UNIVERSITÀ POLITECNICA DELLE MARCHE
Repository ISTITUZIONALE

Natural products in drug discovery: advances and opportunities

note finali coverpage

(Article begins on next page)

07 September 2024

Natural products in drug discovery: advances and opportunities

Abbreviated author list for the printed edition of the manuscript:

Atanas G. Atanasov[1,2,3]*, Claudiu T. Supuran[4]* and the International Natural Product Sciences Taskforce

[1]Institute of Genetics and Animal Breeding of the Polish Academy of Sciences, Jastrzebiec, Poland.

[2]Department of Pharmacognosy, University of Vienna, Vienna, Austria.

[3]Institute of Neurobiology, Bulgarian Academy of Sciences, Sofia, Bulgaria.

[4]Università degli Studi di Firenze, NEUROFARBA Dept., Sezione di Scienze Farmaceutiche, Florence, Italy.

*Emails:

a.atanasov.mailbox@gmail.com

claudiu.supuran@unifi.it

Full author list for the internet version of the manuscript and for indexing in databases such as PubMed, Scopus, WoS, etc.:

Atanas G. Atanasov[1,2,3]*, Bharat B. Aggarwal[4], Nicolas Arkells[5], Maciej Banach[6], Davide Barreca[7], Maurizio Battino[8,9], Rudolf Bauer[10], Edward A. Bayer[11], Ioana Berindan-Neagoe[12,13,14], Anupam Bishayee[15], Valery Bochkov[16], Günther K. Bonn[17], Nady Braidy[18], Franz Bucar[10,19], Alejandro Cifuentes[20], Grazia D'Onofrio[21], Maria Daglia[22], Marc Diederich[23], Albena T. Dinkova-Kostova[24,25], Thomas Efferth[26], Khalid El Bairi[27], Ilkay Erdogan Orhan[28], Tai-Ping Fan[29,30], Bernd L. Fiebich[31], Michael Freissmuth[32], Milen I. Georgiev[33,34], Francesca Giampieri[8,9], Simon Gibbons[35], Keith M. Godfrey[36], Christian W. Gruber[37], Michael Heinrich[38], Lukas A. Huber[39,40], Elena Ibanez[20], Anake Kijjoa[41], Anna K. Kiss[42], Aiping Lu[43],

Francisco A. Macias[44], Mark J.S. Miller[45], Andrei Mocan[46], Rolf Müller[47], Ferdinando Nicoletti[48], George Perry[49], Valeria Pittalà[50], Luca Rastrelli[51], Michael Ristow[52], Judith M. Rollinger[2], Gian Luigi Russo[53], Ana Sanches Silva[54,55], Daniela Schuster[56,57], Helen Sheridan[58], Krystyna Skalicka-Woźniak[59], Leandros Skaltsounis[60], Eduardo Sobarzo-Sánchez[61,62], Marc Stadler[63], Hermann Stuppner[64], Antoni Sureda[65,66], Nikolay T. Tzvetkov[67,68], Rosa Anna Vacca[69], Robert Verpoorte[70], Wolfram Weckwerth[71,72], Michael Wink[73], Jean-Luc Wolfender[74], Jianbo Xiao[75], Andy Wai Kan Yeung[76], Sergey B. Zotchev[2], Gérard Lizard[77], Michael A. Popp[78], Jag Heer[79], Muhammed Majeed[80,81,82], David S. Bredt[83], Michael Bodkin[84], Verena M. Dirsch[2], Claudiu T. Supuran[85]*

[1]Institute of Genetics and Animal Breeding of the Polish Academy of Sciences, Jastrzebiec, Poland.

[2]Department of Pharmacognosy, University of Vienna, Vienna, Austria.

[3]Institute of Neurobiology, Bulgarian Academy of Sciences, Sofia, Bulgaria.

[4]Inflammation Research Center, San Diego, CA, USA.

[5]International Natural Product Sciences Taskforce (INPST), Jastrzebiec, Poland.

[6]Polish Mother's Memorial Hospital Research Institute (PMMHRI), Lodz, Poland.

[7]Department of Chemical, Biological, Pharmaceutical and Environmental Sciences, Università degli studi di Messina, Messina, Italy.

[8]Nutrition and Food Science Group, Dept. of Analytical and Food Chemistry, CITACA, CACTI, University of Vigo - Vigo Campus, Vigo, Spain.

[9]Department of Clinical Sciences, Faculty of Medicine, Università Politecnica delle Marche, Ancona, Italy.

[10]Institute of Pharmaceutical Sciences, Department of Pharmacognosy, University of Graz, Graz, Austria.

[11]Department of Biomolecular Sciences, The Weizmann Institute of Science, Rehovot, Israel.

[12]MEDFUTURE - Research Center for Advanced Medicine, Cluj-Napoca, Romania.

