

Prospects for plant-derived antibacterials

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Can weakly active phytochemicals be combined synergistically to produce new antibacterial treatments?

The scarcity of disease in wild plants indicates that the success of plant defense mechanisms in combating pathogen infection rivals that of mammalian immune systems. Although there are some interesting parallels between plant and animal defense strategies—for instance, both groups sacrifice infected tissues through apoptosis; produce numerous antimicrobial peptides; and attack microbes by creating an acidic environment, hydrogen peroxide and iron chelators—the similarities end there. Instead of pathogen-specific, somatically generated antibodies, plants evolved a relatively limited array of genomically encoded receptors to recognize specific pathogens, and developed an enormous variety of small-molecule antimicrobials. Here, we focus on the latter aspect of the plant defense response and discuss the potential for developing plant antibacterials into useful antibiotics.

action, need to be present at millimolar concentrations to offer adequate protection. For example, large amounts of weak acids, such as salicylate and its derivatives, need to penetrate pathogen cells in a protonated form in order to dissipate the transmembrane pH gradient and the proton motive force. Similarly, hydrogen peroxide and other oxidants, such as phenols, are needed in copious amounts to oxidize their many targets and overcome the oxidation stress responses of pathogens. Yet, looking at a random sampling of plant antimicrobials, one finds many that are not obviously toxic detergent-like molecules or redox agents, or are not necessarily produced in large quantities. A very effective though nonspecific antimicrobial, pyrrithione (**Fig. 1**), is synthesized by the Chinese medicinal plant *Polyalthea nemoralis*² and is active against bacteria and fungi.

Coincidentally, this apparent ionophore was independently synthesized by chemists and is used commercially in shampoo to prevent the growth of yeast that cause dandruff.

Do plants synthesize target-specific antibacterials? Although there are dozens of plant secondary metabolites that show activity in the micro- to submicromolar range, at least against Gram-positive species³, surprisingly little is known about their mechanisms of action. Coumarins appear to be the only group of plant antibacterials for which a specific target has been suggested. This is based on the chemically related antibiotic novobiocin, a systemically used therapeutic agent that targets DNA gyrase and is produced by several different *Streptomyces* species. Although some plant coumarins show excellent activity against *Staphylococcus aureus*, they are

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Plants as a source of antimicrobials

Plants make over 100,000 small-molecule compounds¹, many if not most of which have antimicrobial activity. At the same time, their activity is generally weak—orders of magnitude less than that of common antibiotics produced by bacteria and fungi. Although at first glance this may not seem encouraging for drug development, closer scrutiny suggests room for optimism.

Some plant antimicrobials are produced at high levels and, owing to their mechanism of

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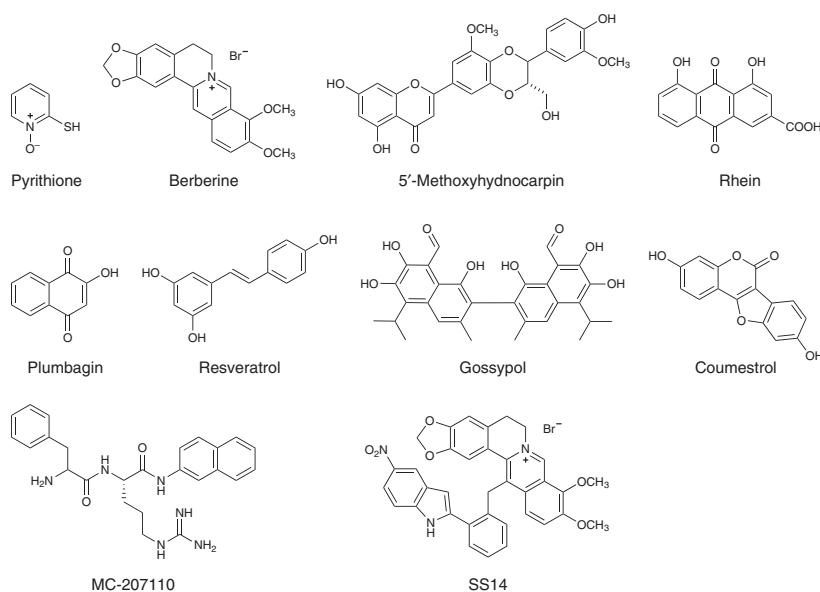


Figure 1 Structures of plant antimicrobials and MDR inhibitors.



