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Current Opinion in Chemical Biology

Natural and engineered precision antibiotics in the context of resistance

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Abstract

Antibiotics are essential weapons in our fight against infectious disease, yet the consequences of broad-spectrum antibiotic use on microbiome stability and pathogen resistance are prompting investigations into more selective alternatives. Echoing the advent of precision medicine in oncology, precision antibiotics with focused activities are emerging as a means of addressing infections without damaging microbiomes or incentivizing resistance. Historically, antibiotic design principles have been gleaned from Nature, and reinvestigation of overlooked antibacterials is now providing scaffolds and targets for the design of pathogen-specific drugs. In this perspective, we summarize the biosynthetic and antibacterial mechanisms used to access these activities, and discuss how such strategies may be co-opted through engineering approaches to afford precision antibiotics.

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Current Opinion in Chemical Biology 2022, **69**:102160

This review comes from a themed issue on **Next Generation Therapeutics (2022)**

Edited by **Luke L. Lairson**

For complete overview of the section, please refer to the article collection Next Generation Therapeutics (2022)

Available online 31 May 2022

https://doi.org/10.1016/j.cbpa.2022.102160

Keywords

Antibiotics, Antagonism, Natural products.

Introduction

Nearly all classes of clinical antibiotics are derived from microbial natural products: small molecules with chemical structures and biological activities honed through natural selection [1]. Following the discovery of penicillin, broad-spectrum antibiotics have remained the most widely used antimicrobial therapeutics, though new insights into the consequences of their use are beginning to erode their value as front-line therapies. Decades of misuse and poor stewardship have incentivized the development of widespread antibiotic resistance, which now endangers the continued utility of our antibacterial arsenal. The indiscriminate nature of these drugs also damages the microbiome [2,3], undermining the efficacy of immunotherapies [4] and potentially driving secondary infections by antibiotic-resistant gut residents like *Clostridium difficile* [5]. Approved narrow-spectrum antibiotics offer a more precise means of treatment [6], and continued refinement of their activity spectra beyond distinguishing between Gram-positive or -negative bacteria will eventually deliver truly pathogen-specific therapeutics.

As a byproduct of decades of bioactivity-guided searches for broad-spectrum antibiotics, researchers have uncovered a number of highly specific antimicrobials, with focused activity spectra centered on individual bacterial phyla, genera, or species [7]. Although their specificity originally caused these compounds to be overlooked, advances in clinical diagnostics have created an opportunity to reinvestigate these natural products as leads for pathogen-specific therapeutics, capable of preserving microbiome ecologies while limiting the potential for community-acquired resistance [8]. As studies of broad-spectrum natural products paved the way for our current antibiotic arsenal, future investigations of the targets and strategies employed by Nature to generate precise antibacterial molecules may be similarly fruitful [9].

Perhaps most relevant to the need for novel antibiotics, selective antibacterial natural products represent an untapped well of evolved chemical scaffolds that exploit new microbial targets. Early returns from mechanistic and biosynthetic studies are beginning to provide insight into how these chemical structures and biological activities are evolved in Nature, and how this process could be recapitulated in the laboratory to generate true narrow-spectrum antibiotics. In this perspective, we explore classes of molecules that display focused antibacterial activities in Nature, and discuss the modes by which they achieve this specificity. Leveraging

insight into the biosynthetic assemblies that create these drug-like molecules, we highlight laboratory methods that can be used to alter their bioactivity and specificity in pursuit of precision antibiotics.

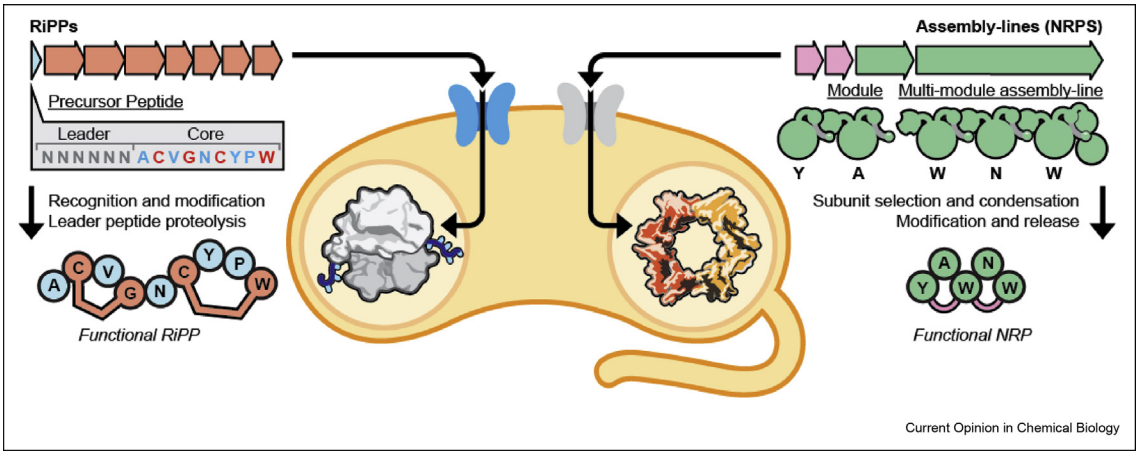
Selective, ribosomally-synthesized antibiotics

