

# Molecular mechanisms that confer antibacterial drug resistance

Christopher Walsh

Biological Chemistry and Molecular Pharmacology Department, Harvard Medical School, Boston, Massachusetts 02115, USA

**Antibiotics — compounds that are literally ‘against life’ — are typically antibacterial drugs, interfering with some structure or process that is essential to bacterial growth or survival without harm to the eukaryotic host harbouring the infecting bacteria. We live in an era when antibiotic resistance has spread at an alarming rate<sup>1–4</sup> and when dire predictions concerning the lack of effective antibacterial drugs occur with increasing frequency. In this context it is apposite to ask a few simple questions about these life-saving molecules. What are antibiotics? Where do they come from? How do they work? Why do they stop being effective? How do we find new antibiotics? And can we slow down the development of antibiotic-resistant superbugs?**

**A**ntibiotics can kill bacteria (bacteriocidal) or sometimes just nullify growth (bacteriostatic). Most antibiotics in human use as antibacterials are natural products, elaborated by one species of microbe (bacteria or fungi) as chemical weapons, often in times of crowding, to kill off other microbes in the neighbouring microenvironment. Over the past 60–70 years most antibiotics have been discovered by screening of soil samples for such natural products that kill bacteria, including known pathogens, first on culture plates and then in animal infections. These include penicillins and cephalosporins from fungi and a host of antibiotics from different strains of the filamentous bacterium *Streptomyces*, such as streptomycin, erythromycin, tetracycline and vancomycin. Semisynthetic modifications have produced second- and third-generation  $\beta$ -lactams of both the penicillin and cephalosporin classes whereas total synthesis has created the second-generation erythromycins — clarithromycin and azithromycin. As of the end of 1999, only the fluoroquinolones (for example, ciprofloxacin) represent a totally synthetic, significant class of antibiotic.

## Targets for the main classes of antibacterial drugs

To understand how antibiotics work and, concomitantly, why they stop being effective requires a brief look at the targets for the main classes of these antibacterial drugs. As summarized in Box 1, there are three proven targets for the main antibacterial drugs: (1) bacterial cell-wall biosynthesis; (2) bacterial protein synthesis; and (3) bacterial DNA replication and repair.

### Cell-wall biosynthesis

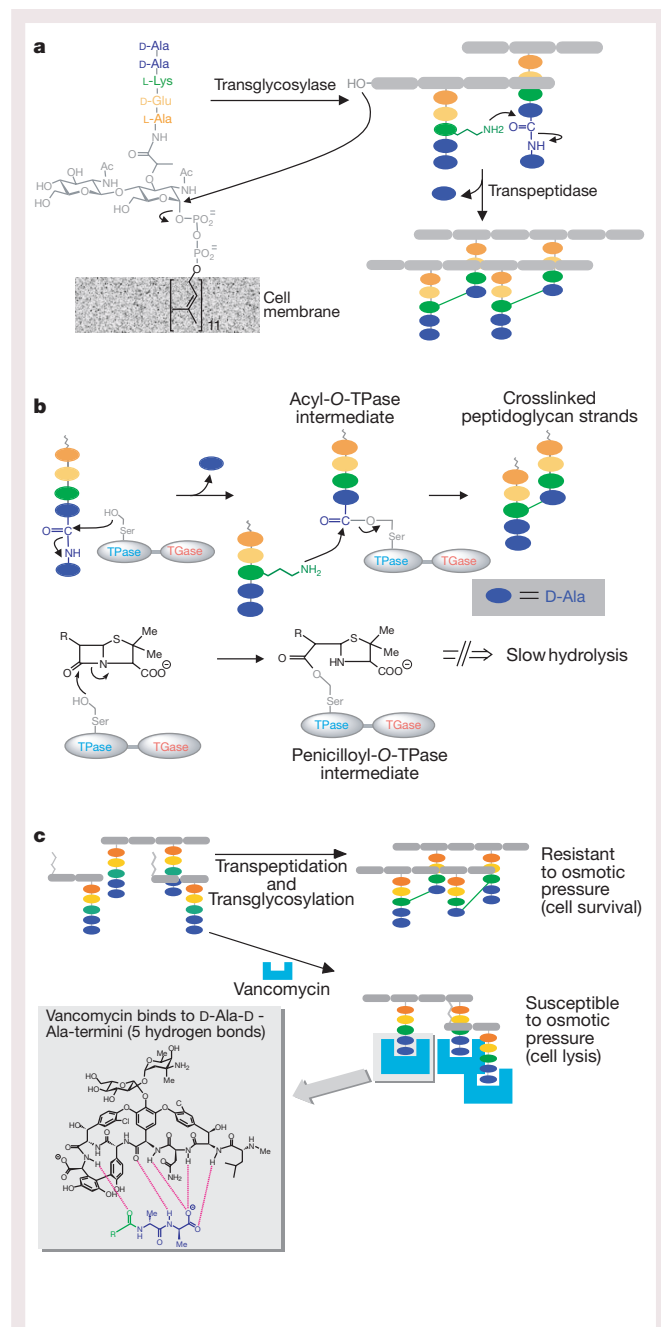
The layer of the bacterial cell wall that confers strength is the peptidoglycan, a meshwork of strands of peptide and glycan that can be covalently crosslinked (Fig. 1a). The larger the fraction of adjacent peptide strands that are connected in amide linkage by action of a family of transpeptidases, the higher the mechanical strength to osmotic lysis. Transglycosylases act on the glycan strands to extend the sugar chains by incorporation of new peptidoglycan units from *N*-acetylglucosamine- $\beta$ -1,4-*N*-acetylmuramyl-pentapeptide-pyrophosphoryl-undecaprenol (lipid II). Bifunctional enzymes containing both transpeptidase and transglycosylase domains are the target sites for the killing of bacteria by the  $\beta$ -lactam-containing penicillins and

