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Novel approaches to discovery of antibacterial agents

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Abstract

Antimicrobial resistance is a rapidly increasing problem impacting the successful treatment of bacterial infectious disease. To combat resistance, the development of new treatment options is required. Recent advances in technology have aided in the discovery of novel antibacterial agents, specifically through genome mining for novel natural product biosynthetic gene clusters and improved small molecule high-throughput screening methods. Novel targets such as lipopolysaccharide and fatty acid biosyntheses have been identified by essential gene studies, representing a shift from traditional antibiotic targets. Finally, inhibiting non-essential genes with small molecules is being explored as a method for rescuing the activity of 'old' antibiotics, providing a novel synergistic approach to antimicrobial discovery.

Keywords: antibiotic resistance, novel targets, genome mining, high-throughput screening, synergy, chemical genetics

Introduction

The development of antimicrobial agents in the 20th century has resulted in a dramatic decline in the number of deaths from infectious diseases. Together with vaccines, antibiotics have had a transformative impact on human and animal health. It seems almost inconceivable that now we may in fact be approaching conditions that are reminiscent of the pre-antibiotic era, at least for certain disease-causing organisms. The great success of these antibiotics has resulted in their widespread use (and misuse), leading to the selection of bacteria that are resistant to many commonly used drugs. The emergence and clinical relevance of methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant enterococci (VRE) in hospitals (Talbot *et al.*, 2006), as well as the spread of MRSA in animals (Leonard and Markey, 2008), have focused ongoing antimicrobial research toward Gram-positive pathogens. In parallel, multidrug-resistant (MDR) Gram-negative pathogens, including *Pseudomonas aeruginosa*, *Acinetobacter baumannii* and *Escherichia coli* in the clinic (Livermore and Woodford, 2006; Talbot *et al.*, 2006) and *Salmonella* spp. in veterinary settings (Dargatz and Traub-Dargatz, 2004), are also increasing in prevalence, requiring new drug development. This review outlines the challenges of antibiotic discovery and the new approaches currently being explored to meet these challenges.

Recap of antibiotic discovery

Over the past 60 years, antibacterial drug development was generally focused on two main strategies: (i) the discovery of compounds from natural sources (in particular soil bacteria of the actinomycetes group and fungi) and (ii) the development of synthetic antimicrobial molecules. Most of the antibiotics still frequently used today were discovered in the middle of the last century, including the β-lactams, aminoglycosides, macrolides, aminocoumarins, tetracyclines, ionophores and glycopeptides and their semisynthetic derivatives. These natural product chemical scaffolds represent the building blocks for the majority of clinically and agriculturally applied antibiotics and are the foundation of modern anti-infective chemotherapy. The rate of discovery of new

