ABSTRACT Bioprospecting is the exploration, extraction and screening of biological material and sometimes indigenous knowledge to discover and develop new drugs and other products. Most antibiotics in current clinical use (e.g. β-lactams, aminoglycosides, tetracyclines, macrolides) were discovered using this approach, and there are strong arguments to reprioritize bioprospecting over other strategies in the search for new antibacterial drugs. Academic institutions should be well positioned to lead the early stages of these efforts given their many thousands of locations globally and because they are not constrained by the same commercial considerations as industry. University groups can lack the full complement of knowledge and skills needed though (e.g. how to tailor screening strategy to biological source material). In this article, we review three key aspects of the bioprospecting literature (source material and in vitro antibacterial and toxicity testing) and present an integrated multidisciplinary perspective on (a) source material selection, (b) legal, taxonomic and other issues related to source material, (c) cultivation methods, (d) bioassay selection, (e) technical standards available, (f) extract/compound dissolution, (g) use of minimum inhibitory concentration and selectivity index values to identify progressible extracts and compounds, and (h) avoidable pitfalls. The review closes with recommendations for future study design and information on subsequent steps in the bioprospecting process.

KEY WORDS biolaw · drug discovery · ecology · ethnomedicine · natural products

ABBREVIATIONS

| Abbreviation | Description |
|--------------|-------------|
| AAF-R110     | Bis-alanyl-alanyl-phenylalanyl-rhodamine |
| ATCC         | American Type Culture Collection |
| BrdU         | Bromodeoxyuridine |
| CBD          | Convention on Biological Diversity |
| CC50         | Half-maximal cytotoxic concentration |
| CFU-GM       | Colony forming unit granulocyte/macrophage |
| CLSI         | Clinical and Laboratory Standards Institute |
| CO-ADD       | Community for Open Antimicrobial Drug Discovery |
| DMSO         | Dimethyl sulfoxide |
| EdU          | Ethynyl deoxyuridine |
| ELC          | Eli Lilly and Company |
| ELISA        | Enzyme-linked immunosorbent assay |
| EMA          | European Medicines Agency |
| EUCAST       | European Committee on Antimicrobial Susceptibility Testing |
| EURL         | European Union Reference Laboratory for Alternatives to Animal Testing |

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INTRODUCTION

Whilst antibiotic resistance continues to emerge and spread, the number of new antibacterial drugs being approved for use is declining. Linezolid (an oxazolidinone), daptomycin (a lipopeptide), bedaquiline (a diarylquinoline) and lefamulin (a pleuromutilin) represent the only new classes of systemic antibiotics introduced in the last 20 years [with the discovery of pleuromutilin (1) and pleuromutilin (2) antibiotics actually dating back much further]. Declining productivity has been attributed, in part, to an over-reliance on synthetic chemical libraries and sub-cellular target-based screening for drug discovery. Synthetic ‘Lipinski-like’ molecules, favored for their amenability to hit-to-lead optimization for other medical conditions, are now recognized to penetrate the bacterial cell envelope poorly (3,4). Also, engineering cell permeability into inhibitors identified by in vitro or in silico target-based screening has proven more difficult than anticipated (5,6). Commercial factors are at play too. Antibiotics, because they are typically used in short courses of treatment and can elicit resistance, provide a smaller, more risky return on investment than other drugs, and many multinational pharmaceutical companies have withdrawn from antibacterial research (7,8).

During the ‘golden age’ of antibiotic discovery (1940 to 1970), the templates for most of the new drug classes (the aminoglycosides, tetracyclines, amphenicols, polymyxins, macrolides, pleuromutilins, glycopeptides, rifamycins, lincosamides, streptogramins and phosphonic acid antibiotics) were identified by screening natural products for activity against whole bacterial cells (9,10). The discovery of the nitroimidazole and quinolone antibiotic classes was less straightforward, but can be traced back to natural products also (azomycin and quinine, respectively) (11,12). Because natural products are natural metabolites, they are more likely than synthetic compounds to be substrates for the transporter systems that facilitate their entry into the bacterial cell (13,14). Natural products are also more architecturally complex than synthetic chemicals, their many chiral centers, ring fusions and functional groups permitting greater specificity towards biological targets (15,16). Whole cell screening, used exclusively until the 1980s (5), is very effective as an antibiotic discovery strategy too. In addition to identifying only those compounds capable of penetrating bacterial cells, this approach is more likely to identify multitarget inhibitors (17), decreasing the ease with which bacteria can evolve antibiotic resistance (5,18).

With Big Pharma reducing investment in antibacterial research, other sectors are attempting to fill the void (1,19). The biopharmaceutical/biotechnology sector is an important example, and several companies (including Adenium Biotech, Motif Bio, NovaBiotics, Paratek Pharmaceuticals and Spero Therapeutics) now have drug candidates in clinical-stage development (20,21). Drug research has also increased in academia, but is held in low regard by many in industry (22). Despite its considerable collective expertise and an unparalleled level of access to the natural resources so important for antibacterial drug discovery, individual research groups can lack the full complement of knowledge and skills needed to identify progressible compounds (23). One problem in particular is the frequent mismatch between natural product source material and screening strategy (24). To begin to redress these issues, we provide here an integrated multidisciplinary perspective on three key aspects of the current bioprospecting literature. Information on natural product source material and in vitro methods of antibacterial and toxicity testing are critically reviewed by specialists in ecology, ethnomedicine, biolaw, taxonomy, natural product chemistry, microbiology, pharmacology and drug development. Avoidable pitfalls, both legal and scientific, are described throughout the text and summarized at the end. In vivo testing is beyond the scope of this review, but useful methods such as the Caenorhabditis elegans rescue assay (25) should not be overlooked when designing bioprospecting programs.

NATURAL PRODUCT SOURCES

Source Selection

Our planet is home to an estimated 1 to 6 billion species (26), of which many different sub-species and strains can exist.
Given the limited pool of (often public or philanthropic) funding available to screen these organisms, it is incumbent on us to prioritize those sources most likely to yield therapeutically useful compounds. Ecological, ethnomedical and historical information can all be used to predict which organisms might produce antibacterial compounds (Supplementary Table 1). Coprophilous fungi, for example, are screened on the basis that they compete with bacteria for a limited nutrient supply, and this creates a selection pressure for antibiotic production (27,28). Medicinal plants are screened on the basis that they have traditionally been used to treat bacterial infection and may produce antibacterial compounds (29), and soil bacteria are screened on the basis that they have historically been a rich source of antibacterial drugs (17). Attempts can also be made to predict which sources are most likely to yield compounds selective in their toxicity (Supplementary Table 1). If, for example, a medicinal plant has been used for many years without reports of adverse effects, this might suggest its constituents will be safe and tolerable in humans (30). The same can be argued for products obtained from commensal and mutualistic species of the human microbiota (31,32), human cells (33), and, to a lesser extent, the microbial symbionts of other eukaryotic organisms (34,35). Such rationales do, of course, have their limitations. Ecological relationships are complex and theories must sometimes be revisited (36,37), and the safety and efficacy attributed to traditional medicines are not always borne out by in vivo studies (38) or clinical trials (39).

Consideration can be given to other factors too (Supplementary Table 1). Prokaryotic diversity exceeds that of the eukaryotes (13) and, if the structural diversity of their natural products parallels this, then the likelihood of finding useful compounds from these organisms will be greater. The extent to which a habitat has been screened previously and whether or not it is aquatic is also relevant. Underexplored ecosystems (eg. deserts, caves, seas, oceans, permafrost soils, plant and animal microbiotas) will more readily yield novel organisms and novel natural products than those that have been heavily screened (35,40–44), and the diluting effect of aquatic environments may, for secreted compounds, create a selection pressure for greater potency (45). Genome mining techniques such as the antiSMASH algorithm can be used to predict which organisms are likely to produce novel antibiotics too (46,47). Lastly, source selection should take into account the facilities available at the research institution. For example, the case for soil actinomycete screening is strong in settings with high throughput screening (17), but where this is absent it may make more sense to test organisms that can be rationally selected (species by species) based on ecological, ethnomedical and/or genomic considerations. The type of taxonomists (microbiologists, botanists, zoologists etc.) and repositories (culture collections, herbaria, zoological museums, cell banks etc.) available is pertinent too.

**Source Collection (Including Legal Issues)**

Under the United Nations ‘Convention on Biological Diversity’ (CBD; ratified in 1993), countries have sovereign rights over the genetic resources in their territories. Prior to collecting biological material from other countries, researchers must therefore obtain informed consent from the source country. This process involves agreement on how any benefits from the subsequent research will be shared with the source country in a fair and equitable way (48). The ‘Nagoya Protocol’ (NP) on Access and Benefit-sharing, a supplementary agreement (ratified in 2014), provides further clarification and extends the rights of the source country to any traditional knowledge associated with their national biota. In addition to providing source countries with legal recourse in the event of biopiracy, the above treaty seeks to facilitate access and research through the establishment of essential infrastructure, legal certainty for investment, etc. (49). An online platform, the ‘Access and Benefit-Sharing Clearing-House’, has been developed by the Secretariat of the Convention on Biological Diversity to connect providers (source countries) and users (researchers) (50). Publicly funded biobanks have also emerged as a means of facilitating access (51).

