Multi-targeting by monotherapeutic antibacterials

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Abstract | Antibacterial discovery research has been driven, medically, commercially and intellectually, by the need for new therapeutics that are not subject to the resistance mechanisms that have evolved to combat previous generations of antibacterial agents. This need has often been equated with the identification and exploitation of novel targets. But efforts towards discovery and development of inhibitors of novel targets have proved frustrating. It might be that the 'good old targets' are qualitatively different from the crop of all possible novel targets. What has been learned from existing targets that can be applied to the quest for new antibacterials?

The respective contributions to the profile of a given antibacterial agent by the target and the physico-chemical characteristics of that agent are listed in TABLE 1. At present, in the search for novel antibacterials, previously unexploited targets are selected on the basis of their essentiality and presence in the desired spectrum of bacteria, lack of human homologues and theoretical druggability. Inhibitors of those targets are generally obtained through screening of chemical or natural-product libraries for lead compounds, using biochemical assays or target-directed whole-cell screens. *De novo* design of inhibitors of novel targets, in the absence of screening leads, is still relatively rare. Past this point, the physico-chemical optimization of lead compounds is aimed at meeting profile requirements (principally cell entry, pharmacokinetics and safety) while retaining target affinity and specificity. Some degree of bacterial entry is assured with leads that are derived from whole-cell screens. For enzyme inhibitors from biochemical screening, chemical modification is necessary, but not always achieved, to enable penetration into cells and/or avoid efflux from them. The other physico-chemical characteristics that are required for therapeutic efficacy and safety are then arrived at through iterative processes of chemical synthesis and biological assay. So far, no antibacterial agent that has been derived from this novel-target-first paradigm has reached the market.

Interestingly, the molecular targets of most of the commonly used classes of systemic antibacterial agents were identified after the first-generation compounds reached the clinic. In the golden age of antibiotic discovery (1940–1960s), antibiotics and synthetic antibacterials were generally discovered empirically, without pre-selection of targets. Many of those compounds were developed for clinical use in systemic monotherapy and were the progenitors of further generations of drugs. Others of those early discoveries did not have optimal profiles and, as a result of toxicity, narrow spectrum, poor pharmacology or rapid resistance development, were either not developed, lost clinical acceptance quickly, or found application in combination therapy or topical use. Therefore the modern day armamentarium of systemic monotherapeutic agents was largely derived from empirically discovered classes of antibacterials by winnowing and modification to meet clinical criteria for their successful use — a process that might be likened to evolution. The clinical criteria that now define the required profile for systemic agents were, in that analogy, elements of natural selection. The targets that are being exploited at present are those for which inhibitors were selected for success in systemic chemotherapy.

The requirements for essentiality, selectivity and spectrum of targets are certainly of great importance and have been covered in many reviews[1–4], but will not be addressed in any systematic way here. Instead, this review focuses on qualities of successful targets that favour low potential for rapid resistance selection, and discusses recent successes in the modification of inhibitors of those targets to overcome resistance. Approaches towards antibacterials that avoid target-based resistance will also be presented.

The multi-target hypothesis

The antibacterial drugs that are commonly used in current systemic monotherapy are shown in TABLE 2. The major mechanisms of resistance are indicated, with those that are due to endogenous mutations in the chromosome of target organisms indicated by an asterisk.

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Table 1 | **Characteristics determining the profile of antibacterial compounds**

Characteristic	Target contributions	Physico-chemical contributions
In vitro		
Antibacterial efficacy	Essentiality of target	Bacterial entry and low efflux potential
Antibacterial spectrum	Presence of target in critical species	Bacterial entry and low efflux potential
Antibacterial potency	Target affinity, 'druggability'	Bacterial entry and low efflux potential
Low resistance potential	Low frequency of single-step and serial-passage mutation to resistance Low potential for bypass mutations Poor fitness of resistant mutants Low probability of compensatory mutations	Low frequency of resistance due to changes in compound-related permeability and efflux
In vivo		
Safety	Selectivity of target (present in bacterium, not in host) Safety of target-interacting pharmacophores	Non-mechanism-related toxicity Safety of target-interacting pharmacophores
Pharmacokinetics and pharmacodynamics	Effect of target inhibition on bacterial physiology related to drug concentration	Favourable solubility, stability, clearance, distribution, tissue penetration, bioavailability and protein binding
Drug interactions	Recognized potential for synergy or antagonism due to mechanism of action	Effects on drug metabolism
Resistance in clinical use	Low potential for stepwise mutations in long-term serial passage Low likelihood of acquisition of mechanisms of target *trans*-modification or target replacement/bypass	Acquired compound-specific degradative, drug-modifying and efflux mechanisms

Endogenous resistance can be due to changes in target or to decreased cell permeability, increased efflux or upregulation of pre-existing degradative enzymes (for example, the type C β-lactamases of many Gram-negative bacteria[5]). A common feature of all of these agents is the low occurrence of high-level resistance through single-step mutation of their targets. None of the targets, except for that of fosfomycin (discussed below), is the product of a single gene. Rather, their targets are the products of multiple genes or are structures that are synthesized by multiple genes.

A number of antibacterial agents that target single enzymes are in clinical use for standard pathogens (TABLE 3). All are subject to high-level resistance resulting from single-step mutation in the target enzyme. But none of these agents are used in standard systemic monotherapy — they are used in combination with other drugs, or topically. This type of analysis led to the hypothesis[6] that, aside from the development of endogenous resistance due to permeability changes, drugs that are susceptible to the single-step development of high-level endogenous resistance are those which interact with a single gene product. Conversely, drugs that have a low likelihood for the development of high-level endogenous resistance are those that interact with multiple molecular sites, the structures of which are determined by multiple genes. This favours the development of multi-target over single-target compounds for systemic monotherapy. Permeability changes are excluded because they are not a function of the target, but of the modifiable chemical properties of the compound. A number of authors have noted the relationship of the multi-target nature of successful antibacterials[7–9] and their lowered potential for target-based resistance.

Exceptions and caveats. With some of the multi-targeted antibacterials, such as macrolides, altered ribosomal proteins have been found to confer resistance by affecting the conformation of the target rRNA (discussed below). The incidence of such resistant isolates is low (although it is increasing), it has taken many years to attain clinical significance, and the most prevalent of these protein alterations is not due to a single-step mutation. This is therefore consistent with the concept that drugs with low endogenous resistance development are those interacting with multiple targets. A caveat, however, is that even multi-targeted drugs might be subject to target-related endogenous resistance development.

Certain single-targeted agents might escape target-based resistance. The second-generation dihydrofolate reductase inhibitor iclaprim, discussed below, is an example of the power of structure-guided design in overcoming resistance. Such design strategies, then, might address the limitations of some single-enzyme targets[10].