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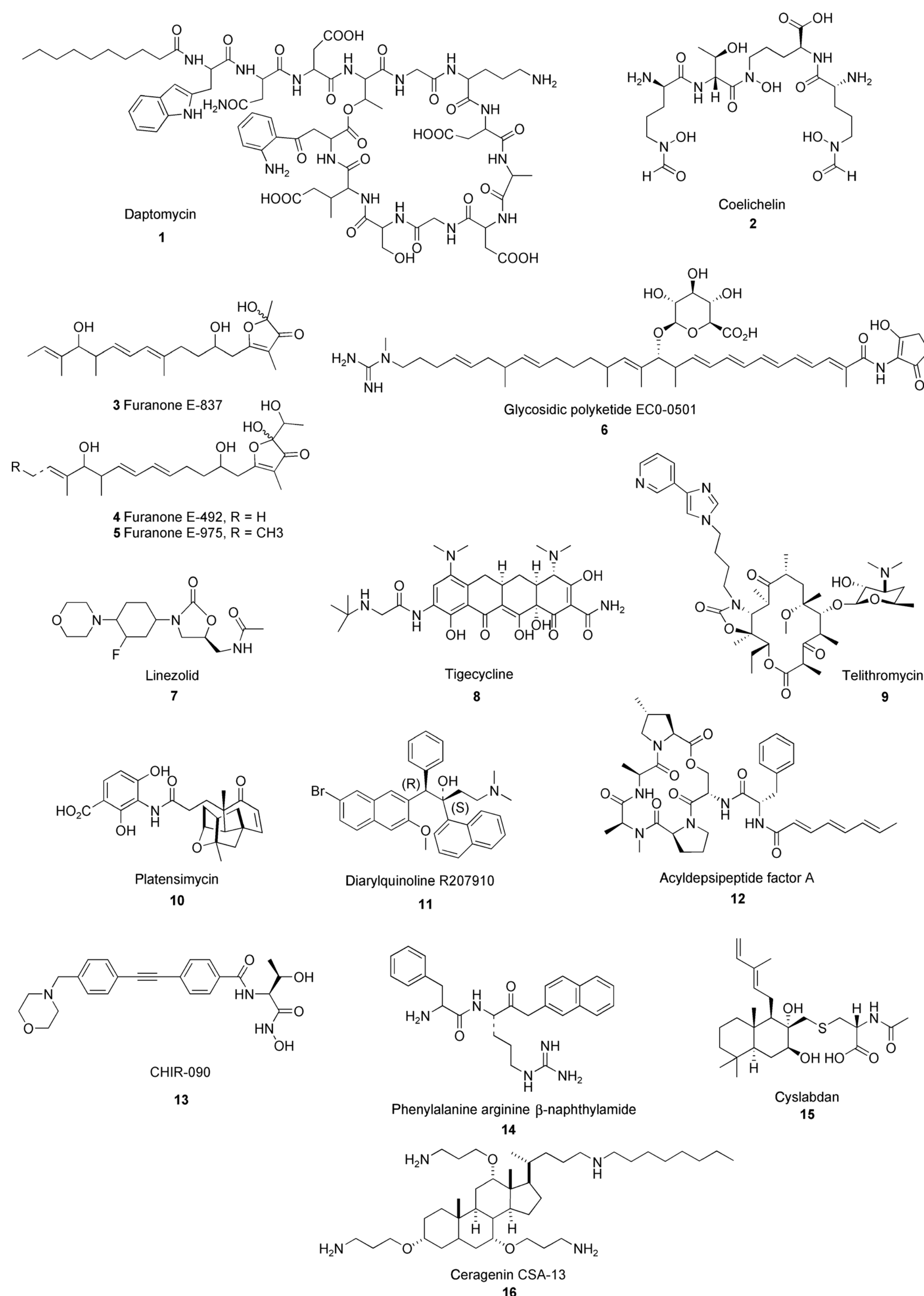

Fig. 1. Chemical structures of novel compounds discussed in this review.

antibiotic chemical classes, however, has been remarkably poor over the past 35 years.

Early in antibiotic development, the challenges of finding novel antibiotics from natural sources, in particular soil bacteria, became apparent. The same chemical classes were re-discovered several times. For example, streptothricin, streptomycin and tetracycline, some of the first natural product antibiotics discovered in the 1940s and 1950s, are respectively produced by approximately 22, 1 and 0.4% of all soil actinomycetes screened for antibiotic activity (Baltz, 2005). In contrast, daptomycin (Fig. 1, compound **1**), discovered in the 1980s, is

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produced in only 0.00001% of all actinomycetes screened (Baltz, 2008). The difficulty in new antibiotic discovery from traditional natural sources thus lies not only in the high volume screening of soil bacteria required to discover novel compounds, but in differentiating the new antibiotics from those already discovered, a task termed dereplication.

There are a number of solutions to this problem. One option is to take advantage of rapid advances in high-throughput small molecule separation and analysis technology (e.g. mass spectrometry), coupled with searchable chemical databases that include mass spectral fingerprints, to rapidly analyze natural product extracts and identify new compounds, while avoiding known ones. In another approach, re-visiting 'old' natural products that were discarded for various reasons (solubility, stability, etc.) during the Golden Age of antibiotic discovery (1940–1960) is warranted. Libraries of such known molecules can be rich sources of novel activities and, in the case of daptomycin (Baltz *et al*., 2005), can be brought to the clinic. Expansion of the screening natural products from bacteria from new sources can also identify new chemical scaffolds. Marine actinomycetes, for example, have recently been shown to produce several new classes of compounds (Fiedler *et al*., 2005; Fenical and Jensen, 2006). Finally, whole genome sequencing of actinomycetes has remarkably revealed that many of these organisms have the genetic programs to produce a large number (>20) of secondary metabolites (Baltz, 2008). Further sequencing of metagenomic libraries from total DNA isolated from environmental sources is also revealing new biosynthetic gene clusters. Some of these may program for compounds with antibiotic activity and, as discussed below, activation of the 'cryptic' gene clusters has already, in fact, resulted in new compounds.

