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Empirical antibacterial drug discovery—Foundation in natural products

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ABSTRACT

Natural products have been a rich source in providing leads for the development of drugs for the treatment of bacterial infections. However, beyond the discovery of the natural product, thienamycin and the synthetic lead, oxazolidinone in the 1970s, there has been a dearth of new compounds. This commentary provides an overview of current antibiotic leads and their mechanism of action, and highlights tools that can be applied to the discovery of new antibiotics.

1. Introduction

Drugs derived from plant sources have been empirically used in the treatment of various human disorders for thousands of years in the form of the traditional Ayurvedic and Chinese medicine. A number of natural products, e.g. aspirin, morphine and quinine (Fig. 1), are still in use today [1–3]. Drugs derived from microbial fermentations have played an equally seminal role in modern discovery and have revolutionized medicine, saving both human and animal lives. The recent history of microbially-derived medicines had its beginning with the serendipitous observation of Fleming in 1929 that bacterial growth was prevented by the growth of *Penicillium notatum*. After a belated start, this led to the isolation, structural elucidation, clinical evaluation and commercialization of penicillins as the first broad spectrum antibacterial agents in the early 1940s, driven to no small extent, by the medical needs of World War II casualties. Antibiotics from natural product discovery provided the means to treat bacterial infections savings millions of individuals who would otherwise have died. For their efforts in the discovery and development of penicillin, Fleming, Florey and Chain received the Nobel Prize in 1945.

The success of penicillin led to unparalleled efforts by government, academia and the pharmaceutical industry to discover other compounds from natural sources for the treatment of bacterial infections resulting in nearly all novel classes of antibiotics being from natural product sourced scaffolds through 1962. These were discovered simply by measuring zones of inhibition of bacterial strains on agar plates by applying whole broth or extracts obtained from microbial ferments. Despite major technological advances, the approach to identify novel antibiotics in the 21st Century is very similar to that used by Fleming in the last although

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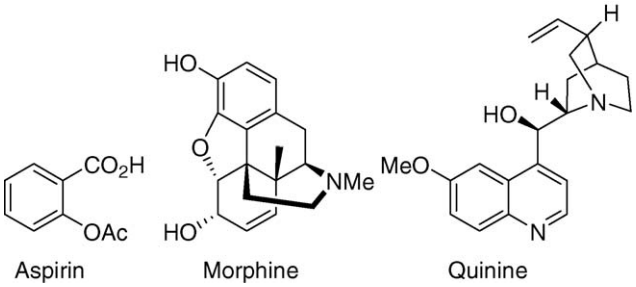

Fig. 1 – Structures of aspirin, morphine, and quinine.

mining of the bacterial genome is providing new avenues to the identification of essential bacterial targets that can be used for the discovery of antibacterial agents that have reduced side effects due to the uniqueness of the bacterial target [4–9].

Advances in microbial isolation, fermentation and natural product chemistry techniques provide tremendous opportunity to collectively apply these tools to the discovery of novel antibiotics that would have been difficult only 5 years ago. This commentary briefly reviews the current antibiotic leads and drugs, their mode of action, and highlight new tools that have proven useful for the discovery of new antibiotics.

2. Sources for antibiotics

Like other areas of drug discovery, there are two sources for antibiotic leads—natural products and synthetic compounds. Natural products have been the mainstay in providing novel chemical scaffolds for many drugs [2] as well as leads that were chemically modified and developed as antibacterial agents. In fact, natural products account for all but three antibiotic classes. The timeline of the discovery of the major classes of antibiotics and their source is shown in Fig. 2 [10].

2.1. *Antibiotics of natural origin*

The discovery of broad spectrum antibiotics began with the discovery of penicillin, the first of the β-lactams which led to the ''Golden age'' (1940–1962) of antibiotic discovery. Many classes of novel natural product antibiotics were discovered (Fig. 3), as well as the synthetically derived product, nalidixic acid. These included the phenyl propanoids (chloramphenicol), polyketides (tetracycline), aminoglycosides (streptomycin, gentamycin), macrolides (erythromycin), glycopeptides (vancomycin) and streptogramins (quinpristin and darfopristin [Synercid®]), and second generation β-lactams (cephalosporins). A third class of β-lactams (carbapenems such as imipenem) were discovered in the early 1970s. Structurally, the three classes of β-lactam antibiotics are distinguished by differences in the second, non-β-lactam ring motif. Penicillins contain a five-membered sulfur heterocycle, cephalosporins a six-membered sulfur heterocycle, and carbapenems a five-membered carbocycle (Fig. 3).

Antibiotics from natural sources range from small molecular weight compounds (e.g., penicillins) to large peptides (e.g., teicoplanin). They generally possess complex architectural scaffolds and densely deployed functional groups, affording the maximal number of interactions with molecular targets, often leading to exquisite selectivity for pathogens versus the host.