Fosfomycin targets UDP-*N*-acetylglucosamine (UDP-GlcNAc) 1-carboxyvinyltransferase (also known as UDP-GlcNAc enolpyruvyl transferase; MurA), which is the first enzyme in the cell-wall synthesis

Table 2 | **Antibacterial compounds commonly used in systemic monotherapy**

Class	Target	Mechanisms of high-level resistance that compromise therapy	
		Target related	Compound-chemistry related
β-lactams	Cell-wall synthesis: multiple penicillin-binding proteins (PBPs)	Horizontal transmission of resistant PBPs	Horizontal transmission of β-lactamase Upregulation of β-lactamase; permeability decrease, general efflux*
Vancomycin	Cell-wall synthesis: D-Ala-D-Ala of peptidoglycan substrate	Bypass pathway (VRE) Stepwise increase in wall thickness*	
Tetracyclines	Protein synthesis: 16S rRNA	Ribosome protection	Compound-specific efflux
Gentamicin	Protein synthesis: 16S rRNA		Inactivating enzymes
Macrolides	Protein synthesis: 23S rRNA	Ribosome protection Stepwise rRNA mutations* Low-frequency alterations in ribosomal proteins*	Compound-specific efflux
Lincosamides	Protein synthesis: 23S rRNA	Ribosome protection	
Chloramphenicol	Protein synthesis: 23S rRNA	Ribosome protection	Inactivating enzyme
Oxazolidinones	Protein synthesis: 23S rRNA	Stepwise rRNA mutations* Ribosome protection	
Fluoroquinolones	DNA replication: topoisomerases, gyrase and topo IV	Point mutations in both targets* Target protection	Compound-specific and general efflux*
Daptomycin	Bacterial membrane	Stepwise changes*	
Metronidazole	DNA alkylation		Loss of reductase
Nitrofurantoin†	DNA and protein alkylation		
Fosfomycin†	Cell-wall synthesis UDP-GlcNAc enolpyruvyl transferase	Inactivating enzymes	Loss of permease*

*Resistance results from mutations in the chromosome (endogenous mechanisms). All other mechanisms occur by horizontal transmission of plasmids or transposons, or by transformation (endogenous mechanisms). †Compounds used, in the US, uniquely in oral form for uncomplicated urinary-tract infections. VRE, vancomycin-resistant enterococci; UDP-GlcNAc, undecaprenyl *N*-acetylglucosamine.

pathway. It is used mainly for oral therapy of uncomplicated urinary-tract infections, and resistance is rare in clinical isolates although it is subject, under laboratory conditions, to high-frequency, high-level resistance in many organisms as a result of compromised fosfomycin transport. This can probably be attributed to the slow growth of such transport mutants in urine in the presence of fosfomycin[11]. It is known that a change to the MurA target enzyme can confer resistance to fosfomycin[12]. The presumably low frequency and possibly lowered fitness of the specific change that is required, in the face of the high frequency of transport mutants, might explain the lack of reported, spontaneous, target-based resistance to fosfomycin. Gram-positive bacteria contain two genes encoding a MurA activity; both are sensitive to fosfomycin and each is capable of supporting growth without the other[13]. So, single-target mutations in Gram-positive bacteria would be unlikely to lead to fosfomycin resistance.

Although laboratory results with fosfomycin seem to have overestimated the effect of resistance mutations on clinical outcomes, low mutation rates with other compounds under evaluation might underestimate future problems. The relationships among mutation frequencies to antibiotic resistance, the amplitude of that resistance, the fitness of mutants and compensatory mutations are complex, yet they are important for predictive modelling of clinical resistance to any new drug. Discussion of these relationships is beyond the scope of this review, but is clearly relevant. The subject is well reviewed by Martinez and Baquero[14].

Targets of successful monotherapies

Penicillin-binding proteins. The β-lactam ring is the shared pharmacophore of penicillin, cephalosporin, cephamycin, monobactam and carbapenem antibiotics, which are produced by bacteria and fungi (FIG. 1a). Penicillin inhibits the crosslinking reaction of muropeptide subunits — undecaprenyl-pyrophosphoryl-*N*-acetylmuramyl-(pentapeptide)-*N*-acetylglucosamine (known as Lipid II)[15–17] — by transpeptidation (FIG. 2b,c). The CO–N bond of the lactam ring is an analogue of acyl-D-Ala-D-Ala, the terminal dipeptide of the substrate that is cleaved during the transpeptidase reaction (FIG. 2a). The lactam is cleaved by the enzyme and forms a penicilloyl–enzyme complex, which is effectively irreversible[16].

Table 3 | **Antibacterials targeting single enzymes in clinical use (excluding mycobacterial infection)**

	Target	Common resistance mechanisms	Clinical use
Rifampin	RNA transcription RNA polymerase	Single mutations in RpoB*	In combination for MDR Gram-positive organisms
Fusidic acid	Protein synthesis Elongation factor G	Single mutations in Ef-G* Compound-specific efflux Exogenous ribosome protection	In combination for MDR Gram-positive organisms
Novobiocin	DNA replication DNA gyrase B subunit	Single mutations in gyrase B*	In combination for MDR Gram-positive organisms
Trimethoprim	Folate synthesis Dihydrofolate reductase (FolA)	Single mutations in FolA* FolA overproduction* Exogenous resistant FolA	In combination with sulphonamide
Sulfonamides	Folate synthesis FolP	Insertion in FolP* FolP overproduction* Exogenous resistant FolP	In combination with trimethoprim
Mupirocin	Protein synthesis Isoleucyl tRNA synthetase New IleS	Mutations in IleS*	Topical

*Mechanisms resulting from endogenous mutations. Ef-G, elongation factor G; FolA, dihydrofolate reductase; FolP, dihydropteroate synthase; IleS, isoleucyl-tRNA synthetase; RpoB, RNA polymerase B; MDR, multi-drug resistant.

Polyacrylamide gel electrophoresis of membrane proteins that are treated with radiolabelled penicillin can be used to identify numerous penicillin-binding proteins (PBPs)[18], the complement of which varies greatly among bacteria, with similar patterns of PBPs appearing in related genera[17,19]. The PBPs of lower molecular mass are monofunctional carboxy- and endo-peptidases, transpeptidases and β-lactamases. The species of higher molecular mass are multimodular, containing a transpeptidase module and a second module, which can be a transglycosylase[20,21]. The many β-lactams were shown (by competition with labelled penicillin) to bind with compound-specific affinity to the various PBPs[22,23]. The penicillin-binding site of the PBPs consists of three conserved motifs, SXXK, SXN and KTG (where X represents any amino acid), which occur in the same order and at roughly the same spacing in all PBPs[20].

The essentiality of PBPs and their role in bacterial killing are complex. In *Escherichia coli*, which contains 12 PBPs[24], the loss of any single PBP does not compromise viability, and the minimal requirements for survival are PBP1a or PBP1b plus PBP2 or PBP3 (REF. 24). In *Staphylococcus aureus*, which contains 4 PBPs, PBP1 (REF. 25) and PBP2 (REF. 26) are essential for viability, but PBP3 (REF. 27) and PBP4 (REF. 28) are inessential. This is not completely consistent with the finding that killing by β-lactams seems to require inhibition of PBP1 and either PBP2 or PBP3 (REF. 29). Determination of the minimal combinations of PBPs that are required for viability might explain the paradox. It seems that inhibition of at least two PBPs is required for efficient killing by β-lactams.

β-lactam resistance due to the acquisition of β-lactamase in *S. aureus* was seen soon after the introduction of penicillin. Modified penicillins, which were insensitive to this enzyme, were then introduced. Clinical use of β-lactams that had improved activity against Gram-negative organisms revealed pre-existing chromosomal Class C β-lactamases[5], and selected for the spread of plasmid-borne Class A β-lactamases. Newer penicillins, cephalosporins, carbapenems and combinations of penicillins and β-lactamase inhibitors have addressed much of that resistance. Nevertheless, target-based resistance due to the horizontal transfer of new β-lactam-resistant PBPs from commensal or environmental species has become a major clinical problem. In naturally transformable organisms, *Neisseria* spp. and *Streptococcus pneumoniae*, β-lactam resistance is generally due to mosaic PBPs with reduced penicillin affinity apparently derived from commensals[30–32]. Non-lactamase resistance to β-lactams in staphylococci is due to a new PBP, *PBP2a*, which is probably derived from the environmental staphylococcal species *S. sciuri*[33]; this species has a very low affinity for β-lactams[34] and therefore functions when other PBPs are inhibited.