The gene cluster encoding coelichelin (Fig. 1, compound **2**) biosynthesis was the first novel secondary metabolite to be discovered entirely using genetic analysis techniques. A search of the *Streptomyces coelicolor* genome sequence has successfully identified a new non-ribosomal peptide synthetase (NRPS) based on homology to known NRPS motifs (Challis and Ravel, 2000). Sequence analysis predicted coelichelin to have a tripeptide structure with siderophore activity. Purification of coelichelin from culture supernatant confirmed the production of a tetrapeptide by a trimodular NRPS (Lautru *et al*., 2005). Siderophores chelate ferric iron from the environment and shuttle it into the bacterial cell. In addition to providing a proof of principle for genome mining in natural product discovery, these natural products are of interest to the infectious disease community for two reasons. First, since iron uptake is essential to bacterial cell survival, the siderophore biosynthetic pathway is an attractive new target for antibiotics. Second, siderophores can be conjugated to antibiotics and recognized by uptake systems, delivering the antibiotic into the cell. Naturally occurring siderophore–antibiotics have been isolated, such as the albomycins, ferromycins and danomycins. These compounds show weak antibiotic activity leading to the development of synthetic siderophore–antibiotic combinations (Miethke and Marahiel, 2007).

Ecopia Biosciences developed a high-throughput method to scan entire genomes for unique gene clusters. Short random genome sequence tags (GSTs) are generated using shotgun sequencing of a genomic DNA library and compared with databases of known bacterial biosynthetic gene clusters. GSTs flagged as potential matches to genes of interest are then used to design screening probes to identify entire gene clusters from cloned subgenomic DNA fragments (Zazopoulos *et al*., 2003). This method has identified over 450 previously unknown actinomycete natural product biosynthetic gene clusters, including two responsible for the synthesis of novel furanones that inhibit electron transport (Fig. 1, compounds **3–5**) (Banskota *et al*., 2006a), and one encoding genes for the production of a glycosidic polyketide (Fig. 1, compound **6**), a novel compound class with bacteriocidal activity against a number of Gram-positive pathogens (Banskota *et al*., 2006b). The implementation of these genome mining techniques has been key to the recent resurrection of natural products as a source of novel antimicrobial agents.

An alternative solution to natural product discovery is the development of completely synthetic antibiotics. Success was found early in the creation of the sulfonamides in the 1930s, followed by the fluoroquinolones and the folate biosynthesis inhibitor trimethoprim in the 1970s. Recently, a new class of synthetic antibiotic, the oxazolidinones, represented by the first-in-class drug linezolid (Zyvox®) (Fig. 1, compound **7**), marks the possible re-emergence of synthetic chemistry as a viable option for novel antibiotic development (Brickner *et al*., 2008). The challenge in the use of synthetic compounds as antibiotics is not in identifying molecules that are sufficiently toxic to the pathogen, as screens of synthetic chemical libraries yield many hits, but in specificity of the compound to the target and toxicity to the host. One reason for these problems is that current synthetic libraries available were not prepared for use as antibiotics, but rather tend to follow general drug-like property guidelines, such as Lipinski's rules for orally available drugs. Efforts to develop similar structural criteria for small molecule antibiotics (O'Shea and Moser, 2008) could improve success in this area. Peptides, including cationic peptides, are another class of synthetic molecules inspired by natural host immunity compounds that have significant promise (Sitaram and Nagaraj, 2002; Hancock and Sahl, 2006). The limited number of new antibacterial agents that have emerged over the past decade, from both synthetic and natural product small molecule screening initiatives highlights the need for novel approaches to antibiotic discovery.