2.2. *Antibiotics of synthetic origin*

Synthetic chemical collections have played a minimal role as sources of leads for antibiotics. The synthetic sulfonamides, discovered in the 1930s, were the first class of antibiotics used in clinical practice before the discovery of penicillins and continue to be used today. The second and highly successful class of synthetic antibiotics were the quinolones discovered in 1962 by empirical screening of the by-product of chloroquine synthesis. This led to the

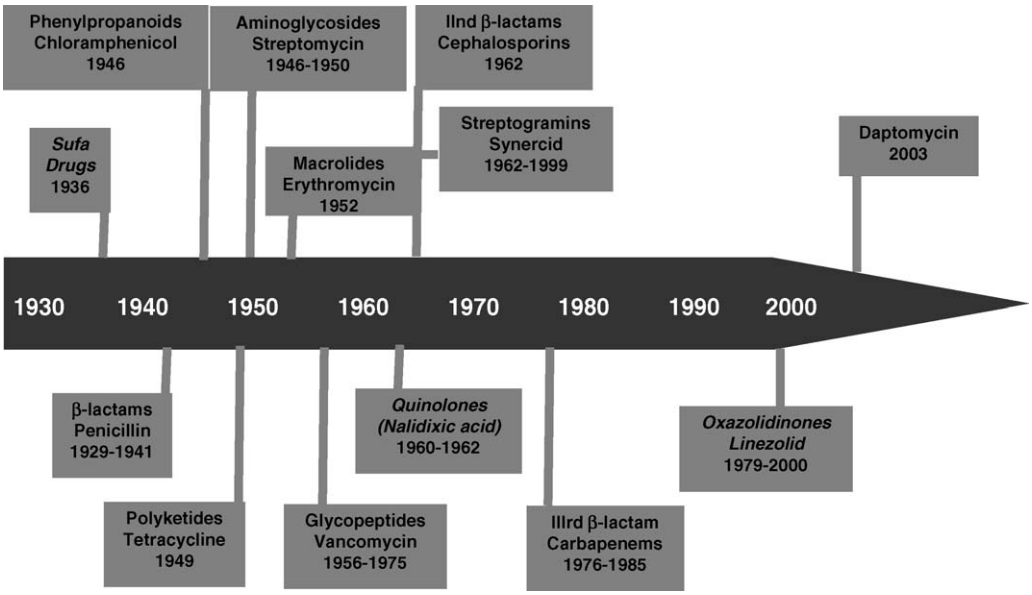

Fig. 2 – Timeline of discovery novel classes of antibiotics and introduction in clinic. Compounds listed in normal fonts are natural product derived and in italics are derived from synthetic origin.