Ribosomal RNA. The bulk of classes of clinically important antibacterials target the ribosome and inhibit protein synthesis (TABLE 1) by binding to the 16S[35,36] or 23S[37,38] rRNA of the 30S or 50S ribosomal subunits, respectively. Clinically significant resistance to these agents has arisen almost exclusively through horizontal transmission of drug inactivation or target-protection mechanisms, many of which have been postulated to have originated in the organisms that produce the antibiotic[39]. In addition to the relatively common ribosome-protection mechanisms such as TetM (mechanism unknown) and MLS_B (*erm* methylation of A2058, causing resistance to macrolides, lincosamides and streptogramin B), a recently identified function, encoded by the *cfr* gene present on a transposon, has been shown to methylate A2503 of 23S RNA[40] and mediate resistance to phenicols, lincosamides, oxazolidinones, pleuromutilins and streptogramin A[40,41]. So far, *cfr* has been seen mostly in animal isolates of *S. aureus*[42] but there has been a recent report of a human *S. aureus* isolate with linezolid resistance due to the presence of *cfr*, the first instance of transmissable oxazolidinone resistance in a clinical isolate[43].

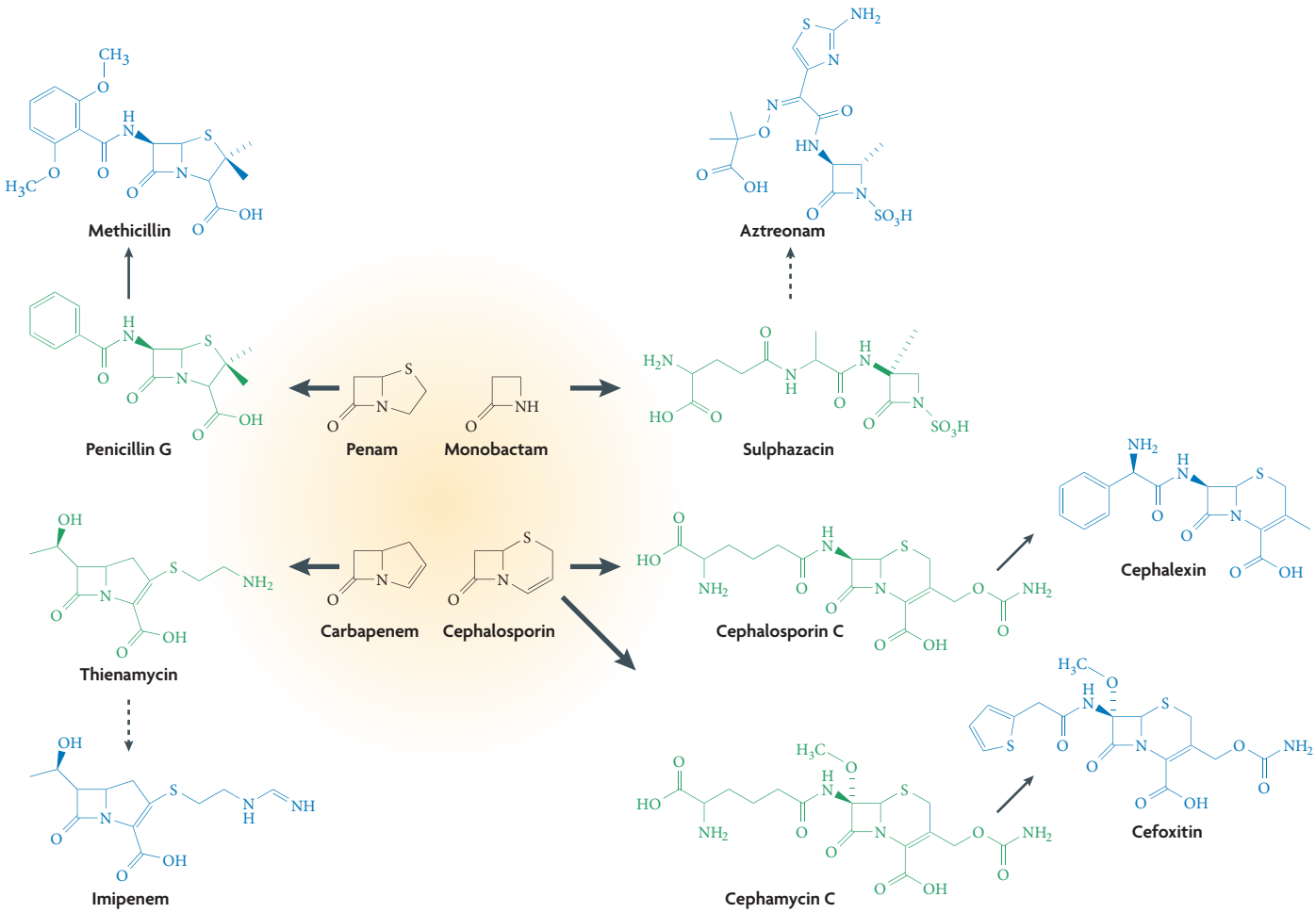

Figure 1 | **β-lactam antibiotics.** The penam, carbapenem, cephem and monobactam nuclei are shown in black. Natural products are shown in green. Penicillin G and cephalosporin C are fungal products. Cephamycin C and thienamycin are streptomycetes (bacterial) products. Sulphazacin is produced by *Pseudomonas acidophila*. The compounds shown in blue are semisynthetic derivatives of the compounds to which they are connected by solid black arrows, or totally synthetic compounds based on the structures to which they are connected by black dashed arrows.

The rRNA targeting of protein-synthesis inhibitors was recognized in the late 1980s[44] and the lack of rRNA single-step mutants is explained by the presence of multiple copies of the rRNA operons in these laboratory strains (and most common pathogens)[45], such that any single mutation alters only a fraction of ribosomes and is effectively recessive[46]. Slow growing mycobacteria contain only a single rRNA operon[47] and are illustrative of this scenario, in that endogenous resistance in *Mycobacterium tuberculosis* to streptomycin, kanamycin and amikacin maps in both ribosomal proteins and 16s rRNA[48]. In *M. avium*, resistance to the macrolide clarithromycin is due to alteration of 23S rRNA[49].

Resistance to the synthetic antibacterial linezolid, an oxazolidinone, resulting from changes in multiple 23S rRNA operons, has been seen, though rarely, in the clinic in enterococcal species and *S. aureus*. Modelled in the laboratory, such changes are seen to be stepwise, and the rise in minimum inhibitory concentration (MIC) is related to the number of operons present[50,51]. Similarly, macrolide-resistant strains with alterations in multiple rRNA operons have been seen in clinical isolates of *S. pneumoniae*[52–54]. In one large study[54], 1.5% of isolates (1,043 total) were macrolide resistant, but did not demonstrate the common efflux or methylation mechanisms. All had alterations in rRNA operons.

In laboratory studies, low-frequency endogenous resistance to some of the ribosomally targeted protein-synthesis inhibitors is due to altered ribosomal proteins. Single-step or serial-passage experiments in *E. coli*, *M. pneumoniae* and *S. pneumoniae*, with macrolides, ketolides, lincosamides and streptogramins, have yielded isolates with mutations in ribosomal proteins L4 or L22[55–58]. At least one L4 mutant had a growth defect[57]. These protein alterations affect the conformation of neighbouring rRNA, leading to altered interaction of the macrolide with its rRNA target[59]. Changes in these proteins have been seen in clinical isolates of *S. pneumoniae*[52–54] and in *S. aureus* isolates from cystic fibrosis patients[60,61]. In *S. pneumoniae* two types of L4 mutant have been described, one with an insertion of three residues, which confers high-level resistance but slow

growth[52]; and a more prevalent mutant with seven base changes that result in the alteration of three contiguous amino-acid residues (GTG to TPS) in L4[52,53]. As these seven changes are clustered, it is tempting to speculate that the GTG-to-TPS alteration in L4 is analogous to the mosaic PBPs that have arisen in transformable organisms (see above). A mutant L22 protein with a six-amino-acid insertion in the carboxyl terminus of the protein has also been described[53]. Such isolates, which were first reported in 2000 (REF. 52), have been seen at low frequency, but might represent an increasing problem. It seems that isolates with alterations in ribosomal proteins occur with lower frequency than rRNA changes. In the study noted above[54], only one of the macrolide-resistant isolates that did not display efflux or methylation-resistance mechanisms had a mutation in ribosomal protein L22, in addition to rRNA changes.