Narrow-spectrum natural products are often deployed in Nature to inhibit sympatric species, facilitating antagonism between related bacteria during competition for a shared ecological niche [7,10]. Studies of competition between Enterobacteriaceae from the microbiome [11] revealed early members of perhaps the most widely distributed superfamily of natural products: the ribosomally synthesized and post-translationally modified peptides (RiPPs) [12]. These structurally and functionally diverse molecules are created through a common scheme, wherein leader sequences of a precursor peptide guide the recognition and modification of a core peptide region by tailoring enzymes, culminating in proteolytic cleavage and liberation of the active modified RiPP (Figure 1). The resultant modifications define RiPPs into chemical families with conserved features and activities [12]. As most tailoring enzymes require only conserved leader peptide motifs to initiate modification, these systems can be combinatorialized to generate new chemical families [13]. The mutability of the precursor peptide, coupled with these adaptable modifying enzymes, makes RiPPs the most readily evolvable natural products. In light of their diversity and distribution, RiPPs can display refined biological activities [8], with selectivity often stemming from features found at the cell surface.

Lasso peptides

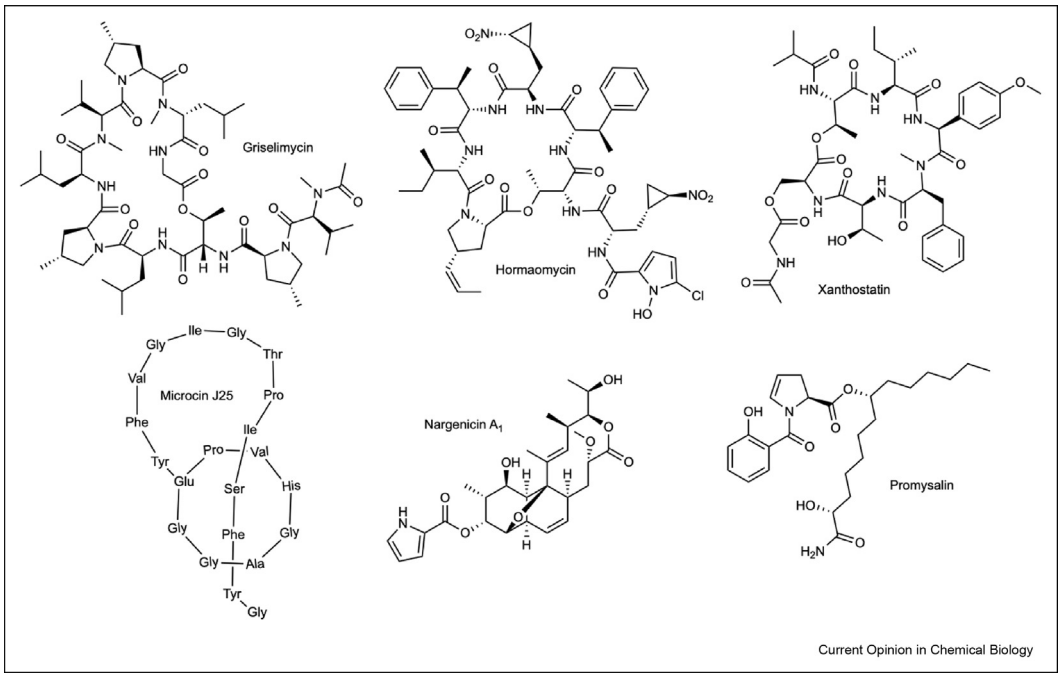
The large size and peptidic nature of RiPPs limits their diffusion through lipid membranes [14]. As a result, the mechanism used by RiPPs to traverse the cellular envelope and gain access to their targets often determines their activity spectra. One such approach is the hijacking of transmembrane transport complexes, a strategy exemplified by the knotted macrolactams known as lasso peptides [15], which classically exploit the outer membrane siderophore importer FhuA to gain access to the periplasm [16]. To transit from the periplasm to the cytoplasm, lasso peptides co-opt a second transporter known as SbmA, a non-essential proton-driven importer that is exploited by diverse antibiotics, antimicrobial peptides, and RiPPs [17]. Among Proteobacteria, lasso peptides like the *Escherichia coli*-derived microcin J25 (McJ25, Figure 2) are used for intra-family antagonism, and have evolved to target specific *fhuA* genotypes [18–23] (Table 1). Once inside the cell, Proteobacterial lasso peptides inhibit RNA polymerase by engaging phylogenetically conserved residues that line the nucleotide uptake tunnel. Lasso peptides therefore display a broad spectrum of activity against purified microbial RNA polymerases *in vitro* [24], which contrasts with their selective activity *in vivo*. Due to a dependence on active import, mutations in *fhuA* and *sbmA* are a common means of lasso peptide resistance in the laboratory [25]. Lasso peptides produced by Gram-positive bacteria have different activities, but are similarly