Figure 2 *Berberis fremontii*, which produces berberine and 5'-methoxyhydrnocarpin.

ineffective against Gram-negative species—a general problem with many antibacterials, as discussed below.

Attempts to find potent, nontoxic, broad-spectrum antibiotics from plants, and more specifically from medicinal plants, have failed, even though large-scale screens have been undertaken both by pharmaceutical firms (e.g., Whitehouse Station, New Jersey, USA-based Merck (L. Silver, LL Silver Consulting, personal communication) and New York-headquartered Pfizer (J. Clancy, Emergent BioSolutions, personal communication)) and by biotech firms (Phytera (Worcester, MA, USA) and Shaman Pharmaceuticals (South San Francisco, CA, USA)). At least some of these screens (specifically at Phytera) were performed using extracts from plants that were stimulated to synthesize antibacterials. A major component of the plant defense response is induced by pathogen attack, presumably because synthesis of antibacterials is energetically costly. It is well established that pathogen infection or treatment with immune elicitors, such as salicylic acid, greatly increases the repertoire of secondary compounds produced⁴.

Although no antibiotics with specific cellular targets have been isolated from plants, a notable precedent for the successful isolation of a compound used to treat an infectious disease is quinine, isolated from the bark of cinchona tree and used as an antimalarial agent by peoples native to South America⁵. Quinine is a metal chelator, and its high potency against the malarial parasite seems to be a lucky coincidence.

Synergy and multidrug resistance pump inhibitors

One explanation for the failure to identify potent broad-spectrum plant-derived antibacterials is that plants may use a different chemical strategy for the control of microbial infections, perhaps to decrease the selective pressure for developing antibiotic resistance. This is not necessarily surprising, as multicellular animals also do not produce potent antibiotics but instead rely on a sophisticated immune response to thwart invading pathogens. For example, plant antibacterials may act in combinations and have little efficacy alone. This idea has been tested in one of our laboratories (K.L.) using the plant alkaloid berberine (**Fig. 1**). Berberine, a hydrophobic cation that increases membrane permeability and intercalates into DNA⁶, is widespread in nature and is present, for instance, in common barberry (*Berberis* species) plants (**Fig. 2**) and the medicinal plant goldenseal (*Hydrastis canadensis*). The fact that both of its targets are immutable makes berberine the perfect antibacterial. Moreover, its positive charge facilitates its active accumulation in bacterial cells⁷. Nevertheless, in spite of these apparently excellent properties, berberine is ineffective as an antibacterial because it is readily extruded by pathogen-encoded multidrug resistance pumps (MDRs)⁸. Hydrophobic cations such as berberine are actually the preferred substrates of all classes of MDRs^{8,9}. Reasoning that plants would benefit from blocking this efflux, an MDR inhibitor was sought in barberry plants, and the compound 5'-methoxyhydrnocarpin (**Fig. 1**) was isolated. 5'-Methoxyhydrnocarpin

blocks so-called major facilitator MDRs of Gram-positive bacteria, which are drug-proton antiporters and have an especially strong bias toward cationic substrates. A combination of 5'-methoxyhydrnocarpin and berberine acted as a potent antibacterial¹⁰.

