Harnessing the potential of chemical defenses from antimicrobial activities

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Summary

Resistance to the drugs used in the treatment of many infectious diseases is increasing, while microbial infections are being found to be responsible for more life-threatening diseases than previously thought. Despite a large investment in the invention and application of high-throughput screening techniques involving miniaturization and automation, and a diverse array of strategies for designing and constructing various chemical libraries, relatively few new drugs have resulted. Natural products, however, have been a major source of drugs for centuries. Since some of them are produced by organisms as a result of selection in favour of improved defense against competing deleterious microorganisms, in principle they would be less likely to incur resistance. Furthermore, the production of those defensive secondary metabolites is inducible because their original function is a response to environmental challenges. Moreover, symbioses, co-habitation associations between two or more different species of organisms, are universal in nature, and the production of secondary metabolites by symbiotic microbes may be an important adaptation allowing microbes to affect their hosts. Therefore, co-culture strategies, using combinations of plant cell–pathogenic microbes, plant cell–endophytes (or symbionts), and symbiont–pathogenic microbes, based on the principles of chemical defense and the known mechanisms of organism interactions, may be an efficient general approach in the search for new anti-microbial drugs.

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Introduction

Since the first use of penicillin in clinical trials in early 1941, medical practice has come to rely tremendously on antibiotics. By definition, natural antibiotics are the products of secondary metabolisms of microorganisms, which are not essential for the living of their producing organisms. The use of antibiotics in clinics has saved many lives, and a diverse array of antibiotics has been used to treat new infectious diseases that emerge and to deal with existing diseases that have developed drug resistance (Fig. 1). However, microbial infections are being recognized with increasing frequency as an important cause of both morbidity and mortality.[1] Current chemotherapy is far from optimal, and there is evidence that resistance to available drugs is increasing.[2–4] Moreover, hospital patients with diseases that impair their natural defenses are susceptible to opportunistic infections and drug resistance is emerging in virtually all nosocomial (hospital) pathogen–antimicrobial combinations. As a result, several bacterial infections may soon become refractory to treatment. These include: methicillin-resistant *Staphylococcus aureus*,[5] vancomycin-resistant *Enterococci*,[6] and numerous gram-negative species resistant to extended-spectrum cephalosporins (see the chemical structures of penicillins, cephalosporins and vancomycin in Fig. 1). In addition, communities have also experienced drug resistance problems in both developing and developed countries. Drug resistance in the developed world has been increasing in food-borne pathogens such as *Campylobacter*[6] and *Salmonellae*.[7] These forms of drug resistance are most likely attributed to the use of antimicrobials in animals bred for food. In addition, microbial infections may cause more life-threatening diseases than we thought previously,[8] for example, gastric ulcers are attributed to a *Helicobacter pylori*, atherosclerosis and coronary disease are caused by *Chlamydia pneumoniae*, hepatic infection by *Helicobacter hepaticus* has been linked to some cases of liver cancer, and even kidney stones may be at least partially caused by infectious bacteria.[9]

These developments highlight the need to stimulate further research into strategies aimed at preserving the effectiveness of currently available antimicrobial agents and finding new classes of antimicrobial agents. In this article, we will discuss the potential of the still largely unknown world of secondary