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Table 1. Summary of essential genes identified from various genomes

Organism	Total number of Open Reading Frames	Number of essential genes	% essentiality	Reference
Escherichia coli K-12	4288	303	7.1	Baba *et al.* (2008), Gong *et al.* (2008)
Bacillus subtilis	4101	271	6.6	Kobayashi *et al.* (2003)
Staphylococcus aureus	3117	168	5.4	Forsyth *et al.* (2002)

Antibiotic targets

Six major antibiotic targets have been identified and exploited: ribosome assembly and action (aminoglycosides, macrolides and tetracyclines); cell wall stability and biosynthesis (β-lactams and glycopeptides); DNA replication (aminocoumarins and fluoroquinolones); DNA transcription (rifamycin); membrane integrity and biosynthesis (ionophores and lipopeptides), and folic acid biosynthesis (sulfonamides and trimethoprim) (Walsh, 2003). Even the most recently approved new classes of antibiotics fall within these six categories (Fig. 1). Linezolid is predicted to interfere with tRNA positioning on the ribosome, disrupting protein synthesis (Wilson *et al.*, 2008), and daptomycin is a membrane-active agent (Baltz *et al.*, 2005). Tigecycline (Fig. 1, compound **8**) and telithromycin (Fig. 1, compound **9**) are semisynthetic derivatives of the tetracycline and macrolide classes of translation inhibitors (Ackermann and Rodloff, 2003; Fraise, 2006; Townsend *et al.*, 2006). These two compounds demonstrate that structural modifications of known antimicrobial chemical scaffolds remain a good source of new generation antibiotics, often with improved pharmacological properties and the ability to overcome existing resistance mechanisms. However, this strategy may be reaching its limits in several classes (e.g. β-lactams and fluoroquinolones), and for this reason, new compound classes with completely different structural scaffolding are currently being sought. Expanding the current target arsenal beyond these six areas in particular is an area of intense activity.

Target-based approaches to drug discovery require that the target is well known and characterized before the search can even begin. For example, dihydrofolate reductase is a well-characterized enzyme and a known antimicrobial target. A recent high-throughput screen of 50,000 small molecules against this enzyme yielded nine previously unidentified inhibitors of unique structure, with potential for development into therapeutics (Zolli-Juran *et al.*, 2003). However, clinical resistance to trimethoprim has been observed when *Enterococcus* spp. incorporate exogenous folates to complete nucleic acid and amino acid synthesis (Wisell *et al.*, 2008). Thus, new inhibitors of this enzyme may provide little gain in terms of combating this form of resistance. While this strategy would seem to be the most straightforward and many screens have successfully yielded hits from this approach, target-based screening needs to be improved upon for the expansion of known antibiotics.

New targets from genomics

The influence of genomics was previously discussed in the discovery of novel biosynthetic gene clusters from secondary metabolite producers. The ability to easily sequence entire bacterial genomes also presents a new opportunity to identify novel antimicrobial targets in pathogens, through the identification of essential genes in various genomes. Here we define an essential gene as one that upon deletion or attenuation results in inhibition of cell growth. This is a functional description that, for the most part, has been explored using rich media and optimal growth conditions in the laboratory, rather than in infection models. As a result, this may be too rigorous a definition for the identification of worthy antibiotic targets. It is worth noting, however, that using these criteria, outstanding targets, such as the penicillin binding proteins in cell wall assembly, may not be included in such lists due to functional redundancy. Nevertheless, gene disruption and deletion studies, promoter replacement, over-expression studies, random transposon footprinting and transcription arrays have all been used to help identify essential genes, each of which, in theory, is a viable antibiotic target (Brown and Wright, 2005). Genetic studies in *E. coli*, *Bacillus subtilis* and *S. aureus* have revealed essential gene sets for each of these organisms (Table 1) (Forsyth *et al.*, 2002; Kobayashi *et al.*, 2003; Baba *et al.*, 2008; Gong *et al.*, 2008). In addition, comparative genomic studies have identified essential genes that are widely conserved among pathogens or divergent from eukaryotic genomes, additional important criteria for an antibiotic target of broad application.

