhances killing by quinolones (except in the case of nalidixic acid)[8,33]. Preventing the activation of the SOS response has also been shown to reduce the formation of drug-resistant mutants by blocking the induction of error-prone DNA polymerases[34], homologous recombination[20] and horizontal transfer of drug-resistance elements[35,36].

Together with studies revealing that co-treatment with quinolones and the protein synthesis inhibitor chloramphenicol inhibits the ability of quinolones to kill bacteria[19,37], there seems to be a clear relationship between the

SOS response
The DNA stress response pathway in *E. coli*, the prototypical network of genes of which is regulated by the transcriptional repressor LexA, and is commonly activated by the co-regulatory protein RecA, which promotes LexA self-cleavage when activated.

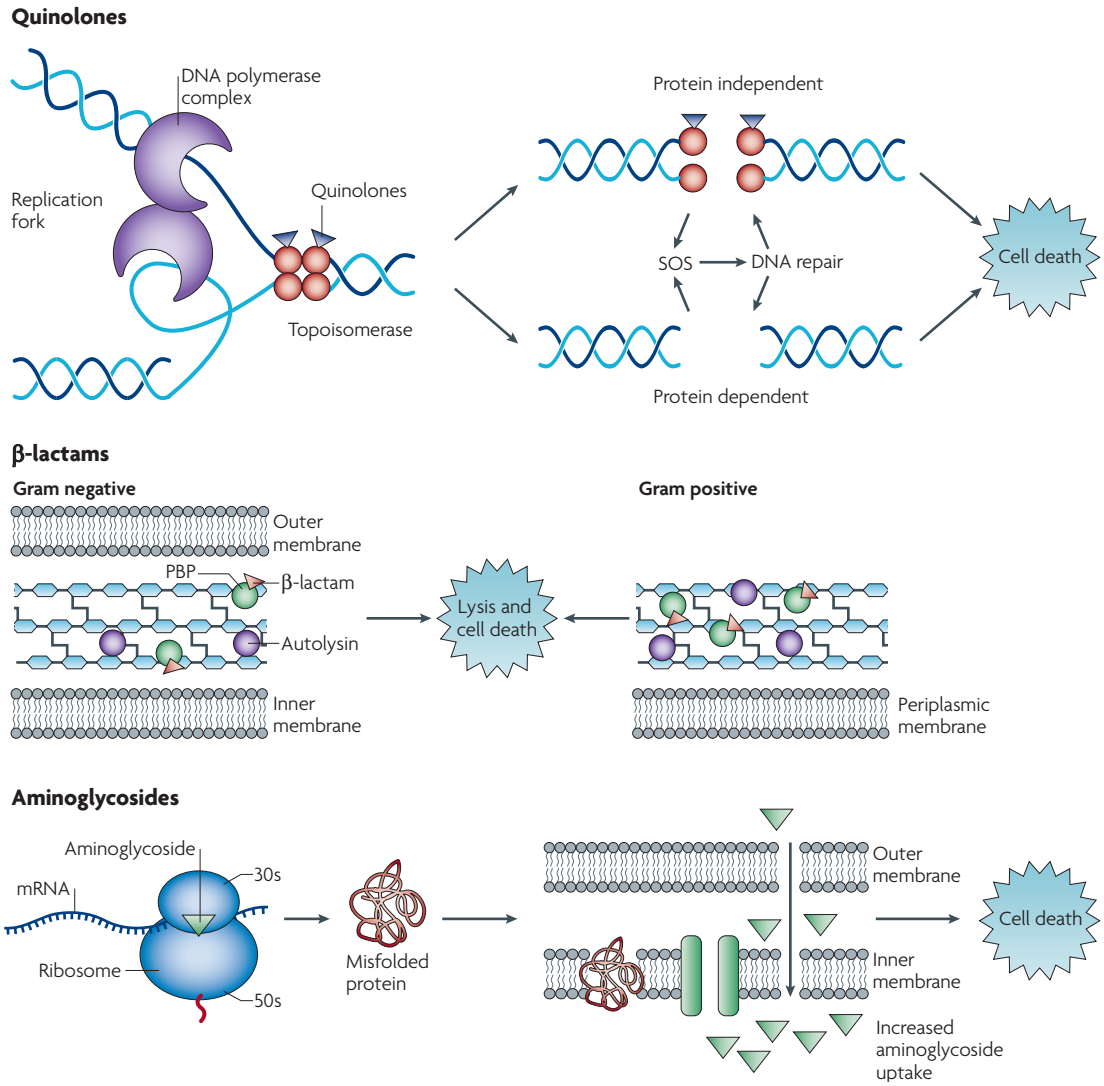


Figure 1 | **Drug-target interactions and associated cell death mechanisms.** Quinolone antibiotics interfere with changes in DNA supercoiling by binding to topoisomerase II or topoisomerase IV. This leads to the formation of double-stranded DNA breaks and cell death in either a protein synthesis-dependent or protein synthesis-independent manner. β-lactams inhibit transpeptidation by binding to penicillin-binding proteins (PBPs) on maturing peptidoglycan strands. The decrease in peptidoglycan synthesis and increase in autolysins leads to lysis and cell death. Aminoglycosides bind to the 30S subunit of the ribosome and cause misincorporation of amino acids into elongating peptides. These mistranslated proteins can misfold, and incorporation of misfolded membrane proteins into the cell envelope leads to increased drug uptake. This, together with an increase in ribosome binding, has been associated with cell death.

primary effects of quinolone–topoisomerase–DNA complex formation and the response of the bacteria (through the stress-induced expression of proteins) to these effects in the bactericidal activity of quinolone antibiotics. For example, the contribution of reactive oxygen species (ROS) to quinolone-mediated cell death has recently been shown to occur in a protein synthesis-dependent manner[38]. Also, the chromosome-encoded toxin MazF has been shown to be required under certain conditions for efficient killing by quinolones owing to its ability to alter protein carbonylation[39], a form of oxidative stress[40].

