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Strategies for target identification of antimicrobial natural products

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Abstract

Despite a pervasive decline in natural product research at many pharmaceutical companies over the last two decades, natural products have undeniably been a prolific and unsurpassed source for new lead antibacterial compounds. Due to their inherent complexity, natural extracts face several hurdles in high-throughout discovery programs, including target identification. Target identification and validation is a crucial process for advancing hits through the discovery pipeline, but has remained a major bottleneck. In the case of natural products, extremely low yields and limited compound supply further impede the process. Here, we review the wealth of target identification strategies that have been proposed and implemented for the characterization of novel antibacterials. Traditionally, these have included genomic and biochemical-based approaches, which, in recent years, have been improved with modern-day technology and better honed for natural product discovery. Further, we discuss the more recent innovative approaches for uncovering the target of new antibacterial natural products, which have resulted from modern advances in chemical biology tools. Finally, we present unique screening platforms implemented to streamline the process of target identification. The different innovative methods to respond to the challenge of characterizing the mode of action for antibacterial natural products have cumulatively built useful frameworks that may advocate a renovated interest in natural product drug discovery programs.

1 Introduction

Nature has provided an unparalleled source of small molecules that have played and continue to play a prominent role in medicine. Approximately two-thirds of clinically used antibacterial therapies are derived from natural products[1]. This success can be attributed in most part to unique properties that have been honed by evolutionary processes to provide the producing organism a selective advantage. Such privileges as intrinsic cell permeability, vast chemical diversity and target specificity[2] are often absent in synthetic chemical compound libraries. Nevertheless, following the revolutionary discoveries of most of the antibacterial drug classes known today, major pharmaceutical companies have almost completely diminished their focus on natural products in the last two decades[3]. To blame are the inherent complexities of natural product drug discovery. Efforts are constrained by the challenges of rediscovery of known scaffolds following laborious purification and characterization, low compound availability and inevitable false-positives due to interference by other substances in extracts[3]. The rationale for natural product discovery programs was further challenged by the advent of high-throughput screening in the 1990's, which revealed that natural product discovery could not keep pace with the fast turnaround in screening synthetic chemical libraries. Ironically, new advancements in modern drug discovery efforts investigating synthetic chemical libraries have had a tremendous impact on natural product-based discovery and have been the driving force for novel technological advances to overcome common technical barriers. For instance, laboratory automation has lead the way for the generation of large libraries of pre-purified fractions or extracts better suited for screening in high-density formats. Further, with means for more rapid and efficient strategies to track

bioactivity[4, 5] and strategies for efficient dereplication of known molecules[5, 6], natural product-based discovery is poised to make a comeback. This comes at a most trying time, when the rapid onset of resistance and thus diminishing effectiveness of antibiotics, are at an all time high.

Despite remarkable achievements in the development of antibacterial natural products, a major bottleneck in the drug discovery process remains target identification[7]. An accurate evaluation of mechanism of action (MOA) is a vital part in the discovery and development of drugs and often the decisive step in both academic and pharmaceutical research. Identifying the full spectrum of targets associated with a bioactive small molecule can lead to faster optimization, help identify unwanted off-target side effects, thus allowing the ability to minimize possible toxicities early in the discovery process[8]. Over the years, several new target identification strategies have been developed and the number of successful examples steadily grows. The lack of generic methodology that can be widely applied to the majority of cases, however, has yet to be established. Furthermore, in the case of natural product drug discovery, an added challenge is low compound availability, hindering the use of several target identification strategies. Here we provide an overview of common approaches for target identification and present validations that historically were most successful for well-known antibacterial natural products. We discuss the techniques currently available to characterize newly identified natural products. We also present the most modern approaches for target identification, first suited and tested for synthetics, that have inevitably inspired the natural product discovery paradigm and provide several representative examples illustrating the state-of-the-art. Finally, we discuss innovative screening approaches that may shape the future

prospects of natural product drug discovery. Growing appreciation of functional assays for target identification will ultimately further contribute to the revival of interest in revisiting natural products for antimicrobial drug discovery[9].

2 Target identification, a look back

Historically, natural products were discovered based on their desired phenotypic effects at the cellular level and relevant protein targets identified using *ad hoc* approaches. Typically, these involved genetic or biochemical strategies. The former approach lends to the concept that identification of a gene(s) causing a resistance phenotype may lead to the target of the small molecule. Although identifying and characterizing drug-resistant clones can be simple and powerful in identifying target, it is typically limited to model microbial systems and may not always succeed as there are multiple ways resistance can arise to a drug. Nevertheless, this approach has proven useful in identifying the target of various well-known natural products. In the case of rifampicin, for example, it was detection of mutations in the *rpoB* gene that encodes the β-subunit of RNA polymerase that revealed its cellular target[10]. For novobiocin, its targets gyrB and parE were also revealed by sequencing analysis of coumarin-resistant mutants[11]. Biochemical strategies, on the other hand, are more cumbersome and generally involve isolating the proteins that directly bind the molecule of interest. Pioneering work in affinity purification involved monitoring chromatographic fractions for enzyme activity after exposure of cell lysates to compound immobilized on a solid matrix[12]. Following elution, bound proteins are analyzed and characterized. This not only requires large amounts of extracts, possibly prefractionated, but also requires chemical modification of the molecule in question, which in turn entails an understanding