In the modern target-based paradigm, the production of the essential gene product, usually an enzyme or a receptor, is followed by the development of an assay suitable for high-throughput compound screening and subsequent identification of small molecules that bind the target (Brown and Wright, 2005). These compounds serve as leads in iterations of chemical synthesis to improve target affinity and drug-like properties for further validation in downstream whole-cell assays and animal models of infection. Despite being an active area of research for the past decade, this approach is yet to result in new antibiotics. Here the published experience of the antibacterial group at GlaxoSmithKline (GSK) is worthy of summary (Payne *et al.*, 2007). Over a 7 year span, 70 different high-throughput screening initiatives (67 target-based, three whole cell) using 260,000–530,000 small

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molecules resulted in only five lead compounds. None of these leads made it through clinical trials. GSK has since adapted its antibacterial research approach to focus on novel chemical structures, rather than targets, with broad-spectrum activity both *in vitro* and *in vivo* that could be engineered to meet pharmaceutical requirements. This strategy has limited discovery efforts to improving known antibiotics, screening limited compound libraries and rational design and has resulted in six leads for further development. One of the main challenges identified by GSK in creating a drug out of a lead, however, is cell permeability. Target-based screening fails to assess whether or not a given hit will be active *in vivo*. The added complexity of the outer membrane (OM) permeability barrier and efflux-mediated resistance in Gram-negative bacteria further decrease the likelihood that a hit will be bioactive. This shortcoming has been instrumental in the current shift away from traditional target-based high-throughput screening.

Chemical genetics: linking lead compounds with the genome

Genomic studies have also enabled a chemical genetics approach to drug discovery. As noted above, the target-based discovery pathway is governed by high-throughput screening approaches where small molecules are screened against single enzymes or pathways *in vitro* to find novel inhibitors or ligands. Due in large part to the explosion of genomic knowledge, small molecules can now be used to simultaneously identify lead compounds, determine target gene and gene product function, and validate these targets by 'synthetic deletion' of the gene. In this new paradigm, high-throughput screening is now employed in whole cells using a forward chemical genetics approach, where the desired phenotype is used to identify the gene/protein of interest. The advantages of this approach are four-fold. First, screening is not limited to single targets, but rather in principle every gene and gene product within the cell. Second, upstream and downstream cellular effects, which would have been impossible to identify in a target-based screen, become apparent. Third, cell viability is immediately assessed, and finally active compounds are instantly validated as cell-permeable, as a compound that cannot access the cell is omitted by the very nature of the screen. The challenge of this approach emerges in trying to precisely identify the target. In many cases, the compound may not have one target, but work against multiple proteins, to effect processes upstream or downstream from the actual target that can be difficult to trace, or work in series against a combination of targets to weaken the cell.

Recent studies have identified novel antimicrobial compounds and their targets in this manner. Platensimycin (Fig. 1, compound **10**), the first in a new class of antibiotics, was discovered by screening 250,000 natural product extracts in a whole-cell assay targeting bacterial type II fatty acid biosynthesis (FASII) (Young *et al*., 2006). The FASII pathway is essential to bacterial cell viability and is markedly different from the corresponding human system, making it an attractive target for novel antibiotics. Essential and conserved enzymes in this pathway include the initiation condensing enzyme (FabH), elongation condensing enzymes (FabF/B) and the enoyl-reductase (FabI) (Young *et al*., 2006; Lu and Tonge, 2008). To target this pathway, specifically FabF and FabH, a two-plate differential sensitivity assay was developed, where one agar plate was inoculated with *S. aureus* strains expressing *fabF* antisense mRNA (AS-RNA) and the other with control cells lacking AS-RNA expression. AS-RNA expressing strains exhibited greater sensitivity to FASII biosynthesis inhibitors due to reduced production of FabF and FabH. Hits were identified based on growth inhibition of the AS-RNA expressing strain compared with the control strain. Platensimycin was isolated from one of the extracts screened and follow-up biochemical assays confirmed that the compound inhibits both FabF and FabH. Further analysis shows that platensimycin has activity against multidrug-sensitive and -resistant Gram-positive pathogens with no observed host toxicity, making it a viable candidate for drug development (Wang *et al*., 2006).