Inhibition of RNA synthesis by rifamycins

The inhibition of RNA synthesis by the rifamycin class of semi-synthetic bactericidal antibiotics, similarly to the inhibition of DNA replication by quinolones, has a catastrophic effect on prokaryotic nucleic acid metabolism and is a potent means of inducing bacterial cell death[5]. Rifamycins inhibit DNA-dependent transcription by stably binding with high affinity to the β-subunit (encoded by *rpoB*) of a DNA-bound and actively transcribing RNA polymerase[41–43] (TABLE 1). The β-subunit is located in the channel that is formed by the RNA polymerase–DNA complex, from which the newly synthesized RNA strand emerges[44]. Rifamycins uniquely require RNA synthesis to not have progressed beyond the addition of two ribonucleotides; this is attributed to the ability of the drug molecule to sterically inhibit nascent RNA strand initialization[45]. It is worth noting that rifamycins are not thought to act by blocking the elongation step of

RNA synthesis, although a recently discovered class of RNA polymerase inhibitors (based on the compound CBR703) could inhibit elongation by allosterically modifying the enzyme[46].

Rifamycins were first isolated[47] from the Gram-positive bacterium *Amycolatopsis mediterranei* (originally known as *Streptomyces mediterranei*) in the 1950s. Mutagenesis of this organism has led to the isolation and characterization of more potent rifamycin forms[48], including the clinically relevant rifamycin SV and rifampicin. Rifamycins are considered bactericidal against Gram-positive bacteria and bacteriostatic against Gram-negative bacteria, a difference that has been attributed to drug uptake and not to affinity of the drug with the RNA polymerase β-subunit[49]. Notably, rifamycins are among the first-line therapies used against mycobacteria because they efficiently induce mycobacterial cell death[50], although rifamycins are often used in combinatorial therapies owing to the rapid nature of resistance development[49,51].

Interestingly, an interaction between DNA and the hydroquinone moiety of RNA polymerase-bound rifamycin has been observed[52], and this interaction has been attributed to the location of the rifamycin molecule in relation to DNA in the DNA–RNA polymerase complex[42]. This proximity, coupled with the reported ability of rifamycin to cycle between a radical and non-radical form (rifamycin SV and rifamycin S[52,53]), may damage DNA through a direct drug–DNA interaction. This hypothesis could account for the observation that rifamycin SV can induce the SOS DNA damage response in *E. coli* and that treatment of *recA*-mutant *E. coli* results in cell death whereas treatment of wild-type *E. coli* leads to bacteriostasis[8].

Inhibition of cell wall synthesis

Lytic cell death. The bacterial cell is encased by layers of peptidoglycan (also known as murein), a covalently cross-linked polymer matrix that is composed of peptide-linked β-(1-4)-*N*-acetyl hexosamine[54]. The mechanical strength afforded by this layer of the cell wall is crucial to a bacterium's ability to survive environmental conditions that can alter prevailing osmotic pressures; of note, the degree of peptidoglycan cross-linking correlates with the structural integrity of the cell[55]. Maintenance of the peptidoglycan layer is accomplished by the activity of transglycosylases and penicillin-binding proteins (PBPs; also known as transpeptidases), which add disaccharide pentapeptides to extend the glycan strands of existing peptidoglycan molecules and cross-link adjacent peptide strands of immature peptidoglycan units, respectively[56].

β-lactams and glycopeptides are among the classes of antibiotics that interfere with specific steps in homeostatic cell wall biosynthesis. Successful treatment with a cell wall synthesis inhibitor can result in changes to cell shape and size, induction of cell stress responses and ultimately cell lysis[6] (FIG. 1). For example, β-lactams (including penicillins, carbapenems and cephalosporins) block the cross-linking of peptidoglycan units by inhibiting the peptide bond formation reaction that is catalysed by PBPs[55,57,58]. This inhibition is achieved by penicilloylation of the PBP active site — the β-lactam (containing a cyclic amide ring) is an analogue of the terminal D-alanyl-D-alanine dipeptide of peptidoglycan and acts as a substrate for the PBP during the acylation phase of cross link formation. Penicilloylation of the PBP active site blocks the hydrolysis of the bond created with the now ring-opened drug, thereby disabling the enzyme[59,60].

By contrast, most actinobacterium-derived glycopeptide antibiotics (for example, vancomycin) inhibit peptidoglycan synthesis by binding peptidoglycan units (at the D-alanyl-D-alanine dipeptide) and by blocking transglycosylase and PBP activity[61]. In this way, glycopeptides (whether free in the periplasm like vancomycin or membrane-anchored like teicoplanin[62]) generally act as steric inhibitors of peptidoglycan maturation and reduce the mechanical strength of the cell, although some chemically modified glycopeptides have been shown to directly interact with the transglycosylase[63]. It is worth noting that β-lactams can be used to treat Gram-positive and Gram-negative bacteria, whereas glycopeptides are effective against only Gram-positive bacteria owing to low permeability (TABLE 1). In addition, antibiotics that inhibit the synthesis (for example, fosfomycin) and transport (for example, bacitracin) of individual peptidoglycan units are also currently in use, as are lipopeptides (for example, daptomycin), which affect structural integrity by inserting themselves into the cell membrane and inducing membrane depolarization.