of its structure-activity relationship. Although powerful, these methods are best suited for high-affinity ligands that bind relatively abundant target proteins. The success of affinity purification is best represented by the classic pull down of penicillin-binding proteins, the group of enzymes involved in cross-linking of bacterial cell wall, as the targets of the naturally produced β-lactam antibiotics.

Macromolecular assays also represent customary methods for the process of target identification. Here, the effect of newly identified antibacterial compounds on the synthesis of macromolecules is assessed by monitoring the incorporation of radiolabeled precursors into major biosynthetic pathways. Typically, these measurements determine whether a compound specifically inhibits DNA, RNA, protein, or cell wall biosynthesis. While informative and used for years by the pharmaceutical community, macromolecular assays have drawbacks. In some cases, the assay loses its utility when dealing with compounds acting via novel mechanisms, as it reports on only a small fraction of potential MOA's. To that end, however, macromolecule assays can be used to report on off-target effects[13]. Another advantage is that the assay distinguishes compounds that affect all processes simultaneously, likely working by non-specific mechanisms. A classic example of the successful use of macromolecular analysis is with the naturally-produced lipopeptide, daptomycin. Analysis of its effect on macromolecular synthesis revealed a small decrease in peptidoglycan synthesis and a greater effect on the incorporation of radiolabelled precursor for lipid biosynthesis[14], uncovering daptomycin's ability to disrupt multiple functional aspects of the cell membrane. The MOA of many more natural products with antibacterial activities have been deduced from similar assays[14-16]. Overall, although macromolecular assays suffer from low

resolution and throughput, they are good starting points in investigating the mode of action of novel compounds.

3 'Old school' methods with modern improvements

The traditional 'old school' method of selecting mutants able to grow in the presence of a lethal concentration of a newly identified natural product remains a powerful target identification strategy. With the advent of next-generation sequencing (NGS), the task of mapping drug-resistant mutations following sequencing and annotation of microbial genomes is now not only comprehensive in nature but also rapid and inexpensive (Table 1) (Fig. 2a). NGS can deliver accurate genome information through platforms that perform massively parallel sequencing, during which millions of fragments of DNA from a single sample are sequenced in unison[17]. Given bacterial genomes are small, many strains, such as isogenic-sensitive and -resistant mutant strains can be sequenced per run and in the timescale of less than one day. Recent work on the naturally-produced antimycobacterial compound, pyridomycin, illustrates the staying power of genomics in target identification with the added technological advancement of NGS. Examination of pyridomycin-resistant mutants of *Mycobacterium tuberculosis* by whole-genome sequencing and subsequent genetic confirmation identified InhA, the NADH-dependent enoyl-acyl carrier protein reductase, as the principal target of pyridomycin[18]. In recent years, similar approaches have continued to link newly identified antibacterial natural products to their cellular targets[19-21]. In some instances, resistant mutants can inform on more than just target. An example lies in the recently discovered arylomycins and related lipoglycopeptides, which are natural product antibiotics that inhibit bacterial type I signal peptidases (SPases)[22]. Sequencing of

resistant mutants revealed that specific SPase mutations were analogous to mutations that are naturally present in many bacteria, explaining its originally perceived narrow spectrum of activity[21]. This analysis inspired the identification of various bacterial species lacking SPases with these mutations and showing sensitivity to arylomycins, therefore expanding the known activity spectrum of the arylomycins[21]. Although whole-genome sequencing can lead to a quick path to a predicted target, in some cases it is often not possible to generate mutants resistant to a drug in question. In other cases, sequencing of resistant mutants reveals other pathways of resistance, distinct from those attributed to specific mutations in target genes. For instance, multidrug efflux pumps and transporters can be overexpressed due to mutations in regulatory regions[23]. Antibacterials can also induce the expression of multidrug resistance efflux pumps by interacting with regulatory systems. For example, in the presence of the common natural product, tetracycline, TET-specific pumps possess regulatory controls that sense the presence of antibiotic and thereby act as an inducer[24], leading to increased levels of drug resistance. In these cases, mapping of resistant mutants may lead to efflux genes instead of drug target genes. Nevertheless, whether sequencing using next-generation technology or standard benchtop cloning approaches, the genetic approach is often able to identify the molecular target of an antibiotic, including the specific amino acid residues important for its interaction. The low compound requirement and simplicity of this approach lends to its staying power in identifying the target of novel natural products.