cephalosporins, which act as pseudosubstrates and acylate the active sites of the transpeptidases (also termed penicillin-binding proteins or PBPs)<sup>5</sup> (Fig. 1b). The ring-opened, penicilloylated transpeptidases deacylate very slowly, and so occupy the enzyme active sites, preventing normal crosslinking of peptide chains in the peptidoglycan layer and leaving it mechanically weak and susceptible to lysis on changes in osmotic pressure.

In addition to penicillins and cephalosporins, the vancomycin family of glycopeptide antibiotics also target the peptidoglycan layer in the cell-wall assembly. But rather than targeting the enzymes involved in peptide crosslinking, vancomycin ties up the peptide substrate<sup>6</sup> and thereby prevents it from reacting with either the transpeptidases or the transglycosylases. The net effect is the same: failure to make peptidoglycan crosslinks leads to a weaker wall that predisposes the treated bacteria to a killing lysis of the cell-wall layer. The cup-shaped undersurface of the vancomycin antibiotic makes five hydrogen bonds to the D-Ala-D-Ala dipeptide terminus of each uncrosslinked peptidoglycan pentapeptide side chain (Fig. 1c), which accounts for the high affinity of the antibiotic for its target, both in partially crosslinked walls and in the lipid II intermediate. Because  $\beta$ -lactams and vancomycin work on adjacent steps — substrate and enzyme — they show synergy when used in combination.

### Protein synthesis

The RNA and protein machinery of the prokaryotic ribosomes is sufficiently distinct from the analogous eukaryotic machinery that there are many inhibitors of protein synthesis, targeting different steps in ribosome action, with selective antibacterial action. These include such important antibiotics as the macrolides of the erythromycin class<sup>7</sup>, the tetracyclines<sup>8</sup> (which are products of the aromatic polyketide biosynthetic pathways) and the aminoglycosides<sup>9</sup> (of which streptomycin was the founding member, supplanted now by later synthetic variants such as kanamycin) (Fig. 2a). Given the large number of molecular steps involved in initiation, elongation and termination of protein assembly by the ribosome, it is not surprising that there would be many steps of binding or catalysis that could be interdicted by these and many other classes of protein-synthesis inhibitors. This multiplicity also indicates that protein synthesis will provide a multifaceted target for new antibiotics and this is the mechanism for the action of oxazolidinones<sup>10</sup>, one of

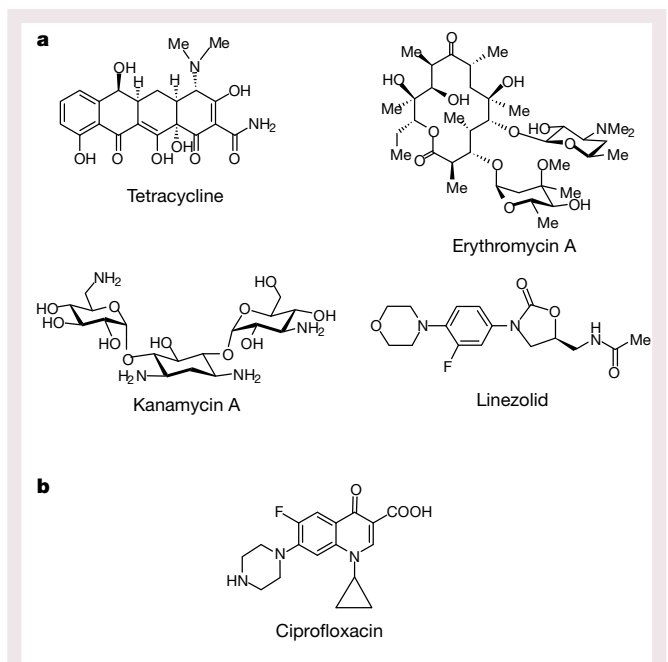


**Figure 1** Blockade of transpeptidation and transglycosylation steps of cell-wall biosynthesis by penicillins and vancomycins. **a**, Interruption of the normal crosslinking and strength-conferring enzymes by antibiotics that inhibit the enzyme (penicillins) or sequester the substrate (vancomycin). **b**, Inhibition of transpeptidase activity by penicillins through formation of a slowly hydrolysing covalent acyl enzyme intermediate. **c**, Complexation of the D-Ala-D-Ala termini of peptidoglycans by vancomycin in a network of five hydrogen bonds.

which has been approved in the United States in the first quarter of 2000.