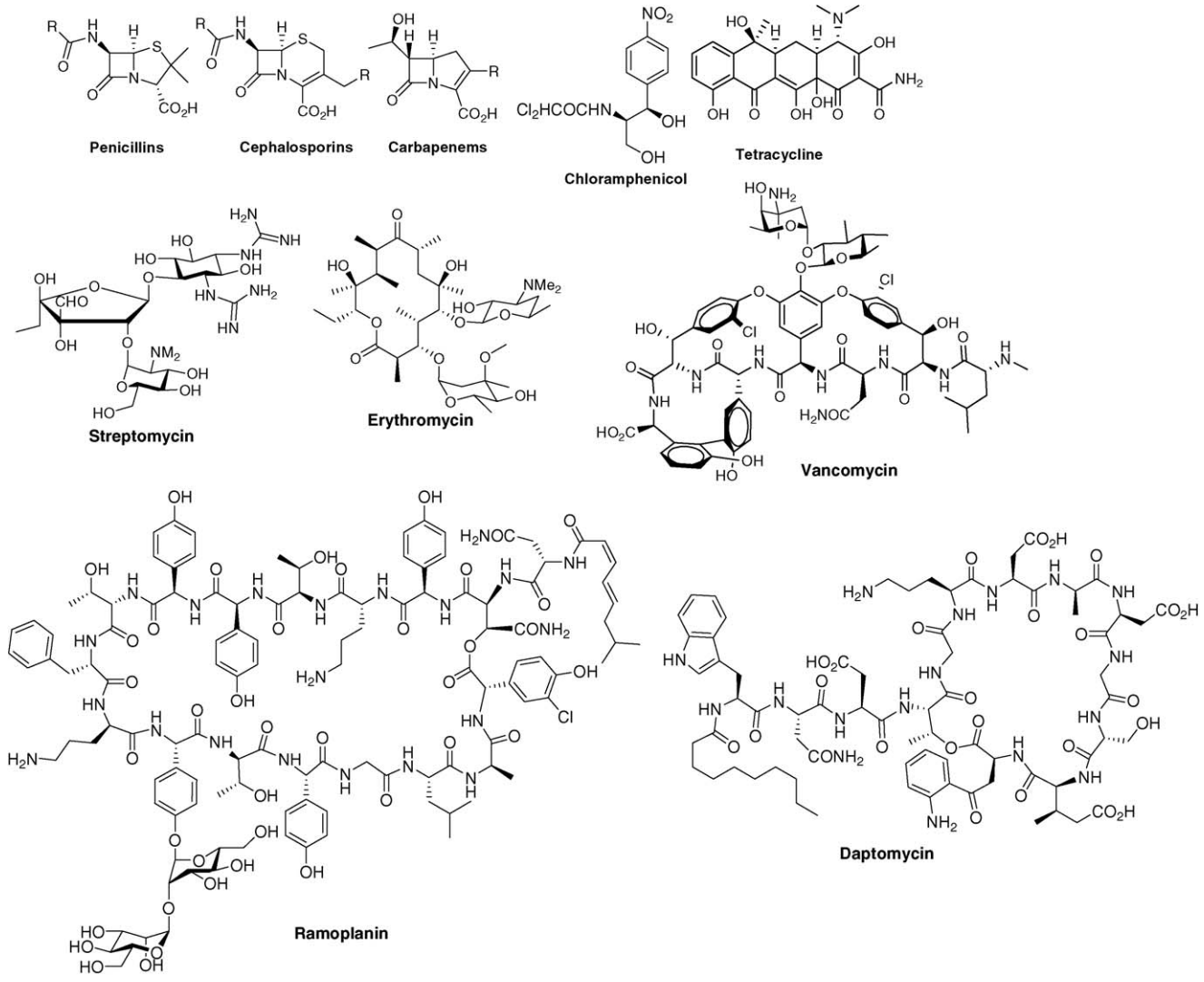

Fig. 3 – Representative antibiotics of natural product origin.

discovery and development of nalidixic acid as the first quinolone antibiotic which eventually evolved to quinolones such as ciprofloxacin and others. Oxazolidinones represent the third class of synthetically derived antibiotic leads discovered in 1979 that led to the clinical development and approval of linezolid in 1999. While quinolones were originally discovered as antibiotics from total synthesis, natural quinolones (e.g., aurachins C and D) [11] were subsequently discovered and could have served as a scaffold (Fig. 4).

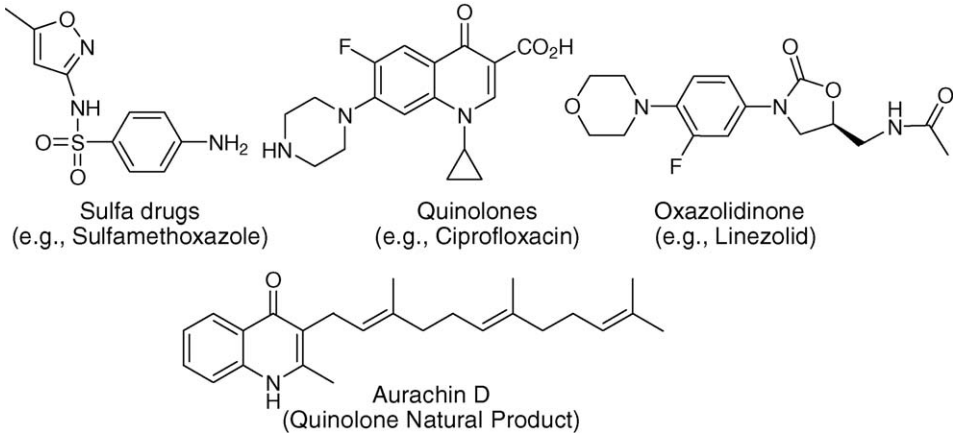

Fig. 4 – Antibiotics with synthetic origin.

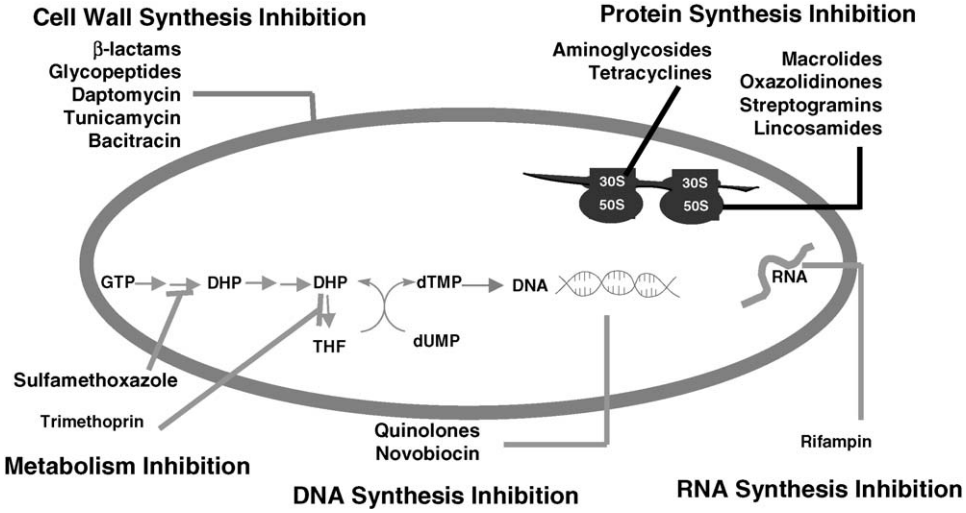

Fig. 5 – Mode of action of antibiotics.

3. Mechanism-of-action (MOA) of antibiotics

The MOA of all antibiotics currently in clinical use was determined following their discovery. The majority of antibiotics exert their action either by inhibition of bacterial cell wall or protein synthesis. Exceptions are the quinolones that inhibit DNA synthesis, and the sulfonamides that inhibit the synthesis of metabolites used for the synthesis of DNA (Fig. 5). The point of emphasis here is that there is no requirement to start with a pre-determined mechanism of inhibition, nor a defined target, and that empirical screening of natural products against whole cell bacteria remains a viable discovery strategy when combined with new technologies for isolation and detection (see below).