Figure 1



Microbial methods for accessing precision antibiotic molecules. The ribosomally-synthesized and post-translationally modified peptides (RiPPs; left) are created from precursor peptides encoded by canonical amino acids. RiPP leader sequences recruit tailoring enzymes, which modify the core sequences to furnish the final active product. RiPPs are frequently deployed for antagonism between related bacteria, wherein specificity is often achieved through selective targeting of extracellular features (e.g. selective uptake). Similarly complex molecules can be accessed through enzymatic assembly-lines, including nonribosomal peptide synthetases (NRPSs; right). In this scheme, multi-domain modules select metabolic subunits and condense them into a growing polymer, before modifying and releasing a functional nonribosomal peptide (NRP). Selective assembly-line products appear to be less frequently restricted by cellular entry and have been shown to achieve specific activity profiles through interaction with phylogenetically unique binding sites and targets.

Figure 2



Structural Diversity of Natural Narrow-spectrum Antibiotics. As discussed throughout this review, nature has evolved many scaffolds capable of affecting specific bacterial taxa, including griselimycins, nargenicins, and proteobacterial lasso peptides such as microcin J25. While we describe several prominent classes, some notable examples were omitted for brevity. Hormaomycin is a cyclic peptide from *Streptomyces* with exceptional activity against related bacteria, such as *Arthrobacter*, which it achieves through an unresolved mechanism [127]. The cyclic peptide xanthostatin demonstrates selective antibacterial activity against *Xanthomonas spp* [75] which is notable considering its extensive structural similarity to salinamide, a broad-spectrum antibiotic and inhibitor of RNA polymerase [74]. Finally, promysalin is an unusual *Pseudomonas* natural product that functions as an inhibitor of succinate dehydrogenase, achieving species-specific killing of *P. aeruginosa* due to this organism's unique metabolic dependence [128]. Additional information for these and other examples can be found in Table 1.

directed towards related microbes. The *Streptomyces* lasso peptide siamycin appears to act by binding lipid II [26], a mechanism that is likely shared by other peptides such as streptomonomicin due to their overlapping resistance profiles [27]. Lassomycin meanwhile, is produced by *Lentzea* actinobacteria, and shows selectivity for related microbes including *Mycobacterium*, operating through activation the ATPase activity of the barrel protease ClpP and uncoupling it from proteolysis [28].

Microcin B17

Uptake-driven specificity is a recurrent theme among RiPPs and reveals features that enable selective targeting of different microbes. A second well characterized effector is the *E. coli*-derived product microcin B17 (McB17), which displays potent antibacterial activity against other Enterobacteriaceae (Table 1). This 43-residue polypeptide is predominantly composed of glycines and post-translationally generated azole heterocycles, and acts as an inhibitor of DNA gyrase in a manner akin to synthetic quinolones [29]. The selectivity of McB17 is defined by uptake, first passing through the OmpF outer membrane porin before transiting to the cytoplasm via SbmA [30]. Clues to the chemical features that determine uptake have been gleaned from homologs found in diverse bacterial genomes [31], including Ps-McB1 from *Pseudomonas syringae*. A key glycine tripeptide in Ps-McB1 enables uptake by related Pseudomonads without impacting the interaction with the intracellular target, and grafting this sequence into McB17 yields a hybrid RiPP that is active against both Enterobacteriaceae and Pseudomonads [31]. Notably, *Pseudomonas* is naturally resistant to McB17 as it lacks a homolog of SbmA, suggesting additional import proteins could be exploited for selective antibiotic delivery.