Collection of biological material from countries that have not signed the CBD or NP may be governed by national laws (52). Collection of biological material from international waters and territories is not covered by the CBD or NP, but by other treaties such as the United Nations ‘Convention on the Law of the Sea’ (Part XIII; ratified in 1994) (33) and the ‘Antarctic Treaty’ (Protocol on Environmental Protection; ratified in 1998) (54). Collection of biological material within one’s own country may also require formal permits if, as in the case of nature reserves or rare species, the land or species is protected (52,55) or, in the case of human or animal samples, specific legislation is in place (56).

When biological material will be studied in a different country to where it was collected, researchers must ensure they have legally obtained the material under the appropriate legislation or regulatory requirements. With material obtained from countries that are signatories of the CBD and NP, for example, researchers must obtain ‘prior informed consent’ for the work they propose to do. Also, the provider country and researchers should arrange ‘mutually agreed terms’ (MAT) including any monetary and/or non-monetary benefits that they may share. For any subsequent work with the biological material not described in the original MAT, either by the original researcher or another research group, the terms and conditions of benefit sharing must be renegotiated with the provider country. Lastly, for researchers not arranging collection of source material directly but receiving this from collaborators, it is important to request a ‘material transfer agreement’ to
ensure all material has been collected and will be used in compliance with the appropriate laws (37,58).

Other advance planning is also needed. Contact with a repository curator should be made (with non-disclosure agreements in place if necessary) prior to collection to ensure sufficient biological material and supporting information is obtained for formal identification, voucher deposition (59–61), and/or any other biobanking requirements (36). Repositories, incidentally, should not be overlooked as a source of biological material themselves. Many microbial culture collections have not been systematically examined for bioactivity, and are becoming increasingly popular for large screening programs (32). An advantage of this approach is that microbial strains collected by the repository prior to ratification of the CBD (ie. strains pre-dating 29 December 1993) will be available without the regulatory restrictions described above. Ethical approval is required for research with higher animals (vertebrates and cephalopods) and, for all animals, consideration should be given to anesthesia, analgesia and/or euthanasia (62,63). Collecting biological material from humans, in addition to requiring ethical approval, requires informed consent (36,64). As with any research, a health and safety assessment is essential (both for organisms that will likely be encountered during collection and processing and any chemicals used) (56,63). Because intra-species variation and environmental factors can affect what chemical entities an organism produces, consideration should also be given to the collection of different sub-species and strains of the same organism (65,66) and collection from different locations at different times (65,67). Lastly, some organisms (eg. bacteria, fungi, algae) can lose viability within 1 to 24 hours of sampling (66,68), may share habitats with faster growing species (28,69) and/or will undergo genetic drift during passaging, so appropriate plans must be in place for transport, isolation, culture and storage (eg. specialized transport containers (70,71), prepared plates of selective media (68), facilities for dilution-to-extinction culturing (28), gravity separation (66) and other types of pretreatment (41,72), and facilities for multi-temperature culture (41) and long-term preservation (73).

**Confirmation of Source Identity and Deposition of Vouchers**

Confirmation of species identity by a qualified expert is essential if the subsequent research is to be accurate and reproducible. Chemical and pharmacological analyses will be a waste of time and money if the source material has been misidentified and, worse still, could mislead other researchers (65). The deposition of vouchers (Fig. 1) with a permanent, curated repository is also important (61,65). A voucher is defined as “a specimen, a sample, or product thereof, and its associated data, that documents the existence of an organism at a given place and time in a manner consistent with disciplinary standards” (75). Deposits must be to publicly accessible repository collections if a new species or subspecies is being reported, whereas confidential deposits to an International Depository Authority are recommended if there is a need to protect intellectual property through patent application (52,76). With primary vouchers, there must be sufficient material to physically and visually document the existence of the organism (75). Guidelines differ between and within taxonomic groups but, by way of examples, botanical specimens (incl. roots, stems, leaves, flowers and/or fruits) are pressed, dried and mounted on acid-free paper (65,77), and entomological specimens are fixed (in cyanide, ethyl acetate or ethanol), then chemically-, critical point- or freeze-dried, and pin-, point- or card-mounted with their larvae fixed (in boiling water) and preserved in ethanol (60,61). Associated data includes information such as scientific name, taxonomic authority, habitat, species size, population size, georeference, collection method, name(s) of the person(s) who collected and identified the species, date and time of collection, date of preservation (60,61), and details of any collection permit or formal agreement with a landowner (32,59). A primary voucher enables species identity to be re-appraised if there is difficulty reproducing a piece of work (eg. not being able to isolate the same active constituents) or reassigned in the event of a taxonomical revision (eg. the division of one species into two) (63). Secondary vouchers are products derived from the organism that provide supplementary information (75). In the case of organisms used in traditional medicine, a secondary voucher (properly cross-referenced against a primary voucher) might include a specimen(s) showing ethno- biologically important features (eg. juvenile leaves of edible herbs), together with a record of both the scientific and common name, cultural characteristics (in the original language and phraseology), etc. Vouchers in ethno- biological studies bridge the gap between folk knowledge and science (59). Repositories assign each voucher a unique accession number and this number, together with the names of the repository and taxonomist, should be included in any subsequent documents or publications describing work with the organism (59,61). Although the practices described above are not always observed (compliance can be less than 10% for some taxonomic groups) (61), this is likely to change as the number of scientific journals rejecting submissions without these details increases (77).

**Preparation Prior to Extraction**

Whilst some organisms can be obtained in quite large quantities at the collection site (eg. plants or plant material), others may need to be cultivated to obtain sufficient quantities for extraction. This can be challenging for some species. For example, endophytes often have such a close relationship with their host plant species that it is necessary to culture them with freshly harvested plant tissue to achieve optimal growth (68).
Even if a routine cultivation method has been established for an organism, it is worth considering alternative options. With fungi, potato dextrose and malt extract media are generally sufficient for maintaining laboratory cultures, but organisms synthesize few bioactive compounds in these media because of their low protein content (28). Ideally, multiple growth conditions should be tested as the profile of chemical compounds produced can vary depending on the mode of nutrition (autotrophic, heterotrophic or mixotrophic) (66), the nutrients supplied (66, 78, 79) or withheld (80, 81), whether the medium is solid or liquid (28), and factors such as pH (80, 82), temperature (79, 80, 82), salt concentration (83), the degree of aeration (78, 79), light intensity (78, 82) and growth phase (79). In microbiology, this is known as the “one strain – many active compounds” (OSMAC) phenomenon (28). Culture conditions (e.g., culture medium, supplements, O₂ concentration) affect the compounds produced by mammalian cells too (33, 84). For the reasons above, consultation with someone experienced cultivating the organism or culturing the cells under investigation is generally recommended (28, 66). The development of innovative cultivation methods such as the iChip is also encouraged to increase the number of species capable of being cultured (40, 85).

When cultivating organisms or culturing cells prior to extraction, another consideration is whether pathogen attack, competition for resources, or other stresses can be simulated. Plants produce some of their antibacterial compounds constitutively (phytoanticipins), but others are only produced in the event of an infection (phytoalexins) (86). Likewise, insects produce some of their antimicrobial peptides constitutively (e.g., stomoxyn), but others are only produced following an encounter with an invading pathogen (e.g., attacins) (87). A similar pattern of results is observed when two microorganisms are competing for nutrients (88). With Aspergillus nidulans, for example, polyketide production is only triggered in the presence of competing bacteria (88). Even when antibacterial compounds are produced constitutively, synthesis may be occurring at low levels if infection is absent (e.g., cathelicidin LL-37 production by human mesenchymal stromal cells) (84). Such regulation is thought to have evolved as a means for organisms to minimize unnecessary or unbeneficial production of energetically costly antibacterial compounds (80, 89). Because it is desirable to detect and identify the full range of constitutive and inducible antibacterial compounds an organism produces, various methods have been developed to induce or simulate infection and competition. This can be achieved in plants by treatment with immune elicitors such as salicylic acid (89, 90), in insects by injection of entomopathogens or their components (e.g., peptidoglycan, lipoteichoic acid, lipopolysaccharide) (91), in cells from higher animals (e.g., cultured bovine, murine and human mesenchymal stromal cells) by co-incubation with bacterial products [e.g., lipopolysaccharide (92), exotoxin (93)] or inflammatory cytokines [e.g., interferon-γ, interleukin-12 (94)], and in bacteria by co-incubation with another organism (88, 95) or its products [e.g., low concentrations of antibiotic (96, 97), siderophore or ionophore (34, 98)]. In addition to the biotic stressors above, abiotic stressors such as rare earth elements, heavy metals and gamma and ultraviolet radiation can be used to stimulate the expression of silent or less-active biosynthetic pathways (99, 100) or increase constitutive antibacterial production (84, 101).