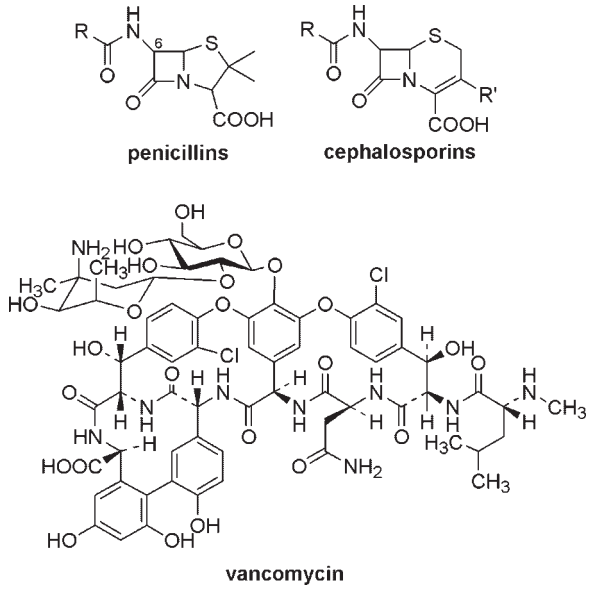


Figure 1. The chemical structures of penicillins, cephalosporins and vancomycin. The penicillins are a group of natural and semisynthetic β-lactam antibiotics. Their different structures are derived by making substitutions at position 6. The cephalosporins are β-lactam antibiotics derived from structural modifications of the fermentation products *Cephalosporium acremonium*. The abundant variants of penicillin and cephalosporin, a great many of which have been used as antibiotics in clinics, were produced to combat successive waves of resistant pathogens, reflecting the innovatory significance of antibiotic natural products, e.g. β-lactam natural products, in antimicrobial drug discovery.

metabolites of plant and/or microbial origins as the irreplaceable sources of potential antimicrobial drugs, and indicate some strategies for uncovering particular ones of value.

The frontiers and dilemma of drug discovery

Drug discovery is an integrated business, which depends on a great deal of communication between multiple disciplines such as chemistry (biochemistry), biology, pharmacology, toxicology and clinical testing. The need for cross-discipline communication can be attributed to two main aspects of the process: screening and production of compounds. Modern drug discovery usually starts with screening, which tests large numbers of compounds to see if they produce an appropriate biochemical, molecular or cellular effect. A positive response in the first round of screening identifies the primary ''hit'' compounds. These molecules are then subjected to more strict screening procedures to see if they have physicochemical and pharmaceutical properties that are not too incompatible with the standards of making a drug. If the compound passes the screening process, a ''hit'' becomes a ''lead''. Lead compounds then undergo further rounds of chemical refinement or modification and pharmacological screenings before finally entering clinical trials. With a good deal of luck, the ''lead'' might eventually be approved as a drug 12–15 years after testing began.[10]

The screening techniques applied by academic and industrial scientists are presently dominated by high-throughput screening deployed with miniaturization and automation.[10] But despite tremendous advances, chiefly the increased use of automation, in all aspects of the screening process, these improvements have not brought about the expected rise in productivity, and the industry's drug pipeline still looks decidedly narrow. But all now agree that, for screening, quality is more important than quantity, making sure that high-quality compounds are going into robust and reproducible assays. One attractive stategy is to evaluate and screen compounds for drug-like properties such as structure feature, solubility and lipophilicity before they ever get into the chemical library. This should give medicinal chemists an easier time by ensuring that lead compounds need less modification to turn them into drugs.

The advances in high-throughput technology highlight the need to increase the number of compounds available for screeing. One of the efficient methods pharmaceutical companies can now use to produce chemical libraries, (a chemical library is a large array of diverse molecular entities) is combinatorial chemistry. This approach, step-by-step, builds up millions of related synthetic compounds by setting the systematic and repetitive connection of a set of different ''building blocks'' of varying structures to each other. Others include: combinatorial biosynthesis,[11,12] engineering of biosynthetic pathways,[13,14] bio-transformations, genomics-guided approach,[15] culture technical including elicitation on plant cell cultures[16] and induction of microbial secondary metabolisms,[17] which have more chances to identify candidates with better drug-like features.

Despite the advent and applications of high-throughput screening and combinatorial chemistry, a large investment has so far returned few results. The bottleneck in drug discovery has shifted from the generation of lead structures to their transformation into orally active drugs. These must have the desired physiological properties and performance results in clinical trials, including, in particular, effectiveness to drug-resistant pathogenic strains. The identification and development of drugs with potential for multiple blockade will be advantageous. Simultaneous inhibition of more than one target renders the emergence of resistance through target site alternations less likely, since mutations may be required in several targets to confer resistance.[18] Natural products qualified as base compound drugs, and this term is often used synonymously with ''secondary metabolite'', and are an established and rich source of drugs. Natural products have served as a major source of drugs for centuries, and about half of the pharmaceuticals in use today are derived from natural

products.[19] Interest in natural products research is strong and can be attributed to several factors, including (1) unmet therapeutic needs, (2) the remarkable diversity of both chemical structures and biological activities of naturally occurring secondary metabolites, (3) the utility of bioactive natural products as biochemical and molecular probes, (4) the development of novel and sensitive techniques to detect biologically active natural products, (5) improved techniques to isolate, purify and structurally characterize these active constituents, and (6) advances in meeting the demand for supply of complex natural products.