Research into the mechanism of killing by peptidoglycan synthesis inhibitors has centred on the lysis event. Initially, it was thought that inhibition of cell wall synthesis by β-lactams caused cell death when internal pressure built up owing to cell growth outpacing cell wall expansion, resulting in lysis[6]. This unbalanced growth hypothesis was based in part on the notion that active protein synthesis is required for lysis to occur following the addition of β-lactams.

The lysis-dependent cell death mechanism, however, has proven to be much more complex, involving many active cellular processes. Seminal work showed that *S. pneumoniae* deficient in amidase activity (possessed by peptidoglycan hydrolase or autolysins) did not grow or die following treatment with a lysis-inducing concentration of a β-lactam, an effect known as antibiotic tolerance[64]. Autolysins are membrane-associated enzymes that break down bonds between and within peptidoglycan strands, making them important during normal cell wall turnover and maintenance of cell shape[55]. Autolysins have also been shown to play a part in lytic cell death in bacterial species that contain numerous peptidoglycan hydrolases, such as *E. coli*[65]. In *E. coli*, a set of putative peptidoglycan hydrolases (LytM domain factors) were shown to be important for rapid ampicillin-mediated lysis[66]. The discovery that autolysins contribute to cell death expanded our understanding of lysis and showed that active degradation of the peptidoglycan layer by peptidoglycan hydrolases, in conjunction with inhibition of peptidoglycan synthesis by a β-lactam antibiotic, triggers lysis[64] (FIG. 1).

Non-lytic cell death. *S. pneumoniae* lacking peptidoglycan hydrolase activity can still be killed by β-lactams, but at a slower rate than autolysin-active cells, indicating that

Lysis
Rupture of the cell envelope leading to the expulsion of intracellular contents into the surrounding environment with eventual disintegration of the cell envelope.

Peptidoglycan hydrolase
An enzyme that introduces cuts between carbon–nitrogen non-peptide bonds while pruning the peptidoglycan layer. It is important for homeostatic peptidoglycan turnover.

Autolysin
An enzyme that hydrolyses the β-linkage between the monosaccharide monomers in peptidoglycan units and can induce lysis when in excess.

there is a lysis-independent mode of killing induced by β-lactams[64,67]. Evidence suggests that some of these non-lytic pathways are regulated by bacterial two-component systems[68]. For example, in *S. pneumoniae*, the VncSR two-component system controls the expression of the autolysin LytA and regulates tolerance to vancomycin and penicillin through lysis-dependent[69] and lysis-independent[70] cell death pathways.

In *Staphylococcus aureus*, the LytSR two-component system can similarly affect cell lysis by regulating autolysin activity[71]. LytR activates the expression of *lrgAB*[72], which was found to inhibit autolysin activity and thereby lead to antibiotic tolerance[73]. LrgA is similar to bacteriophage holin proteins[73], which regulate the access of autolysins to the peptidoglycan layer. Based on this information, an additional holin-like system, *cidAB*, was uncovered in *S. aureus* and found to activate autolysins, rendering *S. aureus* more susceptible to β-lactam-mediated killing[74,75]. Complementation of *cidA* into a *cidA*-null strain reversed the loss of autolysin activity but did not completely restore sensitivity to β-lactams[74].

Role of the SOS response in cell death by β-lactams. Treatment with β-lactams leads to changes in cell morphology that are associated with the primary drug–PBP interaction. Generally speaking, PBP1 inhibitors cause cell elongation and are potent triggers of lysis, PBP2 inhibitors alter cell shape but do not cause lysis and PBP3 inhibitors influence cell division and can induce filamentation[76]. Interestingly, β-lactam subtypes have distinct affinities for certain PBPs, which correlate with the ability of these drugs to stimulate autolysin activity and induce lysis[76,77]. Accordingly, PBP1-binding β-lactams are also the most effective inducers of peptidoglycan hydrolase activity, and PBP2 inhibitors are the least proficient autolysin activators[77].

Filamentation can occur following the activation of the DNA damage-responsive SOS network of genes[78] owing to expression of SulA, a key component of the SOS network that inhibits septation and leads to cell elongation by binding to and inhibiting polymerization of septation-triggering FtsZ monomers[79,80]. Interestingly, β-lactams that inhibit PBP3 and induce filamentation have been shown to stimulate the DpiAB two-component system, which can activate the SOS response[81]. β-lactam lethality can be enhanced by disrupting DpiAB signalling or by knocking out *sulA*. This indicates that SulA may protect against β-lactam killing by shielding FtsZ and limiting a division ring interaction among PBPs and peptidoglycan hydrolases. In support of this idea, SulA expression limits the lysis observed in a strain of *E. coli* that expresses FtsZ84 (a mutant of FtsZ that is active only under certain temperatures and media conditions) and lacks PBP4 and PBP7 (REF. 82).

DNA-damaging antimicrobials that do not directly disrupt peptidoglycan turnover, such as quinolones, also cause filamentation by activating the SOS response[4]. Interestingly, a mutant strain of *E. coli* that is deficient in diaminopimelic acid synthesis (*E. coli* W7), a key building block of peptidoglycan, undergoes lysis following treatment with the fluoroquinolone antimicrobials ofloxacin or pefloxacin[83]. This suggests that peptidoglycan turnover and the SOS response could have a role in antibiotic-mediated lytic killing responses.