Advance consideration should be given to what part(s) or product(s) of the organism will be used for extraction. With microorganisms, bioactive compounds are often exuded rather than stored intracellularly, so it is necessary to extract not just the microbial cells but the medium in which they have been cultured (102). With plants, it has been suggested that subterranean organs (e.g., bulbs, roots) may be more likely to produce antibacterial compounds given their proximity to bacteria (incl. pathogenic bacteria) present in the soil (103). Also, some bioactive compounds can be limited to a single plant organ (104). Consideration should be given too to whether or how the raw material will be processed prior to extraction. Following harvest or euthanasia, degradation of
constituent chemical compounds can occur by the action of enzymes from the organism itself (e.g., polyphenol oxidases in the case of plants) or by the action of enzymes from contaminant saprotrophs (e.g., proteases in the case of fungi) (105), so raw material should be processed without delay. To minimize contamination, organisms such as plants and higher fungi are washed or gently brushed to remove soil and other debris (102). Also, to minimize enzymatic degradation of the constituent compounds, raw material is sometimes frozen (102), freeze-dried (66,102), sun-dried (106) or oven-dried (102). Freeze-drying can be used to concentrate compounds too if, as in the case of secreted human or animal peptides or microbial products, they are present in culture media in low concentrations (33,102). If the raw material is large in size or volume, it is advisable to first divide it into small pieces or aliquots to promote rapid and homogenous freezing and/or drying (102). Some compounds undergo structural alteration when exposed to heat and light, and if these are present, fresh, frozen or freeze-dried material will yield a different profile of chemical compounds to material that has been oven- or sun-dried (106,107). Where available, ethnomedical information (108,109) and knowledge of the classes of chemical compound likely to be present (102) should be used to guide the above decisions. For short-term storage, the frozen and/or dried material is sealed in a container, protected from light and put in a cool dry place (102). Long-term storage is not recommended (110).

SCREENING FOR ANTIBACTERIAL ACTIVITY

When bioprospecting for a specific type of activity, bioassays are usually run in parallel to extraction and separation so that the isolated compounds will be not just new but active (111). Antibacterial screening may be classified according to a number of characteristics. Firstly, it may be classified according to the number of samples (compounds or extracts) tested per day, typically ‘high-throughput’ when 10,000 to 100,000 samples are examined per day, ‘medium-throughput’ when 1000 to 10,000 samples are examined per day, or ‘low-throughput’ when less than 1000 samples are examined per day (112). Screening may also be classified as being either ‘whole-cell’ or ‘sub-cellular’ depending on whether sample activity is assessed against whole bacterial cells or a single molecular target such as an enzyme. Lastly, antibacterial screening can be classified according to the type of activity being detected. This may be direct antibacterial activity (i.e., bacterial growth inhibition or bacterial killing), synergistic activity (i.e., reduction of intrinsic or acquired resistance to an existing antibiotic), or antivirulence activity (i.e., inhibition of a process that contributes to bacterial pathogenesis but is not required for bacterial growth or viability). In this review, we will focus on low-throughput antibacterial screening as medium- and high-throughput screening have been comprehensively discussed in excellent reviews by Fallarero et al. (112) and Niu and Li (113). We will focus on whole-cell rather than sub-cellular screening as this identifies only those compounds capable of either penetrating the cell envelope or exerting their antibacterial effect from outside the cell. Lastly, we will focus on assays of direct antibacterial activity rather than synergistic or antivirulence activities. Assays of synergistic activity often generate discrepant results (114) and, because pathogenesis is a multi-step process that varies between bacterial species, assays of antivirulence activity are so numerous and diverse they would justify a separate review in their own right.

Antibacterial Assays Available and Rationale for Their Selection

Broadly speaking, two categories of assay are available for examining natural products for direct antibacterial activity – those that detect activity (i.e., diffusion-based, bioautographic, and cell morphology-based) and those that quantify activity (i.e., agar dilution, broth macrodilution, and broth microdilution). The use and description of diffusion-based techniques as semi-quantitative assays is, for bioprospecting purposes, almost always inappropriate (115). Disk diffusion can validly be considered semi-quantitative in a diagnostic laboratory setting, where the susceptibility of an unknown bacterial strain (a clinical isolate) to a known chemical compound (e.g., an FDA- or EMA-approved antibiotic) is approximated based on foreknowledge of the compound’s diffusion characteristics in the assay (116,117). In a bioprospecting laboratory, it is the chemical compound(s) (in the form of an extract or isolated constituent) that is being characterized rather than the bacterial strain, and no foreknowledge of the diffusion characteristics of this compound(s) exists. Because weakly antibacterial compounds that are small, polar and/or anionic diffuse through bacterial growth medium more quickly and can generate larger zones of inhibition than potently antibacterial compounds that are large, nonpolar and/or cationic, the activity of test compounds cannot reliably be approximated or compared based on the size of these zones of inhibition (115,118). The only circumstance in which diffusion-based assays can validly be considered semi-quantitative in a bioprospecting laboratory is when the susceptibility of different (often paired) bacterial strains (e.g., a drug-resistant mutant and its parent strain, or an overexpressed target strain and its parent strain) to a single extract or compound are being compared for the purpose of dereplication or target identification (119).

Both non-quantitative and quantitative assays can be used to guide the isolation of antibacterial compounds, and the category selected depends on the strategy of the research group and the resources available to them. If the priority is
to isolate active compounds from source material as quickly as possible, and the research group has access to the human and technological resources to accomplish this, then an assay that detects rather than quantifies antibacterial activity can justifiably be used. If the priority is not to isolate compounds that are merely active but to isolate compounds that are sufficiently potent to justify further investigation, then a quantitative antibacterial assay should be used. Quantitative assays should also be used if a research group does not have the resources to isolate and identify active compounds because, without information on potency, any report describing antibacterial extracts is unlikely to generate sufficient interest for the species to be investigated further. Lastly, regardless of the category of assay(s) used to guide the isolation of antibacterial compounds from crude extract, the activity of those isolated compounds should be quantified.

Three key attributes both the above categories of assay should have are that they do not generate false negative results (e.g., the assay is sufficiently sensitive to detect antibacterial compounds present at low concentration, and does not mistake redox activity or autofluorescence for failure to inhibit bacterial growth) (24,112,119) or false positive results (e.g., the assay does not mistake solvent activity for natural product activity) (120), and that the results obtained are reproducible (i.e., similar results are obtained when the assay is performed on different days, by different users, in different locations, etc.) (24,112). For quantitative assays, an additional requirement is that they generate accurate results (i.e., the assay is able to produce the correct results when reference compounds of known antibacterial potency (usually FDA- or EMA-approved antibiotics) are tested against reference (culture collection) strains of bacteria) (112). Other desirable attributes for antibacterial assays are that they be simple, rapid (112,120), inexpensive (108) and suitable for testing compounds of various polarity (115,120). If possible, they should also be capable of dereplication (i.e., able to differentiate novel natural products from known natural products) (24,119), generating results from small quantities of extract or compound (119,121), differentiating antibacterial activity from non-specific cytotoxicity (119), differentiating bacteriostatic activity from bactericidal activity (108,115), detecting activity against mycobacterial species (103), and identifying antibacterial target (i.e., able to identify what cellular structure the natural product interacts with to exert its antibacterial effect) (122). The relative importance of each of the above attributes will vary from one bioprospecting program to another. For example, when examining source material in which known antibiotics are more likely to be found than novel antibiotics (e.g., soil actinomycetes (17)), an assay capable of dereplication will probably be the priority. However, when examining source material in which redox active compounds are likely to present (e.g., plants (123)), an assay that does not mistake redox activity for failure to inhibit bacterial growth will probably be the priority, and when examining source material likely to harbor general cellular poisons (e.g., cyanobacteria (124), animal venom (125)), an assay capable of differentiating antibacterial activity from non-specific cytotoxicity will probably be the priority.