Chemical defense and the inducible secondary metabolisms

Among the various natural functions of secondary metabolites, their deterring actions against host competitors are most obvious since their source organisms are continuously exposed to a large diversity of potentially harmful biotic and abiotic factors. In fact, such chemical defense is observed for many organisms, from microorganisms to higher plants and animals. Conventionally, it is well accepted that chemical defense is "characteristic of free-living organisms with limited range of movement or limited control over their movement".[20] Defense against pathogens, parasites and other harmful organisms is often enhanced after their invasion, which indicates that chemical defense can be induced.[21] The presence of defensive compounds in plants is virtually universal, ranging from mosses to angiosperms, most of which remain firmly rooted to the ground for most of their lives.

Natural products produced by higher plants evolved by selection for improved defense against microbial attacks. Because plants are continuously exposed to a large diversity of potentially deleterious microorganisms,[22] they are, therefore, less likely to incur resistance. Plants are the sources of enormous numbers of metabolites, whose structures, biological functions and applications have been exploited only superficially. Among the natural products, antifungal secondary metabolites of plants are the most widespread. These compounds may be preformed, can be found in healthy plants and may contribute to the in-built chemical barriers to potential pathogens. Alternatively, these compounds may also be produced in response to a pathogen invasion (phytoalexins), which include a diverse array of secondary metabolites, such as phenolics, saponins, cyanogenic glycosides, cyclic hydroxamic acids, sesquiterpenes, isoflavonoids, sulfur-containing indole derivatives, and many other types of structure.[23]

Symbionts for chemical host defenses

Among the general classes of interaction of organisms, symbiosis is of great interest because it may propel evolution through the engine of chemical interactions, particularly chemical defense. Symbiotic or mutualistic associations that involve interspecific genetic interactions are common in nature because organisms do not exist in isolation from others, and organisms do not occur axenically. Symbioses have incurred evolutionary impacts on speciation and biodiversity, microbial pathogenesis, and the evolutionary arms race between a host and its symbiont. Symbioses affect gene pools through horizontal gene transfer, which has been documented in all forms of organisms, and has had an impact on the evolution of secondary metabolisms. More importantly, the production of secondary metabolites by symbiotic microbes is thought to be an important adaptation allowing microbes to affect their hosts.

Although proven examples are rare, it is not unlikely that symbionts may be important sources of chemical defenses for plants and animals. Aquatic plants and animals are particularly vulnerable to pathogenic microorganisms because of their constant intimate contact. The adaptation of symbiotic microbes may represent an essential survival strategy in freshwater and marine environments. Research into the defending mechanism of embryos of the shrimp *Palaemon macrodactylus* against infection by the crustacean pathogenic fungus *Lagenidium callinectes* revealed that the production and release of the antifungal compound 2,3-indolinedione (isatin, tribulin) by the epibiotic bacteria *Alteromonas* sp. served as a chemical defense for the symbiotic hosts.[24] The common seaweed *Lobophora variegata* has invented the strategy of using a macrolactone, lobophorolide, probably produced by its symbiont to defend against pathogenic and saprophytic marine fungi.[25]

Symbionts also provide defensive functions for their hosts in terrestrial organisms. Symbiotic interactions of C3 grasses with fungal endophytes, *Epichloë* species and their asexual relatives *Neotyphodium*, provide the grass host with chemical defense fitness through the production of four groups of alkaloids: lolines, peramine, ergot alkaloids, and lolitrems.[26] Many antimicrobial natural products are suspected metabolites of uncultured bacterial symbionts. These situations are intriguing and are quite firmly supported on the basis of molecular evidence. The cloning and sequencing of the pederin synthetase gene cluster from an uncultured organism of *Paederus* beetles unraveled the symbiotic origin of this structurally unique insect defense compound.[27]

Chemical defense is also involved in microbial interactions. In both terrestrial and marine biotic biospheres, microbial interactions are common.[28] Soil bacteria of the genus *Pseudomonas* produce a wide array of antibiotics that effectively inhibit the soil-borne fungi that may cause plant root diseases such as black root rot in tobacco.[29] *Pseudomonas fluorescens* Migula F113 was shown to control the soft rot potato pathogen *Erwinia carotovora* subsp. *atroseptica* Dye by production of the antibiotic 2,4-diacetylphloroglucinol.[30] Burgess and coworkers reported that many marine microbial strains that did not normally produce antibiotics could be induced to do so by being challenged with live cells, supernatants from other bacterial cultures or other chemicals.[31]

Overall, secondary metabolites important in the ecological interactions between organisms could be a promising source of novel antimicrobial compounds.