**DNA replication and repair**

The fluoroquinolones, such as ciprofloxacin (Fig. 2b), are synthetic antibiotic structures that kill bacteria by targeting the enzyme DNA gyrase<sup>11</sup> (Box 1), the enzyme responsible for uncoiling the inter-twined circles of double-stranded bacterial DNA that arise after each round of DNA replication. DNA topoisomerases are classified as type I or type II according to whether transient single-strand breaks (type I) or transient double-strand breaks (type II) are made in the DNA



**Figure 2** Structural and functional diversity of antibacterial drugs. **a**, Antibiotics comprise a diverse set of natural product structures, represented by the erythromycin class of macrolides, tetracyclines, the aminoglycosides (represented by kanamycin), and the oxazolidinone linezolid. These products target the 23S rRNA and associated proteins in the peptidyl transferase centre of the ribosome to inhibit steps in the elongation of the protein chain. **b**, The fluoroquinolones, represented by ciprofloxacin, kill bacteria by inhibiting DNA gyrase and the related topoisomerase IV in mid-catalytic cycle, by trapping a doubly cleaved DNA intermediate.

substrate to pass the DNA double helical strands through each other and reduce the linking number (the number of superhelical twists in DNA). Bacterial DNA gyrases are type II topoisomerases and the transient cleavage of both DNA strands involves the reversible attachment of the 5' ends of the cleaved DNA to tyrosyl residues on each of the two GyrA subunits in the active (GyrA)<sub>2</sub>(GyrB)<sub>2</sub> tetramer<sup>12</sup>. Quinolone antibiotics such as ciprofloxacin are mechanism-based inhibitors of DNA gyrase and act by forming a complex with the enzyme and the doubly cleaved DNA that is covalently tethered to the GyrA subunits<sup>11</sup>. In the ciprofloxacin complex, the gyrase cannot religate the cleaved DNA and, as a consequence, double-strand breaks accumulate and ultimately set off the SOS repair system that leads to bacterial cell death. A second type II topoisomerase, known as topoisomerase IV, is also an important target and probably the primary one in *Staphylococcus aureus* infections<sup>13</sup>.

In each of the three main targets — cell wall, and protein and DNA biosynthesis — the antibiotics use comparative biochemical differences between prokaryotic machinery and eukaryotic machinery to act selectively. New classes of antibiotics that may work on additional and new targets will have to display equivalent therapeutic indices and efficacy-to-toxicity ratios to gain regulatory approval and widespread acceptance.

**Bacterial survival strategies to combat antibiotics**

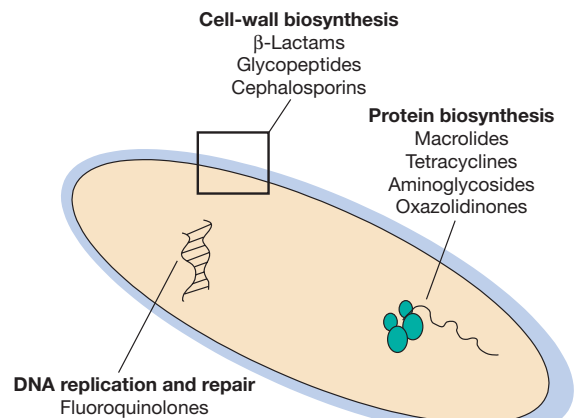
Once an antibiotic is proven to be effective and enters widespread human therapeutic use, its days are numbered. Clinically significant resistance appears in periods of months to years<sup>14</sup>. For penicillin, resistance began to be noted within two years of its introduction in the mid 1940s, which is typical for a resistance mechanism involving the action of one gene product and its time of spread through bacterial populations. Vancomycin resistance, in the context of surging sources of life-threatening vancomycin-resistant enterococci (VRE)

Box 1

Targets, mode of action and mechanisms of resistance of the main classes of antibacterial drugs

Antibiotic	Target	Mode of action	Resistance mechanism
<b>Cell wall</b>			
$\beta$ -Lactams	Transpeptidases/transglycosylases (PBPs)	Blockade of crosslinking enzymes in peptidoglycan layer of cell walls	$\beta$ -Lactamases, PBP mutants
Vancomycin	D-Ala-D-Ala termini of peptidoglycan and of lipid II	Sequestration of substrate required for crosslinking	Reprogramming of D-Ala-D-Ala to D-Ala-D-Lac or D-Ala-D-Ser
<b>Protein synthesis</b>			
Macrolides of the erythromycin class	Peptidyl transferase, centre of the ribosome	Blockade of protein synthesis	rRNA methylation, drug efflux
Tetracyclines	Peptidyl transferase	Blockade of protein synthesis	Drug efflux
Aminoglycosides	Peptidyl transferase	Blockade of protein synthesis	Enzymatic modification of drug
Oxazolidinones	Peptidyl transferase	Blockade of protein synthesis	Unknown
<b>DNA replication/repair</b>			
Fluoroquinolones	DNA gyrase	Blockade of DNA replication	Gyrase mutations to drug resistance

**Box 1 Figure** Proven targets for antibacterial drugs. Cell-wall biosynthesis at the stage of crosslinking of peptidoglycan peptide strands by transpeptidases and transglycosylases is inhibited by the  $\beta$ -lactam antibiotics (penicillins and cephalosporins). Protein biosynthesis at the ribosome is targeted by several classes of antibiotics, including macrolides, tetracyclines, aminoglycosides and oxazolidinones, which block one or more steps involving rRNA and the proteins of the ribosome at the peptidyl transferase centre. The fluoroquinolone antibiotics interrupt DNA replication by trapping a complex of DNA bound to the enzyme DNA Gyrase, a type II topoisomerase.



in hospital wards, became noticeable in 1987 and spread dramatically in the ensuing 4–6 years<sup>15</sup>. VRE have collected five genes that are necessary and sufficient for high-level resistance<sup>16,17</sup> (see below) and this may explain the 29 years' delay between introduction (in 1958) and clinically important resistance.