The presence of 5'-methoxyhydrnocarpin in barberry plants provides support for the hypothesis that, in general, plant antibacterials are individually relatively weak but function in synergy. This leads to a broader question: are plant antibacterials generally limited in their efficacy by MDR efflux? Testing a random collection of compounds against a panel of bacterial pathogens showed that disabling MDRs by mutation, addition of synthetic MDR inhibitors, or both improved antibacterial activity in all cases tested, and for some compounds quite dramatically¹¹. For example, the activity of rhein (**Fig. 1**), the principal antibacterial from rhubarb, was potentiated 100- to 2,000-fold (depending on the bacterial species) by disabling MDRs. Comparable potentiation was observed with plumbagin, resveratrol, gossypol and coumestrol (**Fig. 1**). The extent of potentiation was the largest in the case of Gram-negative bacteria. For example, rhein had no activity against *E. coli* at the limit of solubility (500 µg/ml), whereas disabling MDRs produced a minimum inhibitory concentration (MIC) of 0.25 µg/ml. In principle, MDR efflux could be countered by simply increasing the concentration of the antibacterial above the saturation of the pumps. Indeed, this is how commercial antibiotics are empirically dosed. It is possible that plants may also produce antimicrobials in high concentrations for the same reason, but—as the above example shows—this strategy would not work with rhein.

As mentioned above, MDRs of Gram-positive species are strongly biased toward hydrophobic cations and do not present a serious problem for the penetration of most clinically relevant antibiotics. The NorA (norfloxacin A) major facilitator MDR of *S. aureus* pumps out norfloxacin (Noroxin) and ciprofloxacin (Cipro), which have cationic forms, increasing the MIC two- to four-fold. However, the latest fluoroquinolones bypass the MDRs of Gram-positive bacteria. The cytoplasmic membrane of Gram-positive species, like a simple lipid bilayer, is not a barrier for most amphipathic compounds and can be readily traversed by antibacterials.

In contrast, Gram-negative bacteria have evolved a sophisticated permeability barrier. The surface of the additional, outer membrane of Gram-negative species comprises lipopolysaccharide, and this highly hydrophilic layer restricts penetration of hydrophobic and amphipathic compounds, which encompasses

most drugs. Gram-negative resistance—nodulation—cell division (RND) MDRs efflux amphipathic substances across this barrier. The cytoplasmic membrane, as in Gram-positive bacteria, restricts penetration of hydrophilic compounds. As a result, the Gram-negative cell envelope is designed to restrict penetration of *all* molecules; nutrients enter through porins and specialized transporters^{9,12,13}. This efficient permeability barrier has been largely responsible for the inability of the pharmaceutical industry to produce new classes of broad-spectrum compounds that are equally active against Gram-negative and Gram-positive species. Indeed, the last class of broad-spectrum compounds that were discovered by the pharmaceutical industry, the fluoroquinolones, were developed 40 years ago. Interestingly, this class was derived from nalidixic acid (NegGram), a synthetic inhibitor of DNA gyrase that was synthesized as a precursor of quinine⁵. Tygacil (tigecycline), which was recently developed by Wyeth (Madison, New Jersey, USA), is an excellent broad-spectrum compound that overcomes known resistance to tetracyclines, but it is built on a tetracycline backbone^{14,15}.

It is sobering that we have not been able to synthesize something comparably effective to fluoroquinolones over the past four decades, despite the vastly improved technological capabilities—advanced organic synthesis methods, combinatorial chemistry, high-throughput screening, genomics and proteomics—at our disposal. Evolution produced antibiotics (for example, aminoglycosides, tetracycline and chloramphenicol (Chloromycetin)) that can largely bypass the dual barrier-extrusion mechanism of Gram-negative bacteria, but synthetic compounds almost invariably fail.

Targeting bacterial virulence

In addition to synthesizing relatively weak antibiotics in concert with MDR inhibitors, another chemical strategy that plants may employ to fight bacterial infections is to synthesize compounds that target bacterial virulence rather than bacterial growth. Although relatively little work has been done in this area, it is interesting to speculate that many of the thousands of antibacterial compounds that plants produce target the virulence mechanisms of pathogens.

Evidence for the production of ‘antivirulence’ compounds in plants is the synthesis of quorum-sensing mimics, which interfere with intraspecies bacterial communication that is critical for the regulation of virulence-related genes^{16–18}. The rationale for plants targeting pathogen virulence is the same as that for them synthesizing multiple weak antibiotics along with MDR inhibitors. It may be less likely that

there will be as strong a selection for resistance against compounds that only render pathogens less pathogenic than against compounds that are either bactericidal or bacteriostatic. Natural or synthetic compounds that block the virulence of pathogenic microbes are a largely unexplored class of antibacterial agents.