Sactipeptides

The abundance of RiPP sequences in microbiome genomes [32] suggests that they could serve as effective lead compounds to combat infections within the gut. One such promising class is the sactipeptides, characterized by thioethers formed between cysteine thiols and α-carbons of non-adjacent amino acids. Sactipeptides can facilitate antagonism between Firmicutes, but reported activities can range from species-specific

Table 1

Examples of selective antibacterial natural products.

Compound	Class	Target	Producing organisms	Sensitive organisms	Origin of Specificity	Ref
Griselimycin	NRP	β sliding clamp (DnaN)	*Streptomyces muensis*	Actinomycetia	Unique target interaction	[56]
Xanthostatin	NRP	Unknown	*Streptomyces sp.*	*Xanthomonas sp.*	Uptake system	[74,75,126]
Hormaomycin	NRP	Unknown	*Streptomyces griseoflavus*	*Arthrobacter sp.*	*Unresolved*	[127]
Nargenicin	PK	DNA polymerase α subunit (DnaE)	*Nocardia sp.*	*Staphylococcus sp.*	Unique target interaction	[58]
Promysalin	NRP-PK	Succinate dehydrogenase (Sdh)	*Pseudomonas sp.*	*Pseudomonas aeruginosa*	Metabolic proclivity	[128]
Microcin J25	RiPP (Lasso peptide)	RNA polymerase β' subunit (RpoC)	*Escherichia coli*	Enterobacteriaceae	Uptake system	[22,24]
Lassomycin	RiPP (Lasso peptide)	ClpC1 protease	*Lentzea kentuckyensis*	*Mycobacterium sp.*	Unique target interaction	[28]
Microcin B17	RiPP (Linear azol(in)e-containing peptide)	DNA gyrase	*Escherichia coli*	Enterobacteriaceae	Uptake system	[29]
Phazolicin	RiPP (Linear azol(in)e-containing peptide)	Ribosome	*Rhizobium sp.*	*Rhizobiales*	Uptake system & target interaction	[73]
Thuricin Z	RiPP (Sactipeptide)	Lipid membrane	*Bacillus thuringiensis*	*Bacillus cereus*	*Unresolved*	[33]

[33] to broad spectrum [34]. Surprisingly, these selective antibiotics appear to act on specific features within bacterial membranes, with binding driving permeabilization through an as-yet undefined mechanism [33]. Thuricin CD, a two-component sactipeptide complex from *Bacillus thuringiensis*, has attracted attention for its potent and focused activity directed predominantly against *C. difficile* [35]. While the potency of thuricin CD is comparable to clinical antibiotics, it displays greater species specificity *in vitro* and *in vivo*, limiting changes to the microbiome composition of treated animals [36]. Unfortunately, thuricin CD is rapidly degraded by gastric enzymes, and probiotic strategies do not produce sufficient quantities of the antibiotic *in situ* [37]. In response, targeted discovery of more stable homologs [38,39] has yielded similarly selective molecules capable of eliminating target *Clostridium* spp., presenting avenues to further develop improved sactipeptides as targeted, microbiome-friendly antibacterial therapeutics.

Lanthipeptides

Widely distributed in bacteria and particularly in Gram positive microbes [40], lanthipeptides feature β-thioether bonds that are formed between cysteine thiols and the β-carbons of dehydrated serine or threonine residues (dehydroalanine and dehydrobutyrine, respectively)[11]. As antibacterial "lantibiotics", these diverse RiPPs exploit a limited set of biological mechanisms, working alone or synergistically with other co-expressed lanthipeptides to perforate membranes and bind common membrane components, including lipid II and phosphatidylethanolamine [41]. Given these conserved targets that are accessible at the cell surface, it is unsurprising that the majority of lanthipeptides show broad activity spectra against Gram-positive microbes. However, exceptions have been discovered of highly selective lanthipeptides that enable antagonism between related organisms, suggesting that some generalist lantibiotics could be adapted to eliminate specific threats. In an investigation of microbiome members suspected to affect *Staphylococcus aureus*-driven atopic dermatitis, the related bacterium *Staphylococcus hominis* was shown to limit the growth and colonization of its pathogenic relative through production of lantibiotics. These molecules, *Sh*-lantibiotic-α and β, enabled *S. hominis* to selectively inhibit *S. aureus* growth in skin microbiomes while leaving other members — including *Cutibacterium acnes* and *Staphylococcus epidermidis* — unharmed [42]. Even more selective antagonism has been observed among Bacilli, where the *Bacillus amyloliquefaciens* amylopeptin lanthipeptides can exclusively affect *Bacillus megaterium* but not other Gram-positive microbes, including *Bacillus subtilis* [43]. A similar taxa-dependent activity spectra has been noted for the *Geobacillus thermodinitrificans* lanthipeptide geobacillin II, which selectively inhibits related *Bacillus* species [44].