Development of resistance is not a matter of if but only a matter of when<sup>3,18</sup>. Given the large number of bacteria in an infection cycle, the rapid generation time, and the intrinsic rate of mutation of about 1 in 10<sup>7</sup>, then a pool of 10<sup>10</sup> bacteria would have mutations on average in a thousand loci. If one of those mutations confers resistance to an applied antibiotic, whereas all sensitive bacteria are killed, the resistant one will grow, fill the space vacated by its dead neighbours and become the dominant variant in the population. If an antibiotic treatment is at subtherapeutic levels, outgrowth of resistant bacteria is practically guaranteed.

A principal mechanism for the rapid spread of antibiotic-resistance genes through bacterial populations is that such genes get collected on plasmids<sup>18</sup> that are independently replicated within and passed between bacterial cells and species. Furthermore, some of these genes that reside on plasmids, such as the five genes that specify VRE, may be further segregated within transposons<sup>16</sup> that can actively cut themselves out of one DNA locale and hop into other locales, promiscuously moving their antibiotic resistance-conferring genetic cargo.

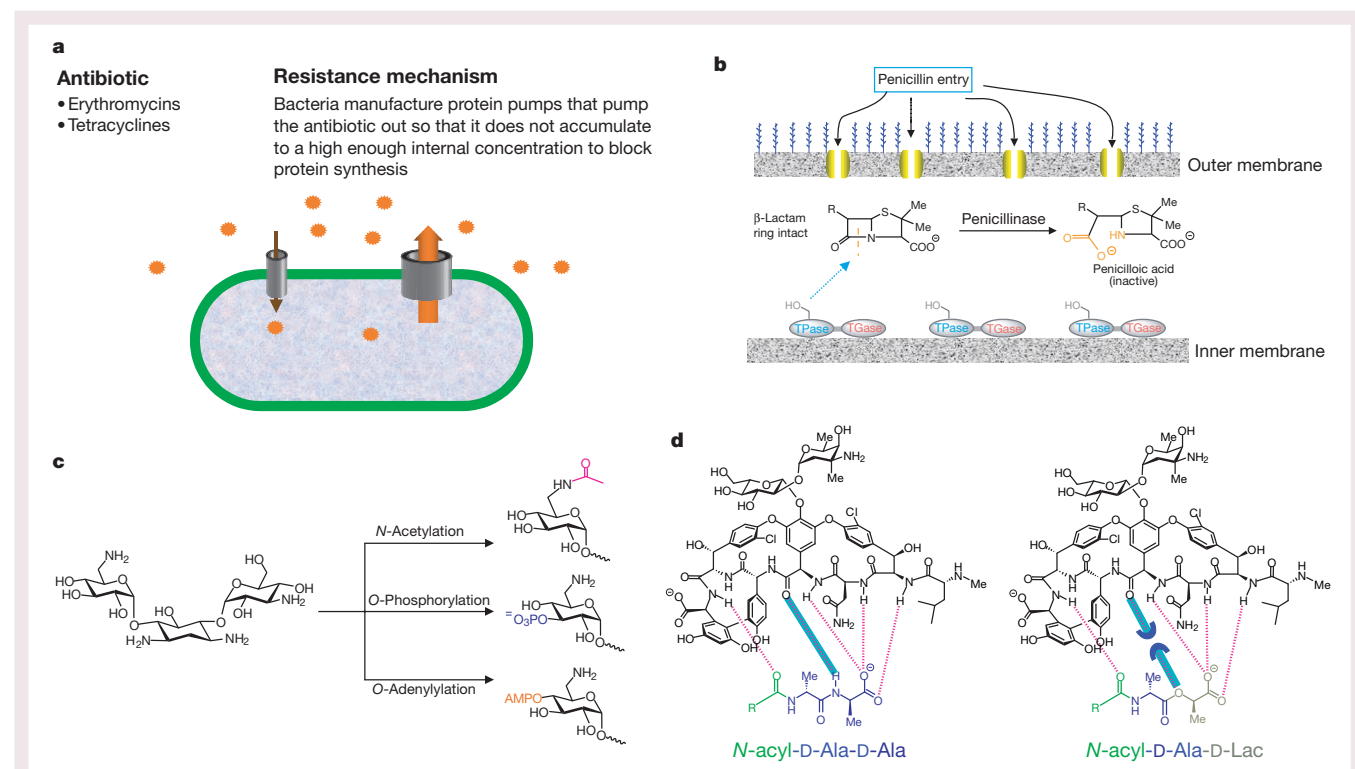
Selective pressures on *S. aureus* and on *Enterococcus faecalis*, pathogens that can invade the abdominal cavity after surgical procedures, have been documented in hospital environments where a *S. aureus* culture, in a minority of patients, can switch from methicillin-susceptible *S. aureus* (MSSA) to methicillin-resistant *S. aureus* (MRSA) in five to seven days. If vancomycin is then given on day eight and cultures are sampled 14 days later, patients with signs of continuing infection are now dominated by VRE<sup>19</sup>. In addition to

showing how antibiotic-resistant bacteria are selected in hospital environments by constant antibiotic pressure, this emphasizes the continuing need for cycles of new antibiotic discovery and development.

Three types of antibiotic-resistance strategies, which deal with most of the main classes of antibacterial drugs listed in Fig. 1 and Box 1, illustrate the strategies by which resistant bacteria strike back and nullify the action of antibiotics that appear in their neighbourhoods<sup>20</sup>.