The successful targeting of a virulence product is illustrated by a recent paper by Hung *et al.*¹⁹, in which a high-throughput screen was used to identify compounds that inhibit the activity of the *Vibrio cholerae* transcriptional regulator ToxT, which is required for expression of cholera toxin and the toxin-co-regulated pilus. A small molecule identified in this screen, 4-[*N*-(1,8-naphthalimide)]-*n*-butyric acid, protected infant mice from intestinal colonization by *V. cholerae*.

Future directions

There seems to be considerable potential to develop at least three different types of antibacterials from plants—traditional antibiotics, MDR inhibitors and compounds that target bacterial virulence. For antibiotics, the obvious question is whether plants synthesize target-specific compounds that have a sufficiently good MIC, at least against Gram-positive species. There has indeed been a considerable effort to discover plant-derived antibacterials active against methicillin-resistant *S. aureus* (MRSA) strains, which have developed resistance to most existing antibiotics³. The alarm level went up when strains resistant to the last line of defense, vancomycin, were isolated from the clinic. Driven by this urgent need for new antibiotics, numerous anti-*S. aureus* plant-derived antibacterials with micromolar MICs have been identified in the past decade by researchers in academia³. To our knowledge, however, this effort did not result in the identification of the mechanism of action or animal testing of any of the compounds, leading to the assumption that these are simply additions to the already existing long list of plant-derived antiseptics.

In order to become a viable lead compound, a plant-derived substance must show specificity against a particular target that is absent in humans. This obviously increases the likelihood of developing a nontoxic antibacterial. At the same time, the drug industry has developed several excellent new antibiotics active against MRSA and other Gram-positive species. These are Zyvox (linezolid; a synthetic protein synthesis inhibitor), Synercid (another protein synthesis inhibitor based on the synergistic action of quinupristin and dalbapristin—derivatives of streptogramins produced by *Streptomyces* species) and Cubicin (daptomycin; a membrane-acting antibiotic

produced by *Streptomyces roseosporus*²⁰). As discussed above, however, the industry has failed to find new broad-spectrum compounds against Gram-negative species.

Modifying existing natural antibiotics to make broad-spectrum compounds has been more successful. Examples are ampicillin, derived from penicillin, and Zithromax (azithromycin), derived from erythromycin. Similarly, one may envisage creating new types of broad-spectrum antibiotics based on plant antibacterials. Given that we do not know of any plant antibacterial (with the exception of the fairly toxic pyrrhione) with high potency against Gram-negative bacteria, broadening the spectrum of existing potent compounds acting against Gram-positive bacteria seems like a reasonable option. Of course, this will depend on whether these potent narrow-spectrum compounds are target-specific, as discussed above.

The second approach for identifying plant-derived antibacterials is discovering therapeutically useful MDR inhibitors. So far, several types of plant-derived inhibitors, such as 5'-methoxyhydrnocarpin (mentioned above), have been reported^{10,21–25}, but all of them are inhibitors of major facilitator MDRs of Gram-positive bacteria. Indeed, plants seem to protect themselves against Gram-positive bacteria very effectively (reflected, perhaps, in the fact that essentially all agriculturally significant bacterial pathogens are Gram-negative species), in part due to multiple weak antibacterials and the presence of major facilitator MDR inhibitors. But do plants make inhibitors that are effective against RND MDRs of Gram-negative species? Developing MDR inhibitors against RND pumps could rapidly enlarge our current arsenal of broad-spectrum antibiotics. Both older antibiotics, such as Rifadin (rifampin) and lincosamides (which are currently ineffective against Gram-negative species), and the newer narrow-spectrum antibiotics discussed above could be combined with an MDR inhibitor and produce an effective therapy. Finding nontoxic plant-synthesized RND MDR inhibitors would be a very effective strategy against a major group of pathogens, but whether plants synthesize such inhibitors remains an intriguing open question.