Modern-day affinity chromatography experiments no longer rely on traditional techniques for its effectiveness. Indeed, the methodology has undergone continual improvements, for example in affinity reagents[25] or analytical frameworks, such as

quantitative and high-resolution mass spectrometry (MS) analysis[26] (Fig. 2b). Considerable efforts have been dedicated to develop new and varied applications of affinity purification and such methods have been comprehensively reviewed in[27]. In recent years, modern strategies of affinity purification have shed light on the molecular targets of the natural product, vancomycin. Affinity chromatography studies revealed direct interaction of immobilized vancomycin derivatives with several membrane proteins involved in peptidoglycan assembly from cell lysates, suggesting the existence of discrete target enzymes[28]. These were later identified as bacterial transglycosylases[29]. Proteomic profiling has also revealed the specific labeling of two previously unknown vancomycin targets that are likely to contribute to its antibiotic activity. Indeed, vancomycin-based affinity probes allowed Eirich *et al.* to identify the major staphylococcal autolysin Atl and an ABC transporter protein as novel interaction partners[30]. Further, a uniquely synthesized vancomycin photo-affinity probe allowed to capture for the first time vancomyin's direct binding to the VanS receptor, which activates the transcription of vancomyin resistance genes, *vanHAX*[31]. Although several key molecular targets have been identified through affinity chromatography[32], it has not been widely applied as a general solution to target identification (Table 1). The main challenge is the preparation of compound to be immobilized or the preparation of affinity reagents that retain the desired cellular activity. Both cases are especially limited for natural products where chemical modifications are not trivial and structures may lack functional handles allowing the generation of affinity agents. Another shortcoming is the method is biased toward high-affinity interactions, which may not be fully representative

of other interactions, which may play significant roles in the pharmacology of a bioactive compound.

Utilized for more than 40 years, macromolecular synthesis assays also continue to define patterns of inhibition of DNA, RNA, protein, and cell wall biosynthesis of newly identified natural products. In fact, improvements in methodology and throughput have been made over the years to address its problems of low throughput (Table 1) (Fig. 2c). Originally, these assays were designed in large culture formats, but have now been made amenable to microplates[13, 33]. These improvements are certainly welcomed for target elucidation of natural products, where quantities of material are often limiting to these studies. Such strategies have passed the test of time as useful starting points to distinguish inhibition of major cellular processes. Indeed, macromolecular assays were recently used as a first pass for MOA determination of the novel natural product, teixobactin, and shown to have a strong effect solely on the synthesis of cell wall, suggesting inhibition of peptidoglycan synthesis[34]. In-depth studies revealed a mechanism of inhibition through binding of a highly conserved motif of lipid II.

4 Challenges of target identification of natural products

Generally, for any novel antibacterial, whether synthetic or natural product, target identification is a notoriously difficult task, but a crucial one for advancing hits through the discovery pipeline, as is knowledge of the mechanisms by which resistance can arise[7]. Difficulty stems from the lack of generic methods intended for all cases and the high degrees of uncertainty that exist for those methods that are available, often making it best to use multiple methods to increase the chances of success. Even following identification of relevant target(s), additional functional studies are required to confirm and validate the

observations. Target identification of natural products is further plagued by problems of reliable compound access and supply (Fig. 1). For many methods, whether traditional or more modern, such as chemical genomics (described below), compounds are generally required in milligram quantities, not to mention additional requirements for prior isolation and structure elucidation. Typically, laboratory cultures can produce compounds at the microgram per liter level. Although technologies for large-scale cultivation can circumvent this problem, such facilities are seldom available in academic settings. In some cases, the identified natural product is an exceedingly minor component of the extract further limiting supply for extraction. While accessing minor metabolites may be solved by heterologous overexpression of their biosynthetic gene cluster[35, 36], assuming it is known, this remains to be routinely attained on a production scale. Further, metabolites are often complex molecular structures posing immense difficulties for production via chemical synthesis. While many target identification strategies exist and continue to be developed[8], the use of several of these for natural products has been curtailed owing to these issues of compound supply. One way to improve the quality of crude libraries for HTS in order to circumvent potential issues such as active components being present in concentrations that are too low to have effects that an be measured or interfering compounds that confound the assay signal, is through the use of pre-fractionation strategies. There are several different methods to obtain pre-fractionated samples and these have been recently reviewed[9]. In terms of compound availability, the continued development of methods to face the difficulties in obtaining adequate amounts of naturally produced compounds[9] for target elucidation work will surely facilitate the discovery and characterization of novel antibacterial natural products. In the meantime,

several methods for target identification, described below, have been modified to accommodate a limited supply of compound. These efforts have led to the characterization of the MOA of several novel antibacterial natural products in recent years (Table 2).