Whole-cell screening using a dual-reporter assay has resulted in a viable inhibitor of bacterial cell division, an essential process that remains untargeted by any clinically available antibiotics (Stokes *et al*., 2005). This compound was found to inhibit FtsZ, a protein involved in the first step of bacterial cytokinesis, and resulting analogues show promising antimicrobial activity. FtsZ has been the focus of most drug discovery programs targeting cell division with limited success prior to this study (Lock and Harry, 2008); however, these recent results reaffirm cell division as a novel antibacterial target worth pursuing.

Another study identified a diarylquinoline (Fig. 1, compound **11**) that potently inhibits both drug-sensitive and drug-resistant *Mycobacterium tuberculosis* by whole-cell screening without a known target (Andries *et al*., 2005). This compound inhibits the F_0 subunit of ATP synthase and the mechanism of action was determined by whole genome sequencing and comparison of resistant mutants with sensitive wild-type bacteria. This study provides a powerful example of how rapidly evolving technology, in this case high-throughput genome sequencing, can be harnessed for antibiotic drug discovery.

The recent discovery of a new class of antibiotics, acyldepsipeptides (ADEPs) (Fig. 1, compound **12**), is an example of how genomics can be used to reclassify compounds (Brotz-Oesterhelt *et al*., 2005). ADEPs were originally isolated from *Streptococcus hawaiiensis* in 1985, with unknown antibiotic action. The need for new antimicrobial therapies led to the re-evaluation of this compound and by generating a genomic library of an ADEP-resistant mutant, ADEP was shown to activate the bacterial protein ClpP, part of the Clp protease complex.

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ClpP on its own is inactive in the absence of Clp ATPase, but in the presence of ADEP, ClpP gains unregulated proteolytic function, resulting in proteolysis of cytoplasmic proteins and cell death.

These studies demonstrate the power of combining traditional compound screening with emerging genomic knowledge to identify potential antibiotics, as well as novel targets.

Compound combinations and synergy

While genomics and chemical genetics programs continue to identify new targets, some innovative approaches to expanding the utility of 'old' antibiotics and uncovering untapped potential of bioactive compounds have recently become the focus of antimicrobial research teams. Combinations of known antibiotics have been applied for decades to combat highly resistant pathogens, specifically against *M. tuberculosis*, where drug regimes range over months, using two to six different antibiotics at a time. The most famous example of compound synergy is between amoxicillin and clavulanic acid (White *et al.*, 2004; Ball, 2007). Amoxicillin, a semisynthetic β-lactam, was discovered to be susceptible to deactivation by β-lactamases through cleavage of the β-lactam ring. Screening programs later discovered that clavulanic acid, itself a weak antibiotic, is able to inhibit β-lactamases; the β-lactam ring binds irreversibly without cleavage. Though clavulanic acid itself is not a marketable drug, in combination with amoxicillin, this drug combination, known as Augmentin®, provides broad-spectrum antimicrobial activity possible only in combination. Not even Augmentin® has escaped the onslaught of drug resistance, as clinical isolates showing clavulanic acid–amoxicillin resistance continue to increase (Oteo *et al.*, 2008).

Using small molecules to target non-essential genes in combination with known, weak antibiotics is one strategy currently being explored to expand the number of available drug targets. For example, drug development campaigns have targeted the lipopolysaccharide (LPS) biosynthetic pathway for novel methods to combat Gram-negative bacteria. LPS constitutes the outer leaflet of the Gram-negative OM and is composed of an invariant inner core consisting of lipid A linked to 3-deoxy-D-*manno*-octulosonic acids. Heptoses are grafted to this core component, followed by genus- and species-specific hexose units, which are in turn decorated with the O-antigen, a long glycan polymer that plays a role in cell adhesion and virulence (Raetz and Whitfield, 2002). The explosion of knowledge regarding the structure and biosynthesis of LPS molecules over the past decade has led researchers to pursue components of LPS, such as lipid A and heptoses, as unique drug targets (Yethon and Whitfield, 2001). Lipid A is required for assembly of the OM and many of the components of the lipid A biosynthetic pathway are well characterized and essential to cell survival.