Non-Quantitative Antibacterial Assays

Diffusion-based, bioautographic, and cell morphology-based methods can all be used to detect antibacterial constituents present in source material. Diffusion-based assays involve incubating the entire surface of an agar plate with a bacterial strain, adding small reservoirs of test extract to the surface-seeded agar plate (e.g., on a disk or in a well), incubating for a predetermined time period, and then examining the agar plate for zones of bacterial growth inhibition around the test extracts (118,119). Diffusion-based methods continue to be refined for the purpose of antibacterial bioprospecting, for example the use of susceptible-resistant pair screening as a means of dereplication (24,119), and the use of over- and under-expressing strains as means of identifying the antibacterial target (119,126). Diffusion-based methods were used extensively during the ‘golden age’ of antibiotic discovery, yielding many of the compounds in clinical use today (112,119). Bioautographic methods use thin layer chromatography (TLC) to separate the compounds present in test extracts, with TLC plates then dipped in a bacterial suspension (direct bioautography), placed in contact with a surface-seeded agar plate (contact bioautography), or covered with molten, seeded agar (agar overlay bioautography), and antibacterial activity detected either by observation of zones of inhibition or the use of redox indicators to assess bacterial metabolic activity (127). Bioautographic methods also continue to be refined, for example adjustments that allow microaerophilic and obligately anaerobic bacteria to be tested (128), and coupling with mass spectrometry for the purpose of dereplication (129). Examples of antibiotics discovered by bioautography include lasalocid and salinomycin (127). Lastly, cell morphology-based assays work on the principle that antibacterial compounds inhibiting some bacterial processes induce an associated morphological change in the bacterial cell, the best known example being spheroplast formation (observable as large round refractile bodies) by peptidoglycan synthesis inhibitors (24). Such compounds can be detected by treating bacterial cells with a test extract and then examining them by light microscopy. Though information on this approach has only been disclosed relatively recently, spheroplasting assays were used by Merck and other pharmaceutical companies in the discovery of antibiotics such as fosfomycin, thienamycin, noemonycin and mureidomycin (24,119). The advantages and disadvantages of all three of the above assay types are presented in Table 1.
Technical Standards and Guidelines for Non-Quantitative Antibacterial Testing

With the exception of the disk diffusion method, standardized methods have not yet been developed for the above assays. The responsibility of ensuring these methods are capable of generating reproducible results therefore falls to the individual research groups using them. This can be achieved by, for example, regularly testing reference compounds of known stability and antibacterial potency against reference strains of bacteria of known susceptibility (108). Because the standardized disk diffusion methods were not developed for the purpose of bioprospecting [but for differentiating antibiotic-susceptible and -resistant clinical isolates in the diagnostic microbiology laboratory (116,117)], researchers also need to verify that this and the other methods are not generating false negative results, false positive results etc. Detection limits can be determined by, for example, testing samples with known concentrations of antibacterial compound. Also, the possibility that false negative results are being caused by redox active natural products can be ruled out by testing extracts with the redox indicator in the absence of bacterial cells to ensure no color change is taking place (112).

Quantitative Antibacterial Assays

Agar dilution, broth macrodilution and broth microdilution methods can all be used to measure the inhibitory activity of test extracts and isolated compounds against bacterial growth. This activity is expressed as a minimum inhibitory concentration (MIC), the lowest concentration of test extract or compound required to inhibit bacterial growth (108,118). The first method, agar dilution, involves preparing different concentrations of the test extract or compound in an agar medium, spot inoculating the agar surface with bacterial strains, incubating for a predetermined time period, and then examining the agar surface for the growth of bacterial colonies (118). The other two methods, broth macrodilution and broth microdilution, involve preparing different concentrations of the test extract or compound in a broth medium (in tubes or microtiter plates, respectively), inoculating each of the tubes/wells with a bacterial strain, incubating, and then examining the tubes/wells for turbidity or bacterial pellets (118). The broth microdilution method has been used extensively for antibacterial bioprospecting (119). Unlike the agar dilution method, both broth dilution methods can also be used to measure bactericidal activity (118,135). This is expressed as a minimum bactericidal concentration (MBC), the lowest concentration of test extract or compound required to kill 99.9% of bacterial cells (118,135). MBCs are determined by transferring clear (growth-free) broth from the MIC and supra-MIC tubes/wells of the macrodilution/microdilution assay, inoculating onto an agar medium containing no test extract or compound, incubating, then performing colony counts and using these counts as a proxy measure of bacterial viability (118,135). The advantages and disadvantages of all three of the above assays are presented in Table 2.

Technical Standards and Guidelines for Quantitative Antibacterial Testing

Standardized methods have been developed for all three of the above assays by the Clinical and Laboratory Standards Institute (CLSI) (135,139), with an additional International Organization for Standardization (ISO) standard available for the broth microdilution method (140,141). Like the disk diffusion method, these guidelines were developed for use in the diagnostic microbiology laboratory rather than for bioprospecting, but there is a strong case to be made for their use. Firstly, because the CLSI standards have been in use for over thirty five years and undergone thirteen multicenter-coordinated iterations of review and revision in this time, they are fairly comprehensive in describing not just the many variables affecting antibacterial susceptibility test results (eg. inoculum size and age, growth medium, agar depth or broth volume, height to which agar plates or microtiter plates are stacked, incubation time) but also what materials or values to use (139). Their employment for the purpose of bioprospecting is likely, therefore, to improve the reproducibility of test results. Another important feature of the CLSI standards is that they include tables of test results that MIC and MBC assays should generate for reference (FDA-approved) antibiotics tested against reference (culture collection) strains of bacteria (135,139). Such quality controls could help ensure MIC and MBC accuracy during bioprospecting. Test result accuracy is important because it helps funding bodies (and sometimes other researchers) prioritize the most potent antibacterial extracts and compounds for further investigation. Test result accuracy is also desirable in that it could allow the antibacterial activity of compounds isolated by different research groups to be directly compared, thereby facilitating the establishment of provisional structure-activity relationships (SARs) (142).

In most cases, the above standards can be used for bioprospecting without modification simply by including additional controls or testing additional bacterial strains as required. With broth macro and microdilution assays, for example, an uninoculated dilution series of test extract/compound and broth can and should be included to detect solubility problems and avoid false negative results due to compound precipitation (108). Also, dereplication can be achieved in all three assays by using paired strains (eg. a drug-resistant mutant and its parent strain) and comparing their susceptibility (24,119). In other cases, small modifications of the technical standards may be justifiable. Because plant-derived antibacterial compounds are prone to bacterial efflux, for example, it has been suggested that plant extracts be screened for activity in combination with efflux pump inhibitors to increase assay sensitivity and reduce the likelihood of false negative results.
Table 1  Advantages and Disadvantages of Different Non-Quantitative Assays of Direct Antibacterial Activity

| Type of assay | Advantages | Disadvantages | References |
|---------------|------------|---------------|------------|
| Diffusion-based (eg. disk diffusion, well diffusion, cylinder diffusion, spot-on-lawn) | • Simple, quick to perform (multiple extracts can be tested on the same plate), & requires no specialized equipment. | • Because test results are based on the observation of bacterial growth, this type of assay takes 18-24 h to generate results. | (10,16,24,115,119,130,131) |
| | • False negative results can be minimized by using hypersensitive bacterial strains (ie. a panel of strains under-expressing conserved essential enzymes such as FabF) or using reporter strains (ie. a panel of strains which emit light when transcription of stress response genes or other genes of interest occurs). | • False negative results are a possibility with all of these assays as nonpolar compounds can fail to diffuse through the agar & produce a zone of inhibition. With the disk diffusion method, false negative results can also occur when testing cationic compounds as these can adsorb to the disk. | |
| | • Dereplication can be achieved by using paired strains (eg. a drug-resistant mutant & its parent strain) & comparing their susceptibility. | • False negative results are a possibility with the presence of multiple antibacterial compounds (eg. one known & one novel antibiotic in the same extract), resulting in further false negative results. | |
| | • Target identification can be achieved by testing a panel of over- or under-expressing target strains & comparing their susceptibility to the parent strain, or by using reporter strains. | | |
| | • False positive results caused by general cellular poisons (eg. membrane disruptors) can also be minimized by using a panel of over-expressing, under-expressing, or reporter strains. | | |
| Bioautographic (ie. TLC-direct bioautography, TLC-contact bioautography, & TLC-agar overlay bioautography) | • Quite simple to perform & when a redox indicator (eg. resazurin or 2,3,5-tetrazolium chloride) is used to detect metabolically active bacteria, assays can generate results in ≤6 hours. | • False negative results are a possibility with some redox indicators (eg. resazurin) because redox-active compounds can react with the indicator, giving the impression test bacteria remain metabolically active. False negative results are also a possibility with the contact & agar overlay methods because nonpolar compounds may not diffuse from the chromatogram to the plate or may migrate poorly through the agar & fail to produce a zone of inhibition. | (112,120,127,129,132,133) |
| | • Dereplication can be achieved because compounds separated by TLC & shown to have activity can be removed from the plate & analyzed by mass spectrometry or other methods to determine structure. | • False positive results are likely because, even after extensive drying, some acidic & alkaline solvents remain on the TLC plate. | |
| | | | |
| Cell morphology-based (eg. spheroplasting assay) | • Because test results are based on the observation of morphological changes (not bacterial growth), this type of assay can generate results within several hours. | • False negative results are likely as assay only detects those antibacterial compounds inducing morphological changes. False negative results can also occur if multiple antibacterial compounds are present in the same extract, as the effects of one can mask the effects of the other (eg. a membrane disruptor lysing the spheroplasts generated by a peptidoglycan synthesis inhibitor). | (24,119,134) |
| | • False negative results caused by low compound potency or concentration are minimized because morphological changes occur at concentrations less than those needed to inhibit bacterial growth. | | |
| | • Target can provisionally be assigned based on a positive test result (eg. natural products inducing spheroplasts often target peptidoglycan synthesis). | | |