4. How they were discovered?

The approach that resulted in the discovery of penicillin was also used for the discovery of other antibiotics and is still used today. The original discovery of penicillin was made from a fungal source; however, most other natural antibiotics are produced by prokaryotic organisms. The process involves growing bacterial strains on agar plates and applying whole fermentation broths or broth extracts of natural products to the agar 'lawn' of bacterial growth. After incubation for a pre-determined time period, the zone of bacterial clearance was measured. A broth or extract that exhibited a zone of killing was then subjected to bioassay-guided chromatographic fractionation, leading to the isolation of active natural product(s), followed by structural elucidation of the active compound.

While this methodology delivered most key antibiotics in use today, it does not assess the MOA, cannot reveal potency at the screening stage, or discriminate known from novel antibiotics, and provides an unacceptably high hit rate (20–30%). Dereplication of hits is a considerable challenge even with state of the art analytical methods, due to the very large number of known antibiotics produced in varying amounts with varying degree of activities.

5. Antibiotic resistance

The successes of the Golden Age of antibiotics led to considerable excitement leading with excessive hyperbole, e.g. "... experts agree that by year 2000, viral, and bacterial diseases will have been eliminated" (Time, February 1966); "... that we had essentially defeated infectious diseases and close the book on infectious diseases" (US Surgeon General, W.H. Stewart in a testimony to the US Congress in 1969). No sooner were these statements made than antibiotic resistance began to emerge, becoming rampant in certain instances resulting in vancomycin becoming the antibiotic of last resort for the treatment of Gram-positive bacterial infections in hospitals. After a number of years of use, emergence of nosocomial vancomycin-resistant *Staphylococcus aureus* and *Enterococcus faecalis* strains have become a common occurrence [12,13].

For whatever reason, the post Golden Age discovery of novel antibiotics was unsuccessful. To fill the void, researchers adopted the strategy of incrementally improving the existing classes of antibiotics, to be active against resistant bacterial pathogens. It is now accepted that resistance is inevitable, and that resistance management will be part of the process for all new antibiotics.

5.1. *Major modes of resistance*

Bacteria adapt various methods to survive antibiotic inhibition or killing. These include: (i) inactivation of antibiotics by enzymatic reactions (e.g., inactivation of β-lactams by β-lactamase); (ii) efflux mechanisms by which antibiotics are transported out of cells by pumps (e.g., tetracyclines subject to *tet*M efflux pumps); (iii) target mutation to decrease binding efficiency of antibiotics (e.g., modification of D-Ala-D-Ala to D-Ala-D-Lac making vancomycin less effective); (iv) overproduction of target (e.g., DHFR); (v) bypass of the metabolic pathway to remove the essentiality of the target (e.g., peptide deformylase in *Streptococcus pneumoniae* or *Haemophilus influenzae*); and (vi) decreased uptake of antibiotics (e.g., *Pseudomonas aeruginosa* loss of its D2 porin).

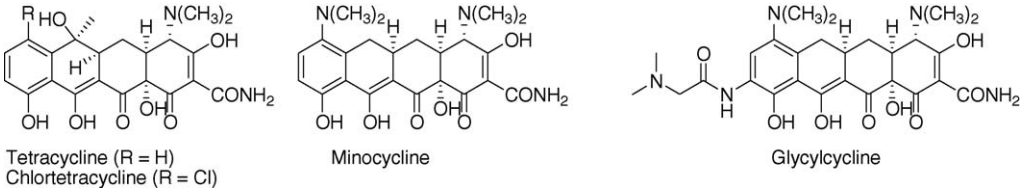

Fig. 6 – Evolution of tetracycline class of antibiotics.

5.2. *Strategies for discovery and development of new antibiotics*

While the discovery of new chemotypes with antibacterial activity was the primary research goal, few innovative approaches were developed in the discovery laboratories, resulting in minimal success. Instead, major industrial efforts were directed towards the incremental improvement of existing chemotypes, improving potency, stability, pharmacokinetics, or adverse reactions by chemical modifications. This led to the development of five generations of penicillins (15+ compounds), four generations of cephalosporins (24+), two generations of carbapenems (4+), aminoglycosides (12+), tetracyclines (8+), macrolides (5+), glycopeptides (3+), quinolones (18+), and an oxazolidinone (1). An alternate strategy for drug discovery involved targeting the mechanism-of-resistance to reverse the decrease in antibiotic potency. This led to the development of β-lactamase inhibitors (e.g., clavulanic acid, sulbactam, tazobactam) that were co-administered with a β-lactam antibiotic (e.g., amoxicillin) leading to combination products such as Augmentin® [14]. Likewise, strategies to block efflux have been successful leading to tigecycline (Fig. 6), in which chemical modifications were made to the core tetracycline scaffold to reduce efflux and resistance. Specifically, the addition of aminoglycyl group in ring A of minocycline blocked antibiotic efflux from bacteria.

