

REVIEW ARTICLE

Prospects for new antibiotics: a molecule-centered perspective

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There is a continuous need for iterative cycles of antibiotic discovery and development to deal with the selection of resistant pathogens that emerge as therapeutic application of an antibiotic becomes widespread. A short golden age of antibiotic discovery from nature followed by a subsequent golden half century of medicinal chemistry optimization of existing molecular scaffolds emphasizes the need for new antibiotic molecular frameworks. We bring a molecule-centered perspective to the questions of where will new scaffolds come from, when will chemogenetic approaches yield useful new antibiotics and what existing bacterial targets merit contemporary re-examination.

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A PERSONAL PATHWAY TO ANTIBIOTICS RESEARCH

For one of us (CTW), a career-long interest in antibiotics¹ was spurred by discussions on the mechanism of action of D-fluoroalanine^{2,3} during a seminar visit, as a second year assistant professor, at Merck in 1974. This visit led to examination of the mechanism of action of mono-, di- and trifluoroalanines as mechanism-based inactivators for the bacterial pyridoxal phosphate-dependent alanine racemase, the first step in assembly of the muramyl pentapeptide of the bacterial peptidoglycan layer.^{4–7} In turn, that led to analysis of other muramyl peptide biosynthetic enzymes, including MurA^{8–11} (the target of fosfomycin) and MurB,^{12–18} which together build the lactyl ether moiety of UDP muramic acid. We progressed on to the enzyme MurF,¹⁹ the D-Ala-D-Ala ligase that adds this D-D-dipeptide to UDP-muramyl tripeptide, to yield UDP-muramyl pentapeptide as the last step in the cytoplasmic phase of peptidoglycan assembly.^{20,21}

A second phase of interest was initiated when CTW was the CEO of the Dana Farber Cancer Institute in the early 1990s at a time when vancomycin was being used as the antibiotic of last resort for treatment of life-threatening Gram-positive bacterial infections of patients undergoing cycles of chemotherapy. The molecular mechanism of resistance of pathogenic vancomycin-resistant enterococci (VRE) turned out to be replacement of the D-Ala-D-Ala termini of muramyl pentapeptide intermediates with D-Ala-D-Lactate, which had 1000-fold lower affinity to vancomycin.²² We established the VanA enzyme in VRE was not the classic D-Ala-D-Ala ligase but a D-Ala-D-Lac ligase^{22–26} and thus determined the molecular basis of drug resistance in VRE.^{27–29}

The rise of VRE through the 1990s focused our attention on how natural product antibiotic scaffolds such as those of vancomycin and the related teicoplanin were assembled,^{29,30} in part to decipher the

chemical logic and molecular machinery and, in part, with the hope that one might learn to reprogram natural antibiotic assembly lines to engineer improved molecular variants.

We have subsequently deciphered many of the rules for nonribosomal peptide (NRP) synthetase assembly lines, including post-translational priming of carrier protein domains by phosphopantetheinylation, and the nature of the chain initiation, elongation and termination steps.^{30–35} These efforts have allowed us and many other groups to undertake full reconstitution of NRP synthetase assembly lines, especially in siderophore biosynthesis.³⁶ From our extensive work on characterization of natural product biosynthesis catalytic machinery and molecular logic, we have had a continued interest in new natural products and the underlying chemistry of molecular scaffold assembly.³⁷

ANTIBIOTICS AS A SPECIAL CLASS OF THERAPEUTICS

One could argue that antibiotics represent a special class of therapeutic agents whose misuse affects not just the individual patient but the broader community. This is due to the almost inevitable selection for antibiotic-resistant bacteria that arise in clinically significant waves at some point after widespread introduction and use of a new antibiotic (both in veterinary and human populations). As Table 1 (modified from a review by Palumbi)³⁸ shows, resistance is inevitable.^{39–45} It is not a question of *if* but rather a question of *when*. Much effort in recent years has gone into definition of the resistome,^{46,47} the collection of antibiotic-resistant genomes harbored in both bacterial pathogens and in environmental bacterial populations. The origins of vancomycin resistance, for example, derive from transfer of *vanHAX* genes from intrinsically resistant soil microbes such as lactobacilli and leuconostoc strains to enterococci where they often reside on transposable elements.^{46,48,49}

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The depth of the resistome reservoir was powerfully demonstrated in the findings of D'Costa *et al.*⁵⁰ in 2006 showing that collections of soil actinomycetes had, on average, resistance genes to 7 antibiotics while some isolates were resistant to 15 drugs.

Because of the mobilization of resistome genes into opportunistic and professional bacterial pathogens, there is always a need for the next generation of antibiotics. There are parallels to antiviral and anticancer therapeutics where the rapid proliferation and the large number of viruses and tumor cells in a patient can mirror the rapid growth and titer of bacterial pathogens. Mutations rates of 10^{-6} per cell division are problematic in all the three therapeutic arenas. We shall return later to a distinction in therapeutic regimens: in antiviral and anticancer drug regimens, combination therapy is the norm while for bacterial infections monotherapy has been the default historical prescribing pattern.

Gram-positive and Gram-negative pathogens present overlapping but distinct target profiles. While Gram-positive pathogens such as VRE and methicillin-resistant *Staphylococcus aureus* (MRSA) have rightly drawn attention over the past two decades, the Gram-negatives, underlined, in the list of ESKAPE pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* species), are in

Table 1 Evolution of resistance to clinical antibiotics

Antibiotic	Year deployed	Clinical resistance observed ^a	Ref.
Sulfonamides	1930s	1940s	38
Penicillin	1943	1946	38
Streptomycin	1943	1959	38
Chloramphenicol	1947	1959	38
Tetracycline	1948	1953	38
Erythromycin	1952	1988	38
Vancomycin	1956	1988	38
Methicillin	1960	1961	38
Ampicillin	1961	1973	38
Cephalosporins	1960s	Late 1960s	38
Nalidixic acid	1962	1962	39
Fluoroquinolones	1980s	1980s	40
Linezolid ^b	1999	1999	41
Daptomycin ^b	2003	2003	42
Retapamulin ^{b,c,d}	2007	2007	43
Fidaxomicin	2011	2011	44
Bedaquiline ^{b,e}	2013	?	45

^aThis table was modified from a previous report by Palumbi.³⁸

^bRepresents a first-in-class drug for human use.

^cApproved for topical use only.

^dResistance to retapamulin was observed in clinical isolates of *S. aureus* without previous exposure to pleuromutilins, but no case of resistance development during retapamulin therapy was found in the literature.⁴³

^eApproved only for use in combination therapy for treatment of MDR TB.

many cases more of a pressing treatment challenge.⁵¹ The approvals of synergicid and daptomycin were largely in response to VRE and MRSA challenges^{52,53} but there has not been a new antibiotic scaffold for Gram-negative pathogens in decades. As a consequence polymyxins (for example, colistin) have become front-line therapy in recent years,^{54,55} even though they were eschewed as too toxic when discovered 40 years ago. Multidrug-resistant tuberculosis pose their own crises for therapy around the world but, finally, promising new molecules (for example, benzothiazinone BTZ043⁵⁶⁻⁶¹ in late preclinical GLP/Tox funded by the European Commission, sponsored by New Medicines for Tuberculosis, and in development by Alere in Germany; nitroimidazole PA-824^{62,63} in Phase II sponsored by the TB Alliance; ethylenediamine SQ109⁶⁴ in Phase II sponsored by Sequella Inc and OOO Infectex; and Sutezolid⁶⁵ (also known as PNU-100480) a thioxazolidinone in Phase II under Pfizer) are progressing through late stage clinical trials or have recently been approved by the Food and Drug Administration (FDA; bedaquiline,⁴⁵ a diarylquinoline inhibitor of ATP-synthase developed by J&J).^{66,67}

This essay is not encyclopedic and doubtless has important omissions about promising molecules in both discovery and development phases. Rather, the choices reflect the particular perspective of the authors about emerging science that seems intriguing and worthy of follow up. In consideration of the underexploited targets noted below, there is a chemocentric set of prejudices: that the availability of promising chemical matter, from Nature or from initial screening campaigns is an important precondition for finding hits, developing them into leads and conducting lead optimization for compounds to be selected as development candidates. Figure 1 is a variant of a typical drug discovery/development flow chart adapted for antibiotics;⁶⁸ the low success rate emphasizes the importance of starting with molecular frameworks that can stand up to the rigors of development. The focus of the remarks in this chapter are all on discovery, not development.