Following the paradigm of target-based drug discovery outlined above, lipid A biosynthetic enzymes therefore make ideal drug targets as they are both essential and absent from eukaryotic cells. For example, LpxC is a zinc-dependent amidase that catalyzes the second step in lipid A biosynthesis. Inhibitors of this enzyme have recently been discovered and, in the case of CHIR-90 (Fig. 1, compound **13**), show antimicrobial activity against both *P. aeruginosa* and *E. coli* (Barb *et al.*, 2007). These inhibitors represent a scaffold upon which future drugs can be built against this novel target.

An unexplored approach is to target non-essential LPS biosynthetic pathways, for example biosynthesis of the unique heptose units. Heptose biosynthesis fails the essentiality test in the laboratory setting as outlined above; nevertheless heptose mutants generate a highly drug susceptible phenotype as a result of impaired LPS structure (Valvano *et al.*, 2002; Loutet *et al.*, 2006). The logic in this strategy is that compounds that inhibit the function of any of the enzymes in this pathway could be used to potentiate hydrophobic antibiotics that otherwise would have little activity toward Gram-negative pathogens. A recent *in vitro* screen of the ADP-heptose pathway resulted in identification of the first documented inhibitor of this pathway; however, *in vivo* studies failed to show bioactivity (De Leon *et al.*, 2006). While recent reports have added to the mechanistic knowledge of these enzymes (McArthur *et al.*, 2005; Taylor *et al.*, 2008), further screening on the ADP–heptose biosynthetic pathways is necessary to probe these highly promising antimicrobial targets.

Efflux proteins are another example of a barrier to antibiotic activity. These proteins span the inner phospholipid membrane to actively transport toxins and antibiotics out of the cell. There are five families of efflux proteins related to antibiotic resistance: the ATP-binding cassette (ABC) superfamily, the major facilitator superfamily (MFS), the multidrug and toxic-compound extrusion (MATE) family, the small multidrug resistance family (SMR) and the resistance-nodulation-division (RND) family (Pages *et al.*, 2005; Piddock, 2006). RND pumps are most often associated with Gram-negative bacterial resistance and form a tripartate system, where the pump itself is linked to two other proteins, which respectively span the periplasmic space and the OM, creating an export channel (Marquez, 2005). *P. aeruginosa* utilizes six active RND pumps: MexAB-OprM, specifically active against β-lactams and fluoroquinolones, is constitutively expressed, whereas the remaining five pumps are upregulated in the presence of antimicrobial substrate (Rice, 2006). In *E. coli*, the major RND pump is ArcAB-TolC, which transports a wide spectrum of antimicrobials, including intrinsic resistance to macrolides, β-lactams, fluoroquinolones and tetracyclines, resulting in resistance when the proteins are over-expressed (Piddock, 2006). Given their role in Gram-negative antimicrobial resistance, efflux pumps have been identified as a novel target for new antimicrobial action.

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There are numerous avenues available to diminish the activity of an RND efflux pump. These include blocking the OM protein channel, introducing competing molecules for the inner membrane pump, removing the pump's source of energy, and physically altering the pump itself (Pages *et al*., 2005). To date, a number of efflux pump inhibitors (EPIs) have been discovered. Thioderivatives of tetracycline compete with tetracycline itself to inhibit the TetB efflux pump. L-Phe-L-Arg-β-naphthalamide (PAβN) (Fig. 1, compound **14**) has been shown to potentiate fluoroquinolones in *P. aeruginosa* and *E. coli* and similar results have been found for arylpiperazines in *E. coli* (Piddock, 2006; Rice, 2006; Mahamoud *et al*., 2007). Screening of natural product libraries has also identified potential RND EPIs, including extracts from *Streptomyces*, which inhibit MexAB-OprM in *P. aeruginosa* (Stavri *et al*., 2007). So far, no EPIs have been approved for treatment.

Several challenges exist in discovering EPIs (Lomovskaya and Bostian, 2006). Broad-spectrum inhibitors will be difficult to obtain, given the structural and specificity differences that exist between pumps, not only in different species, but also within a single organism. Ideally, EPIs could be administered with the antimicrobial they potentiate, to maximize the efficiency of the treatment. However, EPIs would not compensate for other resistance mechanisms that may already be present within the cell. Before EPIs become mainstream antimicrobial therapies, RND pumps need further characterization, such that the development of EPIs is more directed.