6. The impact of molecular biology on antibiotic discovery

Molecular biology has revolutionized the ability for target identification and expression facilitating in vitro screening and lead identification in all disease targets including antibacterial agents. While this method has been successful in many areas of drug discovery it has had limited success in the antibiotic area. While leads have been identified, with few exceptions these leads are unable to cross the bacterial cell wall/membrane and include inhibitors of t-RNA synthetase [15], FabH [16] and carbapenamase [17]. The benzimidazole and indazole gyrase inhibitors [18] are more successful examples, demonstrating that optimization aided by computer modeling of enzyme docking can lead to molecules that are not only potent inhibitors of bacterial gyrase, but also have in vitro and in vivo antibiotic activity.

6.1. *Virtual screening*

This approach involves enzyme purification, crystallization and 3D structural determination of a drug target using X-ray crystallographic or NMR methodologies. Once the active site has been identified, pharmacophore(s) can be screened by in silico docking of structures to the active site. Obviously, this approach is limited to targets amenable to 3D-structure determination. Many targets are too complex and would not lend themselves to this approach. Various known or unknown structures are docked to an active site with those structures having the best fit being selected for ''wet'' screening. This approach can be applied in two ways: (i) de novo docking of yet unknown structures – designing a theoretical chemical space that fits the active site and translating it to a tractable chemical structure – for wet biology testing. In a practical sense however, it is generally very difficult to commit resources and organize a chemistry team to synthesize a complex theoretical structure that may not be biologically active. (ii) The second approach, in which known database structures are docked, is used by many discovery organizations. This technique has significant value when samples of the compounds resulting from virtual screening are available, and can be easily tested. While this rational approach has been useful in lead optimization for a number of targets (e.g., gyrase inhibitors [18,19] and FabH [16], HIV protease [20]), and has considerable potential, it has yet to deliver significant leads for antibacterial targets.

6.2. *Genome-wide antibacterial targets*

Sequencing of the *S. aureus* genome to the prediction that it contained ~265 to 350 essential genes [21]. Of these, about 60% are broadly conserved in key clinically relevant bacteria. Currently marketed drugs target approximately 15 of these essential gene products (Table 1). Selection of the most tractable target(s) from those remaining targets is the subject of considerable debate with a priori prediction being difficult. To putatively validate a target would require identification of meaningful leads. Identification of drugable versus non-drugable targets is a time consuming exercise when conducted one target at a time. This can be circumvented by screening against multiple targets simultaneously in a multiplex or array format using whole-cell assay, improving the odds for success. This allows for the discovery of novel leads active at the predicted target, potentially validating the target and provides leads for further development.

A number of technologies can be used to globally screen all the gene products of a bacterial genome and include antisense 'dial-down' platforms, promoter replacement technology [22], and multi-copy cloning [23]. Each of these technologies can be used to develop target-based whole-cell assays to interrogate one target gene or a group of target genes at a time to identify potential leads.

Table 1 – Known antibiotics and their molecular targets

Drugs	Targets
	Cell wall
β-Lactam	Multiple penicillin binding proteins; synthesis of cell wall peptidoglycan
Vancomycin	D-ala-D-ala of peptidoglycan substrate
Phosphomycin	MurA
Bacitracin	Bactoprenol phosphatase
Cycloserine	Alanine racemase
Daptomycin	Membrane perturbation
Ramoplanin	Transglycosylase
	Protein synthesis
Tetracycline	rRNA of 30s ribosome subunit
Gentamicin	30s ribosome subunit (rRNA)
Streptomycin	30s ribosome/rpsL
Macrolide	rRNA of 50s ribosome subunit
Lincosamide	rRNA of 50s ribosome subunit
Chloramphenicol	rRNA of 50s ribosome subunit
Oxazolidinone	23 S rRNA of 50s ribosome subunit
Fusidic acid	Elongation factor G
Mupirocin	Ile tRNA-synthetase
	RNA synthesis
Rifampcin	RNA polymerase
	DNA synthesis
Quinolone	Bacterial topoisomerases, gyrase A and topoisomerase IV
Novobiocin	Gyrase B
	Metabolism
Trimethoprim	DHFR (FolA)
Sulfamethoxazole	PABA synthase (FolP)
	Fatty acid synthesis
Isoniazid	InhA (FabI)

6.3. *Differential sensitivity screening approach for lead discovery*

Technologies exist to regulate expression of particular gene rendering the organism sensitive to an antibiotic affecting the same target. Differential sensitivity, using wild-type and resistant pairs, is widely used. Screening for inhibitors of a particular target in liquid whole-cell antisense assays [24] is an elegant approach to profile a limited number of compounds but is not amenable to high-throughput screening (HTS) due to upfront titration requirements. This assay has been transformed to an agar-based whole-cell format using antisense and non-antisense wild-type strains. The agar-diffusion assay takes advantage of the diffusion gradient of compounds generated in the agar and thus eliminates a priori titration requirement [25].

The basic concept of antisense screening is that novel antibiotic leads can be detected from the same sources that previously produced no leads owing to the increased sensitivity of the antisense assays. Mechanistically, this is possible due to the lower expression of an essential gene product in an antisense knock-down strain (usually regulated by an exogeneous inducer of the antisense RNA) compared to its isogenic wild-type strain. When a zone of clearing from a natural product broth is compared between both strains, a zone of inhibition against the antisense strain but not the wild-type strain, indicates a mechanism-based inhibitor against the target gene subject to down-regulation (i.e. dial-down). In addition, the antisense strain becomes supersensitive due to the dial-down of an essential gene product—thus providing an increased sensitivity, mechanism-based whole-cell screen for novel antibiotics [24,25]. Thus, one is able to identify specific mechanism-based inhibitors among many other antibiotics and non-specific toxins.