Inhibition of protein synthesis

The process of mRNA translation occurs over three sequential phases (initiation, elongation and termination) that involve the ribosome and a range of cytoplasmic accessory factors[84]. The ribosome is composed of two ribonucleoprotein subunits, the 50S and 30S, which assemble (during the initiation phase) following the formation of a complex between an mRNA transcript, *N*-formylmethionine-charged aminoacyl tRNA, several initiation factors and a free 30S subunit[85]. Drugs that inhibit protein synthesis are among the broadest classes of antibiotics and can be divided into two subclasses: the 50S inhibitors and 30S inhibitors (TABLE 1).

50S ribosome inhibitors include macrolides (for example, erythromycin), lincosamides (for example, clindamycin), streptogramins (for example, dalfopristin–quinupristin), amphenicols (for example, chloramphenicol) and oxazolidinones (for example, linezolid)[86,87]. 50S ribosome inhibitors work by physically blocking either initiation of protein translation (as is the case for oxazolidinones[88]) or translocation of peptidyl tRNAs, which serves to inhibit the peptidyltransferase reaction that elongates the nascent peptide chain. A model for the mechanism by which these drugs act has been formulated by studies of macrolides, lincosamides and streptogramins. The model involves blocking the access of peptidyl tRNAs to the ribosome (to varying degrees), subsequent blockage of the peptidyltransferase elongation reaction by steric inhibition and eventually triggering dissociation of the peptidyl tRNA[89,90]. This model also accounts for the phenomenon that these classes of drugs lose their antibacterial activity when elongation has progressed beyond a crucial length[91].

30S ribosome inhibitors include tetracyclines and aminocyclitols. Tetracyclines work by blocking the access of aminoacyl tRNAs to the ribosome[92]. The aminocyclitol class comprises spectinomycin and aminoglycosides (for example, streptomycin, kanamycin and gentamicin), which bind the 16S rRNA component of the 30S ribosome subunit. Spectinomycin interferes with the stability of peptidyl tRNA binding to the ribosome by inhibiting elongation factor-catalysed translocation, but does not cause protein mistranslation[93–95]. By contrast, the interaction between aminoglycosides and the 16S rRNA can induce an alteration in the conformation of the complex formed between an mRNA codon and its cognate charged aminoacyl tRNA at the ribosome. This promotes tRNA mismatching, which can result in protein mistranslation[96–99].

Among ribosome inhibitors, naturally derived aminoglycosides are the only class that is broadly bactericidal. Macrolides, streptogramins, spectinomycin, tetracyclines, chloramphenicol and macrolides are typically bacteriostatic; however, they can be bactericidal in a species- or treatment-specific manner. For example, chloramphenicol has been shown to kill *S. pneumoniae* and *Neisseria meningitidis* effectively[100], and chloramphenicol

Two-component system
A two-protein signal relay system composed of a sensor histidine kinase and a cognate receiver protein, which is typically a transcription factor.

Box 1 | **Drug synergy**

Combinatorial antibiotic treatments can have diverse effects on bacterial survival. Antibiotics can be more effective as a combination treatment displaying either an additive effect (an effect equal to the sum of the treatments) or a synergistic effect (an effect greater than the sum of the treatments). The combination can also be antagonistic — that is, the effect of the combination treatment is less than the effect of the respective single-drug treatments[136]. Technological advances have allowed high-throughput quantification of drug–drug interactions at the level of cell survival and target binding, thereby opening the door for the systematic study of synergistic and antagonistic drug combinations[137].

The exploration of the survival fitness landscape between drug combinations has allowed the study of the mechanisms by which antibiotics work against bacteria[138] and has also allowed a study of the evolution of drug resistance[137]. Further study of the synergy or antagonism between antibiotics will provide additional insight into the underlying cell death mechanisms for the individual classes of antibiotics. For example, the suppressive interaction between protein synthesis inhibitors and DNA synthesis inhibitors has been shown to be due to non-optimal ribosomal RNA regulation by DNA-inhibiting drugs[139].

The synergy between aminoglycosides and β-lactams has been attributed to β-lactam-mediated membrane damage leading to increased uptake of aminoglycosides[140]. It will be interesting to see whether the synergy between these two drugs is also related to the induction of the envelope stress response that has been observed following treatment with aminoglycosides[10].

and the macrolide azythromycin have exhibited bactericidal activity against *Haemophilus influenzae*[100,101]. This species-specific variability in ribosome inhibitor-mediated cell death probably has to do with sequence differences among bacterial species in the variable regions of the highly conserved ribosomal proteins and RNAs[102]. In addition, high concentrations of macrolides and combinations of streptogramin group A and group B can behave in a bactericidal manner. For the rest of this section, however, we focus on aminoglycosides, which have the best-studied mechanism of killing by ribosome inhibition.

Aminoglycoside uptake and cell death. Binding of aminoglycosides to the ribosome does not bring translation to an immediate standstill. Instead, as noted above, this class of drugs promotes protein mistranslation through the incorporation of inappropriate amino acids into elongating peptide strands[96]; this phenotype is specific for aminoglycosides and contributes to cell killing (FIG. 1).