Trojan-horse antibiotics

As noted earlier, RiPP antibiotics often achieve their narrow activity spectra through selective cellular uptake, hijacking transmembrane transport complexes. The phosphoramidate antibiotics, typified by the *E. coli* product microcin C7 (McC7), exploit molecular mimicry to access the cytosol where metabolic enzymes act on it to liberate an adenylate analog inhibitor of aspartyl-tRNA synthetase [45]. Instead of imitating imported metabolites, McC7 mimics the apparent antimicrobial peptide substrates of the YejABEF inner membrane transport complex to gain entry to the cell, an interaction that is affected by peptide length and N-formylation [46]. Antibiotic activity from this *E. coli* product appears limited to closely related bacteria, including *Salmonella*, *Shigella*, and *Klebsiella*, with specificity determined by cellular uptake [47]. Biosynthetic machinery for related antagonistic antibiotics can be observed in diverse bacteria, with experimental evidence demonstrating a conserved role in intrafamily antagonism [48].

An alternative means of cellular entry exploited by RiPP antibiotics is hijacking the uptake mechanism reserved for iron-binding siderophores. While lasso peptides mimic siderophore structures using carefully positioned amino acids [15], another class of natural antibiotics exploit functional siderophore moieties to smuggle antibacterial warheads into the cell. These natural 'sideromycins' — exemplified by albomycin — have inspired a suite of synthetic antibiotic—siderophore conjugates, yielding inhibitors that can readily transverse the bacterial outer membrane [49]. While microbial consortia produce diverse siderophores, each bacterium often recognizes the precise composition and configuration of its own siderophore-metal complexes. Because of this specificity, siderophores isolated from human pathogens have been used to develop synthetic antibiotic conjugates selective for particular bacteria [49,50]. Smaller modifications using distinct siderophore substructures have also proven successful in improving antibiotic uptake, as seen in the modified cephalosporin cefiderocol [51], which in 2019 became the first clinically-approved sideromycin.

Selective antibiotics from enzymatic assembly-lines

Although RiPPs and other biosynthetic paradigms can yield antibiotics, clinically used antimicrobials have largely been derived from non-ribosomal peptides (NRPs) or polyketides (PKs). These natural products are created by a common and intertwined family of assembly-line enzymes known as non-ribosomal peptide synthetases (NRPSs) and polyketide synthases (PKSs; Figure 1), which build their respective products from adenylate-activated amino acids and malonate-derived acyl subunits [52]. Each assembly-line protein contains multi-domain modules which select, modify, and condense chemical building blocks into a growing polymer. Nascent products progress along the modules of the assembly-line from the N- to C-terminus before being released, with each domain contributing a readily predictable chemical modification. Although NRPS and PKS enzymes use different substrates, their conserved modular architecture and organization ensures a mutual compatibility, resulting in frequent hybrid NRPS-PKS assembly-lines that can access even more chemically diverse products. Unlike the readily mutable RiPPs, these large enzymes appear to evolve through recombination [53], spontaneously generating new chemical structures that can access new activities [54]. A similar link between enzyme sequences and chemical products can be observed in minimal iterative type II PKS pathways, wherein variable aromatic cyclization of polyketide chains can yield divergent biological activities [55]. Collectively, NRPS and PKS enzymes can effectively serve as microbial drug factories that provide chemical solutions to ecological challenges, from overcoming emergent resistance to generating precision antibiotics.

Griselimycin

Like RiPPs, NRPs and PKs often facilitate antagonism between related bacteria [7]. However, as these natural products are generally smaller and more readily capable of entering bacterial cells, specificity is more often determined by interactions with unique targets or binding sites, rather than uptake. This strategy can be seen in griselimycin (Figure 2), a cyclic NRP and preclinical antibiotic lead from *Streptomyces muensis*, thoroughly detailed by Kling et al. [56] (Table 1). Griselimycin displays specific bactericidal activity towards related Actinomycetia and particularly against members of the Corynebacteriales order, including *Mycobacterium*. After its initial development was abandoned following the release of rifampin, this antibiotic has attracted renewed attention as a lead for treating drug-resistant tuberculosis. While griselimycin itself is an effective antibiotic, minimal synthetic modifications yielded a cyclohexyl derivative with enhanced oral bioavailability, metabolic stability, and nanomolar potency *in vitro* and *in vivo*. Mechanistic studies revealed that griselimycin uniquely targets the DNA polymerase sliding clamp DnaN, and disrupts DNA replication by binding to an Actinobacterial-specific hydrophobic groove that typically mediates interactions with the polymerase. In *S. muensis*, self-resistance is provided by an insensitive variant of DnaN. In treated *Mycobacterium*, resistance occurs almost exclusively through spontaneous genomic amplification of the origin of replication, *dnaN*, and other neighboring genes. This unusual resistance mechanism yields concomitant growth defects, driving a reversion to the sensitive genotype in the absence of the drug. As griselimycin and its derivatives affect a unique target, they do not exhibit cross-resistance with established therapies, providing a new lead to combat drug-resistant *Mycobacterium tuberculosis*.