**Note:** Although the above assays cannot be used to determine the antibacterial potency of test extracts and compounds, they can be used to determine spectrum of activity. TLC, thin layer chromatography

(89). Also, when testing panels of over-expressing or under-expressing strains [obtained by genetic manipulation (143) or antisense RNA technology (144)] for the purpose of target identification, it is necessary to include an inducer [eg. arabinose (143), xylose (144)] in the growth medium to regulate target protein levels. Any such modification to CLSI or ISO methods should be kept to a minimum and explained in publications. Lastly, CLSI and ISO standards are only suitable for measuring inhibition of exponentially growing cells. For measuring inhibition of bacterial biofilms, other methods such as the microtiter plate-based resazurin assay (145), Lubbock chronic wound biofilm model assay (146), chronic wound biofilm infection assay (147) and microfluidic wound model assays (146) are required.
Dissolution or Solubilization of Extracts and Compounds for Antibacterial Testing

When dissolving test extracts or isolated compounds prior to antibacterial testing, wasted effort can be minimized if consideration is given to the maximum concentration of test extract or compound that needs to be prepared. For antibacterial assays, this is generally 4000 μg/ml for crude or fractionated extracts and 400 μg/ml for isolated compounds [working on the basis that extracts and isolated compounds incapable of inhibiting bacterial growth at concentrations at or below 1000 μg/ml and 100 μg/ml respectively, are generally considered

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**Table 2** Advantages and Disadvantages of Different Quantitative Assays of Direct Antibacterial Activity.

| Type of assay | Advantages | Disadvantages | References |
|---------------|------------|---------------|------------|
| Agar dilution | • Quite simple & quick to perform because multiple bacterial strains can be tested on the same agar plate.  
• Can be used to test polar (hydrophilic) & nonpolar (hydrophobic) compounds.  
• Can be used to test colored extracts & compounds, & colloids & emulsions as agar discoloration & turbidity are readily distinguishable from bacterial growth occurring on the agar surface.  
• False negative results due to bacterial/fungal contamination are unlikely as this is generally detectable by eye.  
• Bacterial growth on a solid surface is considered more biologically relevant than planktonic growth in liquid. | • Requires quite large quantities of extract or compound.  
• Minimum bactericidal concentrations (MBCs) cannot be determined, so bacteriostatic & bactericidal activity cannot be differentiated. | (108,112,115,118,121,131) |
| Broth macrodilution | • Quite simple to perform.  
• MBCs can be determined, so bacteriostatic & bactericidal activity can be differentiated. | • Slow to perform because individual dilution series need to be prepared for each bacterial strain.  
• Difficult to test nonpolar (hydrophobic) compounds using this method unless a carrier, surfactant or emulsifier is used.  
• Difficult to test darkly colored extracts & compounds, colloids, & emulsions as broth discoloration & turbidity can, respectively, mask or resemble growth.  
• Requires quite large quantities of extract or compound.  
• False negative results can occur due to bacterial/fungal contamination because, unless occurring in uninoculated control tubes, it is not detectable by eye. | (108,115,118,121,131,136,137) |
| Broth microdilution (incl. tetrazolium- & resazurin-based assays for mycobacteria) | • Can be performed with quite small quantities of extract or compound.  
• MBCs can be determined, so bacteriostatic & bactericidal activity can be differentiated.  
• Antimycobacterial activity can be measured by using a fast-growing model species (e.g., Mycobacterium phlei or M. smegmatis), or by using redox indicators (i.e., dyes such as tetrazolium or resazurin that assess the metabolic activity of test mycobacteria), or by using a reporter strain of M. tuberculosis (i.e., a strain expressing green fluorescent protein that allows fluorescence to be used as a proxy measure of bacterial growth). | • More difficult than other methods as multi-channel pipette requires skill, & slow because a dilution series needs to be prepared for each bacterial strain.  
• Difficult to test nonpolar (hydrophobic) compounds using this method.  
• Difficult to test darkly colored extracts & compounds, colloids, & emulsions using this method.  
• False negative results can occur due to contamination because it is not detectable by eye. False negative results can also occur with redox indicators (e.g., resazurin) as redox-active compounds can react with them, & with reporter strains as some compounds autofluoresce. | (103,108,115,118,121,131,136–138) |

As with diffusion-based assays, dereplication & target identification can be achieved & the risk of false-negative and false-positive results can be minimized in all the above assays by using under-expressing, over-expressing, drug-resistant &/or reporter strains of bacteria. Accurate results are also achievable with all the above assays if they are performed in compliance with CLSI or ISO & EUCAST guidelines (an advantage of this being that inter-study comparisons of results are then possible). MBCs, minimum bactericidal concentrations
insufficiently active to merit further investigation ([121,148]). Because most natural products have limited aqueous solubility, use of a solvent is almost always necessary. Many options are available (e.g., acetone, dimethyl formamide, dimethyl sulfoxide (DMSO), ethanol, methanol, polyethylene glycol 400, sodium bicarbonate, sodium carbonate, sodium hydroxide (DMSO), ethanol, methanol, polyethylene glycol 400, sodium bicarbonate, sodium carbonate, sodium hydroxide ([131,149]), but it is DMSO that is most frequently used. Advantages of DMSO for bioassays include its ability to dissolve both polar and nonpolar compounds, and its miscibility with water and growth media ([150]). Also, the high boiling point of DMSO reduces room temperature evaporation, improving the accuracy of the test concentrations prepared ([108]). Lastly, pure DMSO is antimicrobial ([151]) so, if necessary, natural product extracts and compounds can (with adequate controls in place) be tested without filter sterilization, an advantage when working with compounds that might adsorb to the membrane filter ([108]). Bacterial tolerance to DMSO varies between species [MICs of 5 to 30% (v/v) ([151]) but, in the interests of standardization, the final in-assay concentration of this solvent does not normally exceed 2.5% (v/v) ([152]) and is ideally just 1% (v/v) ([108]).

Regardless of which of the above solvents is used, some hydrophobic natural products will precipitate out (either immediately or eventually) upon dilution with water or growth media. Natural product solubility in these situations can sometimes be improved by using carriers such as cyclodextrins ([137]) or serum albumins ([153]). An alternative option is to prepare a colloid or, in the case of essential oils, an emulsion. This involves the use of a surfactant (e.g., polysorbate 80, polyalkylene glycol) ([115,154]) or emulsifier (e.g., agar, lecithin) ([155,156]) to disperse the natural product throughout the assay medium, promoting contact with bacterial cells and reducing the risk of false negative results. Mixed solvents are sometimes used too (e.g., N-methyl-2-pyrrolidone and DMSO) ([154]). For low-throughput screening, the above solutions, colloids and emulsions are usually prepared fresh on the day of the experiment to reduce the risk of compound precipitation or degradation, or vehicle incompatibility or reactivity. Because solvents ([151]), carriers ([157]) and surfactants ([158]) can themselves be antibacterial or alter the activity of the compounds being tested, their use and concentration(s) should always be reported.

Selection of Target Organisms

According to the World Health Organization, future antibiotic research and development efforts should focus on certain key pathogens. Top of this list (critical priority) are *Mycobacterium tuberculosis* (multidrug- and extensively drug-resistant), *Acinetobacter baumannii* (carbapenem-resistant), *Pseudomonas aeruginosa* (carbapenem-resistant) and the Enterobacteriaceae (carbapenem- and third generation cephalosporin-resistant). Next (high priority) are *Enterococcus faecium* (vancomycin-resistant), *Staphylococcus aureus* (methicillin- and vancomycin-resistant), *Helicobacter pylori* (clarithromycin-resistant), *Campylobacter spp.* (fluoroquinolone-resistant), *Salmonella spp.* (fluoroquinolone-resistant) and *Neisseria gonorrhoeae* (third-generation cephalosporin- and fluoroquinolone-resistant), and lastly (medium priority) *Streptococcus pneumoniae* (penicillin non-susceptible), *Haemophilus influenzae* (ampicillin-resistant) and *Shigella spp.* (fluoroquinolone-resistant) ([159]). Testing wild-type (antibiotic-susceptible) reference strains (e.g., American Type Culture Collection, National Collection of Type Cultures) of the above species is usually advisable in the first instance as they are well characterized (making interpretation of results more straightforward), widely used (making inter-study comparisons of results possible) ([108]), and less likely to generate false negative results (due to efflux or other resolvable issues) ([89, 119]). As explained in Tables 1 and 2, there are also advantages with using paired strains, over-expressing or under-expressing strains, and reporter strains of bacteria ([24, 119]). Lastly, whilst the use of surrogate or model species can be very effective (e.g., use of fast-growing *Mycobacterium smegmatis* instead of *M. tuberculosis* in the Janssen Pharmaceutica drug discovery program that yielded bedaquiline), it should be borne in mind that inter-species differences can cause false negative results (e.g., up to 50% of *M. tuberculosis* inhibitors are not detected in screens using *M. smegmatis*) ([160]).