Respiration also has a crucial role in aminoglycoside uptake and lethality[103]. Following the initial step of drug molecule adsorption (in Gram-negative species such as *E. coli*) through electrostatic interaction, changes in membrane potential allow aminoglycosides to access the cell. Respiration-dependent uptake relies on the activity of membrane-associated cytochromes and maintenance of the electrochemical potential through the quinone pool[104,105]. Accordingly, under anaerobic conditions aminoglycoside uptake is severely limited in both Gram-positive and Gram-negative bacteria[106,107], although there is evidence that aminoglycoside uptake can occur under certain anaerobic conditions by a mechanism that is sensitive to nitrate levels. In *E. coli* and *Pseudomonas aeruginosa*, aminoglycoside uptake can take place when nitrate is used as an electron acceptor in place of oxygen, and anaerobic bacteria that have quinones and cytochromes can take up aminoglycosides if sufficient anaerobic electron transport occurs[108].

Quinone pool
Membrane-associated cyclic aromatic-based compounds that shuttle electrons along the electron transport chain.

In *E. coli* aminoglycoside-mediated killing has been linked with alterations to the cell membrane ultrastructure that ultimately increase drug uptake[109,110]. Aminoglycosides can affect membrane composition through the incorporation of mistranslated membrane proteins into the cytoplasmic membrane, thereby increasing cell permeability, which allows increased access of the drug[103] (FIG. 1). Sufficient aminoglycoside uptake resulting in increased ribosome inhibition and cell death could also occur as a function of the changes in membrane integrity owing to the incorporation of mistranslated membrane proteins[103]. An alteration in membrane permeability owing to aminoglycoside-induced membrane damage is thought to be one of the mechanisms by which aminoglycosides cooperate with β-lactams (see BOX 1 for more on drug synergy and antagonism).

Another consequence of mistranslated protein incorporation into the bacterial membrane is the activation of envelope (Cpx) and redox-responsive (Arc) two-component systems. These intracellular signal relay systems regulate the expression of genes that are important for the maintenance of membrane integrity and composition[111], and membrane-coupled energy generation[112,113], respectively. Disruption of Cpx or Arc two-component system signalling (through a series of single-gene knockouts) has recently been shown to reduce the killing efficacy of aminoglycosides, a result associated with findings linking bactericidal antibiotic-induced cell death with drug stress-induced changes in metabolism. Interestingly, disruption of Cpx or Arc two-component system signalling was also shown to reduce the lethality of β-lactam and quinolone antibiotics[10]. Together, these findings point towards a broad role for the envelope stress-responsive and redox-responsive two-component systems in killing by bactericidal drugs (FIG. 2).

Antibiotic network biology

As noted above, antibiotic-mediated cell death is a complex process that only begins with the drug–target interaction and the primary effects of these respective interactions. The development of new antibiotics and the improvement of current antibacterial drug therapies would benefit from a better understanding of the specific sequences of events beginning with the binding of a bactericidal drug to its target and ending in bacterial cell death.

Bioinformatics approaches that use high-throughput genetic screening or gene expression profiling have proven to be valuable tools to explore the response layers of bacteria to different antibiotic treatments[114]. For example, recent screens for antibiotic susceptibility in a single-gene deletion library of non-essential genes in *E. coli*[115] and a transposon mutagenesis library in *P. aeruginosa*[116] have provided important insights into the numbers and types of genes that affect treatment efficiency (bactericidal versus bacteriostatic effects), including those related to drug molecule efflux, uptake or degradation. In addition, monitoring global changes in gene expression patterns, or signatures, resulting from antibiotic treatment over a range of conditions, has advanced our understanding of the off-target effects elicited by primary drug–target interactions[114].