A PRESSING NEED FOR NEW ANTIBIOTIC SCAFFOLDS

Over the past century of antibiotic discovery and development, two parallel and independent lines of discovery have been fruitful. One route has been the identification of antimicrobial chemical weaponry in Nature as small-molecule natural products were observed to have clinically useful antibacterial activity. Among these scaffolds were the penicillins and cephalosporins, the macrolides such as erythromycin, the glycopeptides exemplified by vancomycin and teicoplanin, the tetracyclines and the aminoglycosides. A separate track is represented by the discovery that aromatic sulfa scaffolds originally from the chemical dyes industry frameworks had antibiotic activity.⁶⁹ The resultant sulfa drugs have been in continuous use in one incarnation or another for 80 years. The second example of man-made 'magic bullets' as widely used antibiotics were the fluoroquinolones, first

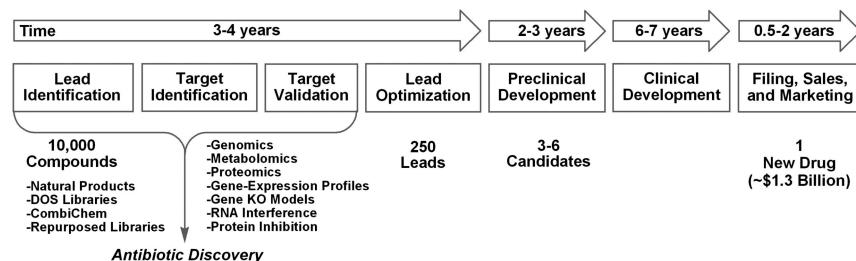


Figure 1 Antibiotic drug discovery and development flow chart adapted from literature versions.⁶⁸

introduced clinically in the 1960s. A third distinct synthetically derived scaffold is represented by the first-generation oxazolidinone linezolid approved in the US in 2000.

The ‘golden age’ of discovery of natural antibiotics of clinical significance was actually a short two decades (Figure 2), between 1940 and 1960. One could argue that the ensuing 50 years has been a ‘golden age’ of antibiotic medicinal chemistry where chemical tailoring of the periphery of major antibiotic classes, while leaving the core intact, has been successful in dealing with successive waves of resistant bacterial pathogens. As illustrated in Figure 3, successive generations of cephalosporins, fluoroquinolones, macrolides and tetracyclines have been developed and commercialized with significant usage. In the tetracycline arena, new synthetic methodologies

have enabled synthesis of pentacyclines,⁷⁰ which may become a fourth generation of this antibiotic class. Analogously, while the informal designation of generations of an antibiotic class are somewhat in the eye of the beholder, one could argue that molecules such as prulifloxacin⁷¹ represent a fifth generation of fluoroquinolones. It is unlikely that medicinal chemistry modifications of these four antibiotic classes can go indefinitely. That prediction argues for a pressing need for new antibiotic scaffolds, either from natural products or from the many strands of modern synthetic chemistries. In the short term, it is understandable that antibiotic development groups and companies choose to reduce risk by working on known scaffolds and known targets, but in the longer term, new molecules and new targets will need to be identified⁷² as bacterial

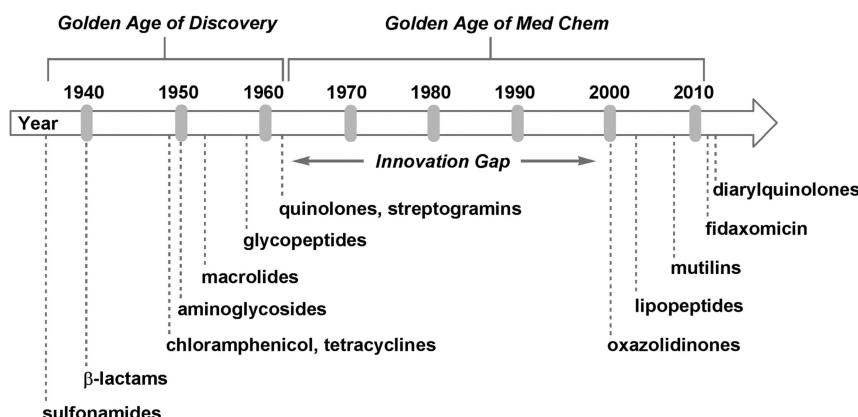


Figure 2 Timeline showing the ‘Golden Age’ of antibiotic discovery (1940–1960) and the ‘Golden Age’ of antibiotic medicinal chemistry (from 1960 to present). No new structural classes of antibiotics were introduced between 1962 and 2000, representing a serious innovation gap during the genomic era.

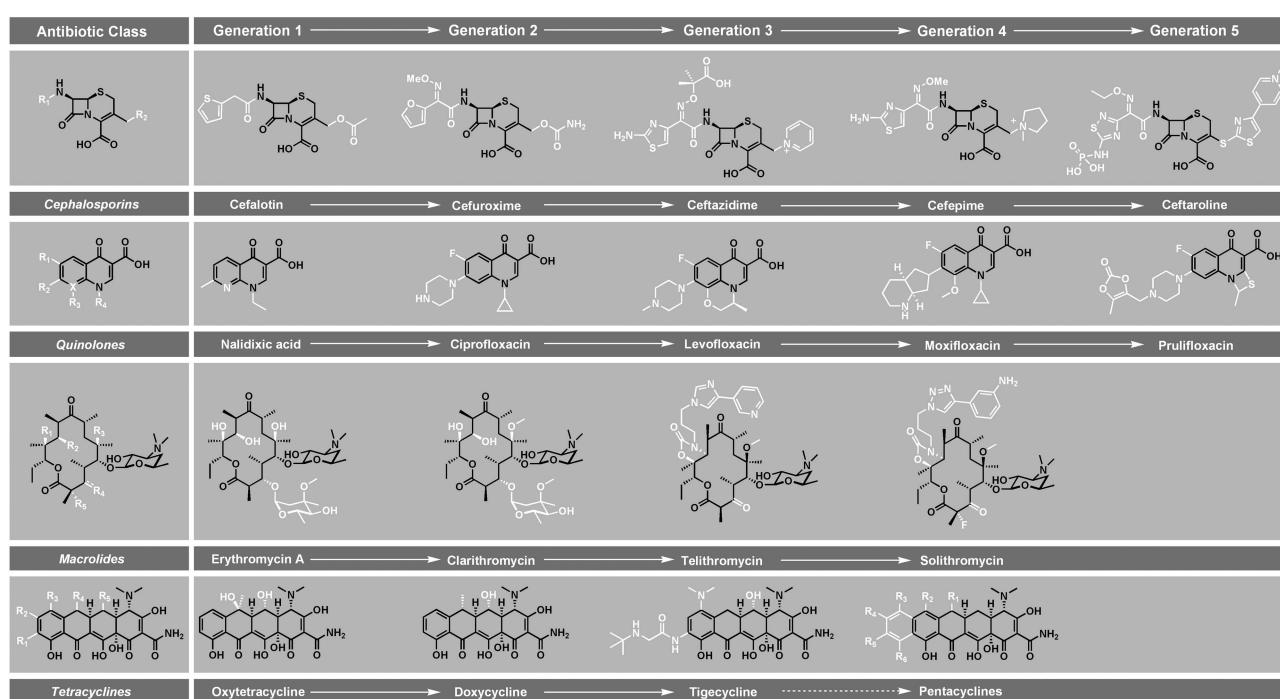


Figure 3 Synthetic tailoring of antibiotic core structures leading to successive generations of antibiotic classes has been the mainstay of antibiotic drug development for the past 50 years. New antibiotic core scaffolds are desperately needed. Core scaffolds are shown in black and peripheral chemical derivatizations are shown in white. The quinolone/fluoroquinolone scaffolds are of synthetic origin, while all other scaffolds are natural products or their semisynthetic derivatives.

pathogens become multidrug resistant as exemplified by some of the ESKAPE pathogens.⁵¹

A 2011 survey of 21 antibiotics launched since 2000⁷³ revealed that the two lines of discovery and development, natural products vs synthetic chemicals, are still operant with two new natural product classes, represented by daptomycin as a stem loop lipopeptide and retapamulin as a member of the terpenoid pleuromutilins. Of the nine antibiotics of synthetic origin launched in the past 12 years, other than the oxazolidinone linezolid, the other eight are fluoroquinolones, stressing the need for scaffold diversification away from such over-reliance on the F-quinolone framework. In that same 2011 survey of five antibiotic candidates in phase III trials, the one synthetic molecule was a second-generation oxazolidinone torezolid phosphate.^{74–76} Another second-generation oxazolidinone, radezolid, is progressing through late stage trials.^{77–80}

WHERE WILL NEW SCAFFOLDS COME FROM

The two historical lines of discovery, synthetic chemical efforts vs isolation of new natural product scaffolds are both still in play. Two contemporary lines of chemical efforts are high throughput screening of chemical libraries and target structure-guided efforts.