**Pump out the antibiotic**

For antibiotics to be effective they must reach their specific bacterial targets and accumulate at concentrations that can act in some reasonable time frame. For example, the protein-synthesis machinery is located in the cytoplasm so antibacterials that are inhibitors of protein synthesis must pass through the cell membranes (outer and inner permeability barriers for Gram-negative bacteria; inner membrane barriers for Gram-positive bacteria) and then accumulate to a high enough concentration to block the particular susceptibility step of protein assembly. Both Gram-positive and Gram-negative bacteria that become resistant to tetracyclines commonly overproduce related membrane proteins (with relative molecular masses of 42,000) that act as an export or efflux pump for the drug<sup>21,22</sup>. As schematized in Fig. 3a, the drug is pumped out faster than it can diffuse in, so intrabacterial concentrations are kept low and ineffectual; bacterial protein synthesis proceeds at largely unimpeded rates. The pumps are variants of membrane pumps possessed by all bacteria to move lipophilic or amphipathic molecules in and out of the cells. Some are used by antibiotic producers to pump antibiotics out of the cells as fast as they are made and so constitute an immunity or protective mechanism for the bacteria to prevent being killed by their own chemical weapons. Equivalent drug efflux pumps have been observed in several bacteria, including staphylococci, which become



**Figure 3** Principal resistance strategies for bacterial survival. **a**, Drugs such as tetracyclines or erythromycins are pumped back out of bacterial cells through efflux pump proteins to keep intracellular drug concentrations below therapeutic level. **b**, The antibiotic is destroyed by chemical modification by an enzyme that is elaborated by the resistant bacteria. This is exemplified here by the  $\beta$ -lactamase secreted into the periplasmic space to hydrolyse penicillin molecules before they reach their PBP targets in the cytoplasmic membrane of this Gram-negative bacterium. **c**, The aminoglycoside antibiotic kanamycin can be enzymatically modified at three sites by three kinds of enzymatic processing — *N*-acetylation, *O*-phosphorylation or *O*-adenylation — to block recognition by its target on the ribosome. **d**, The target structure in the bacterium can be reprogrammed to have a low affinity for antibiotic recognition. Here the switch from the amide linkage in the D-Ala-D-Ala peptidoglycan termini to the ester linkage in the D-Ala-D-Lac termini is accompanied by a 1,000-fold drop in drug-binding affinity.

resistant to the erythromycin class of macrolide antibiotics<sup>22,23</sup>.

**Destroy the antibiotic warhead**

Although the above mechanism prevents the antibiotic from accumulating in the desired compartment, it leaves the antibiotic unchanged; a second strategy of resistance is destruction of the chemical warhead in the antibiotic. The classic case is the hydrolytic deactivation of the  $\beta$ -lactam ring in the penicillins and cephalosporins by elaboration of the hydrolytic enzyme  $\beta$ -lactamase by resistant bacteria<sup>24</sup> (Fig. 3b; and see discussion in refs 1, 20). Because the four-membered, strained lactam ring is the chemically activated functionality in the drugs that acylate and irreversibly modify the cell wall-crosslinking PBPs, the hydrolysed, ring-opened penicilloic acid product is now deactivated and nonfunctional as a PBP pseudosubstrate and useless as an antibiotic. The lactamase-producing bacteria secrete this enzymatic weapon into the periplasm to destroy  $\beta$ -lactam antibiotics before they can reach the PBP targets in the cytoplasmic membrane. A single  $\beta$ -lactamase molecule can hydrolyse  $10^3$  penicillin molecules per second. So if  $10^5$  enzymes are secreted per resistant cell, then 100 million molecules of penicillin are destroyed every second, which is clearly an effective strategy.

Other antibiotic classes, such as the aminoglycosides, do not contain such hydrolytically labile groups. These protein-synthesis inhibitors are still neutralized by deactivating enzymes but now the enzymes decorate the periphery of the aminoglycosides with three types of chemical substituents<sup>25</sup> that interrupt the binding to the RNA targets in the ribosome. As shown in Fig. 3c, aminoglycoside-resistance enzymes can be adenyl transferases, which add AMP moieties, phosphoryl transferases, which add  $-PO_3$  groups, or acetyl transferases, which acetylate the amino groups of the antibiotic. The modified aminoglycoside products have considerably lower affinity for RNA and so do not bind and interrupt protein synthesis.

The X-ray structure of an antibiotic phosphotransferase indicates an evolutionary relationship to a protein kinase<sup>26</sup>, defining a route by which bacteria may have recruited an enzyme for the resistance brigade.

**Reprogramme the target structure**

A third resistance strategy focuses not on removal or destruction of the antibiotic but on a reprogramming or camouflaging of the target in the now resistant bacteria. In the erythromycin-resistance manifolds, in addition to efflux pumps, resistant bacteria have emerged that have learned to mono- or dimethylate a specific adenine residue, A2058, in the peptidyl transferase loop of the 23S RNA component of the ribosome. This modification is carried out by a methyl transferase enzyme Erm<sup>27</sup> that does not impair protein biosynthesis but does lower the affinity of all the members of the erythromycin class of drugs for the RNA, as well as for the pristinamycin class described below. The Erm mechanism is the main resistance route in drug-resistant clinical isolates of *S. aureus* and is present in erythromycin-producing organisms as a self-immunity mechanism.