6.4. *Natural product-based antibiotic discovery*

Natural product-based drug discovery programs involve a synergistic interaction of at least three component areas: (i) sensitive and robust biological targets/assays; (ii) sources of natural products; (iii) isolation and structural characterization of natural products.

6.4.1. *Natural product sources*

Novel natural products of marked structural diversity and complexity with biological activities are found in almost all biological sources including terrestrial plants, lichens, marine macro organisms (656 new compounds in 2003 [26]). Marine organisms continue to be productive sources for novel drug leads for cancer and many other disease targets and a number of them are in clinical development [27–29]. However, they are less popular as antibiotic leads. While plant sources have not delivered clinically relevant antibiotics plant derived natural products have shown anti-staphylococcal activities (Gibbons [30]).

Sourcing for a microbiological material involves the collection of soil and environment samples (e.g., leaf litter, animal dung, etc.) from diverse geographical areas and a variety of habitats. This seemingly simple task happens to be one of the most critical parts for the discovery of novel natural products. This process has become very complex after the Rio De Janeiro treaty of ''Convention on Biological Diversity'' [31] which requires the meticulous documentation of collecting the sample and tracking any disposition of the sample. While this treaty is very important to ensure the rights of countries to be properly rewarded, it has slowed the progression of tapping into natural products due to the additional expense and legal requirements in sourcing throughout the world. A global process to move forward needs to be developed to harness the compounds of natural origin that can unleash their power for treating the potentially untreatable diseases which benefit the mankind. Fortunately, the biosynthesis and production of compounds are not exclusive and limited to unique biota and same compounds are produced by different genera distributed throughout the world [32–34].

Microorganisms from soil or other environmental samples are recovered by various methods. Classical methods for recovery of producer organisms have favored fast growing organisms, and have been reasonably successful in the identification of both novel producing strains and novel compounds. Specifically, the classical approach has been to plate microorganisms on an agar lawn with or without antibiotics (to selectively prevent overgrowth by a major grower) and to detect and isolate the microorganism, for subsequent assessment of their ability to produce a bioactive compound. This assessment has classically been by zones of clearing of spent-media broths and/or extracts of the

fermented culture. Key to success has been to suppress the overgrowth of fast growing microorganism which would otherwise prevent the slower growing strains from reaching a critical mass to present as a potential producer strain that can be harvested.

Recent improvements in the microbial isolation techniques, using high dilution extinction culturing conditions, allow the isolation of single strains of producer strains regardless of their growth rates. This seemingly insignificant but innovative protocol change has facilitated the isolation and fermentation of novel organisms [35]. Similar in strategy to the approach of limited dilutions in monoclonal hybridoma procedures, 96-well microtiter plates can be used for the isolation of microorganisms in high dilution conditions; however statistically only ~13% of wells contain microorganisms and the remaining wells are empty. This is expected as the principle is to dilute until one or less than one microorganism (just as with monoclonal-producing hybridomas) are in each unique well. Modifications in the procedures have circumvented this problem by applying a bead capture technology to this limited dilution protocol in which captured individual organisms can be sorted by flow cytometry. This process allows to capture beads with organisms in individual wells of 96-well plates and discard beads that do not contain any microorganisms [36]. While high dilution extinction culturing in 96-well has been only reported for prokaryotic organisms it can also be applied to eukaryotic organisms.

After the microorganisms are isolated they are ''dereplicated'' which is the process of identification and characterization of the isolated microbial strain, and subsequent matching to a pre-characterized genus/species, or naming of a new genus/species. The most common methods for dereplication of fungal species are morphological characteristics. While the broader dereplication of prokaryotic organisms is accomplished with less difficulty, usually based on morphology, size and growth pattern on select media, the precise dereplication of *Actinoplanes* and *Streptomyces* requires significant work, involving fatty acid analysis (FAME) and 16S rRNA analysis, which is both time consuming and faces the constant challenge of harvesting redundant isolates. More advanced efforts has included the use of Fourier Transform Infra Red (FTIR) to dereplicate microbial isolates [36] which has increased throughput but has yet to be proven successful [36].

Experience has indicated that the secondary metabolites of a growing culture of microorganisms frequently leads to the production of secondary metabolites that are often made by the producer organism to protect its existence in a given environment. As such, different environments (e.g., different media or growth conditions) can alter growth and production of secondary metabolites—and the artificial manipulation of the growth conditions can enhance the diversity of secondary metabolites. One clever example of moving this process to high-throughput optimization of media growth conditions has been reported by the Duetz group [37–39], in which a 96-well high-throughput system has been designed to allow for the simultaneous growth of a single microbial isolate in dozens of varied micro-environments on a miniaturized scale of ~1 mL samples [37–39]. However, there is no true 'model' of secondary metabolite production that guides the research lab to invest in increased unique isolates or an increased variety of growth condition. The 'brute-force' nature of this tedious and somewhat unsophisticated science of natural product sourcing has contributed to the movement away from natural products as a lead-seeking paradigm.