A comprehensive screening effort against genetically validated essential targets, on the part of many companies, most notably detailed by a GSK group in 2007,⁸¹ effectively failed to find new chemical matter worthy of development. This has properly dampened enthusiasm for continued screening campaigns. On the other hand, the development of focused libraries, more suited for specific target classes, could hold more promise. A Pfizer group repurposed a library built for eukaryotic kinase projects and identified hits and optimized leads against the bacterial form of the ATP-dependent biotin carboxylase, which generates malonyl CoA from acetyl CoA at the start of fatty acid biosynthesis.⁸²

Chemical libraries that mimic the scaffold complexity of natural products in three dimensional architectures, regiochemical constraints, multiple stereogenic centers and high density of functional groups have not been available over the first two decades of combichem efforts. Such libraries, even relatively small and focused around particular scaffolds, may be requisite for screening efforts to increase in success rates against bacterial targets.^{83–86}

A recent report of a structure-guided antibiotic discovery effort comes from an Astra Zeneca team on thymidylate kinase inhibition. Reasoning that this kinase lies at the intersection of both salvage and *de novo* formation of dTMP, essential for DNA synthesis, Keating *et al.*^{87,88} designed scaffolds to bind to the dTMP subsite rather than the ATP subsite, using the X-ray structure of the enzyme as guide. TK-666 (Figure 4a) represents a thymine-sugar mimic with fragment coupling to two additional rings. The molecule is rapidly bacteriocidal against Gram-positives such as *S. aureus*, with MIC of $0.25 \mu\text{g ml}^{-1}$ and a K_i against pure *S. aureus* dTMP kinase of 0.33 nM . Inhibition of thymidylate kinase from a variety of pathogenic microbes is being pursued by several groups using modified thymidine scaffolds and mimetics.^{89–93}

The parallel search for new scaffolds in Nature bears fruit when new assays are developed, new cultivation methods established^{94–96} and new niches explored.^{97,98} As will be noted in a subsequent section, the platensin and platensimycin scaffolds (Figure 4b) were discovered^{99,100} in antisense screens that made FabF the rate-limiting step in *S. aureus* lipid biosynthesis and made bacterial growth sensitive to inhibition of this step. The initial enthusiasm over these potent Fab enzyme inhibitors has been partially muted by the observation that some bacteria can scavenge host lipids during infection.¹⁰¹ With a collection of 245 *S. aureus* strains similarly manipulated to lower the bacterial cell levels of encoded enzymes, a Merck group¹⁰² reported the isolation of kibdelomycin (Figure 4c) as a DNA gyrase subunit A inhibitor, the first new natural product scaffold against gyrase in the past several decades. This antisense approach is discussed further in a subsequent section.

Exploration of new niches has traditionally yielded new molecular frameworks. In this vein, isolation of microbes from the abyss in the Sea of Japan led to the novel isoprenoid abyssomycins, which inhibit *p*-aminobenzoate biosynthesis in the folate biosynthetic pathway¹⁰³ (Figure 4d). The genome sequences of several thousand bacteria reveal up to two dozen silent clusters for natural product biosynthesis, typically polyketide (PK), NRP and hybrid PK-NRP frameworks.^{104–110} Fungi similarly harbor multiple biosynthetic gene clusters that are cryptic under normal laboratory culture conditions.¹¹¹ A variety of approaches have been used to turn on silent biosynthetic gene clusters to evaluate the novelty and activity of the resultant small molecules.^{111–113}

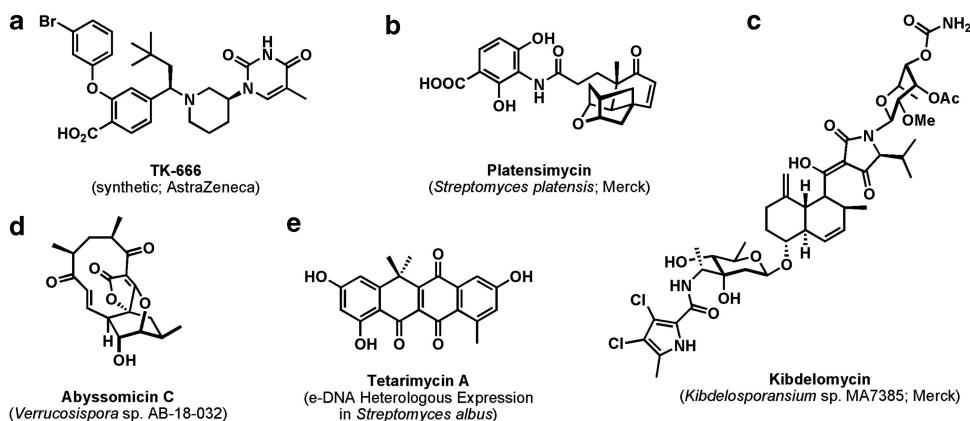


Figure 4 (a) Structure of TK-666, a potent synthetic inhibitor of bacterial thymidylate kinase developed by researchers at AstraZeneca. (b) Structure of platensimycin, a potent inhibitor of bacterial β -ketoacyl-ACP synthase type I/II (FabF/B) isolated from *Streptomyces platensis* by a group at Merck. (c) Structure of kibdelomycin, an inhibitor of DNA gyrase subunit A isolated from *Kibdelosporangium* sp. MA7385 by a group at Merck. (d) Structure of abyssomicin C, an isoprenoid inhibitor of *p*-aminobenzoate biosynthesis in the folate biosynthetic pathway. (e) Structure of tetramycin A, an anti-MRSA tetracyclic molecule discovered through heterologous expression of a gene cluster found in environmental DNA.

Progress also continues in examination of the genomes of uncultivated microbes through induced expression of environmental DNA gene clusters.^{96,98,114} One recent example is the isolation of tetracycline A (Figure 4e), a tetracyclic molecule active against MRSA. This was accomplished by insertion of e-DNA containing the PK gene cluster along with a regulatory protein promoter, into the generic host *Streptomyces albus* under the control of a constitutive erythromycin-resistance promoter.¹¹⁵

CHALLENGES OF A TARGET-POOR THERAPEUTIC AREA

Historically, the treatment of human bacterial infections by antibiotics has been a target-poor therapeutic arena. Despite thousands of molecules shown to have antibiotic properties, there are essentially five major clinically validated antibacterial targets/pathways.¹ As schematized in Figure 5, these include bacterial peptidoglycan/cell wall biosynthesis, targeted by both the β -lactams and the vancomycin-type glycopeptides. A second set of targets is encompassed by bacterial protein synthesis, with most antibiotics targeting the ribosome. Daniel Wilson has provided an elegant, structurally oriented compendium of the A–Z of molecules targeting the small and large ribosomal subunits and the interface between them.¹¹⁶ A third target area is the blockade of DNA replication and transcription to RNA, most notably the targeting of DNA gyrase and RNA polymerase. The fourth is the folate biosynthetic pathway, which provides the one carbon unit required for the deoxythymidylate building block for DNA synthesis. The sulfa drugs and the folate analogs, used in combination, block two sequential steps in that biosynthetic pathway.¹¹⁷ The fifth and perhaps the most recent target of widespread clinical utility has been daptomycin disruption of bacterial membrane integrity.^{118,119}

WHEN WILL CHEMOGENETIC APPROACHES PAY OFF?

Over the decades since the 1960s, the antibiotics research community has sought to broaden the number of clinically validated targets, using many rational as well as screening approaches. A particularly concerted effort went on with the advent of genome sequences of the most common bacterial pathogens and the determination of essential genes. Their protein products were intensively examined in a first wave of screens in the chemogenetics era. The hallmark paper from the GSK group in 2007⁸¹ noted above, summarizing more than 100 screens, starkly described the failure of that first wave. Among the resultant questions were the utility of *in vitro* screening of a target vs whole-cell assays and also the utility of chemical library collections in pharmaceutical companies for screening prokaryotic rather than the eukaryotic targets, for which many of those libraries were biased.

SMART SCREENS: LEADS FOR COMBINATION THERAPIES?

Among the smart screens to be employed in recent years are a series of publications^{102,120–123} from the Merck groups on a 245 siRNA collection to lower the levels of essential gene expressions in MRSA, one at a time. These siRNAs may thereby render that particular step in a pathway sufficiently rate determining to give a growth impairment signal when inhibited by a small molecule. Screening of such strains with these and related gene knock-down collections in other pathogens has turned up new scaffolds from both natural and synthetic sources. This approach led to discovery of the platensin and platensimycin (Figure 4b) novel terpenoid architectural frameworks that target fatty acid biosynthesis,^{96,99} although their clinical utility has been questioned.¹⁰¹ This approach also turned up kibdelomycin (Figure 4c) as the first new natural product scaffold for gyrase inhibition in 60 years.¹⁰² The coumarin antibiotics (which are not

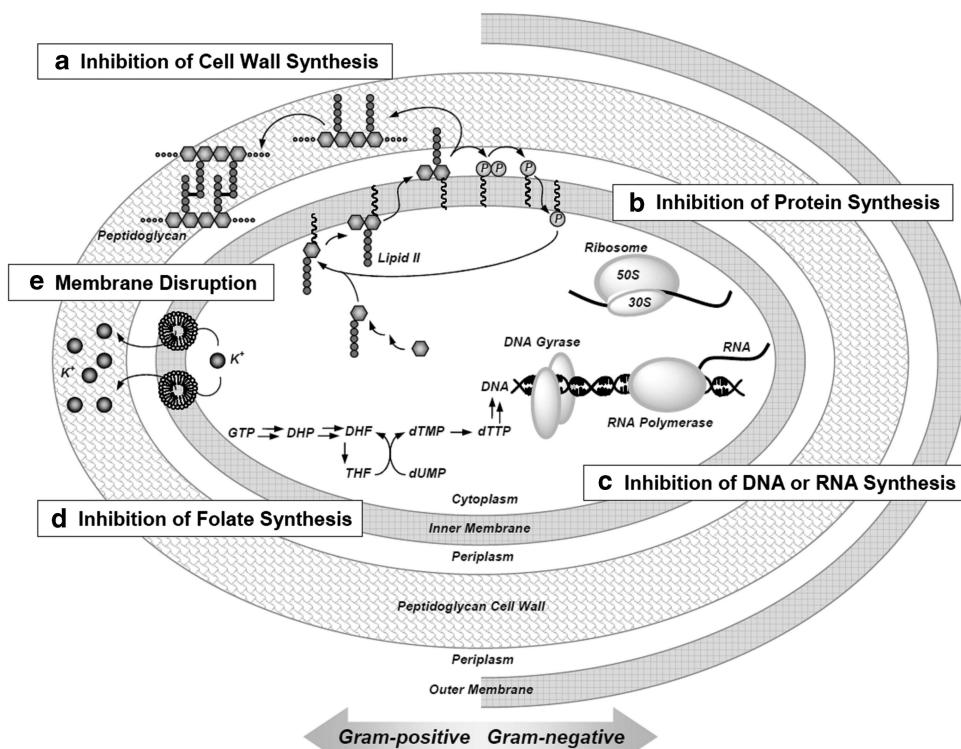


Figure 5 The five major clinically validated antibacterial targets/pathways. (a) Inhibition of cell wall biosynthesis. (b) Inhibition of protein synthesis. (c) Inhibition of DNA or RNA synthesis. (d) Inhibition of folate biosynthesis. (e) Disruption of membrane integrity. Modified from the cover of Walsh.¹

used in human bacterial infections) target the ATPase domain in the GyrB subunit of DNA gyrase.¹ Although structurally related to the coumarin antibiotics, kibdelomycin shows no cross resistance indicating that it targets a distinct subsite of DNA gyrase, possibly within the GyrA subunit or the catalytic active site of GyrB.¹⁰² The kibdelomycin scaffold is forbiddingly complex for structure-activity relationship (SAR) work, but there is the eribulin anticancer drug precedent^{86,124} to show that a complex (32 step) synthesis can be commercially viable.