5 Modern target identification

5.1 Chemical-genomic strategies

In recent years, the field of chemical genomics, established in response to a need to link gene to function and drug to gene product[37], has had considerable success in uncovering the molecular target of biologically active molecules. Modern efforts have exploited the systematic screening of antimicrobial agents against genome-wide collections of overexpression and deletion clone sets, such that drug susceptibility phenotypes can be rapidly mapped to specific genes[38-40]. These methods often use a principle of genetic interaction, relying on the idea of genetic modifiers (enhancers and suppressors) to generate hypotheses regarding the MOA of novel compounds. For example, downregulation of *fabF* in *Staphylococcus aureus*, encoding a ketoacyl carrier protein synthase, caused hypersensitivity to the natural product antibiotic, cerulenin[41], while downregulation of *yidC* in *Escherichia coli* by antisense RNA resulted in sensitization to antibacterial essential oils eugenol and carvacrol[42]. Generating a chemical genetic profile of a bioactive agent allows a diagnostic interaction fingerprint about the target of the compound and in the case of nonessential gene mutants, information about related buffering pathways. Such comprehensive genomic collections, originally founded in the model yeast *Saccharomyces cerevisiae*[43], are now available for diverse bacterial pathogens, including *S. aureus*[44, 45], *E. coli* [46, 47] and *Pseudomonas aeruginosa*[48, 49]. These

panels of mutants come in various types, all lending to the concept of altered gene dosage and ready to be challenged with particular inhibitory molecules. In 2012, Xiao *et al.* used the principle of overexpression, employing an ordered overexpression library of essential genes in *E. coli* (ASKA library[46]) to identify clones resistant to a novel natural product, myxovirescin[50]. The study proved successful in identifying the type II signal peptidase as the cellular target. Notably, availability of purified myxovirescin was limited, so the authors devised a unique approach to overcome supply problems. Specifically, the producing strain was grown as a lawn then overlaid with soft agar onto which the AKSA library was transferred, the idea being that the producer strain will make the antibiotic and resistant clones could be identified. Indeed, the compound supply problem is the main drawback for the use of chemical genomics for MOA determination of antibacterial natural products. Such methods require substantial amounts of purified compound, yet newly isolated natural products are often only available in small quantities (Table 1). This is likely the reason chemical genomics has been increasingly applied for target identification of synthetic small molecules, where material is generally not limited.

One chemical genomic strategy that allows comprehensive analyses while using minimal amounts of material is through the use of elegant strain-specific barcodes that have been engineered to uniquely identify individual mutants, thus enabling parallel screening in pools[51]. First used in yeast[52], following screening in coculture, the technology allows the relative abundance of strain-specific barcoded to be quantified by microarray hybridization[53] or more recently, by direct sequencing of the barcodes[54]. An added benefit here for natural product discovery is that the assays can also be done within

crude extracts. These strategies were first exploited for target identification and validation of natural product antifungals; unsurprisingly as advancements in yeast chemical genomics have surpassed those seen for bacteria. In fact, Merck exploited such fitness test methodologies for years, successfully mechanistically annotating several naturally produced antifungals, even within crude extracts[55]. An example is the a new class of natural product antifungals, parnafungins[56, 57] which were uncovered as inhibitors of poly(A) polymerase-mediated mRNA processing[56, 58]. In recent years, the use of bacterial fitness tests began to rise and similarly led to successful identification of targets of novel antibacterial natural products. For instance, the *S. aureus* fitness test[44] was applied to identify the targets of two classes of natural product antibiotics, a cyclic depsipeptide (krisynomycin) and a lipoglycopeptide (actinocarbasin), both compounds having synergistic effects with imipenem against methicillin-resistant *S. aureus*[59]. Their cellular target, the bacterial type I signal peptidase SpsB, a serine protease that is required for the secretion of proteins, was apparent as the one antisense strain hypersensitive to the action of the compounds.

Chemical genomic screening methods often rely on growing various strains of a model organism on a solid agar surface in a typical grid pattern to allow reliable parallel comparison and quantification, with methods for data analysis continually being developed and improved[60, 61]. Even with the small genomes of these bacterial species, the libraries contain thousands of genes that require multiple agar plates, thus requiring ample purified natural product. At present, screening systems allow for up to 1536 colonies per agar plate[62]. Advancements, albeit in yeast genomics, are ongoing and recent work has enhanced screening throughput by enabling growth and analysis of 6144 mutant

yeast colonies on a single agar plate[63]. Such developments will surely allow an increased use of chemical genomic strategies and facilitate the characterization of a greater percentage of antibacterial natural products.