Nargenicin

In contrast to the intrinsic anti-Actinobacterial activity of griselimycin, other malleable scaffolds can be naturally tailored to access divergent and specific activity spectra. Notable examples are the ether-bridged decalin PKs, including the C8—C13 ether macrolactones represented by nargenicin (Figure 2) [57]. Nargenicin was discovered through its selective nanomolar inhibition of methicillin-resistant *Staphylococcus*, and mechanistic investigations later established it as the first known inhibitor of the DNA polymerase alpha subunit DnaE [58]. Similar to griselimycin, specificity is determined at the level of the target, as nargenicins are >3000-fold more selective for the *S. aureus* enzyme relative to the *E. coli* counterpart. Synthetic diversification revealed that this specificity can be altered while maintaining potency, with triiodation of a key pyrrole yielding a derivative selective for *Streptococcus* (US Patent No. 10,144,741 B2). Chemical alterations that drive divergent activity spectra can also be achieved through evolution, as the closely related C9—C13 ether macrodilactones are instead selective for anaerobic and microaerophilic bacteria, displaying activity towards *Clostridium*, *Haemophilus*, and *Neisseria* [59] not observed in macrolactones (WO 2015/028094 Al, US Patent. No. 4,148,883). Although the absence of the aforementioned pyrrole in the macrodilactones largely explains their ineffectiveness against *Staphylococcus* [60], it remains unclear how activity towards Gram-positive and -negative anaerobes is achieved.

Arylomycin

As antibacterial natural products have evolved to affect essential biological targets, overlooked molecules with undesirable specificities or efficacies may nonetheless provide useful starting points for further synthetic refinement. This point is exemplified by the development of the arylomycin antibiotics, NRPs that feature limited activity towards a small collection of microbes [61]. Early investigations established arylomycins as inhibitors of bacterial type 1 signal peptidase, an essential and ubiquitous surface-exposed protease [62]. Unlike previously discussed selective antibiotics, the activity spectrum of arylomycins is constrained by a widespread resistant polymorphism within the target, such that an engineered genetic reversion is sufficient to re-sensitize Gram-positive and -negative bacteria [63]. Given their broad-spectrum activities, these latent antibiotics have been the subject of extensive medicinal chemistry campaigns by several pharmaceutical companies [64]. These efforts culminated in the recent development of G0775, a potent arylomycin derivative that overcomes the aforementioned polymorphism and acts as an irreversible inhibitor of signal peptidase [65]. In contrast to the natural product, G0775 is capable of broad-spectrum killing of drug resistant Gram-negative bacteria *in vitro* and *in vivo* with a low frequency of spontaneous resistance, presenting an exceptionally promising lead for future development. As broad-spectrum antibiotic scaffolds are increasingly exhausted by widespread resistance, these overlooked narrow-spectrum molecules present an untapped source of new clinically-relevant leads.

Targeted analog discovery and synthetic diversification

The evolutionarily conserved structure—function relationships in assembly-line NRPS and PKS enzymes can enable accurate, automated predictions of chemical structures, and facilitate the targeted discovery and prioritization of molecules with desired bioactivities [66]. Following the case of griselimycin, insensitive copies of the antibiotic target can often be found in the associated biosynthetic pathway, a feature that has enabled predictive mining of antibiotics with desired mechanisms [67—69] (Table 1). Combining bioinformatic predictions of structural novelty with known antibiotic resistance profiles can facilitate the identification of antibacterial natural products with new activities [70], distinguishing them from more established and related antibiotics [71]. Genome-guided methods can also be used to specifically explore individual families of antibiotics, an approach that can yield new chemical entities that overcome emerging resistance [72] or display uniquely selective activity spectra [73]. Continued efforts to describe the targets and mechanisms of selective antibiotics may offer new insight into how such conversions between broad- [74] and narrow-spectrum [75] molecules may be achieved.