The Merck 245 siRNA collection targeting MRSA screens may also be useful in finding rational choices for new combination therapies.^{122,123} Interactome analysis in such collections challenged with different subsets of β -lactam antibiotics turned up molecules that resensitize MRSA to carbapenems. A 256-fold gain in sensitivity to imipenem was seen in the presence of a small-molecule inhibitor of glucosamine synthase.¹²¹ For example, L-norvaline-N³-(4-methoxyfumaroyl)-L-2,3-diaminopropanoic acid (Nva-FMDP) is a mimetic of the natural product dapdiamide, which captures the active site Cys thiolate of the glutaminase domain of glucosamine synthase.^{125,126} Deprivation of glucosamine units required for peptidoglycan assembly generates a defective cell wall that shows synergistic susceptibility to imipenem. A distinct but parallel approach to resensitize MRSA to β -lactam antibiotics is conferred by tunicamycin, which blocks wall teichoic acid biosynthesis.¹²⁷ A third example involved a screen for inhibitors of lipid II flippase, with a synthetic library.¹²² Again, a molecule was detected that was read out as a regain of sensitivity to carbapenems.

Inter alia, these results, with both natural product and synthetic chemical libraries, bring into high relief the whole issue of default monotherapy for treatment of bacterial infections vs combinations of antibiotics. These sensitization-screening approaches may offer good algorithms for choice of the combinations and might be extended; for example, to molecules that could combine with colistin for treating MDR Gram-negative pathogens. Although consecutive deployment of antibiotics one at a time to treat persistent bacterial infections has been a long held approach, there are multiple examples reflecting combination therapy with antibiotics that may presage a future where combinations are applied simultaneously, with real-time knowledge of the pathogen genotypes as a guide. Those combinations include β -lactams and β -lactamase inhibitors such as augmentin (amoxicillin and clavulanate), sulfa drugs plus trimethoprim to block two consecutive steps in folate biosynthesis and the standard drug cocktails used in front-line tuberculosis therapy which include rifampin, isoniazid, pyrazinamide and ethambutol.¹

Second-generation combinations of β -lactam and β -lactamase inhibitor are progressing through clinical trials. The lactamase inhibitors are not clavulanate or the penicillin sulfones used in the first- and second-generation combinations but rather bicyclic

diazabicyclooctanes (Figure 6) in which the warhead is not the traditional four membered β -lactam but instead a five member γ -lactam bearing an O-sulfate substituent on the nitrogen¹²⁸ in the clinical candidates avibactam and MK-7655. Studies on avibactam with purified lactamase show that γ -lactam is opened by the active site Ser-OH to yield a variant of the typical O-acyl-lactamase covalent intermediate.¹²⁹ Over time, the lactam ring can reform and induce deacylation and regain lactamase activity, but the residence time suffices for either ceftaroline or ceftazidime, cephalosporins administered in combination with avibactam to reach their penicillin-binding proteins and function as killing antibiotics. Merck's MK-7655 is being clinically evaluated in combination with the carbapenem imipenem.¹²⁸

SHOULD SOME UNDEREXPLORED TARGETS BE REVISITED?

Peptidoglycan biosynthesis

In peptidoglycan assembly, the nascent disaccharyl pentapeptide units are incorporated into existing cell wall by two kinds of enzymes, the PG transpeptidases (TPases), long the targets of clinically useful β -lactam and glycopeptides antibiotics, and the PG transglycosylases (TGases; Figure 7a). The transpeptidases are robust targets, in part, because their active sites are accessible from the periplasmic face in Gram-negative membranes and from the outside environment in Gram-positives. Bifunctional versions of the TPases and TGases are common killing targets in many bacteria, where inhibition of the TPase leaves mechanically weakened cell wall layers.¹

Although β -lactam antibiotics have been in use for 70 years, by contrast, no TGase inhibitors, which would have the same outcome in accumulation of uncrosslinked peptidoglycan units, are in human clinical use. Nature has turned up one TGase-targeting scaffold in the glycolipid moenomycins, metabolites from streptomycetes. Moenomycins are structurally complex natural products with five sugars and an isoprenoid C25 lipid tail connected by a glycerol phosphate linker.¹³⁰ They are used in veterinary medicine but have poor pharmacokinetic properties, which curtail their use in humans.¹³⁰ Nonetheless, recent advances of note are the determination of the X-ray structure of the PG transglycosylase, now from three different research groups^{131–136} working with three distinct organisms (*E. coli*, *S. aureus*, *A. aeolius*), some with moenomycin bound^{131,134–136} (Figure 7b). This structural information along with advances in chemical synthesis,¹³⁷ biosynthesis¹³⁸ and assay development¹³⁹ should enable structure-driven optimization/simplification of the moenomycin scaffold (by synthesis and/or by pathway engineering) to get to a minimal structural core with potential amelioration of physical and pharmacodynamic properties that would move a moenomycin framework from veterinary to human indications.

Generations:	1: Clavulanates	2: Penicillin Sulfones		3: Diazabicyclooctanes (DBOs)		
β -Lactamase Inhibitors						
	Clavulanate	Sulbactam	Tazobactam	Avibactam	MK-7655	NXL105
Development Status	Approved (Augmentin) GlaxoSmithKline	Approved (Cefobid) Pfizer	Approved (Zosyn) Pfizer	Phase II/III AstraZeneca/Forest	Phase I/II Merck	Preclinical AstraZeneca

Figure 6 Next generation 'non- β -lactam' β -lactamase inhibitors (diazabicyclooctanes; DBOs) in clinical development.