Interpreting Antibacterial Test Results

Although the CLSI and ISO standards describe methods that can readily be repurposed for bioprospecting, they are on their own insufficient for interpreting results. Additional information is required to guide the isolation of antibacterial compounds from crude extracts, and to determine whether isolated compounds are sufficiently potent to merit further investigation. Universal consensus is lacking on what level of activity should be present before a crude extract is advanced for semi-purification, but it has been proposed that only extracts with MICs $\leq 1000 \mu g/ml$ should be considered active ([108, 120, 121]). Working on the principle that the antibacterial activity of a crude extract is due to the sum of the activities of its individual constituents, then semi-purified extracts obtained by fractionation should show an improvement in activity (lower MICs), allowing further purification and isolation of the active compound(s) ([109]). In practice, an improvement in activity is not always seen. This may be because compounds in the original crude extract were acting synergistically ([108,109]), or because structural modification of the active compound(s) has occurred during fractionation ([108,109]). Structural degradation of active compounds or transformation to a less active form can occur if there is a reaction with the solvent being used (e.g., esterification of acid groups on the molecule by an alcoholic solvent) or if oxidation occurs (e.g., phenolic compounds reacting with oxygen dissolved in mobile
phases) (109). If isolation of an antibacterial compound is achieved, it must generally have an MIC ≤100 μg/ml to be considered progressible (120,121,131,148). This is because there are limitations to which structural modification can improve the antibacterial activity of isolated compounds, and because achievement of MIC (and preferably supra-MIC) levels of compound in the blood plasma must be feasible if it is to be effective as a systemic therapeutic agent (109,118). Endpoint criteria are even more stringent within the pharmaceutical industry (119) and public-private partnerships (161), with isolated compounds unlikely to attract interest unless their MICs are <10 μg/ml (103) or <10 μM (161). For compounds that will be used topically rather than systemically (eg. for skin decolonization or the treatment of skin infections), slightly higher MICs may be acceptable as the compound can be delivered directly to the target site. If MBCs are also determined during testing, then compounds with an MBC no more than 4 times the MIC can provisionally be considered bactericidal (162,163) unless they belong to a known cell-aggregating class of natural products such as the flavonoids (164,165). Following the successful isolation of an antibacterial compound from a particular organism, consideration is sometimes given to the screening of other closely related species as minor evolutionary variations in the biosynthetic pathway of the compound can generate structural variants with improved activity (103).

**TOXICITY TESTING**

When antibacterial compounds are isolated as part of a bioprospecting program, it is important they be tested for toxicity (166,167). Contrary to views sometimes expressed in the literature, compounds from nature are no less likely to be toxic than of synthetic origin. Many of the world’s most lethal poisons, for example ricin, batrachotoxin, maitotoxin and botulinum, are actually naturally occurring (168). Toxicity testing is particularly important when whole-cell screens (Tables 1 and 2) have been used for bioprospecting because, with the exception of some cell morphology-based assays and assays using under-expressing, over-expressing or reporter strains of bacteria, these screens do not distinguish between specific (bacterial) and nonspecific (general) cell toxicity (119). The number of in vitro assays available for toxicity testing has grown considerably in recent years and continues to grow, driven by public concern for animal welfare (169,170) and legislative control of animal use (171,172), and a desire on the part of pharmaceutical companies and regulatory agencies to reduce false-negative and false-positive test results caused by species-specific toxicity (173,174), to reduce the cost and duration of toxicity testing (171,175), to generate data for SAR analysis and pharmacokinetic and pharmacodynamic models (176), and to detect toxicity problems earlier in the drug development process (170,177).

**Toxicity Assays Available and Rationale for Their Selection**

At the time of writing, in vitro assays are available to detect cytotoxicity (including hepatotoxicity, nephrotoxicity, neurotoxicity, cardiotoxicity, immunotoxicity and hemotoxicity), mitochondrial toxicity, genotoxicity (including mutagenicity and carcinogenicity), phospholipidosis, steatosis and cholestasis (177). The type of toxicity initially tested for can be prioritized based on several factors. These include any known or predicted toxicity problems associated with the class of natural product being tested, the bacterial pathogen(s) being targeted, and whether a future drug derived from the natural product is likely to be used topically or systemically. Many of the pyrrolizidine alkaloids, for example, are hepatotoxic, many furoquinolones are mutagenic (104) and many cationic amphiphilic compounds induce phospholipidosis (178), so it makes sense to prioritize hepatotoxicity, mutagenicity and phospholipidosis testing with these classes of compound. Testing can also be prioritized based on the findings of predictive toxicity software such as Derek Nexus. Antimycobacterial compounds are usually tested for toxicity against macrophages (179,180) because Mycobacterium tuberculosis resides in these cells during infections. Lastly, drugs likely to be used topically are tested against epithelial, keratinocyte and/or dermal fibroblast cells (181,182), while those intended for systemic use are tested against erythrocytes (183,184) and cells from organs commonly affected by drug toxicity such as the kidney (185,186), liver (187,188) and heart (189,190).

Key attributes toxicity assays should have are that they do not generate false negative or false positive results (177,191), and that the results obtained are accurate [ie. the assay is able to produce the correct results when reference compounds of known toxicity are tested (192,193)], reproducible [ie. similar results are obtained when the assay is performed on different days, by different users, in different locations, etc.] (191,194), and comparable between studies (108). At this point, it is also important the toxicity test is fully quantitative (170) so that a selectivity index/indices (discussed later) can be calculated and a risk-benefit assessment made. The larger the value of the selectivity index, the more likely the compound will gain approval for development (161), and the more likely the final drug will benefit the patient without undue risk (18). Other desirable attributes for toxicity assays are that they be simple, rapid (174,195), safe (196), inexpensive (174,195), capable of generating results from small quantities of compound (197), not dependent upon ethical approval or difficult-to-source materials (173), and yielding information on mechanism of toxicity (167,174). The relative
importance of each of the above attributes will vary from one bioprospecting program to another. When competing for research funding, for example, it may be desirable to demonstrate superior selectivity over other antibacterial compounds, in which case an assay that generates reproducible results comparable to other studies will be the priority. However, when testing a class of natural products with known toxicity issues (e.g., pyrrolizidine alkaloids), an assay that closely resembles in vivo conditions and generates fewer false negative results may be the priority.

Many different toxicity assays are available. These can be categorized according to the type of cells used, the measures and markers used to predict toxicity, and the technology used to monitor changes in the test cells. The two main cell types used are primary cells and immortalized cells (or cell lines), primary cells being cells that have been isolated directly from human or animal tissue, and immortalized cells being cells that have been derived from tumors or treated with viruses to make them easier to culture (198). Potential target organs of toxicity can be identified by testing primary and immortalized cells of different origin (hepatocytes, cardiocytes etc.). Common measures of toxicity against such cells include reduced growth, reduced viability, reduced metabolic activity, reduced membrane integrity, and other forms of cellular disruption. Cell growth can be monitored using total protein (199,200) or DNA synthesis (201,202) as markers, cell viability can be monitored using intracellular protease activity (167,195) or pH gradient (203,204) as markers, metabolic activity can be monitored using redox activity or ATP as markers (167,195), membrane integrity can be monitored using dye exclusion (205) or intracellular enzyme retention as markers (197,206), and general cell health and integrity can be monitored using cell morphology as a marker (173,207). Other more specialized tests, too numerous to describe here in detail, include the hERG potassium channel assays (for cardiotoxicity) (189,190), the hemolysis (208) and colony forming unit granulocyte/macrophage (CFU-GM) (209) assays (for hematotoxicity), the measurement of mitochondrial membrane potential (for mitochondrial toxicity) (210), the Ames (211), chromosomal aberration, micronucleus (193) and mouse lymphoma (192,193) assays (for genotoxicity), and the use of specialized stains such as N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine (NBD-PE), Nile Red and LipidTox (for measuring phospholipidosis, steatosis and cholestasis) (177,207). Lastly, in terms of technology, changes in the test cells can be monitored either spectrophotometrically (167) or by high content analysis (177,207). The advantages and disadvantages of some of the most commonly used types of toxicity assay are presented in Table 3. In resource limited settings where these toxicity assays may not all be available, testing can be outsourced to initiatives such as the Community for Open Antimicrobial Drug Discovery (CO-ADD) or to contract research organizations.