In the meantime, biotech firm Microcide Pharmaceuticals (which in 2001 merged with Altheus to form the now defunct Essential Therapeutics; Waltham, Massachusetts, USA) discovered a synthetic broad-spectrum RND inhibitor, MC-207110 (*N*-phenyl-2(*R*)-phenylalanyl-2(*S*)-(guanidinopropyl)glycylamide), in a high-throughput screen. Its derivatives, MC-002595 (3-[1-oxo-2-[(1-oxo-2(*S*)-aminopropyl)ethylamino]-2(*R*)-phenyl-

propyl]ethylaminoquinoline) and MC-004124 (2*R*,4*R*)-*N*-((*R*)-1-(quinolin-6-ylcarbonyl)-3-phenylpropyl)-4-(aminomethyl)pyrrolidine-2-carboxamide), have proven efficacious in animal models²⁶. Resistance to this class of inhibitors occurred at a rate of approximately 10⁻⁹, which is equivalent to what is observed for clinically relevant antibiotics. Although prolonged administration of MC-004124 caused nephrotoxicity and its further development was terminated, this work provided an important proof-of-principle for the feasibility of developing an MDR inhibitor. Derivatives with diminished nephrotoxicity are currently under development by Mpex Pharmaceuticals (San Diego, California, USA; O. Lomovskaya, personal communication).

A potential problem with any dual-compound therapy is the difficulty in matching the pharmacokinetics of two different molecules. This, however, has not prevented the development of Augmentin, a combination of amoxicillin and clavulanic acid (an inhibitor of β -lactamase) into the most widely used antibiotic. It is also possible to produce a well penetrating conjugate between an MDR inhibitor and an antibacterial. This has recently been demonstrated for a conjugate, named SS14, between the synthetic major facilitator MDR inhibitor INF55 and berberine²⁷ (Fig. 1).

The third approach to identifying plant-derived antibacterials is to screen for compounds that specifically block pathogen virulence by targeting a key toxin or a particularly potent virulence factor. Developing high throughput assays for antibiotics or MDR inhibitors is relatively straightforward, although, as discussed above, it appears advisable to screen for antibiotics in the presence of appropriate MDR inhibitors. In contrast, isolating from plants new antibacterials that have specific virulence-related targets is significantly more challenging because in many cases the effect of this category of anti-infective compound can only be observed in the context of a whole-animal infection model. This is particularly true for many

nosocomial pathogens that infect immunocompromised patients, including *Enterococcus faecalis* and *Pseudomonas aeruginosa*, where no single essential virulence factor has been identified. Without the ability to target a specific known virulence factor, identifying anti-infective compounds becomes a daunting prospect that requires screening *in vivo*.

As described in two recent publications^{27,28}, our laboratories have developed a general method for identifying anti-infective compounds that involves a high-throughput screen in an animal—the nematode *Caenorhabditis elegans*. The screen is based on the observation that a remarkably large number of human pathogenic bacteria infect and kill *C. elegans*. Because *C. elegans* is small and can be manipulated in high-throughput screens using robotics and standard 96- or 384-well microtiter plate technology, it is possible to screen directly for compounds that ‘cure’ an infected nematode of a lethal bacterial infection. In the assays developed to date, the endpoint is nematode survival. Advantages of this screening method are the ability to discover compounds with no antibacterial activity *in vitro*, including prodrugs or compounds that target functions only important for *in vivo* survival or virulence, or activators of innate immunity. Because the assay endpoint is host survival, an additional advantage of this *in vivo* screen is that it eliminates a large background of compounds that are toxic or ineffective when used therapeutically due to poor pharmacokinetics. In work published to date²⁸, 6,000 synthetic compounds and 1,136 natural extracts (mostly from plants) have been screened using a *C. elegans*–*E. faecalis* infection model, leading to the identification of 16 synthetic compounds and 9 extracts that are effective at preventing *E. faecalis*–mediated killing of *C. elegans* but which have little or no activity in preventing the growth of *E. faecalis* *in vitro*. The identified compounds and extracts, some of which may potentially target virulence rather than bacterial growth, illustrate the potential of identifying novel plant-derived compounds that may someday be used as human therapeutics.

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