5.2 Inferences from comparative profiling

The success of target identification studies using chemical genomics has inevitably led to profiling experiments for linking targets to newly discovered antibacterial compounds. In a proof-of-principle study, Boone and colleagues demonstrated that chemical-genetic and genetic interaction profiles overlap substantially for several different inhibitory compounds and their target genes[64]. Profiling studies can be powerful tools for target determination; cellular targets can be inferred by comparing the observed phenotype induced by a compound of interest with known phenotypes from comprehensive phenotype collections. The latter are not only derived from chemical-genetic profiles with large collections of genetic interactions from, for example, gene knockout or RNA interference experiments, but also from gene expression, chemical combinations, morphology and biological activity. While still far from a mature technology, inferring target through profiling strategies has been increasingly used to elucidate the MOA of inhibitors from synthetic libraries and, although less frequently - likely due to the supply problem - those of antibacterial natural products (Table 1).

For instance, in 2011, Merck reported on the discovery of a novel natural product, kibdelomycin and studied its MOA through chemical genetic fitness test profiling in *S. aureus*[65]. They made use of a previously built collection of 245 inducible antisense RNA strains[44] engineered for reduced expression of essential genes, such that the reduced copy of target gene product leads to differential sensitivity of cells to compounds that inhibit

the targeted protein or related functions. Profiling with known inhibitors revealed the MOA for kibdelomycin, one involved in the inhibition of the ATPase activity of type II DNA topoisomerases, which leads to obstruction of DNA synthesis and cell death[65]. Using whole-genome microarray data, Freiberg *et al.* similarly compared the profile of the natural product moiramide B to a reference compendium built from 14 different antibiotics and a set of conditional mutants[66]. Analysis of moiramide B's expression profiles led to a hypothesis for its MOA, one of inhibition of the bacterial acetyl coenzyme A carboxylase.

Recent efforts have also exploited the use of chemical-chemical combinations to facilitate small molecule MOA determination. Analogous to chemical-genetic fingerprints, chemical-chemical combinations with diverse antibiotics can too generate fingerprints that aid in inferring mode of action[67-69]. Indeed, chemically induced growth phenotypes, such as synergistic or antagonistic interactions, from a combination of bioactive compound and known antibiotic can provide important clues as to the drug target of the unknown compound. This approach, although not yet utilized for natural products, seems quite tenable for this purpose, particularly given that relatively little compound is required[67].

Other profiling target identification methods developed in recent years have included "bacterial cytological profiling" (BCP), which uses state-of-the art microscopy to discriminate between compounds with different MOA and can accurately predict the MOA of newly isolated compounds[70]. Indeed, the cellular pathway targeted by novel molecules can be predicted based on comparisons to distinct cytological profiles generated following exposure to various known inhibitors. BCP identified the MOA of

spirohexenolide A, a natural product compound that kills MRSA and other species through a disruption of the cytoplasmic membrane[70]. While the throughput of this approach is limited, the assay was developed in microwell plates, thus requiring small quantities of compound, which is advantageous in the case of natural products. Another recently developed profiling tool is known as anti*bio*tic *m*ode of *a*ction *p*rofile (BioMAP) screening. Here, antibiotics of varying classes were profiled against a panel of clinically relevant bacterial strains to create unique fingerprints of susceptibility[71]. In this study, the authors demonstrated that BioMAP profiles are highly diagnostic for the known structural classes of antibiotics and can be used to infer on the MOA of novel compounds. A screen of chemical extracts from natural sources not only accurately predicted the presence of known antibiotics in the extracts, but also led to the discovery and characterization of a novel antibiotic compound, named arromycin[71].

Profiling strategies can be powerful tools for dissecting pathways targeted by novel inhibitory natural products. A major limitation of profiling experiments, however, is that the assays do not identify precise targets, just pathways. Although narrowing potential candidate pathways does ease the process, follow-up studies remain crucial to determine and validate the exact target. Further, while most methodologies to date have involved high-throughput platforms, they still require substantial amounts of purified compound. While some have shown that natural product pre-fractions clustered well with pure compounds from the training set, a requirement was that each natural product extract contain just a single dominant antibiotic constituent[71], which is not always the case. As such, prefractionation of crude extracts is preferable for these methods as it simplifies the constitution of screening materials and reduces the likelihood of impacting

several targets, resulting in unclear and difficult-to-resolve phenotypes. If compound availability is not a bottleneck, such large-scale investigations can provide very informative biological characterization of compound and, at a minimum, starting points in pinpointing MOA.

6. Strategies to streamline target identification

6.1 A return to target-based screening

Target-based drug discovery has, of course, dominated modern drug discovery paradigms where synthetic compound collections have been the primary source of chemical matter. While target-based approaches have not been the convention in natural product drug discovery, this approach is emerging as a proficient one, particularly for antibacterial discovery. Notwithstanding the modest track record of this approach[72], the associated platforms can be immensely powerful tools, certainly in streamlining target identification, as MOA is clearly defined from the onset (Table 1). This is nicely exemplified by the design and implementation of a cell-free fluorescent FtsZ polymerization assay utilized by Merck that enabled discovery of the first FtsZ inhibitor, the natural product, viriditoxin[73]. Similarly, screens for inhibitors of cytoplasmic enzymes involved in peptidoglycan synthesis through HPLC-based assays against microbial extracts led to discovery, and fast characterization of target, of several natural product inhibitors of various steps in peptidoglycan synthesis[74-76]. Protein synthesis has also been a focus of target-based approaches. Cell-free platforms for inhibition of translation from microbial product extracts have uncovered several novel natural products[77-79]. Another reported biochemical screen looked for inhibitors specific to SbnE and AsbA, which are involved in the biosynthesis of siderophores in *S. aureus* and of *B.*