**Technical Standards and Other Useful Protocols for Toxicity Testing**

Protocols have been published by the National Institutes of Health (NIH), Eli Lilly and Company (ELC) and others for many of the general toxicity assays including the protease assay (167,195), neutral red uptake assay (204), tetrazolium and resazurin reduction assays (167), ATP assay (167), and trypan blue exclusion assay (214). These include useful guidance notes on reagent storage, cell density, positive and negative controls, temperature maintenance etc. Moving on to more specialized toxicity assessments, numerous technical standards have been developed, validated and published by the European Union Reference Laboratory for Alternatives to Animal Testing (EURL), the International Cooperation on Alternative Test Methods (ICTAM) and the Organisation for Economic Co-operation and Development (OECD) including tests for ocular toxicity (eg. OECD Test Guidelines 491 and 492), skin irritation and corrosion (eg. OECD Test Guidelines 439 and 431), and genotoxicity (eg. OECD Test Guidelines 471, 473, 476, 487 and 490) (193,218). As with the CLSI and ISO standards described for antibacterial testing, adoption of these NIH, ELC, EURL, ICTAM and OECD protocols and standards is likely to improve the reproducibility, accuracy, comparability and credibility of the toxicity data generated by bioprospecting programs.

**Dissolution or Solubilization of Compounds for Toxicity Testing**

As discussed earlier, DMSO has many properties that make it a useful bioassay solvent, and it is a popular choice for dissolving compounds for toxicity assays (172). However, it is important to verify experimentally that DMSO does not interfere with the human/mammalian cells being tested. With some cells and cell lines, low DMSO concentrations [≤0.07% (v/v)] can stimulate cell growth (219,220), and higher DMSO concentrations [≥0.5% (v/v)] can inhibit (221) or kill cells (220). If DMSO is found to affect the growth or viability of the human/mammalian cells being tested, then other solvents [eg, acetone, acetonitrile, ethanol, isooctane, isopropanol, sodium hydroxide, tetrahydrofuran (172,219,222,223), surfactants (224), cosurfactants (225), cyclodextrins (226) or dendrimers (227) can be investigated as alternative means of
| Type of assay                                      | Advantages                                                                 | Disadvantages *                                                                 | References                          |
|--------------------------------------------------|-----------------------------------------------------------------------------|---------------------------------------------------------------------------------|-------------------------------------|
| (a) Cell type †                                  |                                                                             |                                                                                 |                                     |
| Primary cells                                    | • More of the key metabolic enzymes (eg, cytochrome P450 enzymes) are present in primary cells than in cell lines, so there is greater in vivo-like functionality & a lower risk of false negative & false positive results. | • Requires ethical approval, & may be expensive & difficult to procure sufficient quantities of cells. | (171,173,176,191,207)              |
|                                                  |                                                                             | • Primary cells have a limited lifespan & begin to dedifferentiate after 24-48 h. |                                     |
|                                                  |                                                                             | • There is donor-to-donor variability in primary cells, so test results may not be reproducible & inter-study comparisons of toxicity are not possible. |                                     |
|                                                  |                                                                             | • Primary cells have a low proliferative rate & can undergo morphological changes during culture, so neither inhibition of cell growth nor morphological changes can readily be used as measures of toxicity. |                                     |
| Immortalized cells (cell lines):‡                | • Cell lines are readily available & ethical approval is not needed to use them. | • Fewer of the key metabolic enzymes are present in cell lines than in primary cells, so there is less in vivo-like functionality & a higher risk of false negative & false positive results. | (171,173,176,191,195)              |
|                                                  | • Cell lines remain viable for longer than primary cells.                    | • Cell lines can become cross-contaminated during extended use, so cell line authentication is important. |                                     |
|                                                  | • Test results are more reproducible with cell lines than primary cells & inter-study comparisons are possible. |                                                                                 |                                     |
|                                                  | • Cell lines have a high proliferative rate, so inhibition of cell growth can be used as a measure of toxicity. |                                                                                 |                                     |
| (b) Markers of toxicity                          |                                                                             |                                                                                 |                                     |
| Total protein [sulforhodamine B (SRB) & Lowry photometric method] | • Small quantities of compound can be tested if microtiter plate-based assays are used. | • The Lowry photometric method requires time-consuming dilution of samples if protein content is high. | (199,200,212)                      |
| DNA synthesis [eg. bromodeoxy-uridine (BrdU) assay, ethynyl deoxyuridine (EdU) assay] | • Small quantities of compound can be tested if microtiter plate-based assays are used. | • The EdU assay is quite expensive.                                              | (201,202)                          |
|                                                  | • Small quantities of compound can be tested if microtiter plate-based assays are used. | • The BrdU assay is quite time-consuming because test cell DNA must be denatured & incubated with anti-BrdU antibody. |                                     |
|                                                  | • The SRB & Lowry photometric assays are not expensive.                     | • EdU can induce DNA damage & cell death in test cells during prolonged culture. |                                     |
|                                                  | • The SRB assay is simple & rapid.                                          |                                                                                 |                                     |
|                                                  | • Cytostatic & cytocidal activity can be differentiated if cells are incubated with fresh medium after treatment with the test compound. |                                                                                 |                                     |
|                                                  | • Assay kits are commercially available.                                   |                                                                                 |                                     |
| Intracellular protease activity (eg. GF-AFC assay) | • More rapid than redox reagent-based assays because cells can be incubated with GF-AFC for ≤1 h. | • More expensive than tetrazolium- & resazurin-based redox activity assays.       | (167,195)                          |
|                                                  | • GF-AFC is non-toxic to cells, so assay duration can be optimized more easily than with redox reagents. |                                                                                 |                                     |
|                                                  | • In addition to quantifying viable cells, the assay can quantify non-viable cells if protease leakage is measured (eg, using AAF-R110). |                                                                                 |                                     |
|                                                  | • An assay kit is commercially available.                                   |                                                                                 |                                     |
| pH gradient between cytoplasm & lysosomes (neutral red uptake assay) | • Small quantities of compound can be tested if microtiter plate-based assays are used. | • Assay is slower than ATP-based assays as treated cells must be incubated with neutral red for 2h. | (196,203,204)                      |
|                                                  | • This assay is less expensive than total protein- & lactate dehydrogenase-based assays. | • Neutral red is itself toxic to cells, so reagent concentration & assay duration must be limited. |                                     |
| Redox activity (tetrazolium- & resazurin-based assays) | • Small quantities of compound can be tested if microtiter plate-based assays are used. | • Assays are slower than ATP-based assays as viable cells take 1-4h to induce color change or fluorescence of redox reagents (longer if cells have low metabolic activity). | (112,167,195,213)                |
Table 3 (continued)