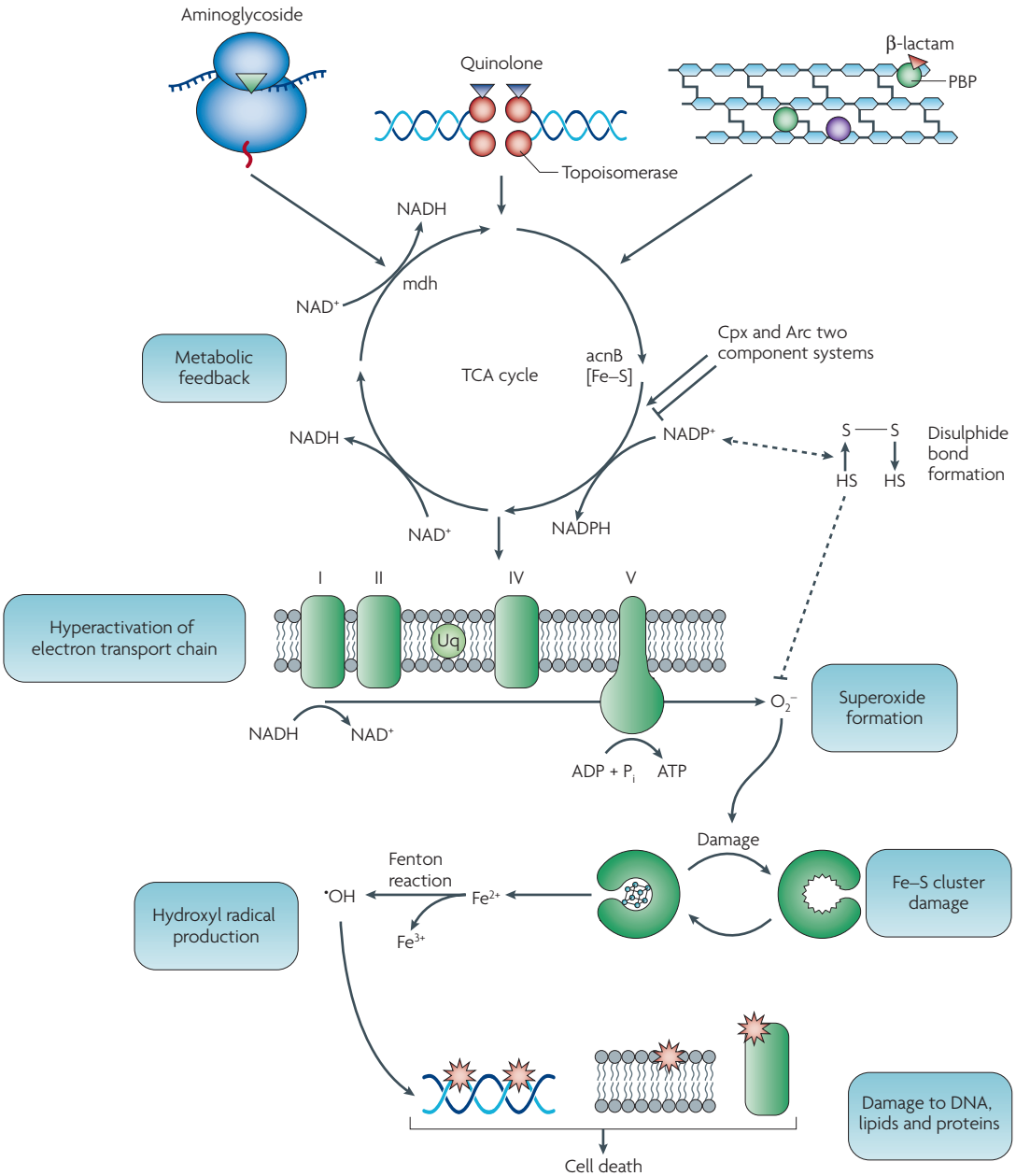


Figure 2 | **Common mechanism of cell death induced by bactericidal antibiotics.** The primary drug–target interactions (aminoglycoside with the ribosome, quinolone with topoisomerase, and β-lactam with penicillin-binding proteins (PBPs)) stimulate the oxidation of NADH through the electron transport chain, which is dependent on the tricarboxylic acid (TCA) cycle. Hyperactivation of the electron transport chain stimulates superoxide (O_2^-) formation. Superoxide damages Fe–S clusters, making ferrous iron available for oxidation by the Fenton reaction. The Fenton reaction leads to the formation of hydroxyl radicals (•OH), which damage DNA, lipids and proteins. This contributes to antibiotic-induced cell death. Quinolones, β-lactams and aminoglycosides also trigger hydroxyl radical formation and cell death through the envelope (Cpx) and redox-responsive (Arc) two-component systems. It is also possible that redox-sensitive proteins, such as those containing disulphides, contribute in undetermined way to the common mechanism (dashed lines). Figure modified, with permission, from REF. 8 © (2007) Elsevier Science. acnb, aconitase b; mdh, malate dehydrogenase; uq, ubiquinone.

A need also exists for the application of network biology methods to discern and resolve the potential interplay between genes and proteins coordinating bacterial stress response pathways. Typically, such methods incorporate gene expression profiling data and the results of high-throughput genetic screens, along with the contents of databases detailing experimentally identified regulatory connections and biochemical pathway classifications, to functionally enrich datasets and predict relationships that exist among genes under tested conditions. As such, biological network studies of drug-treated bacteria can be used to advance our understanding of how groups of genes interact functionally, rather than in isolation, when cells react to antibiotic stress[117].