Mirroring the activity-dependent evolution of natural products, medicinal chemistry has been an effective tool for modifying established antibiotics and keeping pace in the race against resistance. As resistance necessitates the exploration of increasingly challenging semi-synthetic modifications to clinically-relevant scaffolds [76], renewed effort towards this problem has recently provided valuable returns through total syntheses. Mechanism and structure-guided investigations have recently yielded effective variants of clindamycins [77], streptogramins [78] and vancomycin [79,80] that bypass specific resistance mechanisms. Given its versatility, it is unsurprising that medicinal chemistry can also yield derivatives of broad-spectrum drugs with significantly more focused activity spectra, such as sarecycline, a modified tetracycline antibiotic approved for selective treatment of *Cutibacterium acnes* [81].

Engineered precision antimicrobials

Engineered antibiotic assembly-lines

The modular nature of NRPS and PKS enzymes, along with the diversity and activity of their products, has led many to view these systems as programmable platforms for microbial chemistry. After decades of enzymatic and structural study, gaps in our understanding of these

complex enzymes are beginning to be resolved, presenting early successes in engineering custom products [82]. Mirroring the biological processes of Darwinian evolution, directed evolution in the laboratory via site directed mutagenesis [83] alongside recombination [84,85] can create products with altered building block specificity [86] or broadened substrate scope [87]. These efforts began by identifying key NRPS residues responsible for diverse substrate selection [88], whereas PKSs are evolutionarily constrained by relatively fewer building blocks that are diversified by dedicated tailoring enzymes [89]. In a striking example of such improvement following engineering, andrimid analogs with up to >25-fold MIC improvement against *S. aureus* were discovered using unbiased mutagenesis and analysis of >14,000 variants [90]. Other recent innovations in domain and sub-domain swapping approaches [91] can now provide assembly line enzymes with tailored end products or biophysical properties [92], highlighting how this general strategy can be used to create new-to-nature biomolecules. With the advent of bioinformatic [93] and genome editing [94] methods to alter NRPS and PKS product structures, diversified analogs can be readily accessed to overcome antibiotic resistance, as demonstrated using novel erythromycin pathway tailoring [95]. While these methods are still being actively developed and remain limited by bioengineering and productivity bottlenecks, future efforts will undoubtedly advance our ability to harness Nature's antibiotic factories to create new structures that selectively address key pathogens.

Engineered RiPPs and peptide pheromone antibiotics

Bolstered by genetically-encoded scaffolds and programmable post-translational modifications, RiPPs have attracted attention as engineerable antibiotics — a topic recently reviewed in detail by Montalbán-López et al. [8]. RiPPs can be readily modified by changing their precursor peptide cores, while alterations to leader peptides can facilitate the recruitment of different modifying enzymes to yield hybrid structures [9]. Site saturation mutagenesis offers a simple method to explore the sequence—function relationships of RiPPs and has been exploited to great effect. Site saturation mutagenesis of the thiocillin BGC enabled the identification of a variant with 8-fold increased potency against *B. subtilis* 168 [96]. A similar approach applied to thiopeptide GE37468 yielded an improved variant with activity against MRSA [97]. Fusion of RiPP-derived sequences can also afford new-to-nature functions, as was shown through fusion of nisin and ripcin-derived sequences to create new lanthipeptides that could potently affect Gram-negative microbes whereas the independent sequences could not [98]. Of the selective antibacterial RiPPs discussed earlier, lasso peptides have attracted significant attention for engineering, owing to their relatively simple biosynthetic pathways, ease of heterologous expression, and well understood mechanisms of action. Using the *E. coli* product McJ25, structure—activity relationship studies have identified a number of mutable positions [99,100] that can be varied to create derivatives with improved activities towards *Salmonella* or sensitive *E. coli* [101]. With increasing insight into the natural mechanism of import, it may be possible to create analogs that co-opt alternative receptors by taking inspiration from homologous peptides [102], or through incorporation of membrane-permeabilizing peptides to re-sensitize activity against resistant cells [103].

Beyond this, genetic code expansion may also be used to improve on RiPP bioactivity through the site-directed incorporation of non-canonical amino acids (ncAAs), enabling further exploration of new-to-nature structures [104]: introduction of naphthylalanine in lacticin 481 improved bioactivity approximately 2-fold against *Lactococcus lactis* [105], and similar results were found with the unrelated ncAA o-NO2-Phe [106]. In fact, related methods for site-directed or proteome-wide ncAA incorporation have been used to create analogs of cyanobactin [107], MccJ25 [104], lichenicidin [108], nisin [109], thiocillin [110], capistruin [111], among others. By combining these approaches, large libraries of engineered RiPPs can be readily generated for use during *in vivo* selections, providing a means of identifying novel structures with desirable activities [112].