An additional example of the reprogramming strategy is used by VRE to escape from vancomycin. In VRE the *vanHAX* genes encode a new pathway of enzymes that reduces pyruvate to D-lactate (*vanH*), adds D-alanine and D-lactate together to produce D-Ala-D-Lac (*vanA*), and then hydrolyses the normal metabolite D-Ala-D-Ala while sparing D-Ala-D-Lac (*vanX*)<sup>17</sup>. In this cell, only the D-Ala-D-Lac accumulates and serves as a substrate to be elongated and presented at the termini of the peptidoglycan strands (Fig. 3d). The reprogramming of peptidoglycan to end in D-Ala-D-Lac rather than the normal D-Ala-D-Ala has no effect on the crosslinking efficiency carried out by the transpeptidating PBPs, but the switch from the D,D-dipeptide terminus to D,D-depsipeptide terminus lowers the binding affinity of vancomycin by 1,000-fold<sup>28</sup> and enables the VRE to grow at

1,000-fold-higher levels of antibiotic. Finally, penicillin resistance can arise not only by  $\beta$ -lactamase expression, but also by mutation of penicillin-binding proteins to lower-affinity forms as well as by expression of new PBPs with low affinity for antibiotic. The acquisition by *S. aureus* of the *mecA* gene that encodes a PBP2' protein with low affinity for all  $\beta$ -lactam antibiotics provides the molecular basis for the MRSA phenotype<sup>29,30,34</sup> that is now widely disseminated.

### Development of new antibiotics that circumvent resistance

#### Targeting the resistance mechanisms

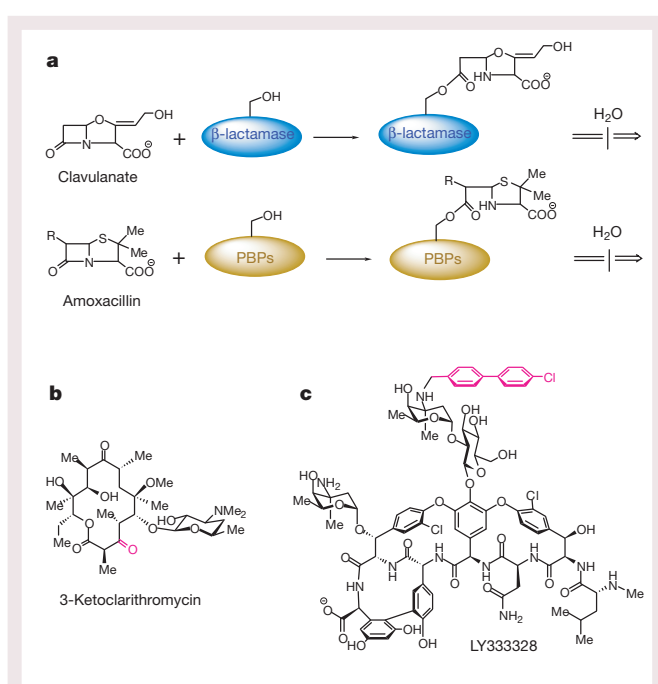
Given the inevitability that resistant strains of bacteria will emerge in response to widespread use of a particular antibiotic and limit its lifetime, knowledge of the principal and specific resistance mechanisms, as described above, can provide insights into strategies for development of new therapeutics. Historically, when resistance to a  $\beta$ -lactam antibiotic arose, medicinal chemists tinkered with the periphery of the  $\beta$ -lactam warhead to obtain a variant that was effective, for a time. When  $\beta$ -lactamase-producing strains became a significant clinical menace, attention switched to approaches to neutralize the antibiotic-destroying hydrolase, both by screening against lactamase producers and by mechanism-based inhibition of the active-site serine hydrolases. Both strategies worked. Clavulanate, a natural product from a streptomycete, was not an effective antibiotic by itself but was a suicide substrate for the lactamase<sup>31</sup>. In combination, clavulanate allowed the classical  $\beta$ -lactam drug amoxicillin to be augmented in its antibacterial range. The combination of clavulanate and amoxicillin is called Augmentin (Fig. 4a) and has become front line therapy<sup>32</sup>. In parallel, the study of a penicillin analogue with a five-ring sulphur atom oxidized to the sulphone revealed that this derivative, called sulbactam, now had a weaker C–S bond that disposed the ring of the acyl-lactamase intermediate to open and create a long-lived covalent enzyme intermediate that was inactive<sup>31</sup>. The combination of sulbactam and ampicillin is Unasyn, which is also an important antibacterial combination<sup>32</sup>. Two other combinations of lactamase inactivator and  $\beta$ -lactam antibiotic, Timentin and Zocin, are also widely used antibacterial combinations that exemplify the utility of this approach.

Analogous logic could be used to screen for or design analogues of tetracyclines<sup>33,34</sup> and erythromycins that would be less susceptible to recognition and transit by the efflux pumps. Third-generation forms of erythromycins, for example, the 16 ketolides<sup>35</sup> (Fig. 4b) that are currently in clinical testing, are less prone to induce the Erm type of methylation resistance. A combination strategy akin to the logic used for Augmentin is under study, where a separate inhibitor of efflux pumps, once identified as having sufficient potency and safety, could be combined with the macrolide antibiotic or the tetracycline.