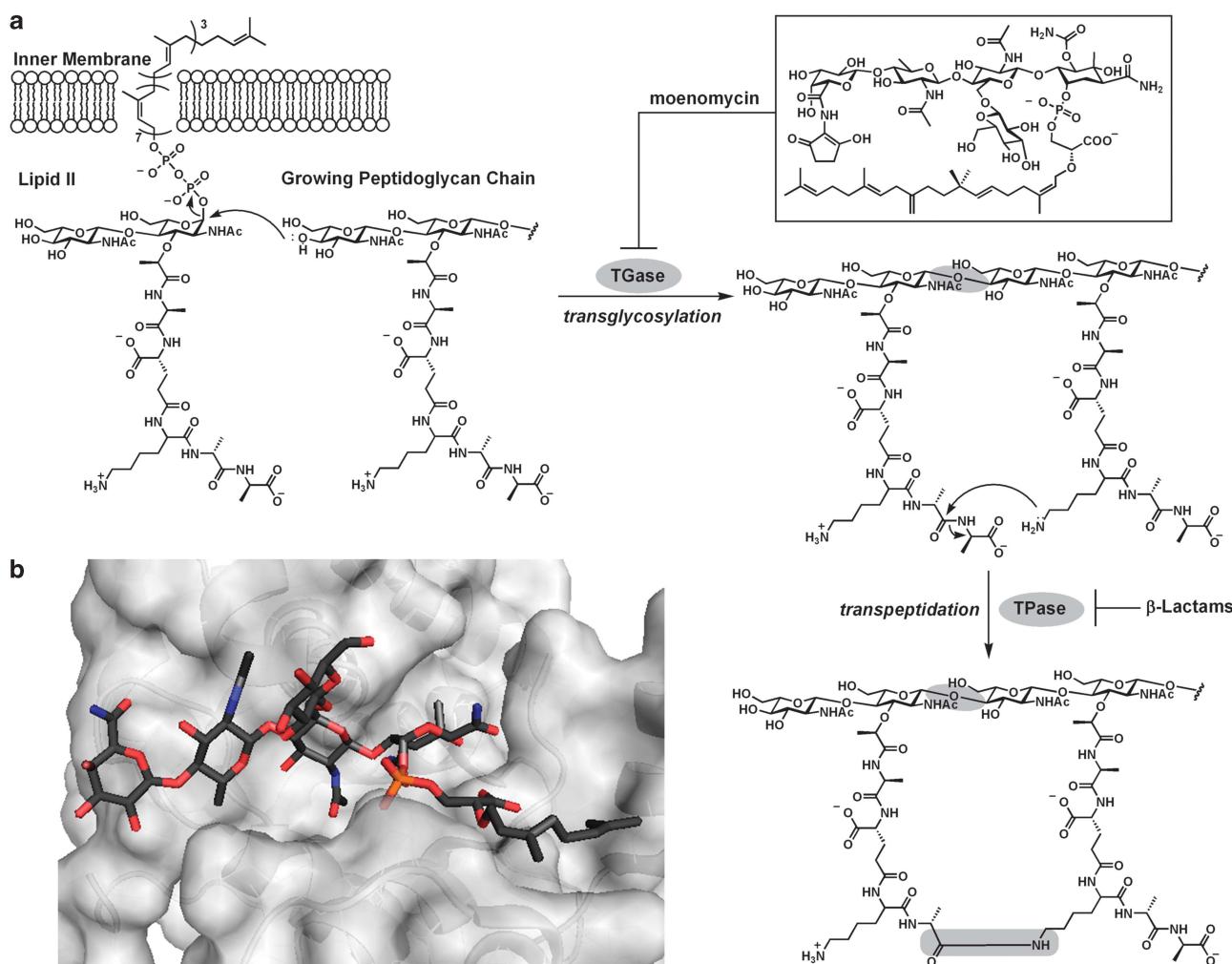


Figure 7 (a) Inhibition of PG transglycosylases (TGases) by moenomycin and inhibition of PG transpeptidases (TPases) by β -lactams. (b) X-ray crystal structure of a moenomycin analog containing a 10-carbon neryl chain bound to the TGase domain of PBP1A from *Aquifex aeolicus*.¹³⁴ The α -amino- β -hydroxy-cyclopentenol ring of the moenomycin analog was highly distorted and is therefore omitted from the structure. Image was generated from PDB entry 3D3H¹³⁴ using PyMOL (The PyMOL Molecular Graphics System, Version 1.4, Schrödinger, LLC).

Lipid II

Lipid II, at a few thousand molecules per bacterial cell, is the C₅₅ isoprenoid carrier that receives the muramyl pentapeptide at the end of the cytoplasmic phase of peptidoglycan biosynthesis. While still on the inner face of the cytoplasmic membrane, the muramyl-penta-peptidyl-lipid II is a substrate for glycosylation by MurG. Then the disaccharyl-penta-peptidyl-lipid II is translocated by membrane flipases¹⁴⁰ to the outer face of the cytoplasmic membrane. On the outward membrane face, it can be a substrate for the TPases and TGases that add these peptidoglycan-building blocks to existing PG strands. When the disaccharyl-penta-peptidyl-lipid II (Figure 8) is exposed at the outer surface of the membrane, the lipid II moiety is targeted at different subregions of its scaffold by two kinds of antibiotics.¹⁴¹ The vancomycin family glycopeptide antibiotics interact with the D-Ala-D-Ala terminus^{29,142} while the antibiotics, such as the food preservative nisin, instead bind to the pyrophosphate subregion¹⁴³ of Lipid II. A third class of natural molecules, the defensin family of peptides/small proteins, also bind to lipid II; the interaction of plectasin with lipid II has been characterized by NMR,¹⁴⁴ and this structure may serve as starting point for

peptidomimetics that may span multiple subregions of lipid II. These antimicrobial peptides by themselves have presented challenges for development,¹⁴⁵ but we shall return to the peptidomimetic approach in a subsequent section on LptD in the lipopolysaccharide (LPS) export pathway.

One recent advance of note is the work of Boger's group to generate the aglycone form of an amidine derivative of vancomycin^{146,147} (Figure 9). The synthetic design took inspiration from the fact that VRE lower the affinity of vancomycin family glycopeptides for the uncrosslinked PG termini by substitution of D-Ala-D-Ala for D-Ala-D-lactate.²² The loss of one H-bond and the electronic repulsion of the D-Ala-D-Lac lone pair on the oxygen of the ester can be compensated in the amidine derivative. Not only did the amidine regain activity against VRE but also maintained acceptable activity against the wild-type D-Ala-D-Ala PG terminus. Addition of the glucosyl-1,2-vancosamine disaccharide moiety to the amidine aglycone, by chemical or enzymatic means, might result in a useful third-generation glycopeptide antibiotic (where the recently approved telavancin¹⁴⁸ would be considered a second-generation molecule).

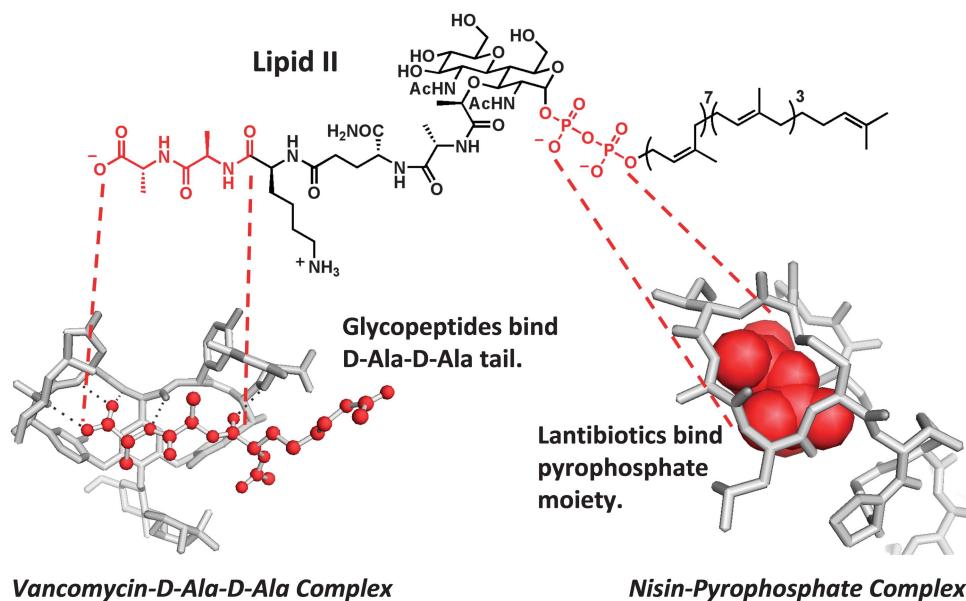


Figure 8 Structure of disaccharyl-pentapeptidyl-lipid II with the D-Ala-D-Ala-vancomycin and the nisin-pyrophosphate binding sites highlighted. Images were created using PyMOL from PDB entries 1FVM¹⁴² (vancomycin-Di-acetyl-L-Lys-D-Ala-D-Ala complex) and IWCO¹⁴³ (nisin-lipid II complex).

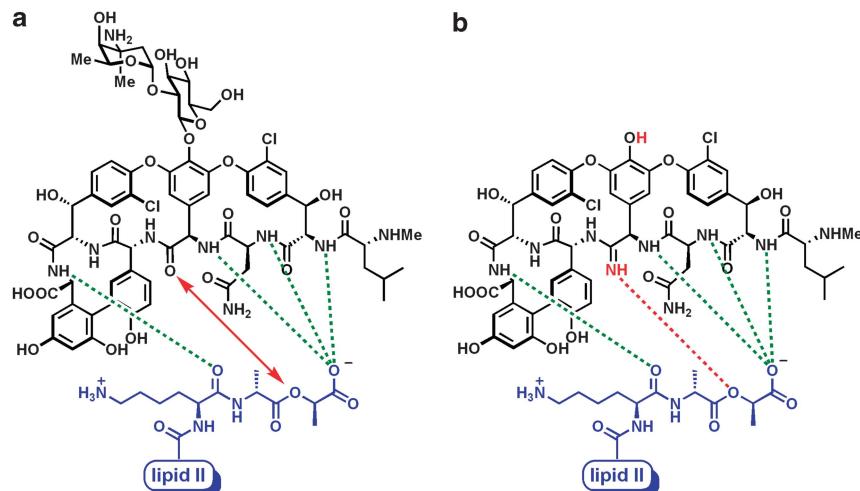


Figure 9 Diagram of the vancomycin-D-Ala-D-Lactate (a) and Boger's synthetic amidine vancomycin aglycone-D-Ala-D-Lactate (b) hydrogen bonding networks. Boger's amidine vancomycin aglycone increases the binding affinity for the uncrosslinked D-Ala-D-Lactate moiety found in modified lipid II of the peptidoglycan (normal lipid II contains a terminal D-Ala-D-Ala) by restoring a key hydrogen bond and compensating for electrostatic repulsion created by this increasingly common resistance conferring lipid II structural modification.