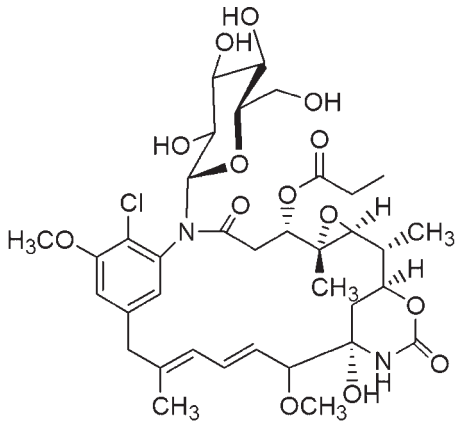
Co-culture may be an efficient approach: using the force of natural selection to produce antimicrobial compounds

The widespread nature of secondary metabolites and the preservation of their sophisticated biosynthetic pathways indicate that secondary metabolites serve survival functions in the organisms that produce them. It is well known that secondary metabolites are most useful to the organisms producing them as competitive weapons, communication "words" and more functions of secondary metabolites that have yet to be discovered. Genome analysis of actinomycetes has revealed the presence of gene clusters encoding more putative natural products than those that have been identified.[32,33] It is not known, however, whether these abundant cryptic loci just remain dormant until appropriate signal inductions or are relics of evolution. Proteomics and metabolomics, in particular, have revealed the unbelievable diversities of secondary metabolites, produced by post-translational protein modifications,[34] their low enzyme specificity,[35] and the fact that their productions are environment sensitive.[36] Moreover, knowledge of genetics and molecular evolution helps us to understand how biosynthesis of many classes of secondary metabolites evolved. This supports the hypothesis that secondary metabolism has arisen through the modification of existing primary metabolic reactions, namely "inventive evolution".[37] The concept of inventive evolution invokes duplication of genes and mutation of the gene copies, amongst other genetic events. The modified duplicate genes, in conjunction with other genetic events, may give rise to new enzymes, which, in turn, may generate new products, some of which may be selected for. Natural selection caused the shape and size of the secondary "metabolome" to be extraordinarily variable so that it can provide the producing organism with comprehensive functions and increase an organism's fitness.

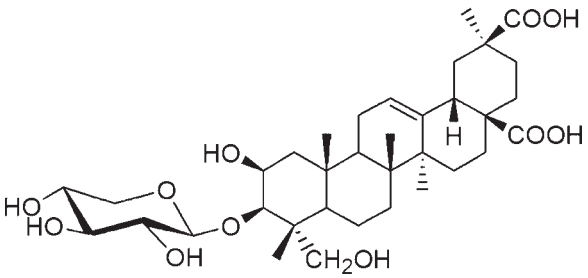
According to the results of genome analysis of actinomycetes, there are numerous cryptic gene clusters encoding more putative natural products,[32,33] indicating that those strains have the potential to generate a diverse array of secondary metabolites. While the recombinant DNA approaches performed by institutions with good financial support can bypass the limitations imposed by traditional cultivation; these recombinant approaches are unaffordable by many academic researchers. To develop as many compounds as possible from limited sources, methodologies of fermentation are critical to drug discovery. Zeeck et al proposed an "OSMAC (One Strain-Many Compounds)" approach, which derived from the observation that very small changes in the cultivation condition can completely shift the metabolic profile.[36] It is an efficient way of improving secondary metabolic diversity though it cannot be designed rationally at the present moment because of our insufficient knowledge about the complex biosynthetic and regulative, intracellular and intercellular, networks. Conventionally, secondary metabolites are mostly produced under submerged liquid fermentation (SLF) in industry because the processes associated with scale-up are straightforward. Recently, solid state fermentation (SSF) is becoming more and more attractive for the production of antibiotics because it produces a high product concentration with a relatively low energy requirement in shorter time period.[38] Moreover, our latest finding indicated that the maytansinoid, ansamitocin-producing strain *Actinosynnema pretiosum* ssp. *auranticum* ATCC 31565 produced both ansamitocins and their N-glucosides (Fig. 2) under SSF conditions but only produced ansamitocins under normal SLF conditions, revealing that fermentation methodology has great potential in manipulating secondary metabolism.[39]