Topoisomerases. The bacterial topoisomerase, DNA gyrase (Gyr), was discovered[62] independently of its inhibitor — the synthetic naphthyridine nalidixic acid[63] — but the connection was quickly made between inhibitor (already known to block DNA replication[64,65]) and target[66,67] (FIG. 3). Medicinal chemistry based on the

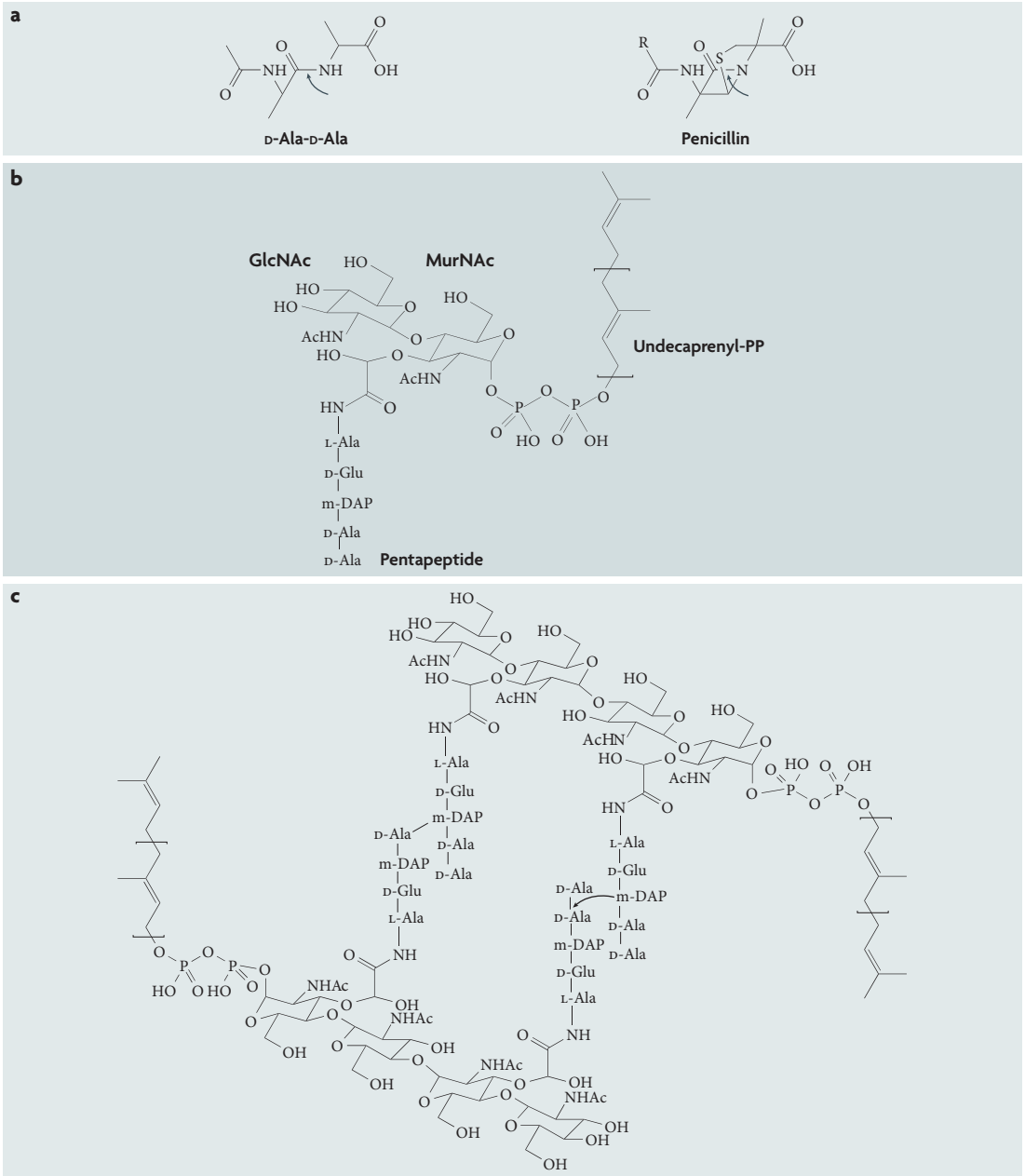

Figure 2 | **Trans-peptidation. a** | Penicillin is an analogue of D-Ala-D-Ala. The bond broken by transpeptidase activity is indicated by the arrow. **b** | Lipid II (undecaprenyl-pyrophosphoryl-*N*-acetylmuramyl-(pentapeptide)-*N* acetylglucosamine). **c** | Transpeptidation (crosslinking reaction). Crosslinking of meso-diaminopimelic acid to D-Ala is indicated by the curved arrow.

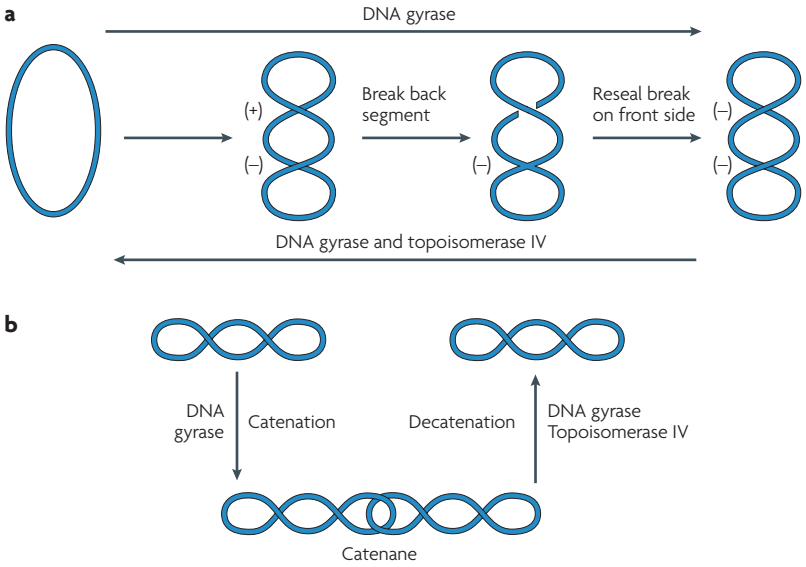

Figure 3 | **Reactions of topoisomerases. a** | In eubacteria, DNA gyrase imparts negative supercoils into closed circular DNA by the concerted breaking and rejoining of double strands. Both DNA gyrase and topoisomerase IV can remove super coils. **b** | Supercoiled DNA is catenated by DNA gyrase and decatenated by both DNA gyrase and topoisomerase IV.

naphthyridine antibacterials led to the fluoroquinolone class, many of which have exceedingly broad spectra and high potency. One of the earliest, ciprofloxacin, has been highly successful in clinical use. Fluoroquinolones, which were thought to target only the A subunit of DNA gyrase (GyrA), seemed to be a major exception to the 1993 hypothesis that good systemic monotherapeutic agents require multiple targets[6], in that mutations in the targeted GyrA did not give rise to high-level single-step resistance. However, it was shown soon thereafter that fluoroquinolones can inhibit both GyrA and the A subunit of topoisomerase IV (ParC), and resistance mutations were found to map in analogous sequences in the genes encoding both enzymes[68–70]. In *E. coli*, GyrA seemed to be the more sensitive target to ciprofloxacin[70], but in *S. aureus*, ParC was the more sensitive[68]. Genetic and biochemical studies of many fluoroquinolones showed that the relative targeting of the enzymes was organism and compound specific[8,71–73]. In *S. aureus*, for example, although the primary target of ciprofloxacin and levofloxacin is ParC, the primary target of sparfloxacin is GyrA, and for moxifloxacin and gatifloxacin, the activity seems balanced[73].