When drug targets have been altered as in VRE, screening for molecules that are now active against the vancomycin-resistant strains have identified compounds such as LY333328<sup>36</sup> (Fig. 4c), a semisynthetic analogue of vancomycin. LY333328 contains a hydrophobic biphenyl substituent on the vancosamine sugar and is more hydrophobic and may partition the analogue more to the membrane<sup>37</sup>, as well as alter its ratio of inhibition between transpeptidases and transglycosylases<sup>38,39</sup>. Pristinamycin (trade name Synercid) is a combination of two non-ribosomal peptide natural products, quinupristin and dalbapristin, which act synergistically to inhibit protein synthesis in a bacteriocidal manner<sup>40</sup>. The drug has recently been approved with the intent of treating VRE infections<sup>41</sup>.

#### Development of new classes of antibiotics

In addition to defining new targets in bacteria for new antibiotics (see below), there has been progress in development of a new structural class of synthetic molecules with broad spectrum and acceptable potency: the oxazolidinones. The oxazolidinones also inhibit protein biosynthesis, specifically by interaction with the 23S ribosomal RNA at or near the peptidyl transferase centre of the ribosome<sup>10</sup>. One such oxazolidinone, linezolid, has now progressed through clinical trials<sup>42</sup> to approval in the United States. Linezolid has been described as the



**Figure 4** Counter-survival strategies. To combat the development of antibiotic resistance requires a knowledge of the specific mechanism of resistance and then development of a new class of compounds that are active against the resistant bacteria. **a**, Augmentin is a combination of clavulanate, which inactivates the  $\beta$ -lactamase by forming a slowly hydrolysing acyl enzyme intermediate, and amoxicillin, which blocks the cell wall-crosslinking transpeptidase, also by forming a slowly hydrolysing covalent acyl enzyme intermediate. **b**, Introduction of a 3-keto group into the macrolide ring in 3-keto clarithromycin (a ketolide) alters the conformation of the macrolactone and alters susceptibility to efflux and to induction of methylation resistance machinery. **c**, Screening against vancomycin-resistant enterococci (VRE) with semisynthetic variants of vancomycin indicates that hydrophobic substituents on the vancosamine sugar, as in LY333328 which has a biphenyl alkyl substituent on the amino sugar, restore about two logs of potency against VRE.

first new structural class of antibiotics introduced in three decades and will join the quinolones as evidence that synthetic compounds can be elaborated that match the antibiotic potency and selectivity of natural products. Also in development is a glycolipopeptide (17 amino acids cyclized to a macrolactone) called ramoplanin<sup>43</sup>. This works against VRE by forming a complex with the lipid pentapeptide intermediates in cell-wall biosynthesis, acting in a somewhat analogous way to vancomycin by targeting a substrate rather than an enzyme in the peptidoglycan assembly pathway.

### New approaches to antibiotic development and resistance

In this last section I take up the two questions: how do we find or make new antibiotics and can we slow down the development of resistance? The first question encompasses the searches both for new bacterial targets that will be killing sites and for new molecules with the killing (antibacterial) properties. The second question includes prescriptions for rethinking the ways that antibiotics are prescribed and used.

#### Using genomics to find new bacterial targets

Bacterial genomics has progressed to the extent that about three dozen bacterial genomes have been completely sequenced in the past few years, including the full gene sequence in such pathogens as *S. aureus*, *Streptococcus pneumoniae*, *Mycobacterium tuberculosis*, *Helicobacter pylori*, *Pseudomonas aeruginosa* and *Vibrio cholerae*. The genome of a prototypic streptomycete, *Streptomyces coelicolor*, which produces several types of polyketide antibiotics, is also close to being finished and should provide additional insights into the organization of antibiotic biosynthetic operons and their regulation. Approaches

involving gene disruption have begun to narrow the list of bacterial genes in pathogens that are essential either for virulence or for survival to perhaps a few hundred genes, some of known or putative function and some unknown. The products of these essential genes constitute an initial set of validated protein targets that can be loaded into optimized, automated screens. Chemical libraries can be assessed for inhibitors that score as hits, and these can then be elaborated into leads of useful potency and specificity, first *in vitro* and then in animal models of infections as antibacterial agents. These kinds of approaches offer significant promise for expansion of the current small number of targets that are the sites of action of contemporary antibiotics<sup>44</sup>.

For example the enzyme that removes the formyl group from the amino-terminal formylmethionyl residue in bacterial proteins, peptide deformylase<sup>45</sup>, is a metalloproteinase that is an essential gene product in many bacteria and for which potent inhibitors have recently been described<sup>46</sup>. Several gene products that are manifestations of virulence responses by pathogenic bacteria<sup>47</sup> are being examined, including peptides involved in bacterial secretion pathways<sup>48</sup> and the two main types of signalling networks: two-component systems (transmembrane sensor kinase and response-regulatory gene-transcription factor)<sup>49</sup>, and quorum sensing pathways that regulate selective gene activation in cell density-dependent responses<sup>50,51</sup>.

The prospect of a much larger set of validated targets in bacteria will create opportunities for the development of novel therapeutics, such that the discovery of new molecules with antibiotic potential will gain in importance. Library approaches are being explored both with synthetic compounds and natural products. The synthetic libraries can be large, with millions of members, or focused around particular pharmacophores<sup>52</sup> and contain a few hundred to thousand members in any iteration. Several of the chemical libraries seek to build in the identity and density of the functional groups and the three-dimensional architecture of compounds, demonstrating convergence towards the complexity of natural products<sup>53</sup>.