| Type of assay                      | Advantages                                                                                           | Disadvantages *                                                                                           | References |
|-----------------------------------|-------------------------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------------|------------|
| **ATP detection (luciferase-based assays)**                                      | ▪ Small quantities of compound can be tested if microtiter plate-based assays are used.              | ▪ Tetrazolium & resazurin compounds are toxic if incubated with cells for longer than 3-4h, so (i) assay duration can be difficult to optimize (it must be long enough to detect redox activity, but short enough to avoid redox reagent-induced toxicity) & (ii) it may not be possible to test cells with low metabolic activity. |
|                                   | ▪ More rapid than redox reagent- & protease activity-based assays, as cells do not need to be incubated with reagent after treatment with test compound. | ▪ False negative results are possible if the test compound reduces the redox reagents directly or, with resazurin, if the test compound is fluorescent. |
|                                   | ▪ Early cytotoxic events can be monitored & mechanism of toxicity can be studied because the assay is available in a real time continuous-read format in which RNA is left intact enough to detect stress response gene expression. | ▪ False positive results are possible if the test compound alters the pH of the culture medium. |
|                                   | ▪ Assay kits are commercially available.                                                              | ▪ The luciferase enzymatic reaction is affected by temperature, so temperature of assay must be carefully controlled. |
|                                   | ▪ Dye exclusion assays are simple & inexpensive.                                                      | ▪ False positive results are possible if the test compound inhibits luciferase. |
|                                   | ▪ When a fluorescent stain (eg. propidium iodide) is used, it is possible to apply a counterstain (eg. calcein AM) so that not just membrane-compromised cells but also membrane-intact cells are quantified. | ▪ False negative results are possible if the test compound inhibits ATPases. |
|                                   | ▪ These assays can detect toxicity regardless of the rate of proliferation of test cells or whether or not the test cells are dividing at all. | ▪ More expensive than tetrazolium- & resazurin-based redox activity assays. |
| **Dye exclusion** (eg. propidium iodide staining, trypan blue staining)          | ▪ Examination of treated cells is labor-intensive unless flow or image cytometry is available.     | (167,195,196) |
|                                   | ▪ False negative results are possible if (i) lethally damaged cells do not lose membrane integrity within the timeframe of the assay or (ii) cells do not just lose membrane integrity but disintegrate completely. | |
|                                   | ▪ Trypan blue is itself toxic to cells, so reagent concentration & assay duration must be limited.    | |
|                                   | ▪ Propidium iodide is a suspected carcinogen & must be handled with care.                           | |
| **Retention of intracellular enzymes** (eg. lactate dehydrogenase assay, dead-cell protease assay) | ▪ False negative results are possible if (i) the test compound inhibits the leaked enzyme being monitored or (ii) enzyme leakage is measured too early (before membrane damage has occurred). | (196,205,214) |
|                                   | ▪ Small quantities of compound can be tested if microtiter plate-based assays (eg. dead-cell protease assay) are used. | |
|                                   | ▪ Data normalization is possible with the dead-cell protease assay, so the results generated are reproducible & comparable between studies. | |
|                                   | ▪ These assays can detect toxicity regardless of the rate of proliferation of test cells or whether or not the test cells are dividing at all. | |
|                                   | ▪ Assay kits are commercially available.                                                              | |
| **Cell morphology** [eg. morphological highest tolerated dose (HTD) assay, high-content analysis] | ▪ Examination of treated cells is labor-intensive unless high-content analysis equipment & software is available. | (197,206) |
|                                   | ▪ Morphological changes (eg. cells becoming round, cells increasing or decreasing in size) occur before loss of cell viability & are therefore a more sensitive measure of toxicity. | |
| **(c) Test system** Spectrophoto-, fluoro- & luminometric analysis                | ▪ Can be performed with standard laboratory equipment such as a microplate spectrophotometer or flow cytometer. | ▪ Detects toxic compounds with low sensitivity (~46% according to one study), predominantly just those compounds disrupting cellular proliferation or viability. | (177) |
| **High-content analysis**                                                     | ▪ Detects toxic compounds with high sensitivity (~93% according to one study) due to ability to detect genotoxicity, phospholipidosis, steatosis & cholestasis. | ▪ Requires specialized equipment & data analysis software. | (177,207) |
dissolution or solubilization. Because concentration-dependent stimulatory and inhibitory effects have been detected with DMSO (219–221) and other solvents (222,228), it is recommended that solvent concentrations always be kept constant in serial dilutions of test compounds (226).

**Interpreting Toxicity Test Results**

While the decision to progress a compound beyond *in vitro* antibacterial testing is determined largely by potency cut-offs (eg. MICs <10 μg/ml or <10 μM for prospective systemic drugs), the subsequent decision to progress that compound beyond *in vitro* toxicity testing is based on a different measure. Referred to by some authors as the ‘selectivity index’ (229) and others as the ‘safety margin’ (230), ‘selectivity’ (231) or ‘selectivity window’ (161), this is the ratio of off-target (human/mammalian) to target (bacterial) toxicity (18,230). In antibacterial research, the selectivity index is usually obtained by dividing a test compound’s half-maximal cytotoxic concentration (CC<sub>50</sub>); the concentration of compound required to reduce human/mammalian cell viability by 50% by the compound’s MIC against bacteria (229). Depending on the toxicity assay used, other values such as the half-maximal inhibitory concentration [IC<sub>50</sub>]; the concentration of compound required to inhibit a given biological process (eg. human/mammalian cell growth or enzyme activity) by 50% (230) or half-maximal hemolytic concentration (HC<sub>50</sub>; the concentration of compound required to lyse 50% of red blood cells) (231) may be used instead of the CC<sub>50</sub>. Because multiple bacterial species and multiple human/mammalian cell types are tested as part of most bioprospecting programs, multiple selectivity indices are generated, and these can be presented in a 2-dimensional table called a ‘heatmap grid’ (230).

Evaluating selectivity indices is a critical juncture in the drug discovery process. If a cut-off is set too high, useful compounds may be discarded, but if a cut-off is set too low, time and resources may be wasted on a compound with no commercial or therapeutic value (161). Where the cut-off is set is a complex decision dependent on many factors. These include (a) whether the drug candidate addresses an unmet medical need, (b) whether the drug candidate is likely to have superior efficacy or safety over the current standard of care, (c) whether the drug candidate is intended for use as a short- or long-term therapy, (d) whether the drug candidate is intended for use in patients with co-infections or comorbidities that would put them at increased risk of drug-drug interactions or other adverse drug reactions, (e) the type of *in vitro* toxicity detected and (f) information on how predictive (sensitive and specific) the *in vitro* toxicity tests used are (230). It should be apparent from the above text that no single cut-off value is universally applicable to all antibacterial compounds (230). For prospective antimycobacterial drugs, for example, it has been proposed that the selectivity index should be greater than ten before they are considered for further development, but this value is usually higher for other indications (161). It should also be apparent from the above text that cut-off values for specific indications change over time because the competitive drug landscape changes over time (230). An important final point to make here is that the ratio of off-target to target toxicity (known as the ‘therapeutic index’ rather than the ‘selectivity index’ in subsequent *in vivo* and clinical studies) usually decreases as a compound progresses through the discovery and development pipeline (Fig. 2). This is, in part, because rarer toxicities are detected as animals and then human subjects are tested for longer durations and in increasingly large numbers (230). Efficacy decreases too, in many cases because antibacterial activity is growth rate-dependent and bacterial cells grow more slowly *in vivo* than they do *in vitro* (232). Such factors must also be borne in mind when deciding to progress a compound beyond *in vitro* toxicity testing.
CONCLUDING REMARKS

Most antibacterial drugs in current clinical use are natural products (or semi-synthetic derivatives thereof), and these have the potential to re-emerge as an important starting point for drug discovery. Given the limited market incentives for development of antibacterial drugs however, and our increasing reliance on public and philanthropic sources of funding for this type of research, it is imperative that future bioprospecting programs are optimized for efficiency. This can be achieved in a number of ways including the selection of study designs appropriate to institutional resources. In settings unequipped for high throughput screening, for example, random testing of soil actinomycetes is unlikely to be successful because less than 1 in 10 million bacteria produce novel antibiotics. In such settings, it may be more productive to select source material based on ecological, ethnomedical and/or genomic information, or to investigate the impact novel culture conditions (eg. different nutrients, simulated pathogen attack, abiotic stress) have on the chemical compounds organisms or cells produce. Antibacterial and toxicity assays should also be rationally selected based on any pertinent foreknowledge of the natural product source material. For example, when screening cyanobacterial extracts for antibacterial activity (or other source material likely to harbor general cellular poisons), the use of panels of over-expressing, under-expressing or reporter strains of bacteria should be considered to differentiate specific and non-specific cytotoxicity. Also, when an antibacterial compound belonging to a toxicity-prone class of natural products (eg. the pyrrolizidine alkaloids) has been isolated and is being assessed for toxicity, the inclusion of primary cells in the panel of test cells should be considered because these generate fewer false negative results than immortalized cells. Where technical standards or protocols are available for these bioassays (from the CLSI, ISO, EURL etc.), their use is recommended as this will improve data robustness and facilitate inter-study comparisons. Lastly, it is important to avoid the many pitfalls associated with each of the discussed aspects of antibacterial bioprospecting. These pitfalls are summarized in Supplementary Table 2.

In this review, we have provided a multidisciplinary perspective on some of the initial steps in natural product-based antibacterial drug discovery. All of the information included is correct at the time of writing, but new bioassays, bioassay guidelines and cell lines continue to be developed, and communication/collaboration with appropriate specialists is always good practice (167,233–234). Space restrictions prevented us from describing within this review all of the steps required for ‘hit validation’, the process of confirming isolated antibacterial compounds have the potential for modification to an entity with properties suitable for clinical use. Hit validation involves additional laboratory and legal work including (a) preparation of concentration response curves and measurement of resistance frequency, (b) elucidation of antibacterial mechanism of action, (c) confirmation of compound identity and purity, (d) confirmation of the druggability of the chemical class (by assessing solubility, stability, reactivity, synthetic feasibility etc.) and (e) confirmation that no serious intellectual property conflicts exist and there is ‘freedom to operate’ (167). Readers are directed to other reviews (134,235–237) and the NIH Assay Guidance Manual (167) for information on these steps, and a review by Katsuno et al. (161) for example hit validation criteria. Further testing, for example medicinal chemistry optimization and SAR assessment, screening for pan-assay interference (PAIN) behaviors, and screening for cytochrome P450 inhibition is not usually performed in depth until compounds have progressed into the subsequent ‘hit to lead’ stage of drug discovery (161,238). However, it is useful to check the research literature for information on the bioactivity of the isolated compound and structurally related compounds, both to build up preliminary knowledge of SAR and assess the risk of PAIN behavior. If a class of natural products is reported to have many different biological activities, then this could suggest these compounds are promiscuous and lack target specificity (22).
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