Targeted antimicrobials can also be developed by taking inspiration from bipartite antibiotics like sideromycins. Rather than relying on siderophore transporters, pheromone importers can be co-opted for intracellular delivery of antibacterial molecules. This approach has been demonstrated by fusing the *Staphylococcus*-specific AgrD1 RiPP pheromone to the broad-spectrum colicin Ia, thereby creating a bactericidal reagent that selectively targets *S. aureus* but not the related *S. epidermidis* or *Streptococcus pneumoniae* [113]. A similar approach was used to derive *Enterococcus faecalis*-specific antimicrobials by fusing the *E. faecalis* pheromone cCF10 to colicin Ia, killing both naive and vancomycin-resistant *E. faecalis* [114]. Importantly, this general approach can selectively deplete the target pathogenic microbe from a multispecies community, highlighting how rationally engineered antimicrobials may be used to edit the human microbiota [115].

Engineered antimicrobial peptides (AMPs)

Membrane-targeting antibiotics are often capable of surprisingly selective activities, as previously illustrated with the naturally-derived sactipeptides. This phenomenon has been thoroughly explored in synthetic AMPs — short, unmodified peptides that can achieve narrow activity spectra through subtle changes in their primary sequences [116]. Given their simple architecture, AMPs have received considerable attention as engineerable therapeutics, and these efforts have

benefited from their wide distribution in Nature. For example, the engineered peptide MAD1 selectively kills *M. tuberculosis* but not other Gram-positive or -negative microbes. Despite a high tryptophan content (KRWHWWRRHWVVW-NH2) that is not uncommon for promiscuous membrane-disrupting AMPs, MAD1 was engineered *de novo* to selectively bind to membranes rich in mycolic acid, where it is posited to create supramolecular membrane defects as the mechanism of killing [117].

Beyond *de novo* engineered AMPs, multicellular eukaryotes biosynthesize a staggering multitude of AMPs to control invading pathogen infections [118]. While these peptides often show unfavorable properties for clinical applications, they can serve as exceptional starting points for the development of selective antimicrobials. One product of this approach is the promising precision antibiotic murepavadin (POL7080), a cyclic peptidomimetic derived from the mammalian AMP protegrin I [119]. This synthetic cyclic peptide selectively affects *Pseudomonas aeruginosa* through a unique mechanism, binding to the outer membrane lipopolysaccharide (LPS) exporter protein LptD. The targeted microbial spectrum can be altered through amino acid substitutions [120], or broadened through conjugation to the LPS-binding antibiotic colistin, yielding derivatives with potent antibacterial activity against multidrug-resistant Gram-negative pathogens [121]. With the advent of robust high-throughput [122] and computational methods (particularly deep learning), large repertoires of pathogen-selective antimicrobial peptides have been described [123], with many variants showing limited resistance acquisition [124] or enhanced biostability through the rational introduction of D-amino acids to reduce proteolysis [125].

Concluding remarks and future directions

Following the discovery of penicillin, microbial natural products have been an essential source of drug-like molecules for treating infectious disease. In the years following the discovery of this transformative medicine, additional broad-spectrum molecules were adopted (and assumed necessary) as antimicrobial therapeutics. Now, poor stewardship and an overreliance on seemingly exhausted broad-spectrum scaffolds has led to widespread clinical resistance to most — if not all — classes of antibiotics. In some cases, our overreliance on broad-spectrum antibiotics has incentivized the evolution of microorganisms that can rapidly accumulate staggering arrays of resistance cassettes, and future investments into similarly indiscriminate antibiotics is expected to make this phenotype even more prevalent in the clinic.

Despite decades of rigorous discovery and development efforts, the diversity of approved antibiotic scaffolds represents only a fraction of the evolved molecules present in Nature. Echoing the successes in mining natural products that drove the first Golden Age of antibiotics, a renewed effort to understand how Nature accesses precision antibiotics may offer pathogen-specific drugs that can overcome resistance. A revised framework for antibiotic development is now critical, and should mirror how we approach other human maladies: pursuing targeted, context-dependent therapies that take advantage of ever-expanding capabilities in diagnostics and next-generation sequencing. As we continue to realize our symbiotic relationship with microbes and microbiomes, precise methods for bacterial control offer solutions not only in treating infectious disease, but also for the improvement of human and environmental health.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This work was supported by the Cancer Prevention and Research Institute of Texas (CPRIT RR210066 to C.W.J.; C.W.J. is a CPRIT Scholar in Cancer Research), the National Institutes of Health Director's Early Independence Award (DP5-OD-024590 to A.H.B.), and The Scripps Research Institute.

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** of outstanding interest

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