Hooper recognized that the presence of two fluoroquinolone targets presented the possibility of designing agents that are 'dual-targeted'[8,74]. The aim would be to balance the activity of a given compound such that it would be potent and equivalent on both enzymes at intracellular concentrations and conditions. In such a case, a mutation in one enzyme would not lead to a rise in MIC, as the other target would provide susceptibility at that concentration. With unbalanced activities, mutation in the most sensitive enzyme would lead to a rise in MIC to the level of the next most sensitive target, which would then be subject to selection. This yields the stepwise pattern for fluoroquinolone resistance that is generally seen[75]. The dual-target hypothesis was explicitly tested by determination of resistance frequencies for single-step mutations by selection (at twofold MIC increments) in six independent cultures of a single strain of *S. aureus* using fluoroquinolones for which the primary target was either GyrA or ParC, alone and in combination[76]. Although single-step mutants could be selected at twofold MIC with the single agents (7.8×10^{-6} to 1.9×10^{-9}), no mutants ($<2.6 \times 10^{-11}$) could be selected with the combination.

Even with balanced inhibitors, however, resistance development can be demonstrated through serial passage at sub-inhibitory concentrations of fluoroquinolones[77]. Therefore, the selecting concentration (sub-inhibitory or above MIC) is important. Drlica proposed the concept of the mutant-protection concentration (MPC), which represents the concentration of drug at which two concurrent mutations are required to overcome inhibition[78]. Drlica reasoned that by selecting proper dosing parameters, *in vivo* selection of resistant mutants might be largely prevented[79] if dosing to maintain drug levels above the MPC is feasible.

Peptidoglycan intermediate structures. Glycopeptides, such as vancomycin and teicoplanin, bind to the terminal dipeptide, D-Ala-D-Ala, of the product of the entire cytoplasmic and membrane-bound stages of peptidoglycan synthesis — Lipid II (FIG. 2b) — and sequester the substrate from its use by synthetic enzymes transglycosylase[80] and/or transpeptidase[81]. No simple change in a synthetic enzyme of the pathway produces a glycopeptide-resistant Lipid II. Vancomycin was in clinical use for almost 35 years before the first isolates of vancomycin-resistant enterococci (VRE) were described[82]. This high-level resistance is mediated by the substitution of an operon (such as VanA) that encodes a partial pathway for synthesis of MurNAc-pentapeptide — the product of the cytoplasmic portion of the peptidoglycan-synthesis pathway. The depsipeptide D-Ala-D-lactate, to which vancomycin binds poorly, replaces the D-Ala-D-Ala terminus. These vancomycin-resistance operons are highly related to similar genes found in glycopeptide-producing organisms[83].

A number of other antibiotics bind to peptidoglycan intermediates. Ramoplanin binds to Lipid I and Lipid II (targeting PP-MurNAc-L-Ala-D-Glu[84]) and has been in clinical trials for use in prevention of colonization by VRE and *Clostridium difficile*-associated diarrhoea[85]. The lantibiotics nisin (used as a food preservative) and mersacidin seem to target the pyrophosphate linkage of Lipid II[86]. AC98-6446, a derivative of mannopeptimycin, which binds to Lipid II and is not displaced by mersacidin or vancomycin, has been shown to have low resistance potential and good *in vivo* efficacy[87], but has not yet entered clinical development. Bacitracin, which binds to undecaprenyl-PP and prevents its recycling to undecaprenyl-P, is a common topical treatment. It is unlikely that the structures to which all these antibiotics bind could be changed by single-step mutations.

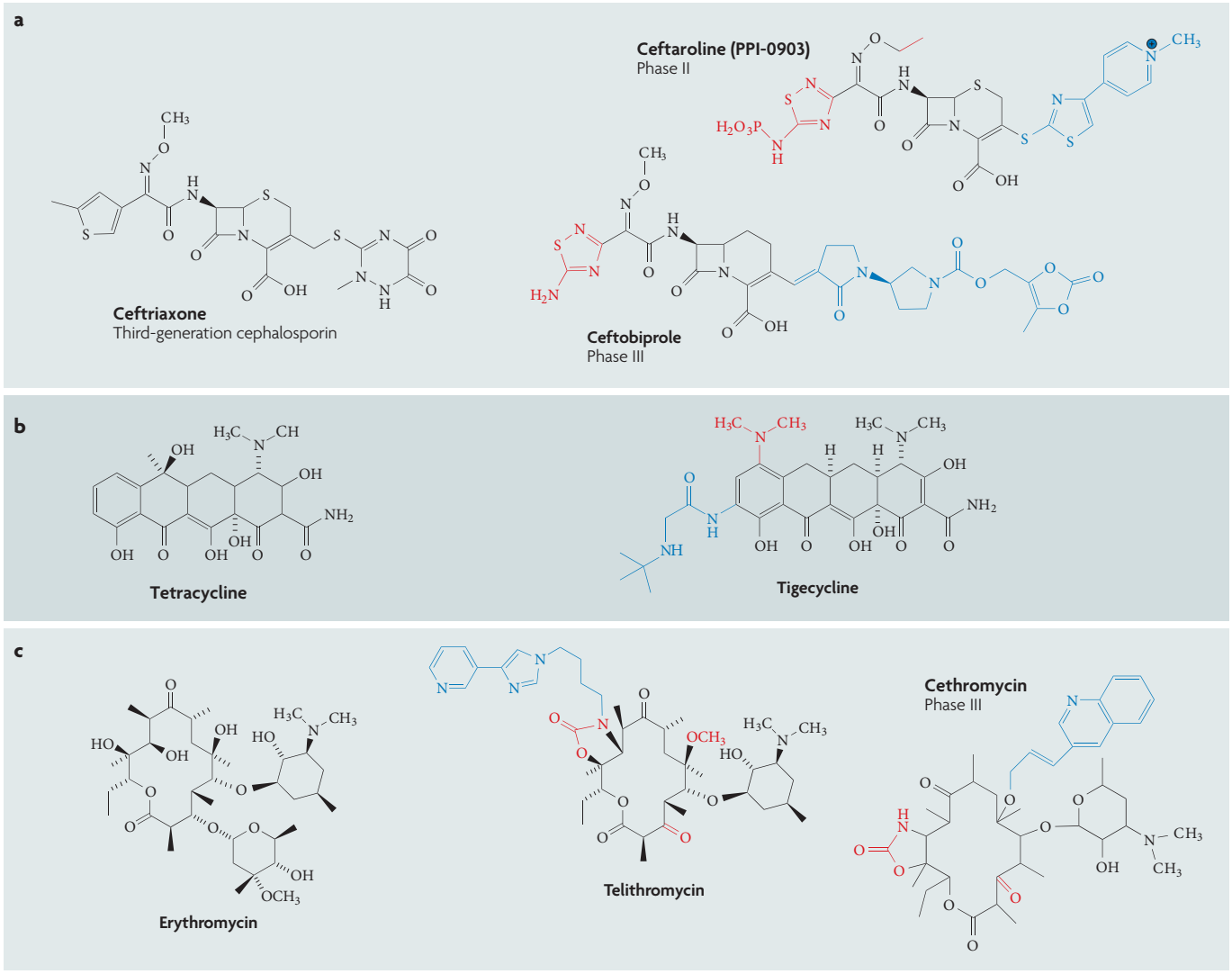

Figure 4 | **Next-generation compounds overcoming resistance.** Compounds on the left are generics of antibiotic classes that are subject to resistance mechanisms, which are overcome by the compounds on the right that are in late stages of development or are already licensed. Blue structures are pharmacophores that make contacts with new binding sites on the target. Other changes to these compounds are in red. **a** | Cephalosporins with activity against methicillin-resistant *Staphylococcus aureus* (MRSA) are compared with a third-generation cephalosporin, ceftriaxone. **b** | Tigecycline, which is active against tetracycline-resistant organisms, is compared with tetracycline. **c** | Ketolides that can overcome some macrolide resistance are compared with erythromycin.