anthracis, respectively[80]. The screen identified baulamycins A and B as broad-spectrum natural product antibiotics. Target-based screens have the appeal of easy target identification. Hits from these screens, however, have to be followed up by determinations of antibacterial activity, where inhibitors may lack whole-cell antibacterial activity due to inability to reach their intracellular targets owing to poor penetration and/or active efflux[81].

6.2 Hypersensitive whole-cell screening platforms

One way to streamline the process of target identification and ensure whole-cell activity is to conduct innovative target-based screens in whole-cells. Devising novel hypersensitive assays in microbial cells in order to identify compounds acting on a desired target(s) accelerates follow-up and makes better use of available resources, especially in the case of natural products (Table 1). Such assays essentially couple the process of antimicrobial screening and target identification, all the while requiring little material. While there are clear advantages for target identification, a cleverly designed hypersensitivity screen that focuses on non-traditional targets has the important benefit of facilitating de-replication of known compounds. Indeed, empiric cell-based growth assays are well known to lead to high rediscovery rates and have impeded progress natural product discovery for decades.

Strategies of this sort have primarily relied on specific reporter gene assays, comparisons of phenotypes of modified strains of interest, either by overexpressing or depleting a gene of interest, changes in morphology or specific cellular phenotypes caused by blocking particular pathways[82]. While cell-based antimicrobial assays that respond to the inhibition of specific targets has engendered great success with

synthetics[82], the number of examples when screening microbial extracts is steadily growing.

A classic example of functional screening dates back to the 1960s with a screening platform that specifically identified natural product inhibitors of cell wall synthesis. With Lederberg's initial report that penicillin caused Gram-negative rods to form spherical cells (spheroplasts) in hypertonic medium[83], came the development of spheroplasting assays at Merck which used microscopy to identify new agents capable of inducing spheroplast formation. Classic cell-wall active natural products discovered through this method include fosfomycin, cephamycin C, thienamycin, moenomycin, mureidocin and tunicamycin[84]. Another cell wall specific assay, the L-form screen, identified compounds that differentially inhibited the growth of wild-type cells but not L-forms, which lack cell walls[85]. This hypersensitive assay yielded the antibacterial natural products teicoplanin and ramoplanin, which both bind to lipid II. Such screening platforms, which require little material, can streamline the process of target identification by detecting inhibitors of, in principle, any step in the cell wall pathway. Validation and mechanistic diagnosis are certainly still needed. A more recent application of the use of morphological alterations (Fig. 3a) involved a sophisticated high-throughput microscopy platform to detect inhibitors of biofilm formation in *P. aeruginosa*. A screen of prefractionated natural products uncovered the natural products skyllamycins B and C, representing the first known class of cyclic depsipeptide biofilm inhibitors/dispersers[86]. In both examples, the approaches benefited from established secondary assays to confirm and better define the MOA of the identified natural products.

Faster routes to elucidating the MOA in the context of natural products have heavily relied on antisense technology, whereby translation of the mRNA encoding a desired essential target is downregulated by its cognate antisense RNA. As such, inhibitors of that target should have a hypersensitive impact on bacterial growth (Fig. 3b). Thus, screens of microbial extracts for growth inhibition of the target strain, expressing the antisense RNA, in contrast to the isogenic control strain, can identify inhibition of the target in question. These assays have been widely applied at Merck on microbial extracts and have resulted in the identification of the target of platensimycin, an inhibitor of fatty acid biosynthesis[87], of the peptide, philipimycin[88] and of lucensimycin[89], both selective inhibitors of protein synthesis. It is noteworthy that these compounds are produced by relatively common strains, such as *Streptomyces platensis*, *Actinoplanes philippinensis* and *Streptomyces lucensis*, respectively, supporting the idea that sensitive whole-cell target-based screening assays can allow the discovery of molecules that have escaped previous detection. This is also nicely exemplified by the design and implementation of a whole-cell assay specific for the identification of fatty acid synthesis inhibitors, based on antisense RNA interference technology. The natural products, phomallenic acids, new inhibitors of FabF, were so discovered[90].