More natural lipopeptides and variants

With the success of the lipopeptide daptomycin (Figure 10a) in treatment of bacterial infections in humans over the past decade,^{118,119} there has been renewed interest in natural and semisynthetic versions of other lipopeptides as antibiotics. We take up acyldepsipeptides (ADEPS) below in the context of ATP-utilizing bacterial enzymes but note here the inhibition of signal peptidases (SPs) by arylomycins,^{149,150} actinocarbasins,¹⁵¹ krisynomycin,¹⁵¹ the polyketide macrolactone antibiotic TA¹⁵² (also known as myxovirescin) and globomycin^{153,154} (Figures 10b and c). Each of these natural products has a constrained conformation due to macrocyclization that is imposed during biosynthesis. The arylomycins and actinocarbasins have a dihydroxybiphenyl moiety that arises from coupling of two tyrosine side chains in the precursor peptide,

presumably through a radical coupling mediated by an iron-enzyme that can make high valent oxo-iron intermediates.¹⁵⁵ This C-C coupling is reminiscent of the coupling of residues 5 and 7 in vancomycin. The natural fatty acyl chain in the N-terminal tail of the actinocarbasins can be replaced semisynthetically with typical med chem biphenyl chains.^{151,156} Gram-negative bacteria have two signal peptidases.¹⁵⁷ Type I signal peptidase (SPI) is a serine protease with a catalytic mechanism distinct from eukaryotic serine proteases for trimming the signal sequence from secreted proteins, outer membrane proteins and periplasmic proteins. Type II signal peptidase (SII) are completely absent in eukaryotes, making this an ideal antibiotic target, and function as an aspartyl protease that trims the precursor forms of lipoproteins found in the outer membranes of Gram-negative bacteria. While arylomycins target

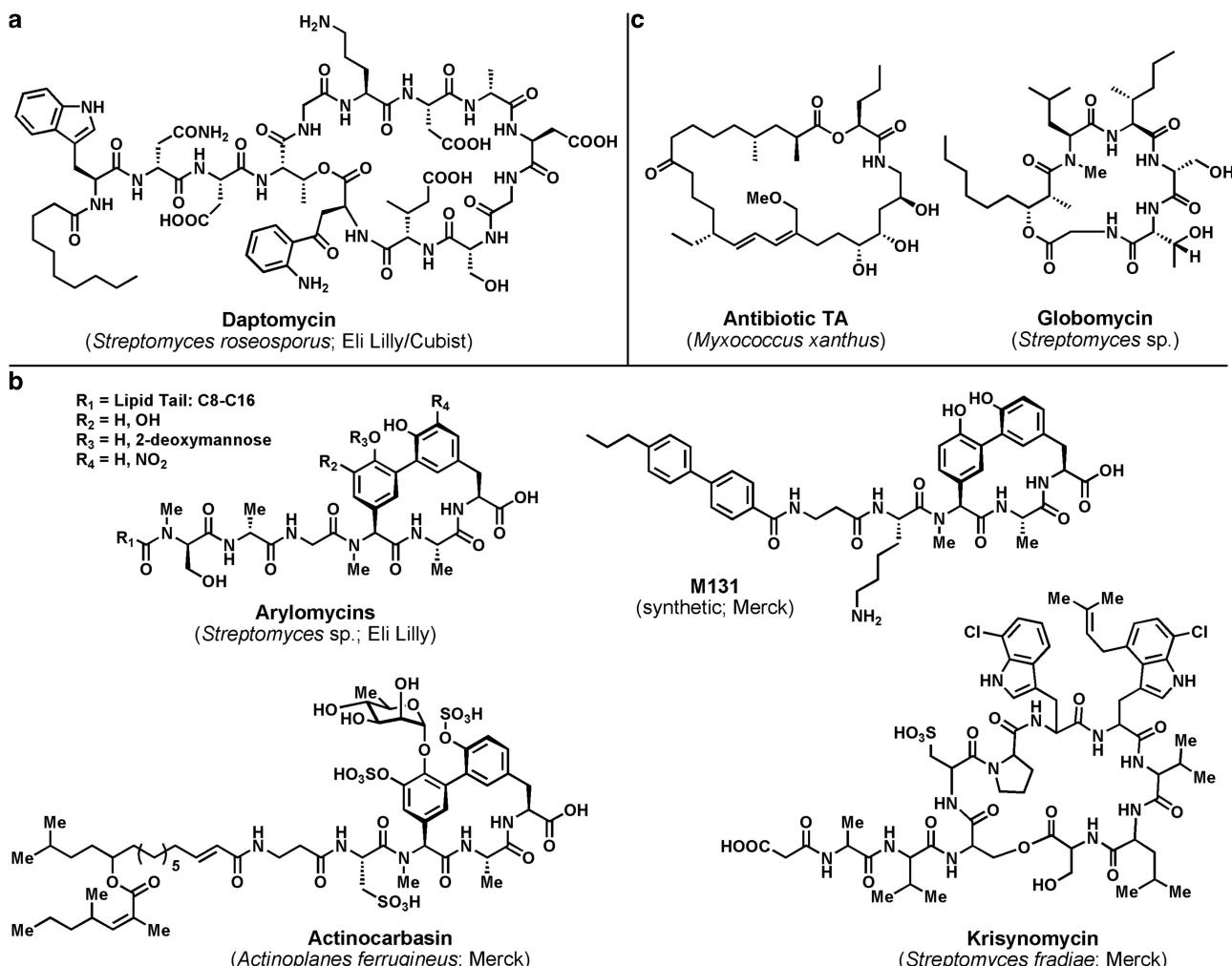


Figure 10 (a) Structure of daptomycin, a lipopeptide antibiotic produced by *Streptomyces roseosporus*, discovered by Eli Lilly and Co. in the late 1980s, acquired and developed by Cubist Pharmaceuticals in 1997 and FDA approved in 2003. (b) Structures of the arylomycin, actinocarbasin and krisynomycin macrocyclic antibiotics that target type I bacterial signal peptidases. (c) Structures of antibiotic TA (also known as myxovirescin) and globomycin macrolactone antibiotics that target type II bacterial signal peptidases.

SPI, antibiotic TA and globomycin target SPII and are bacteriocidal at low concentrations.¹⁵² One feature of potential interest is that arylomycins synergize with aminoglycosides¹⁵⁰ while actinocarbasins and krisynomycin do so with β -lactams.¹⁵¹ These natural products may therefore serve as starting points for further semisynthetic modifications of the frameworks through synthesis^{156,158–161} and pathway engineering,^{155,162–166} with an eye towards combination therapy.

LPS: IS THE TIME RIPE FOR A RENEWED ASSAULT ON THOSE ENZYMES?

One of the holy grails of antibiotic discovery efforts in Gram-negative pathogens has been the search for a broadly active inhibitor of lps biosynthesis and/or export. For decades, investigators have focused on the rate-determining step in lps biogenesis, the second committed step catalyzed by the zinc-dependent deacetylase LpxC (Figure 11a).¹⁶⁷ The central metabolite UDP-GlcNAc undergoes long chain acylation at the 3'-OH of the GlcNAc by LpxA and then LpxC mediates deacetylation of the acetyl moiety on the 2'-amino group. A series of acylations, glycosylations and phosphoryl transfers then ensue on the way to the lipid A core of lps.¹⁶⁸ A prototype

alkynyl hydroxamate CHIR-090 has been characterized for mechanism and structure with LpxC,¹⁶⁹ but narrow spectrum and pathogen mutation to resistance have been problematic for its development. Recent structure–function analysis has led to a diacetylenic congener LPC-009,¹⁷⁰ which overcome the loss of affinity in point mutations that negate CHIR-090 binding (Figure 11b). Perhaps there is finally momentum towards a clinical candidate, at which point toxicology and pharmacology in human hosts will become crucial gating parameters.

There has also been dramatic progress in recent years in delineation of the roles and structures of the periplasmic and outer membrane proteins LptA-E that function as chaperones for lps molecules to get across the periplasmic space.^{171–182} The Lpt protein machinery also effects insertion of the translocated lps molecules and localization in the outer leaflet of the outer membrane in most Gram-negatives. In that context, the outer membrane β -barrel protein LptD has been known to be inhibited by an 18 residue antimicrobial peptide (AMP) protegrin I from porcine leukocytes.¹⁸³ John Robinson and coworkers used this as a starting point for building a peptidomimetic scaffold (Figure 12).^{183–185} The natural bridging disulfide was removed, the rest of protegrin β -sheet was used as template and the framework