Bacterial membrane. Cytoplasmic membranes of bacteria and eukaryotes are subject to depolarization and disruption by many chemicals. Resistance development to these compounds is found to be slow and often stepwise. For use in systemic antibacterial therapy, such agents must show a high preference for bacterial over mammalian membranes. The recently licensed anionic lipopeptide, daptomycin (Cubicin; Cubist) acts by depolarizing the bacterial membrane[88]. It has seen increasing use in the clinic and resistance development has so far been low. Although a protein receptor is not excluded, daptomycin is thought to interact directly with the phospholipid bilayer in a calcium-dependent manner. A recent paper on the development of step-wise resistance to daptomycin[89] showed slow, incremental increases in MIC that occurred over multiple passages. Mutations in *mprF* led to the initial MIC increase. MprF mutants fail to add lysine to membrane phosphatidylglycerol, presumably decreasing the negative surface charge of the membrane[90]. Daptomycin selectivity for bacterial membranes can probably be attributed to the presence of phosphatidylglycerol (J. A. Silverman, personal communication). Although clinical resistance to daptomycin has been infrequent so far, there is a correlation seen in clinical VISA (vancomycin intermediate *S. aureus*) strains between increased vancomycin MICs, cell-wall thickening and reduced susceptibility to daptomycin[91]. The decreased susceptibility to daptomycin might be a result of decreased penetration of the drug to the membrane target.

Improved versions of successful drugs

Although the successful monotherapeutics are multi-targeted and avoid endogenous resistance, they are certainly subject to exogenous horizontally transmitted resistance mechanisms. Indeed, there have been many programmes to discover or design new structural classes of inhibitors of these targets which should escape existing resistance mechanisms, such as the structure-based approaches to design new rRNA-targeted inhibitors, described below. So far, the disclosed compounds have been improvements on previous agents. The improvement of successful drug classes to overcome pre-existing resistance has involved the addition of pharmacophores to introduce new intramolecular binding sites.

MRSA β-lactams. By the mid-1990s, the rate of methicillin-resistant *S. aureus* (MRSA) among clinical *S. aureus* isolates was >50%. β-lactam insusceptibility in MRSA, as explained above, is due to the presence of a new PBP gene, *PBP2a*, which can replace the activity of the otherwise-inhibited essential PBPs. Several companies undertook major programmes that were directed towards modifying carbapenems[92–94] and cephalosporins[95] to increase their PBP2a-binding properties while maintaining their affinity to the other PBPs. Many interesting compounds that showed excellent *in vitro* activity and *in vivo* efficacy were discovered for which binding to PBP2a was greatly increased, generally by addition of a lipophilic sidechain to the 2 position in carbapenems or the 3 position in cephalosporins. None of those compounds was developed past Phase I clinical development because of safety or pharmacokinetic shortcomings. Recently, two cephalosporins with excellent anti-MRSA activity, safety and efficacy have reached later stages of clinical development, Johnson & Johnson's ceftobiprole[96,97] has reached in Phase III, and Cerexa's ceftaroline (formerly PPI-0903)[97,98] has reached Phase II clinical trials (FIG. 4a). Roche's carbapenem RO-4908643 (REFS 97,99), with activity against MRSA, is also in clinical trials.

Improved rRNA-targeting drugs. The tetracyclines have been a stalwart of antibacterial therapy since the time of their discovery in the 1940s, but widespread plasmid-mediated tetracycline resistance has limited their use[100]. The glycylcycline tigecycline (recently registered as Tygacil (Wyeth) FIG. 4b), a derivative of minocycline, is active against tetracycline-resistant organisms. Its 9-*t*-butyl-glycylamido side chain adds rRNA-binding sites to overcome ribosome protection[101] and prevents its recognition by Tet-efflux pumps[102]. Tigecycline is a parenteral drug with broad activity against many Gram-positive and Gram-negative aerobes, as well as many anaerobes and atypical organisms[103], but it is not as effective against many *Pseudomonas aeruginosa* clinical isolates, a limitation that is due to MexAB-OprM and MexCD-OprJ efflux pumps[104]. PTK-0796, a glycylcycline discovered by Paratek, and similar in spectrum to tigecycline[105], has been licensed to Merck for development and is in Phase I testing for both parenteral and oral administration[106].

The macrolide antibiotics in clinical use, such as erythromycin, azithromycin and clarithromycin are subject to resistance caused by *erm*-mediated methylation of 23S RNA at nucleotide A2058 in region V of 23S RNA and by macrolide-specific *mef*-mediated efflux. The ketolides (FIG. 4c) are a semisynthetic group of macrolide derivatives, which have a keto group in place of the cladinose at C-3 that leads to reduced induction of *erm*[107,108] and reduced ribosome binding. This binding reduction is illustrated by the 100-fold decrease in ribosome binding by the ketolide analogue of clarithromycin (RU56006) relative to clarithromycin[108]. The addition of a long side chain overcomes the binding deficit and improves ribosome binding relative to erythromycin (and clarithromycin). In the marketed ketolide, telithromycin (Ketek; Sanofi-Aventis), this side chain is an alkyl–aryl group tethered to a C-10–C-12 carbamate. In chemical-footprinting experiments with *E. coli* ribosomes[109], and in crystallographic studies with *D. radiodurans* 50S subunits[110], telithromycin has been shown to bind to region V, with its alkyl–aryl group interacting with an additional site in region II[110]. This additional interaction improves net ribosome binding tenfold over erythromycin and compensates for reduced binding to region V in wild-type and *erm*-methylated ribosomes[108]. There is evidence for species specificity in ketolide binding, as results of crystallographic studies with archaebacterium *Haloarcula marismortui* 50S subunits indicate that the alkyl–aryl group of telithromycin interacts with an additional site in region V, rather than a site in region II[111]. Telithromycin retains clinically useful activity against *mef* efflux, inducible and constitutive *erm* resistance in *S. pneumoniae*, and inducible *erm* resistance in *S. aureus*. Additionally, the methoxyl group at C-6 stabilizes telithromycin to acid and improves tolerability.

Structure-based design has been applied to the discovery of new agents which target the 23S and 16S rRNA[112]. So far, the compounds disclosed are based on pre-existing drugs, and are therefore included here. Novel synthetic aminoglycosides that target the neomycin-binding site on 16S rRNA have been described that are not subject to common inactivating enzymes[113,114], but no clinical candidate has yet emerged. Rib-X, a company that was founded to exploit the fine-structure mapping of 23S RNA for drug discovery, has synthesized new potent 'designer' oxazolidinones[112] that have extended contacts with the ribosome, one of which, RX-01-423 has been shown to be active against linezolid-resistant ribosomes[115] and has reached Phase I trials[116]. AstraZeneca has recently disclosed an isoxazolino oxazolidinone with greater potency than linezolid and activity against linezolid-resistant enterococcal isolates[117].

Addressing the single-target problem

If single targets are not optimum, what are the options? Several programmes have addressed this conundrum and four main avenues of investigation have been identified: single pharmacophore/multi-target compounds, hybrids of two pharmacophores, combinations of single-target inhibitors to avoid resistance development, and structure-based drug design to create multiple intramolecular drug–target interactions.