Other hypersensitive assays involve the creation of reporter assays that have easily measurable characteristics, such as fluorescence or luminescence indicators (Fig. 3c). The genes of interest and the reporter genes are introduced into the same DNA construct, often times within a plasmid introduced into cells. Scientists at Wyeth developed a sensitive assay method for detecting homoserine lactone (HSL)-related compounds in microbial extracts[91]. Such compounds would be expected to interfere with

quorum sensing in Gram-negative bacteria, where acyl-HSL is believed to be a key player. In other cases, predetermined cellular biosensors, which can signal the presence of many different types of inhibitory molecules with precise MOAs, can be screened in the presence of microbial extracts in the hopes of inhibiting desired targets[92]. Others have relied on unique phenotypes, for example, such as colorimetric assays of pH that specifically identify inhibitors of bacterial sugar metabolism. A screen of 39,000 crude extracts led to the discovery of three novel natural products, one of which, mirandamycin, has broad-spectrum antibacterial activity against various important bacterial pathogens[93]. It remains to be seen whether its rather low potency can be improved by structural modifications. Nevertheless, this example of functional screening demonstrates the power of simple high-throughput screens with rapid inferences as to potential target(s).

Whole-cell screens in defined growth conditions for differential screening have also emerged in recent years[94, 95] (Fig. 3d). Not only can target-specific inhibitors be detected, but these would also most likely not have been previously detected in conventional screens, a great advantage in the context of natural products. A recent example of altered screening conditions comes from the study by Zlitni *et al*. that prioritized synthetic small molecules capable of selectively inhibiting the growth of *E. coli* in defined minimal media in order to identify inhibitors of bacterial metabolism[95]. In fact, interest in antimetabolites and targeting central metabolism as leads for the development of new antibiotics is on the rise[96]. Some natural product antimetabolites include roseoflavin, an antimetabolite of riboflavin (vitamin B2), identified in *Streptomyces davawensis,* potently inhibits the human bacterial pathogen *Listeria monocytogenes*[97]. Similarly, the natural product CJ-15,801, which shares structural

similarity with pantothenic acid, the vitamin precursor of coenzyme A (CoA), and therefore likely blocks a step in CoA biosynthesis or utilization, was found to inhibit *S. aureus*[98]. The search for natural product antimetabolites could be best accomplished in more defined media, thus minimizing potential targets and streamlining the process of MOA.

6.3 Combination screens to streamline target identification

An additional approach to streamline target identification of natural products is to conduct rational combination screens, where prior knowledge of potential hits is well established. The number of success stories utilizing this approach is steadily growing, certainly in the case of synthetics. Intricate interactions in the process of cell wall biosynthesis of *S. aureus*, for example, has inspired the design and development of multiple rational combination screens, to find inhibitors of the synthesis of wall teichoic acid polymers[68] and undecaprenyl phosphate[99]. A recent example of rational combination screening of microbial extracts explored the ability to potentiate the action of meropenem against a metallo-β-lactamase (MBL)-positive carbapenem-resistant strain of *Klebsiella pneumoniae* to uncover potential inhibitors of MBLs. King *et al.* conducted a screen of natural product extracts for those that restore the activity of meropenem and identified aspergillomarasmine A (AMA) as a rapid and potent inhibitor of the NDM-1 MBL enzyme[100]. Narrowing the spectrum of possible targets through rational combination screens can certainly help ease the process of target identification (Table 1). In the case of synergy, it also inherently identifies potential antibacterial synergistic combinations.

Many research groups are exploring how to make better use of screening that is based on whole organisms, and there is a renewed interest in using natural products in these screening platforms. Streamlining the process of target identification through hypersensitive assays in whole-cell contexts may solve problems of supply, time and in some cases dereplication. Indeed, in some cases, such unconventional and hypersensitive screening strategies may allow the identification of previously undetected natural products.

7 Concluding remarks

Over the past few decades, the daunting task of identifying the target of bioactive small molecules has spawned a great deal of inventive work culminating in a number of unique approaches for elucidating the MOA of novel antibacterial natural products. Throughout, the traditional approaches employing genetic or biochemical means have proven their staying power as methods to identify target(s). Further, technological improvements have addressed some of the original shortcomings and rendered these approaches very powerful in modern-day discovery efforts. The most recent developments in this area have been, in particular, in the field of chemical genomics and profiling experiments. Several recent applications for various natural products have confirmed the potential of these approaches for the identification of cellular target(s). Such platforms represent technically impressive achievements and have, in some cases, been honed to deal particularly with difficulties associated with natural product discovery. Further, many research groups are exploring how to make better use of screening so as to be based on whole organisms and tailored to specifically hit target(s) of interest, thus streamlining the process of target identification and requiring less material.

The supply problem in target identification of natural product bioactive likely remains the rate-limiting step in applying the full power of these more modern approaches to elucidate MOA. Approaches to address this issue will inadvertently accelerate the discovery and characterization of novel antibacterial natural products. In many cases, it is also evident that a combination of approaches may be required to fully characterize on-target and off-target effects of a novel compound, requiring even more material. Overall, new opportunities for natural product development made possible by novel and efficient strategies for target identification should encourage a return to natural extracts as integral parts of academic and pharmaceutical screening programs. With luck, this area can continue to provide new therapies towards unmet medical needs.