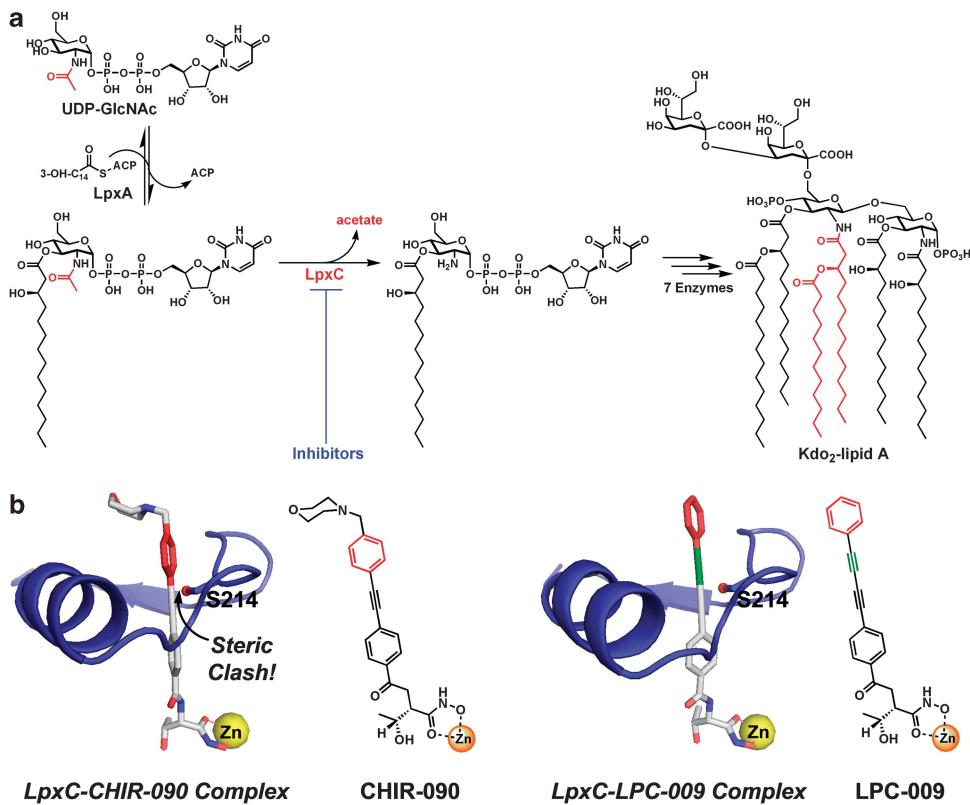


Figure 11 (a) LpxC inhibitors block the first committed step of lipid A biosynthesis, a pathway specific to Gram-negative bacteria. (b) Structure-guided efforts inspired by the prototype alkynyl hydroxamate LpxC inhibitor CHIR-090 revealed the next generation diacetylenic inhibitor LPC-009, which overcomes point mutations (G214 to S214 in *Rhizobium leguminosarum*) associated with CHIR-090 resistance in Gram-negative pathogens. Images were created using PyMOL from PDB entries 2JT2¹⁶⁹ (LpxC-CHIR-090 complex) and 3P3C¹⁷⁰ (LpxC-LPC-009 complex). For both the cases, the structure was solved for WT LpxC from *Aquifex aeolicus* complexed to the inhibitor and a point mutation of G198 to S198 was inserted computationally into the PyMOL structure to demonstrate how this mutation leads to steric repulsion in the LpxC-CHIR-090 complex.

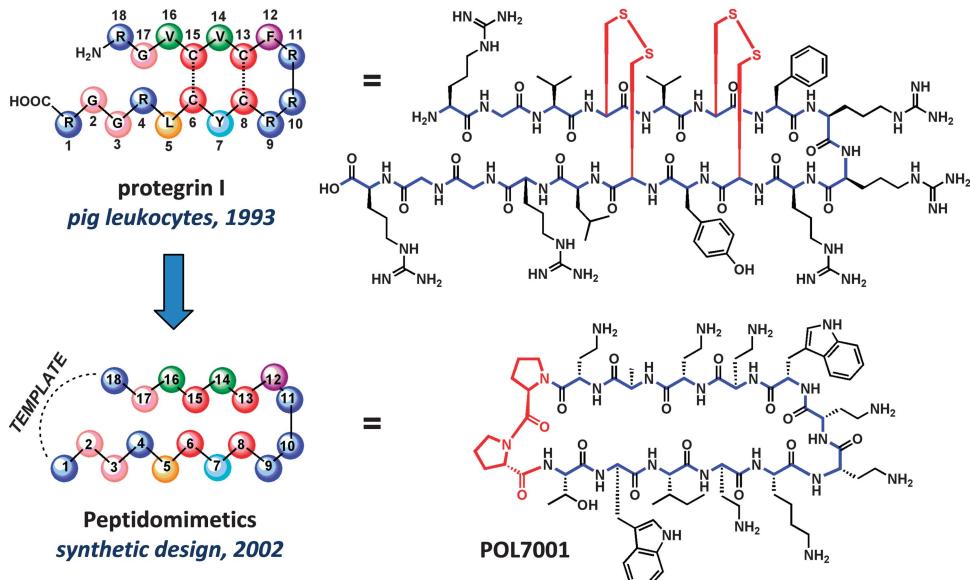


Figure 12 Protegrin I is a naturally occurring disulfide-stabilized beta-turn antimicrobial peptide that targets LptD and served as inspiration for the rational design of template-stabilized beta-turn peptidomimetic scaffolds, such as POL7001, progressing towards clinically useful antimicrobial peptides targeting LptD.

cyclized with a diproline insert to give a potent inhibitor of Lps trafficking in pseudomonas.¹⁸³ Challenges remain to optimize broad spectrum Gram-negative activity. If the peptide framework is not sufficiently stable to protease breakdown, there is the traditionally difficult challenge to go from a peptide-based to a nonpeptidic scaffold to improve *in vivo* lifetimes while preserving activity. Although LptD is in the outer membrane and perhaps thereby particularly accessible to external agents, the other components of the Lpt assembly pathway also seem reasonable targets, and structural information is accruing on their bridge functions, architecture and roles in moving Lps through the periplasm.^{171–182}

BACTERIAL RNA POLYMERASE: MANY NATURAL PRODUCTS BIND IN ADJACENT SUBSITES

Bacterial RNA polymerase has been a validated antibacterial target for decades. It is the target for rifampicin and derivatives as front-line agents in combination therapy against *Mycobacterium tuberculosis*. In fact, a wide range of natural product scaffolds have been examined and found to inhibit bacterial transcription by targeting RNA polymerase.¹⁸⁶ One of the most recently approved antibiotics fidaxomicin, an 18 membered polyketide macrolactone (Figure 13a) from *Dactylosporangium auranticum*¹⁸⁷ likewise blocks RNA transcription and has been approved by the FDA for the treatment of *Clostridium difficile* infections.¹⁸⁸ The drug is poorly adsorbed from the gastrointestinal tract, supporting its use against *C. difficile*. As shown in Figure 13b, rifamycins¹⁸⁹ and the natural product sorangicin¹⁹⁰ bind to almost identical subsites of the enzyme while

streptolydigin¹⁹¹ and myxopyronin¹⁹² bind in separate subsites.¹⁹³ The bicyclic phosphate metabolite tagetitoxin from the phytopathogenic *Pseudomonas syringae* pv *tagetis* binds in yet a fourth subsite coordinating the Mg²⁺ in the active site.¹⁹⁴ Nature clearly presents a rich variety of small molecular frameworks to target different pockets in RNAP. The specific binding subsite for fidaxomycin is not yet determined. One anticipates that other scaffolds may be found for additional or overlapping RNAP subsites and that structure-based evaluation might guide combinations directed against RNAP beyond Tb and *C. difficile* colitis.

ATP-DEPENDENT ENZYME TARGETS

For the past 15 years, inhibition of eukaryotic ATP-dependent protein kinases has been a mainstay of pharmaceutical and biotech research and development activity across a broad swath of human disease areas.^{195,196} Although protein tyrosine kinase equivalent catalysts are almost absent in bacterial life, Ser/Thr kinases have become more prominent with knowledge of bacterial genomes. The histidine kinases that are the front end of dozens of two component signaling systems in bacteria have been investigated multiple times but, as yet, no robust, optimizable chemical scaffolds have emerged.¹⁹⁷ Nonetheless, the ATP-dependent biotin carboxylase component of bacterial acetyl CoA carboxylase, the first committed step in fatty acid biosynthesis, has been targeted with specificity and potency by repurposed kinase libraries by a team at Pfizer.⁸² There is no *a priori* reason to think this need be an isolated success against a bacterial ATP-generating/consuming enzyme (for example, the Lps

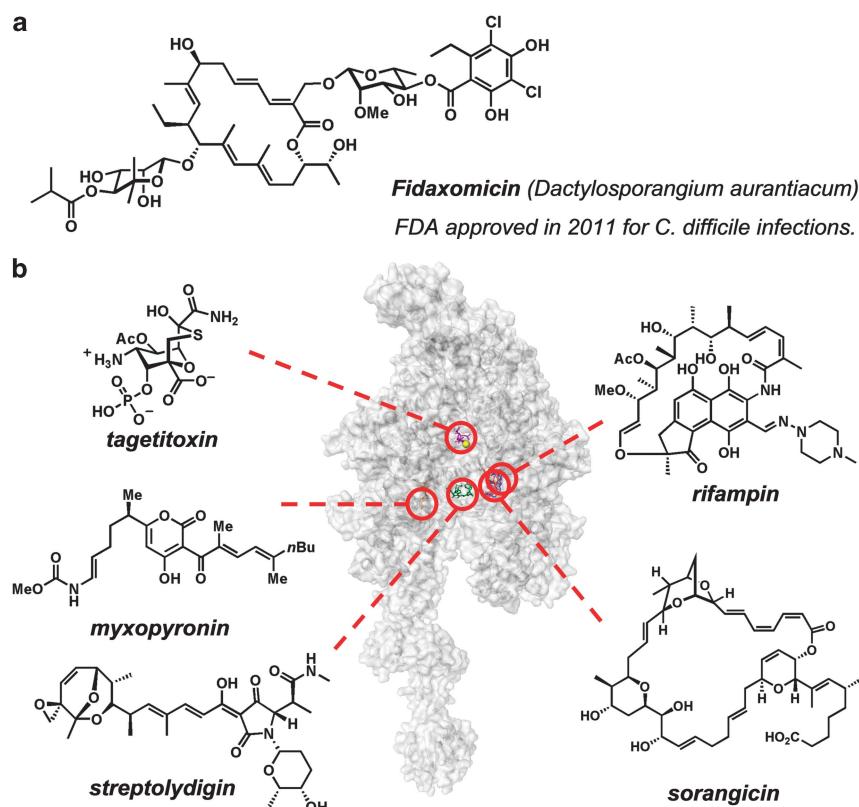


Figure 13 (a) Structure of fidaxomicin, an 18-membered polyketide macrolactone bacterial RNAP inhibitor produced by *Dactylosporangium aurantiacum* approved by the FDA in 2011 for the treatment of *C. difficile* infections. (b) Surface structure of bacterial RNA polymerase from *Thermus aquaticus* showing the binding sites of a representative panel of natural product inhibitors (rifampin, sorangicin, streptolydigin, myxopyronin and tagetitoxin). Image was generated from PDB entries 1YNN¹⁸⁹ (RNAP-rifampin complex; used for alignment of all structures), 1YNJ¹⁹⁰ (RNAP-sorangicin complex), 1ZYR¹⁹¹ (RNAP-streptolydigin complex), 3DXJ¹⁹² (RNAP-myxopyronin complex) and 2BE5¹⁹⁴ (RNAP-tagetitoxin complex) using PyMOL software.

kinases that install the phosphates on lipid A)¹⁶⁸ but does suggest investment in library design may be required.