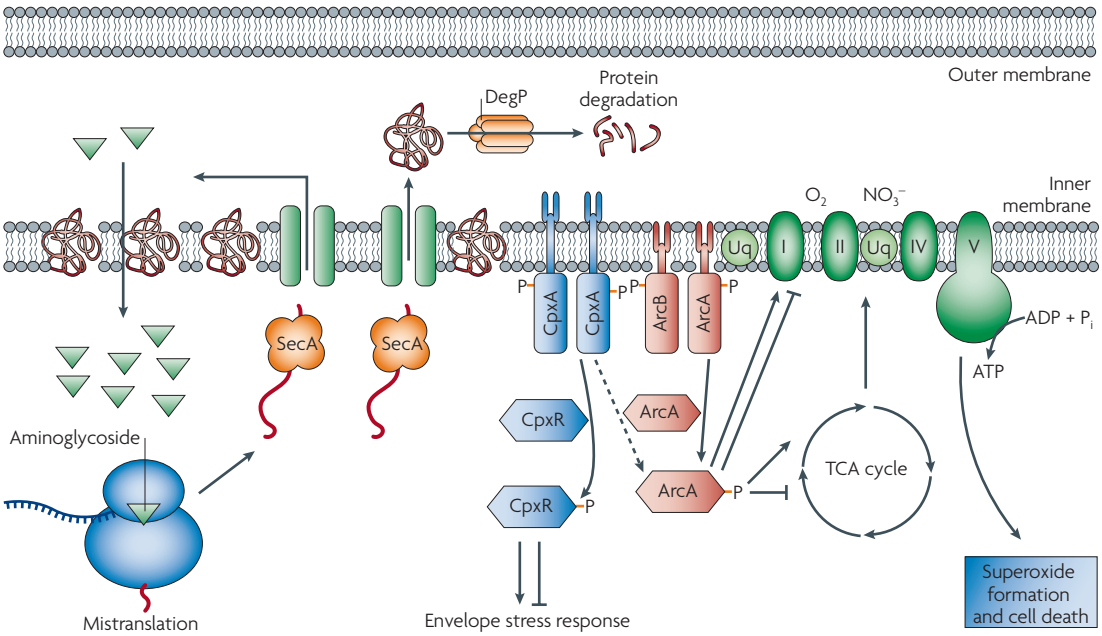


Figure 3 | **Aminoglycosides trigger hydroxyl radical-mediated cell death.** The interaction between aminoglycosides and the ribosome causes mistranslation and misfolding of membrane proteins. Incorporation of mistranslated, misfolded proteins into the cell membrane stimulates the envelope (Cpx) and redox-responsive (Arc) two-component systems. Activation of these systems perturbs cell metabolism and the membrane potential, resulting in the formation of lethal hydroxyl radicals. Figure modified, with permission, from REF. 10 © (2008) Elsevier Science. TCA, tricarboxylic acid; uq, ubiquinone.

To help address this problem, researchers have developed methods to construct quantitative models of regulatory networks[118–122] and have recently used these reconstructed network models to identify the sets of genes, associated functional groups and biochemical pathways that act in concert to mediate bacterial responses to antibiotics[8–10,119]. Below we highlight some mechanistic insights that have been obtained from antibiotic network biology, and discuss some opportunities and challenges for this emerging area of research.

A common mechanism for antibiotic-mediated cell death. As an example of the utility of studying bacterial stress responses at the systems level, biological network analysis methods were recently employed to identify new mechanisms that contribute to bacterial cell death following topoisomerase II inhibition by the fluoroquinolone antibiotic norfloxacin[9]. As noted above, quinolones are known to induce cell death through the introduction of double-stranded DNA breaks following arrest of topoisomerase function[4]. To identify additional contributions to cell death resulting from topoisomerase II poisoning, reconstruction of stress response networks was carried out following treatment of *E. coli* with lethal concentrations of norfloxacin. This work identified an oxidative damage-mediated cell death pathway, which involves ROS generation and a breakdown in iron regulatory dynamics following norfloxacin-induced DNA damage. More specifically, norfloxacin treatment was found to promote superoxide generation soon after topoisomerase II poisoning and to ultimately result in the generation of highly destructive hydroxyl radicals through the Fenton reaction[123]. Under these conditions, the Fenton reaction was found to be fuelled by superoxide-mediated destabilization of Fe–S cluster catalytic sites, repair of these damaged Fe–S clusters and related changes in iron-related gene expression[9].

Building on this work, it was later shown that all major classes of bactericidal antibiotics (including β-lactams, aminoglycosides and quinolones) promote the generation of lethal hydroxyl radicals in both Gram-negative and Gram-positive bacteria, despite the stark differences in their primary drug–target interactions[8]. Stress response network analysis methods used in this study suggested that antibiotic-induced hydroxyl radical formation is the end product of a common mechanism, in which alterations in central metabolism related to NADH consumption (increased TCA cycle and respiratory activity) are crucial to superoxide-mediated iron–sulphur cluster destabilization and stimulation of the Fenton reaction. These predictions were validated by the results of additional phenotypic experiments, biochemical assays and gene expression measurements, confirming that lethal levels of bactericidal antibacterials trigger a common oxidative damage cellular death pathway, which contributes to killing by these drugs (FIG. 2).

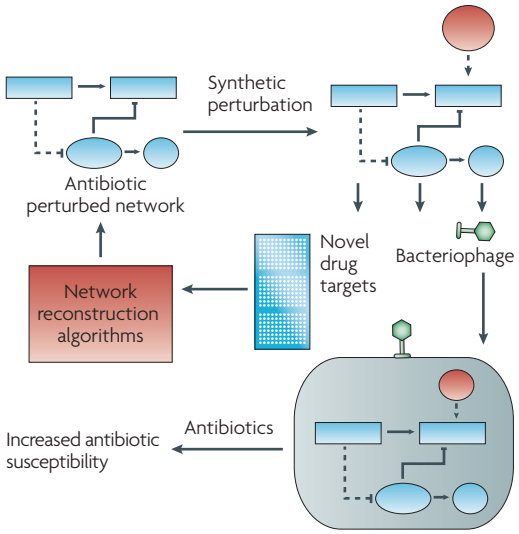
Most recently, the study of antibiotic-induced stress response networks has been aimed at determining exactly how the primary effect of a given bactericidal triggers aspects of cell death that are common to all bactericidal drugs. For example, a comparative analysis of stress response networks, reconstructed using gene expression data from *E. coli* treated with aminocyclitols (spectinomycin, gentamicin and kanamycin),

Fenton reaction
Reaction of ferrous iron (Fe^{2+}) with hydrogen peroxide to produce ferric iron (Fe^{3+}) and a hydroxyl radical.

Box 2 | **Synthetic biology for antibacterial applications**

The study of complex antibiotic-related cell death systems can be aided by synthetic biology. Delivery of engineered gene circuits that alter response network behaviour can serve as a tool to experimentally examine antibiotic-mediated cell death pathways, as well as a means to enhance killing by an antibiotic (see the figure).