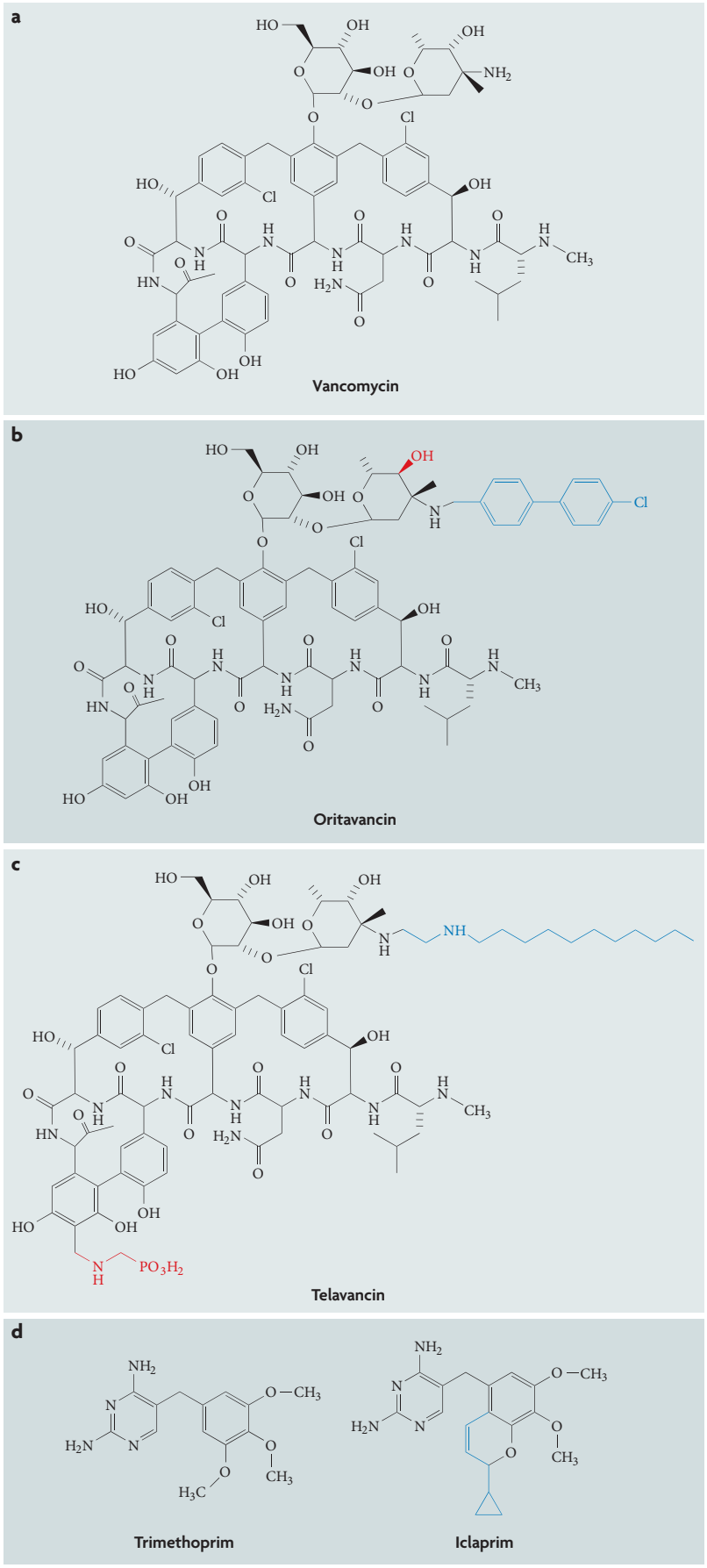

Figure 5 | **Hybrid and single-target agents overcoming resistance.** Blue structures are pharmacophores that make contacts with new binding sites. Other changes to these compounds are in red. **a** | Vancomycin. **b**,**c** | Glycopeptides with improved activity against vancomycin-resistant enterococci (VRE), created by adding a second mechanism (membrane permeabilization) to that of vancomycin (**a**). **d** | Iclaprim (right) overcomes resistance to trimethoprim (left).

Single pharmacophore, multiple targets. The dual targeting by fluoroquinolones of GyrA and ParC contributes to the slow increase of resistance to these compounds in the clinic. The natural products novobiocin, coumermycin and clorobiocin inhibit gyrase by blocking the ATPase activity of the B subunit of DNA gyrase (GyrB)[118–121]. GyrB mutant strains that are resistant to novobiocin, which has been developed for clinical use, arise at relatively high frequency *in vitro* (about 10^{-7} in *E. coli*[120] and *S. aureus*[122]) and the drug is ineffective for monotherapy[123]. Recent work has shown that the apparent inactivity of novobiocin in inhibiting the B subunit of topoisomerase IV (ParE) of *E. coli* can be attributed to a single residue[124]. In a sense, the native topoisomerase IV is intrinsically novobiocin resistant. Indeed, it was later shown that novobiocin resistance in *S. aureus* could be obtained by sequential mutation in *gyrB* followed (at much higher novobiocin concentration) by cognate mutations in *parE*, then *gyrB* — indicating that the primary target is indeed GyrB and that ParE is a secondary target[122]. Recently, a group at Vertex has presented data on aminobenzimidazole compounds that inhibit GyrB and ParE[125,126]. Optimization of the compounds to balance the degree of inhibition of both enzymes has proceeded and compounds with good antibacterial activity and reduced resistance development relative to novobiocin have been obtained.

Hydroxyphenylazo uracil (HPUra), an early inhibitor of the low-GC Gram-positive-specific DNA polymerase, PolC, was described in 1973 and acts as a dGTP analogue[127,128]. More potent HPUra-derived inhibitors, which retain specificity to bacterial over mammalian polymerases, have since been described by Microbiotix. For example, members of the ethyl methyl anilino uracils (EMAUs) have been identified that showed good bactericidal activity[129] with relatively low rates (10^{-8}–10^{-10}) of single-step high-level resistance shown under eightfold MIC selection conditions. These resistance mutations map in *polC*[130], and the compounds show some *in vivo* protection[131]. A related compound was described by Bayer that had slow resistance development in serial passage — probably as a result of single mutations mapping in *polC* — and modest *in vivo* efficacy[132]. Given that these have a single, mutable target, they might be predicted to yield to clinical resistance rapidly. Therefore, it is notable that substituted dichlorobenzyl guanines have been described with potent and equivalent activity against PolC and the other Gram-positive replicative DNA polymerase, DnaE[133]. Such inhibitors, if they have antibacterial activity, would be expected to have lower resistance potential.

Table 4 | **Antimycobacterial drugs**

Antimycobacterial drugs	Inhibited pathway (target)	Chromosomal resistance*
Mycobacterium tuberculosis		
Rifampicin	RNA transcription (RNA polymerase)	Single-step mutation in RpoB†
Isoniazid	Mycolic acid synthesis (InhA)	Single-step mutation in or upregulation of InhA or KasA†, or KatG required for activation (KatG mutants contain compensatory mutations upregulating AhpC)
Ethambutol	Arabinan synthesis, EmbB	Single-step mutation in EmbB†
Pyrazinamide	Nicotinamide antimetabolite	Single-step mutation in PncA (required for activation)
Aminoglycosides (streptomycin, kanamycin and amikacin)	Protein synthesis (30S ribosomal subunit)	Streptomycin: single mutations in RpsL† All: single mutations in 16S rRNA† Permeability changes
Basic cyclic peptides (capreomycin and viomycin)	Protein synthesis (30S and 50S ribosomal subunits)	Single-step mutation in TlaY, a mycobacterium-specific cytosine methylase of single nucleotides in 16S and 23S rRNA†
Ethionamide	Mycolic acid synthesis (InhA)	Single-step mutations in or upregulation of InhA† Loss of oxidative activation by the monooxidase EtaA
Fluoroquinolones	DNA replication (GyrA)	Single-step mutation in GyrA†
Para-amino salicylic acid	Folate synthesis	Single-step mutation in ThyA (bypass by suppression of folate-synthesis inhibition)†
Cycloserine	Cell-wall synthesis (Ddl and possibly Alr)	Possibility of Alr overproduction†
Non-tuberculus mycobacteria		
Clofazamine	Membrane disrupter	
Thiacetazone	Unknown	Loss of oxidative activation by EtaA
Macrolides (clarithromycin, azithromycin)	Protein synthesis (rRNA of 50S ribosome subunit)	Single-step mutation in 23S rRNA†
Dapsone	Folate synthesis (FolP)	Single mutations in FolP†

*All mechanisms are endogenous. †Mutations either in the target or that lead to bypass of the target. AhpC, alkyl hydroperoxide reductase; Alr, alanine racemase; Ddl, D-alanylalanine synthetase; EmbB, arabinosyl transferase; FolP, dihydropteroate synthase; GyrA, gyrase A subunit; InhA, enoyl-ACP reductase; KasA, 3-oxoacyl-(acyl carrier protein) synthase; KatG, catalase-peroxidase-peroxynitritase T; PncA, pyrizinamidase; RpoB, RNA polymerase B subunit; RpsL, 30S ribosomal protein S12; ThyA, thymidylate synthase; TlaY, cytosine methylase.