Figure 14 introduces three additional such targets in bacteria. DNA gyrase, with type II topoisomerase activity for unwinding concatenated DNA at the end of replication, could be considered the grandfather of this ATP-targeting paradigm. In these topoisomerases, ATP hydrolysis powers the mechanical work of strand cleavage, passage and repair.¹ The venerable and contemporary fluoroquinolones block the GyrA subunit and possibly the newly identified kibdelomycin family of natural products targets this same gyrase subunit,¹⁰² while the coumarin¹ and cyclothialidine¹⁹⁸ families of natural products inhibit the ATPase activity conferred by the GyrB subunit.

A second successful example of an antibiotic targeting an ATP-dependent bacterial enzyme is bedaquiline, a diarylquinoline identified in a screening campaign for molecules that could kill *M. tuberculosis*.¹⁹⁹ The mechanism of action was determined by generation of resistant mutants and mapping them by whole-genome sequencing, allowing identification of the F_o subunit of the Mtb ATP synthase.^{200,201} The molecule is specific for mycobacterial ATP synthase²⁰² and has proven efficacy through phase II trials,^{199,203} with a new drug application filed in July 2012 and approval granted at the start of 2013.⁴⁵ Perhaps there are still more ATP-utilizing proteins within the Mtb proteome that might surface as valid therapeutic targets.²⁰⁴

The third potential antibacterial target, the chambered proteases of the ClpP family,^{205,206} are acted on by a family of related lipo-peptidolactones, known as ADEPS,^{207,208} originally isolated from *Streptococcus hawaiiensis* in 1985 by scientists at Eli Lilly and Co.²⁰⁹ Semisynthetic tailoring of the scaffold has led to increased potency; for example, in ADEP2 (Figure 14).²⁰⁵ These bacterial chambered proteases, in mechanistic and structural analogy to eukaryotic chambered proteasomes, require ATP hydrolysis to unfold and thread protein substrates into the central cavity containing the

protease sites. ADEPS are intriguing because they are actually *activators*, not inhibitors, of the basal protease activity of ClpP and so uncouple protease activity from ATP hydrolysis.^{205,206} This occurs by allosteric activation^{207,208} on the outer surface of the multisubunit proteases. Turning up unregulated protease activity is harmful to cells that contain active ClpPs. Of recent note is the connection of ADEP-mediated ClpP activity to disruption of cell division. One of the early key structural proteins to form the cell division ring is FtsZ. It is poorly folded and subject to the inappropriately activated ClpP-mediated degradation in ADEP-treated cells.²¹⁰ In turn, the cell division septum does not form and/or is degraded and cell division abrogated. Although ADEPS may have liabilities in monotherapy, such as rates of mutation of the allosteric site to insensitivity, they may have value in combination regimens.

FtsZ itself is a homolog of eukaryotic tubulin and shows GTPase activity involved in assembly of the divisome components.²¹¹ A molecule known as PC19073²¹² (Figure 15) has been reported to be an FtsZ inhibitor by polymerization assays²¹³ and X-ray crystallography.²¹⁴ However, more recently, Shaw and coworkers²¹⁵ failed to detect inhibition of GTPase activity. They also noted that many structures cited in preliminary reports as FtsZ inhibitors are either aggregators and/or nonspecific GTPase inhibitors. They pointed out zantrin Z3 (Figure 15) as a *bona fide*, specific albeit weak ($IC_{50} = 20 \mu\text{M}$) starting lead for future SAR work.²¹⁵

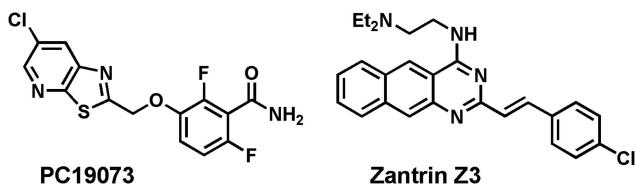


Figure 15 Inhibitors of FtsZ, a bacterial homolog of eukaryotic tubulin with GTPase activity.

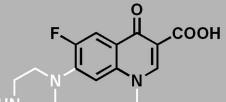
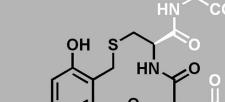
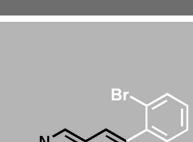
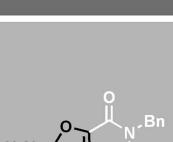
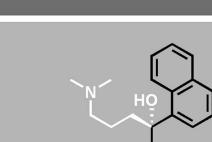
ATP-Dependent Enzyme	DNA Gyrase		
Targeting Antibiotic Scaffolds			
	Fluoroquinolones	Aminocoumarins	Cyclothalidines
ATP-Dependent Enzyme	Biotin Carboxylase	ATP Synthase	Chambered Protease
Targeting Antibiotic Scaffolds			
	Pyridopyrimidines	Aminooxazoles	Diarylquinolones
			Acyldepsipeptides (ADEPs)

Figure 14 Molecular scaffolds targeting bacterial ATP-dependent enzymes as potential antibiotic therapeutic approaches.

PROTEIN SYNTHESIS INHIBITORS

We noted previously that inhibition of protein synthesis is one of the five major clinically validated antibacterial pathways (Figure 5), with most antibiotics in this class targeting the ribosome.¹¹⁶ These have spurred at least one company Ribex to use structure-based approaches to design next-generation ribosome-directed antibiotics. We also noted new developments in tetracycline chemistry⁷⁰ and still more structural information is being reported on this class of antibiotics.²¹⁶ The clinical progression of second-generation oxazolidinones into phase II studies was also mentioned.^{74–80} Achaogen is working to bring forward new aminoglycoside protein synthesis inhibitors, such as plazomicin (ACHN-490), which successfully completed a phase II trial for urinary tract infections.²¹⁷ Novartis has converted the natural thiopeptide GE2270A, an inhibitor of elongation factor Tu,²¹⁸ to a semisynthetic version with a 10⁵ increase in aqueous solubility and moved it into phase II trials for *C. difficile* infections.²¹⁹ Other protein synthesis halting antibacterials currently in various stages of clinical development include members of the pleuromutilin (BC-3781, BC-7013, BC-3205) and macrolide (Cethromycin) structural classes.⁷³

SUMMARY THOUGHTS

This perspective has argued that there is a continuing need for new cycles of antibiotic discovery and development. The genes found in the global resistomes are mobilizable with different frequencies to propel waves of resistant bacteria to each generation of newly introduced antibiotics. Given that the past half century has turned up few new molecular scaffolds, the fine tuning of existing frameworks by medicinal chemists may be approaching an asymptotic limit.

One small surprise has been the rise in usage/rediscovery of lipopeptides made by NRP synthetase machineries as antibiotics. Although daptomycin is the poster child of the past decade, ADEPS show unanticipated mechanisms, not working through membrane disruption. Even the polymyxins have come back into prominence, given the multidrug-resistant profiles of Gram-negative pathogens. There are certainly many more lipopeptide natural products in the biosphere, and they may be starting points for combination therapies. New approaches to interrogation of the biosynthetic capacity of the microbial world for conditional metabolites suggests new scaffolds will be found, presumably opening up additional rounds of both chemical and bioengineering optimization of those novel molecular scaffolds. Different approaches to synthetic library design may also enrich the hit rates in whole bacterial screens, especially to combat the permeability barriers and efflux pumps of the ESKAPE pathogens.

In parallel, we have opined on a select, possibly representative set of targets that we suggest are underexploited based on chemocentric past experience that promising, specific chemical matter has been identified, often in the form of natural product scaffolds. Improvements in biological assay designs and configurations almost always turn up new molecules. Smart screens have already shown that activity-guided isolation of novel natural molecular architectures is still possible. Several of these screens have assayed for resensitization and/or synergy with existing antibiotics to which pathogens have become insensitive. These augur for early guidance for combination therapies against particular pathogens.

More combination therapy seems a likely way forward, paralleling the trend of therapeutic modalities in viral infections and in cancer treatments. In that eventuality, coupling real-time diagnostics of the pathogen population in each patient to a proven or predictively effective combination of antibiotics may be a way forward, although Kishony and coworkers have noted the downside potential for resistance development in combination approaches.²²⁰

CONFLICT OF INTEREST

CTW is on the Board of Directors of Achaogen, which has plazomicin in clinical trials.

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