Whole-cell screens that demonstrate potentiation of hydrophobic antibiotics have the potential to identify new permeabilizing agents. Recent studies suggest, though, that inhibiting non-essential gene targets in combination with known antimicrobials can greatly enhance efficacy. Cyslabdan (Fig. 1, compound **15**), an actinomycete-produced natural product, was identified as a potentiator of imipenem activity against MRSA, using synergy-based screening methods (Fukumoto *et al*., 2008). Similarly, Khalil *et al.* showed that polyethylenimine was not bacteriocidal toward resistant clinical strains of *P. aeruginosa*, but significantly reduced the MICs, as much as 56-fold, of other known antibiotics when used in combination (Khalil *et al*., 2008). Finally, erythromycin has been traditionally ineffectual against Gram-negative pathogens owing to its inability to permeate the OM. Saha *et al.* showed that the MIC of erythromycin was reduced 92% in the presence of ceragenin (Fig. 1, compound **16**), a synthetically produced cholic acid derivative known to permeabilize the OM (Saha *et al*., 2008).

Another non-essential pathway currently being targeted by the drug discovery community is bacterial virulence. While these genes are required for interaction with, and therefore infection of the host, they are not usually essential to bacterial survival. Adhesion and colonization mechanisms, such as pili formation and type III secretion systems, toxin productions pathways, biofilm formation and quorum sensing, are all virulence mechanisms with potential for therapeutic intervention (Cegelski *et al*., 2008; Escaich, 2008). Quorum sensing, especially in the opportunistic pathogen *P. aeruginosa*, has been shown to be involved in the regulation of virulence factors responsible for pathogenicity. Whole-cell studies have demonstrated that in addition to inhibiting production of virulence factors, quorum sensing inhibitors can also increase susceptibility of biofilm bacteria to traditional antibiotic therapies. Screening attempts to discover quorum sensing inhibitors have resulted in identification of several novel compounds, although none are suitable for human use at this time (Rasmussen and Givskov, 2006).

A discussion on the inhibition of non-essential genes allows for a second, intriguing development in identifying novel antibacterial targets: the creation of conditional essential genes by synthetic lethality. The definition of an essential gene in an organism would be altered in this case to one that upon deletion or attenuation results in inhibition of cell growth, when a second gene has already been deleted or attenuated. Using this theory, the number of drugable targets in a cell can be increased well beyond the original number of essential genes to include the targets of any combination of small molecules that result in cell death. Studies in model organisms to elucidate the double deletion mortality profile are already under way (Butland *et al*., 2008).

Conclusions

There is little doubt that with the discovery of each new antimicrobial agent, a new resistance mechanism will follow soon after. By relentlessly probing and discovering both new antimicrobials and new targets, it is possible to continue to stay ahead in this never-ending race for survival. Ironically, one of the major problems facing antimicrobial drug discovery is not a lack of avenues to pursue. Even though bacterial infection remains one of the top causes of death worldwide, pharmaceutical companies often place low priority on antimicrobial research and development, for both scientific and business reasons, as seen in the GlaxoKlineSmith case study. New therapeutic options will only continue to present themselves, as demonstrated in this review, as long as researchers continue to search them out. That being said, the roadblocks to novel antibiotic discovery are numerous and daunting. The limited number of new drugs to reach the clinic over the last 30 years speaks volumes about the difficulty of successfully taking a compound from hit to drug. This review serves to showcase some of the creative ways in which groups are tackling a problem that will have global repercussions. Novel screening methods, made possible by advances in genomics high-throughput screening, represent the cutting edge of drug discovery. LPS biosynthesis inhibition and efflux pump inhibition demonstrate new, and viable, targets to combat

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Gram-negative infection, while small molecule combination therapies are continually under development. Thinking outside of traditional practices and definitions, such as what makes an essential gene, could be a turning point in how compound screening is conducted in the future and could very well pull antimicrobial drug discovery out of its current recession.

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