Hybrid molecules. The concept of synthesizing compounds that are hybrids of existing marketed antibacterials or their analogues to broaden the spectrum, address resistance and improve overall pharmacokinetics and toxicity was initiated by Roche with β-lactam-fluoroquinolone molecules[134]. *In vitro* studies on one of these compounds, Ro 23-9424, demonstrated its activity on bacteria that are resistant to either or both of the components[135]. Although its spectrum was reasonably broad[136], its potency was compromised in some species, notably *P. aeruginosa*[136]. When Ro 23-9424 was evaluated in a human Phase I trial, it showed a relatively short half-life, which probably resulted from the chemical and enzymatic instability of the ester linkage between the cephalosporin and quinolone moieties (J. L. Pace, personal communication).

The hybrid approach has been continued by several companies, focusing foremost on the possibility of overcoming resistance to the components. The oxazolidinone/fluoroquinolone compounds discovered by Morphochem (now Biovertis) are active against bacterial strains that are resistant to either linezolid or ciprofloxacin or both[137,138]. Cumbre Pharmaceuticals has applied for patents on hybrids of rifamycin and fluoroquinolones that are highly active on rifampin-resistant and fluoroquinolone-resistant strains[139,140]. Recently, hybrids of the PolC-targeting EMAUs and a fluoroquinolone were disclosed by GL Synthesis. Some of these have activity comparable to that of oxazolidinones against a wide spectrum of Gram-positive organisms, including fluoroquinolone-resistant strains[141].

The explicit intention of Theravance is the creation of compounds which bind to numerous binding sites on the same or multiple targets — 'multivalency', in their parlance. Among early Theravance programmes are multivalent protein-synthesis inhibitors[142], and hybrids of a glycopeptide and a cephalosporin[143], one of which, TD-1792, is now in Phase I[144]. The most

advanced programme, which is in Phase III, is the development of telavancin (FIG. 5c), a derivative of vancomycin that retains activity against VRE and adds a new mode of action — membrane depolarization and permeabilization — to the glycopeptide core. Membrane permeabilization in *S. aureus* is antagonized by Lys-D-Ala-D-Ala, which suggests that the membrane activity might be mediated by its interaction with Lipid II rather than with the phospholipid bilayer[145], possibly explaining telavancin's apparent specificity for bacteria. Targanta's oritavancin (FIG. 5b), a glycopeptide derivative that is in Phase III trials at present, also adds membrane permeabilization to the glycopeptide-mediated mechanism of cell-wall-synthesis inhibition, and is active against VRE[146].

Combinations to overcome resistance. Combination therapy with single-target inhibitors is standard therapy against HIV, *Helicobacter pylori* and *M. tuberculosis* (MTB). For standard bacterial pathogens, it might be less acceptable to develop a combination of two single agents, neither of which is optimal as a single agent due to resistance development. However, fixed combinations, such as trimethoprim-sulphamethoxazole and Synercid (dalfopristin/quinupristin; Monarch Pharmaceuticals), which demonstrate synergy between the components, are accepted as standard antibacterial therapy. Recently, Replidyne announced its intention of developing, for topical use, a combination of their new Met-tRNA synthetase inhibitor, REP8839, with the generic Ile-tRNA synthetase inhibitor, mupirocin[147]. Although it is not a systemic application, this would be a combination that is designed purely with the aim of overcoming resistance development.

Combinations are always used in MTB therapy, both to overcome resistance development and to target pathogen subpopulations in differing states or locales. The drugs used to treat MTB (TABLE 4) are susceptible to resistance through single-step mutation in the target[148,149], bypassing the target[150], or mutations in functions that are required for drug activation[148,149,151–153]. Most of the targets, where known, are single enzymes. MTB is not susceptible to the early antibiotic therapies for standard pathogens, such as penicillin and sulphonamides, and resistance arose rapidly to ribosomally targeted agents, as MTB has only one rRNA operon. Once other agents were discovered (para-aminosalicylic acid, isoniazid, pyrazinamide, rifampin and ethambutol, all of which are subject to single-step resistance) combination therapy became the norm[154]. The newer fluoroquinolones are active against MTB, but they too have a single target (DNA gyrase) as MTB lacks topoisomerase IV[155].

With the challenge of multi-drug-resistance in MTB, aggressive drug-discovery efforts have resulted in new entities in the pipeline[156]. The situation with MTB (as well as HIV and *H. pylori*), for which combination therapy is the norm, might presage the future of therapy for other pathogens if the standard multi-targeted monotherapies fall to resistance. In the intensive care unit, empiric therapy (treatment before the pathogen is identified) using two or several drugs is often used to cover the spectrum of possible aetiologies. There are few positive clinical data or controlled trials to support the use of combinations of resistance-prone agents to avoid resistance development in common pathogens[157], but further clinical trials have been recommended[157,158]. Development of fixed combinations of novel single-target agents (to be discovered) to prevent or overcome resistance in standard pathogens might be in our future.

Single-target improvement by design. The power of rational drug design is illustrated in the case of iclaprim, a second-generation dihydrofolate reductase inhibitor that is active against a trimethoprim-resistant target enzyme, and which is subject to particularly slow resistance development from wild-type (no change after 17 passages) or trimethoprim-resistant enzymes[106,159]. In addition to increasing the affinity to the trimethoprim binding site, iclaprim (FIG. 5b) forms hydrogen bonds with two residues in the target to which trimethoprim does not bind, and therefore overcomes the alteration that reduces trimethoprim activity[159]. A parenteral formulation of iclaprim is in Phase III clinical development[106]. So, the resistance potential of single-targeted agents might be lowered by increasing drug–enzyme interactions, thereby adding new intramolecular targets, as has been the case for the improved generations of multi-targeted agents.

Conclusions

A distinguishing quality of the existing targets of monotherapeutic antibacterials is their low potential for rapid endogenous resistance development, which seems to be based on their capacity to act at multiple targets. These compounds bind to sites for which the structure is determined by more than one gene, such that single-step high-level resistance should not occur. Improvements on these agents to overcome pre-existing resistance have been attempted by the addition of pharmacophores which interact with additional intramolecular or (in the case of hybrids) intermolecular binding sites. Inhibitors of unexploited multiple targets are under study, and combinations of single-target inhibitors might be developed specifically to overcome resistance. So, although much of antibacterial drug discovery has lately been directed towards novel targets, there has been significant ongoing work explicitly addressing the underlying problem of 'overnight evolution' of endogenous resistance. Although not covered in this review, structure-guided drug design has great potential. Iterative rounds of structure-based drug design that are directed towards overcoming potential endogenous resistance might well be successful[10,160]. As such heavily engineered drug-target interactions might narrow the potential bacterial spectrum, targets must be selected that have active sites which are highly conserved among bacteria. The key to overcoming endogenous resistance potential, then, is ensuring multiple drug–target interactions, be they intra- or intermolecular.

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CORRIGENDA

Multi-targeting by monotherapeutic antibacterials

Lynn L. Silver

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On page 48, the sentence "MprF mutants fail to add lysine to membrane phosphatidylglycerol, presumably decreasing the negative surface charge of the membrane[90]." should read "MprF mutants fail to add lysine to membrane phosphatidylglycerol, presumably decreasing the postive surface charge of the membrane[90]."