It has been postulated that antibiotics are secondary metabolites that are produced by microorganisms as defense mechanisms from either co-existing life forms or environmental predators [40]. The changing environmental conditions, whether in situ in nature or artificial in the laboratory, select for the fittest microorganism(s) that produce new antibiotics. This in turn, may select for microorganisms that produce yet another set of metabolites to fight these newer 'predators' microorganisms that have a growth advantage by virtue of their ability to better survive in the environment. Furthermore, it has already been postulated that antibiotic producers adopt difference self-defense mechanisms to avoid their own suicide (when producing antibiotics), protecting themselves against their own produced and secreted extracellular 'drugs' [41]. Among these protection mechanism are: inactivating their antibiotic products; modifying the antibiotic target sites (such as enzymes or ribosomes); or blocking the entrance of the active compounds into the cell similar to antibiotic resistance mechanisms [40,41].

Major advances have been made for the isolation and successful fermentation of marine microorganisms [42,43]. Other recent developments are pathway engineering and combinatorial biosynthesis [44–46], expression of environmental DNA in a heterologous host [47–49] that may allow 1 day successful culturing of yet un-culturable organisms, and technology to exploit microbial genomics to guide the discovery of novel secondary metabolites of complex polyketides and non-ribosomal peptides [50]. It is predicted that less than 1% of prokaryotic and ~7% of fungal strains have been isolated and cultured, thus these technologies provide tremendous potential for discovery of novel natural products and antibacterial agents from as yet untapped sources [51]. Despite a significant decline in industrial natural products programs, many thousands of novel natural products are reported each year [52].

6.4.2. *Natural products chemistry*

Natural products chemistry is focused on the isolation of active secondary metabolites from biologically active fermentations consisting of the complex mixture. The compound responsible for biological activity is often present in trace amounts, against a background of large amounts of biosynthetically related and structurally unrelated metabolites, making the process challenging. Purification generally involves bioassay-guided fractionation, an iterative approach where each chromatographic step is followed-up by testing fractions in biological assays. Obviously, the isolation of the active entity is most effective when accomplished in the least number of steps, mostly orthogonal steps, producing the least number of fractions [33,34,53–55]. When effectively implemented, the pure compound can be easily isolated in two iterations and in less than 2 weeks. Once pure compound is isolated, its identity can be determined by spectroscopic methods including mass and NMR spectroscopic studies. Ultraviolet and infrared spectral data provide information about chromophore and functional groups. If the molecule is

extremely complex and/or does not contain sufficient hydrogen in the molecule its structure cannot be completely determined by this method. Final structural proof can be obtained from X-ray crystallographic analysis, if the compounds can be crystallized. In the presence of a heavy atom (e.g., bromine, iodine or occasionally chlorine), whether already present in natural product or chemically added, X-ray method provides the structure with absolute configuration. Alternatively relative configuration is generally elucidated by NMR methods and absolute configurations by chemical derivatization (e.g., Mosher ester) followed by NMR analysis.

In recent years significant improvements have been made in the isolation methods, including discovery and development of chromatographic supports (both normal and reversed phase), allowing much finer chromatographic separations. However, most important improvements have been made in automation. Parallel chromatographic (i.e. at least 10 channels) systems and liquid handling systems now allow high-throughput fractionations on a moderate scale. Combining these new methods and equipment for purification of natural products has lead to significant gain in throughput. Small scale fractionations, particularly when only modest resolutions are desired, can be now efficiently performed in 96-well formats packed with reversed phase resins. This is cost and time effective in profiling, prioritization and modest purification. When the titer of the active principle is low, (e.g., less than 1 mg/L) and the mixture is highly complex, isolation can be very challenging and may require a series of purification steps. A number of other purification techniques (e.g., counter current chromatography (CCC), supercritical fluid chromatography (SFC) and capillary electrophoresis (CE)) are now often used for the purification of natural products.

Structure elucidation techniques have improved in recent years especially in LCMS and NMR methods. The LC-ICR/FTMS (Fourier transform ion cyclotron resonance mass spectrometer coupled with liquid chromatogram) [56], is highly sensitive, accurate and capable of measuring molecular mass with exceptional accuracy leading to the accurate generation of molecular formula with sub-microgram quantities of material. While mass spectral data is critical for the molecular formula determination, NMR plays much bigger role in the determination of the structure of natural products. Recent introduction of capillary and cryoprobes allow the structure elucidation of natural products with 5–10 μg of material a reality. However, while structure elucidation from this low amount of material has been demonstrated this is neither routine nor useful in drug discovery, particularly in antibacterial discovery, where biological assays require significantly more material. Structure elucidation of a reasonable natural product is most efficient, time and cost effective when ~3 to 5 mg material is used for NMR analysis allowing the collection of a full range of high quality, high resolution NMR data in less than 48–72 h. Interpretation of the data to a structure remains a highly manual- and expertise-dependent undertaking leading to *highly* variable efficiencies. With reasonable expertise, greater than 90% of novel structures can be elucidated in less than 2 weeks after data collection. Attempts to automate structure elucidation lack wide acceptance as raw data from the NMR spectrometer currently cannot be automatically extracted without significant manual intervention, an issue compounded by the lack of a perfect data set, higher order of structural complexity, and overlapping or poorly resolved signals.