The complementary approach of *in situ* library generation — combinatorial biosynthesis of natural products that act as antibiotics — is underway, especially for polyketide and mixed polyketide/non-ribosomal peptide antibiotics<sup>54–57</sup> where all the biosynthetic genes are typically clustered together within a 50–100-kilobase region of DNA. This makes cloning of new biosynthetic operons for polyketides relatively straightforward, even from the large number of microbes that cannot at present be cultured in the laboratory environment. Meanwhile the multimodular organization of the lines of assembly of polyketide synthase enable domain and module swap, replacement and reprogramming strategies to make small libraries of modified polyketides by fermentation. Several erythromycin derivatives have been reported with up to three segments replaced combinatorially, which illustrates the potential of this approach<sup>58</sup>. The rules need to be determined as to what kinds of point mutations, catalytic-domain replacements and multidomain module swaps can be tolerated and combined into a hybrid assembly line with good throughput of new products. For many of the macrolide antibiotics, represented by erythromycin, the sugars that are added to the macrocyclic lactone scaffolding by committed, tailoring enzymes are crucial determinants of biological activity. Efforts to provide alternate deoxy and amino sugars for *in vivo* alternate tailoring<sup>59</sup> have been reported and would be a second, multiplicative step for expanding the biosynthetic libraries of this class of antibiotics.

### Strategies for extending antibiotic lifespan

Given that the emergence of antibiotic-resistant bacterial strains is an inevitable response to the widespread application of both current and future antibiotics, explicit rethinking of strategies for preserving and extending the useful life of antibacterial drugs has been central in this era of multiple drug-resistant bacterial recrudescence. Guidelines for altering the behaviour of both patients and physicians in antibiotic consumption and prescription have been advanced by

**Table 1 Guidelines for extending the useful lifetimes of antibacterial drugs**

- Optimal use of all antimicrobials
- Selective removal, control or restriction of antimicrobial agents or classes
- Use of antimicrobials in rotation or cyclic patterns
- Use of combination antimicrobial therapy to prevent the emergence of resistance

Adapted from the recommendations in ref. 60.

infectious disease specialists<sup>60</sup> (Table 1). In developed countries, the goal is to reduce the inappropriate prescription of antibiotics (estimates of up to 50% of prescriptions may be written for patients with viral infections that cannot respond to the antibacterial drugs). In developing countries, availability of antibiotics may be intermittent, the quality and potency uncertain, and the tendency to self-medicate without specialist input from physicians can be widespread. The net result is that individuals often take subtherapeutic doses of antibiotics; symptoms may disappear but resistant strains of bacteria are thereby selected and may only become acutely problematic to the individual in a subsequent medical crisis where an infection proves resistant. The threat to the larger population is that reservoirs of drug-resistant bacteria abound.

Among the guidelines considered to alter social norms about antibacterial drugs (Table 1) is that of rotating the use of antibiotics to preserve the efficacy of antibiotics of last resort, such as vancomycin in the treatment of life-threatening infections due to MRSA. The spectre of high-level vancomycin resistance transposed into MRSA is one of the doomsday scenarios in infectious disease<sup>61</sup>. There has also been the call to consider wider use of combinations of antibiotics in primary therapy, not just the Augmentin and Synercid approaches where two components work together to neutralize a single target, but combinations of distinct antibiotic classes that work on different targets concurrently<sup>60</sup>. This combination approach has been the norm for curative regimens of anticancer drugs and more recently the multiple-component HAART (highly active antiretroviral therapy) regimens for control of AIDS progression<sup>62</sup>. But set against with the potential benefits of such an approach is the liability that if subtherapeutic doses are effected in combination therapy, multiple drug resistance will emerge more rapidly. The epidemic of multiple drug-resistant tuberculosis most probably arises from patient non-compliance during the extended period of drug therapy.

The use of antibacterial drugs as growth promoters for animals has been a topic of heated debate for well over a decade<sup>63</sup>, as antibiotic prophylaxis in cattle, pigs, chicken and aquaculture grew to quantities that could dwarf human consumption of the same or related antibacterial drugs. As in humans, subtherapeutic doses in animals can select for resistant strains; if the bacteria cross from animal hosts to human hosts then reservoirs of resistance may markedly reduce the effective lifetime of human antibiotics. In Denmark in 1994, 24,000 kg of a vancomycin derivative called avoparcin was used for animal health, 1,000 times higher than the 24 kg of vancomycin used to treat human infections in the same year<sup>63</sup>. When pigs treated with avoparcin were analysed for VRE strains, they contained the same five gene operons that encode vancomycin resistance in enterococci isolated from nonresponsive human patients. The Danish government later banned the use of avoparcin as an additive to animal feed. In a second example, VRE that were resistant to the recently approved quinupristin/dalfopristin combination were found to have arisen during drug treatment<sup>64</sup>; this may have occurred through the effect of an animal reservoir involving an acetyl transferase carried by bacteria resistant to the related antibiotic virginiamycin, which has been used in animal feeds in Europe for 20 years<sup>63</sup>. At the very least, principal drug classes, especially new ones that have therapeutic potential as human antibacterials should be carefully evaluated before being 'wasted' in animal feed uses.

In conclusion, the increased molecular knowledge about essential bacterial genes and the ability to screen such candidate targets with libraries of new synthetic and natural products to find hits that can be

subjected to iterative cycles of improvement of structure and function indicate that new antibacterial agents against non-traditional bacterial targets may be forthcoming. But new antibiotics by themselves will not alter the kinetics of the cycles of resistance development. Indeed, wider and more indiscriminate use could actually shorten the cycle time unless behaviour changes, which are difficult but not impossible to achieve, occur with regard to valuing antibiotics as precious and finite resources. □

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