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Table 1. Extent of ease, throughput, resolution and requirement for further follow-up to verify target of various strategies for target identification of natural products

	Ease[a]	Throughput[b]	Resolution[c]	Requirement for follow-up[d]
Resistant mutants	+++	+	++	++
Affinity purification	+	+	+++	+
Macromolecular assays	++	+	+	+++
Chemical genomics	+	+++	++	+++
Profiling: chemical-genetic	+	+++	+	++
Profiling: chemical-chemical	+++	+++	+	+++
Profiling: morphology	+	+	+	++
Profiling: activity based	++	++	+	+++
Hypersensitive screening	+++	+++	++	++

[a] Ease: + difficult and cumbersome assay; +++ simple and easy-to-perform assay
[b] Throughput: + low throughput experiment, often requiring large culture flasks; +++ high-throughput assay which can be conducted in microtiter plates
[c] Resolution: + low resolution method often identifying general pathways inhibited rather than precise molecular targets; +++ higher resolution strategy which can pinpoint molecular target
[d] Requirement for follow-up: + approach doesn't require ample follow-up as molecular target is readily identified; +++ follow-up required as strategy only identifies a general bacterial pathway

Table 2. Summary of new natural product classes identified in the last decade and the primary method used for their target identification

	Target identification method*								Bacterial target/ process
	RM	AP	MA	CG	IP	TBS	HS	CS	
A-102395[74]						X			Bacterial Translocase I
Actinocarbasin[59]				X					Bacterial type I signal peptidase
Arylomycin[22]	X								Inhibitor of type I signal peptidase
Aspergillomarasmine A[100]								X	Inhibitor of NDM-1 MBL
Baulamycins[80]						X			Synthesis of siderophores
Fasamycins[19]	X								Inhibitor of fatty acid synthesis
GE81112[78]						X			Inhibitor of translation
Kibdelomycin[65]					X				Type II DNA topoisomerases
Krisynomycin[59]				X					Bacterial type I signal peptidase
Lucensimycin[89]							X		Inhibitor of protein synthesis
Mirandamycin[93]							X		Inhibitor of bacterial sugar metabolism
Moiramide B[66]					X				Inhibitor of acetyl coenzyme A carboxylase
Myxovirescin[50]				X					Inhibitor of type II signal peptidase
Nocathiacins[20]	X								Inhibitor of protein synthesis
Orthoformimycin[79]						X			Inhibitor of translation
Philipimycin[88]							X		Inhibitor of protein synthesis
Phomallenic acids[90]							X		Inhibitor of fatty acid synthesis
Platensimycin[87]							X		Inhibitor of fatty acid synthesis
Pyridomycin[18]	X								Inhibitor of InhA
Skyllamycins[86]							X		Biofilm inhibitor
Spirohexenolide A[70]					X				Cytoplasmic membrane
Teixobactin[34]			X						Cell wall synthesis
Vancomycin[30,31]		X							Binding to Atl, DNA transporter, VanS
Viriditoxin[73]						X			Inhibitor of FtsZ

*Shown is the main target identification strategy used to describe the bacterial process targeted by the natural product. It is important to note that often, more than one method was used to definitively decipher the compound's bacterial target. RM: isolation of resistant mutants; AP: affinity purification; MA: macromolecular assay; CG: chemical genomics; IP: Inference from profiling (genetic/chemical/phenotypic); TBS: target-based screen; HS: hypersensitive target-based whole-cell assays; CS: combination screening

Figure legends

Fig. 1 Overview of relative compound quantity and purity for various target identification strategies for antibacterial natural products, where RM: isolation of resistant mutants; AP: affinity purification; MA: macromolecular assay; CG: chemical genomics; IP: Inference from profiling (genetic/chemical/phenotypic); HS: hypersensitive target-based whole-cell assays.

Fig. 2 Traditional methods for target identification with modern improvements (a) Next-generation sequencing can be used as a means to quickly characterize drug resistant mutants (b) Affinity-based target identification with the use of affinity probes and analytical platforms enhance the sensitivity of target detection (c) Miniaturized macromolecular assays allow the analysis of incorporation of radiolabelled precursors of key biosynthetic macromolecules in microtiter formats.

Fig. 3 Schematic representation of common strategies for hypersensitive target-based whole-cell assays, based on (a) high-throughput microscopy, whereby the pathway affected by a novel natural product can be predicted based on comparisons to distinct cytological profiles among known molecules (b) antisense interference, which can be used to elucidate target in cases where reduced copy of target gene product leads to differential sensitivity of cells to compounds (c) reporter assays, which can be used to identify inhibitors of a specific pathway for which a measurable reporter system is utilized (d) defined growth conditions for differential screening, whereby inhibitors unique to certain growth conditions relating to a specific target can be detected.