6.4.3. *Extract profiling and dereplication*

A key aspect of natural product antibiotic discovery is the development of an algorithm that allows the efficient and accurate elimination of known compounds so that efforts can be directed to the discovery of novel active compounds. This is an arduous task, and has been one of the causes for the de-emphasis of many industrial natural product efforts. It is currently possible to chemically profile extracts by comparing the major components, often identifying them pre- or post-screening, with LCMS. This helps in the prioritization and grouping of extracts. However, extract profiling should not be confused with dereplication. Dereplication is a process that allows the linkage of a compound structure with the biological activity. This process becomes extremely challenging when the sensitivity of the biological assay is higher than that of the analytical methods. One way to overcome this difficulty is by making a list of the compounds that have been found active against the given target and use these to search by single ion plot of high-resolution mass ion, a process called *targeted dereplication* [34]. This process can be automated and extended for known compound classes that may be expected to show up as positives in a particular assay. For this process, a list of compounds with adjusted high-resolution mass values can be generated that can then be used for single ion plot and searching of the observed mass spectral data set generated by LC-ICR-FTMS. After isolation of a new active compound the list is appended. This is very effective tool for dereplication of specifically targeted compounds.

6.4.4. *Lead optimization to clinical candidate*

The process of lead optimization has become routine regardless of the lead source (i.e. synthetic or natural product). While the process is not trivial, the target is using the standard encyclopedia of chemical reactions to modify a lead compound, relying on the same biological assays to measure relevant biological parameters (e.g. in vitro target inhibition, MICs, in vivo efficacy, cytotoxicity, PK, etc.). Natural products identified as antibacterial leads typically have bacterial permeability (i.e. access to the target) and a purpose for production by the microorganism producer, thus avoiding the need of engineering in bacterial membrane and cell wall permeability, a situation often encountered with synthetic leads. Natural product lead optimization requires derivatization of the existing natural product structure that requires the production of larger (10–1000 g) of the natural product. It is therefore an imperative to have access to resources (biological material, capacity for large scale fermentation and chemical purification) that allow for large scale production of natural products.

7. Future of antibiotic discovery—The successful way and the future paradigm

With the passing of the Golden Age of antibiotic drug discovery the many natural product-based antibiotics and their scaf-

folds from the 1950s and 1960s are becoming less useful due to evolving resistance. Concomitantly, natural product sourcing and high-throughput antibacterial screening is virtually things of the past. In what is becoming evident as a misstep, the de-emphasis of natural products as potential drug scaffolds for medicinal chemistry, despite technology advances that have improved, if not removed, key technical difficulties that limited selection to only the so-called 'low-lying fruit'.

Scaffolds, from which medicinal chemists can create a structure–activity relationship, are key in drug discovery and in optimizing antibiotics for human use. The abundant scaffold diversity in natural products is coupled with 'purposeful design' – most microbes make by-products with a purpose – usually to afford an advantage for survival in environments threatening their growth and/or survival. It is reasoned that these ecological defense systems, produced to combat competing microbial life forms, would have some antimicrobial activity that gives the producer organism an advantage and as such, are antimicrobials to begin with [40,41,51,57]. In the search for novel antibiotics, it would be difficult to imagine a more specific source of naturally-occurring antimicrobials than in nature itself. In addition, there is little value placed in the knowledge that the far majority of natural product leads have both target-based inhibitory activity and are antimicrobial to begin with, versus the majority of nanomolar inhibitors identified from screening library collections that have limited or no antimicrobial activity, and for which there is no known SAR to build-in cell permeability.

8. Conclusions

While there is no a priori reason why one would expect non-antimicrobial chemicals that reside in chemical warehouses to have inherent antibacterial activity, there is good reason for the secondary metabolites produced in nature by microorganisms competing for an ecological niche to have inherent antimicrobial activity. So why have we virtually abandoned natural products as a starting point for antibacterial/antibiotic lead optimization? This may be due to confusion between the lack of effort by most industrial discovery organizations and the lack of successful execution in the process (i.e. mining natural products for lead compounds). History tells us that natural products have identified the far majority of quality antimicrobial scaffolds over the past 50 years, yet the dearth of quality, novel natural product leads combined with the wave of 'me-too' semi-synthetic antibiotics has lessened the value of searching for novel antibiotic scaffolds. The overall cost to a sponsoring company using natural product sourcing/screening/identification in antibiotic research is upfront, requiring the minimal post-lead identification medicinal chemistry effort. With more recent advancements in both screening and isolation of natural product actives, the time may be right for reconsideration of the investment in natural product screening for antibiotic leads.

While the majority of pharmaceutical companies have deemphasized their natural product screening efforts, many breakthrough compounds have originated from this source [58] including cholesterol lowering agents (e.g., compactin and lovastatin), immunosupressants (e.g., cyclosporin and FK506), CCK receptor antagonists (e.g., asperlicin) and antiparasitics agent (e.g., avermectin [2]). With new targets and advances in natural product-based technologies, the time is ripe to re-focus efforts on natural products, to revitalize antibiotics discovery. The use of bacterial genomic data together with innovative natural products approaches, will create a second ''Golden Age'' of antibacterial agents, both addressing bacterial resistance and commercial limitations of current approaches [59,60].

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