Bacteriophages, which are bacterium-specific viruses, show promise as an effective means to deliver network perturbations to bacteria to improve antibiotic lethality[141,142]. Bacteriophages have been used to enhance killing of *Escherichia coli* by bactericidal antibiotics through the delivery of proteins that modify the oxidative stress response or inhibit DNA damage repair systems[142]. Bacteriophages are species specific, so it may be possible to use engineered bacteriophages to deliver antibiotic-enhancing synthetic gene networks, therapeutic proteins or antimicrobial peptides that are highly specific for an infecting organism. This would allow efficient treatment of a bacterial infection, while sparing the typical commensal body flora (see the figure).



was used to identify the incorporation of mistranslated proteins into the cell membrane as the trigger for aminoglycoside-induced oxidative stress[10] (FIG. 3). Interestingly, mistranslated membrane proteins were shown to stimulate radical formation by activating the Cpx and Arc two-component systems, ultimately altering TCA cycle metabolism; the TCA cycle had previously been implicated in bacterial susceptibility to aminoglycosides[8,124].

The discovery of the common oxidative damage cellular death pathway has important implications for the development of more effective antibacterial therapies. Specifically, it indicates that all major classes of bactericidal drugs can be potentiated by inhibition of the DNA stress response network (that is, the SOS response), which plays a key part in the repair of hydroxyl radical-induced DNA damage. This may be accomplished through the development of small molecules (for example, RecA inhibitors[125]) or synthetic biology approaches (BOX 2).

ROS, such as superoxide and hydroxyl radicals, are highly toxic and have deleterious effects on bacterial physiology[123,126,127], even under steady-state conditions. There is still much to be learned about how oxidative stress-related changes in bacterial physiology affect antibiotic-mediated cell death and the emergence of resistance[128,129]. For example, it was recently discovered that endogenous nitric oxide produced by bacteria with nitric oxide synthases can protect against ROS-mediated cell death[130]. In addition, considering bacteria have developed mechanisms to avoid ROS produced by phagocytes of the immune system[131], it will be interesting to explore, from a systems-level perspective, the relationship between immune-mediated and drug-mediated cell death.

Opportunities and challenges for antibiotic network biology. One of the more intriguing aspects of antibacterial therapies is that not all bacterial species respond in the same way to antibiotic treatment. Network biology approaches, which provide the field of antibiotic research with an opportunity to view response mechanisms of different bacterial species to various classes of antibiotics, could be extended to the context of particular infectious species, persistent infections or disease settings. As an example, it is generally accepted that Gram-negative bacteria are not susceptible to the glycopeptide vancomycin or the depolarizing lipopeptide daptomycin; however, a single gene, *yfgL*, was recently found that can make *E. coli* susceptible to glycolipid derivatives of vancomycin[132]. Gene expression profiling of daptomycin-treated *S. aureus* has revealed that daptomycin perturbs peptidoglycan synthesis through a mechanism involving the activation of cell wall stress systems and membrane depolarization[133]. Given these findings, we might be able to combine our knowledge of β-lactam- and aminoglycoside-induced gene signatures with the results of high-throughput screens at various drug doses to reconstruct drug-specific cell death networks that use YgfL as a network anchor. Predicted functional and regulatory relationships between enriched genes could then be used to determine the secondary effects of lipopeptide antibiotics and gain insight into the different properties of this drug in Gram-negative and Gram-positive bacteria.

Moreover, the development of comparative network biology techniques will be essential to further our understanding of how species-specific differences manifest themselves in divergent drug-specific cell death networks and variations in physiological responses. These methods could be particularly useful when examining pathogenic bacteria with sparse systems-level data (such as *Shigella* or *Salmonella* spp.) that are closely related to well-studied bacteria (such as *E. coli*). Through a greater understanding of the biological networks that are related to an individual drug target, we eventually might be able to search for meaningful network homologues among species in

Antimicrobial peptide
A short, naturally occurring cationic peptide that has antibacterial properties through its ability to interfere with bacterial membranes.

the same spirit as we currently search for gene homologues. Network-based efforts could also lead to the development of species-specific treatments, including synthetic biology-derived therapies (BOX 2), which could be useful in killing off harmful, invasive bacteria, while leaving our normal bacterial flora intact.

Finally, bacterial network analyses will also be useful in the study of non-classical antibacterial agents that induce cell death. Antimicrobial peptides are short cationic peptides that are thought to kill through interactions with the membrane that result in pore formation[134,135]. However, the mode of action of many antimicrobial peptides could, in fact, be more complex, and cell death networks uncovered for existing antibiotics could be used as mechanistic templates to study cellular responses induced by antimicrobial peptides.

Concluding remarks

Drug-resistant bacterial infections are becoming more prevalent and are a major health issue facing us today. This rise in resistance has limited our repertoire of effective antimicrobials, creating a problematic situation that has been exacerbated by the small number of new antibiotics introduced in recent years. The complex effects of bactericidal antibiotics discussed in this Review provide a large playing field for the development of new antibacterial compounds, as well as adjuvant molecules and synthetic biology constructs, that could enhance the potency of current antibiotics. It will be important to translate our growing understanding of antibiotic mechanisms into new clinical treatments and approaches so that we can effectively fight the growing threat from resistant pathogens.

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Acknowledgments
We thank the anonymous reviewers for their helpful comments and suggestions. This work was supported by the National Institutes of Health (NIH) through the NIH Director's Pioneer Award Program, grant number DP1 OD003644, and the Howard Hughes Medical Institute.

Competing interests statement
The authors declare no competing financial interests.

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