Co-cultures with combinations of plant cell–pathogenic microbes, plant cell–endophytes (or symbionts), and symbiont–pathogenic microbes, designed on the principles of chemical defense and the known mechanisms of organism interactions, are at their nascent stage due to the lack of knowledge about the complex networks of biotic interactions. However, several promising results have emerged recently. Burgess et al.[31] reported that microbial antagonism could be a viable approach to creating bioactive secondary metabolites, and anticipated that "many strains which did not normally produce antibiotics could be induced to do so by exposing them to small amounts of live cells, supernatants from other bacterial cultures or other chemicals". They tested various combinations between marine surface-associated bacteria and culture supernatants of other marine epiphytes, and the elicited production of antimicrobial compounds was observed.

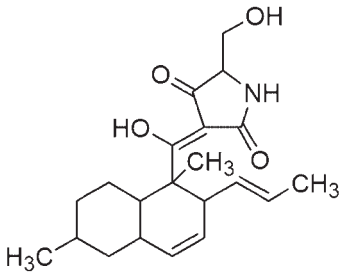
Bacteriocins are antimicrobial peptides produced by many lactic acid bacteria, which may be produced for the competitive purpose of inhibiting the growth of related species or species with the same nutritive requirements that are present in the same ecological niche.[40] *Lactobacillus plantarum* NC8 produced a new bacteriocin named plantaricin NC8 when dually cultivated with *Lactococcus lactis* MG1363 or *Pediococcus pentosaceus* FBB63,[41] indicating that the productions of antimicrobial compounds such as bacteriocins are induced by the presence of competing microorganisms; this should produce antimicrobial compounds more efficiently since their production is targeted. The co-cultures of *Phytolacca americana* callus and *Botrytis fabae*, and *Trichoderma harzianum* and *Catharanthus roseus* callus produced the antifungal compounds phytolaccoside B[42] and trichosetin[43] (Fig. 2), respectively. Trichosetin showed remarkable antimicrobial activities against the Gram-positive *Staphylococcus aureus* and *Bacillus subtilis*.



amide N-glucoside of sansamitocin P-2



phytolaccoside B



trichosetin

Figure 2. The chemical structures of three selected novel compounds discovered in microbial solid state fermentation (SSF) tests or by co-cultures of plant cells and microorganisms. The isolation of amide N-glucoside of sansamitocin P-2, which has prominent antifungal and anticancer activities, from the ansamitocin-producing strain *Actinosynnema pretiosum* ssp. *auranticum* ATCC 31565, under SSF conditions, indicated the potential of fermentation methodology in manipulating secondary metabolism. Phytolaccoside B and trichosetin, which showed evident antifungal activities, were produced by the co-cultures of *Phytolacca americana* callus and *Botrytis fabae*, and *Trichoderma harzianum* and *Catharanthus roseus* callus, respectively. These examples indicate that the production of some bioactive secondary metabolites reflects, at least partially, the consequence of the adaptation of their source organisms to environmental variations, in particular, chemical defense roles. These approaches may have great potential in the search for novel antimicrobial drugs.

Conclusions

It is well known that biosynthesis of secondary metabolites is a high-energy-consuming process and for this reason is well controlled by molecular regulatory systems. Living organisms, particularly sedentary species must be able to adapt their metabolism to the changing environments. This adaptation requires that organisms sense the multitude of extracellular signals (or inputs) and respond by controlling the expression of an appropriate repertoire of genes (outputs). In the various co-culture systems of plant cells and pathogenic microbes, plant cells and endophytes (or symbiont), and symbionts and pathogenic microbes, organisms adapt their productions of secondary metabolites to respond to the changing environmental conditions, therefore, affording antimicrobial activities. Secondary metabolites having defensive activities are usually target produced and, therefore, their productions can be induced through rationally designed co-cultures. These co-cultures should be designed according to the principles of chemical defense and the known mechanisms of organism interactions. Once we fully understand the complex networks of biotic interactions such as environment perceptions by organisms and their responsive mechanisms, and the intracellular signal transduction cascades, the approach of using these naturally produced chemical defenses to obtain antimicrobial compounds for drug applications should become increasingly useful. Finally, new technologies concerning plant cell and/or microbe cultures,[44,45] and the advance in other disciplines of drug discovery will accelerate the progress of this research.[46]

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