[13]Research Center for Functional Genomics, Biomedicine and Translational Medicine, Institute of Doctoral Studies, ""Iuliu Hatieganu"" University of Medicine and Pharmacy, Cluj-Napoca, Romania.

[14]Department of Experimental Pathology, ""Prof. Dr. Ion Chiricuta"", The Oncology Institute, Cluj-Napoca, Romania.

[15]Lake Erie College of Osteopathic Medicine, Bradenton, FL, USA.

[16]Institute of Pharmaceutical Sciences, Department of Pharmaceutical Chemistry, University of Graz, Graz, Austria.

[17]Institute of Analytical Chemistry and Radiochemistry, Leopold-Franzens University of Innsbruck und Austrian Drug Screening Institute - ADSI, CCB— Center of Chemistry and Biomedicine, Innsbruck, Austria.

[18]Centre for Healthy Brain Ageing, Neuropsychiatric Institute, Euroa Centre, Prince of Wales Hospital, Australia.

[19]BioTechMed-Graz, Austria.

[20]Laboratory of Foodomics, Bioactivity and Food Analysis Department, Institute of Food Science Research CIAL (UAM-CSIC), Madrid, Spain.

[21]Complex Structure of Geriatrics, Department of Medical Sciences, IRCCS "Casa Sollievo della Sofferenza", San Giovanni Rotondo, Foggia, Italy.

[22]Department of Pharmacy, University of Naples Federico II, Naples, Italy.

[23]Department of Pharmacy, College of Pharmacy, Seoul National University, Seoul, South Korea.

[24]Jacqui Wood Cancer Centre, Division of Cellular Medicine, School of Medicine, University of Dundee, Dundee, Scotland, UK.

[25]Department of Pharmacology and Molecular Sciences and Department of Medicine, Johns Hopkins University School of Medicine, Baltimore, MD, USA.

[26]Department of Pharmaceutical Biology, Institute of Pharmacy and Biochemistry, Johannes Gutenberg University, Mainz, Germany.

[27]Cancer Biomarkers Working Group, Mohamed 1st University, Oujda, Morocco.

[28]Department of Pharmacognosy, Faculty of Pharmacy, Gazi University, Ankara, Turkey.

[29]College of Life Sciences, Northwest University, Xi'an, China.

[30]Department of Pharmacology, University of Cambridge, Cambridge, UK.

[31]Neuroimmunology and Neurochemistry Research Group, Department of Psychiatry and Psychotherapy, Medical Center – University of Freiburg, Faculty of Medicine, University of Freiburg, Freiburg, Germany.

[32]Institute of Pharmacology and the Gaston H. Glock Research Laboratories for Exploratory Drug Development, Center of Physiology and Pharmacology, Medical University of Vienna, Vienna, Austria.

[33]Group of Plant Cell Biotechnology and Metabolomics, The Stephan Angeloff Institute of Microbiology, Bulgarian Academy of Sciences, Plovdiv, Bulgaria.

[34]Center of Plant Systems Biology and Biotechnology, Plovdiv, Bulgaria.

[35]Research Department of Pharmaceutical and Biological Chemistry, UCL School of Pharmacy, London, UK.

[36]MRC Lifecourse Epidemiology Unit and NIHR Southampton Biomedical Research Centre, University of Southampton and University Hospital Southampton NHS Foundation Trust, Southampton, UK.

[37]Center for Physiology and Pharmacology, Medical University of Vienna, Vienna, Austria.

[38]Research Group 'Pharmacognosy and Phytotherapy', UCL School of Pharmacy, London, UK.

[39]Cell Biology, Biocenter, Innsbruck Medical University, Innsbruck, Austria.

[40]Austrian Drucg Screening Institute-ADSI, Innsbruck, Austria.

[41]ICBAS-Instituto de Ciências Biomédicas Abel Salazar & CIIMAR, Universidade do Porto, Porto, Portugal.

[42]Department of Pharmacognosy and Molecular Basis of Phytotherapy, Medical University of Warsaw, Warsaw, Poland.

[43]School of Chinese Medicine, Hong Kong Baptist University, Hong Kong, China.

[44]Allelopathy Group, Department of Organic Chemistry, Institute of Biomolecules (INBIO), Campus de Excelencia Internacional (ceiA3), School of Science, University of Cadiz, Cadiz, Spain.

[45]Kaiviti Consulting, LLC, Dallas, TX, USA.

[46]Department of Pharmaceutical Botany, "Iuliu Haţieganu" University of Medicine and Pharmacy, Cluj-Napoca, Romania.

[47]ThematicTranslational Unit Novel Antibiotics, German Center for Infection Research (DZIF), Braunschweig, Germany.

[48]Department of Biomedical and Biotechnological Sciences, University of Catania, Catania, Italy.

[49]Department of Biology, The University of Texas at San Antonio, San Antonio, TX, USA.

[50]Department of Drug Science, University of Catania, Catania, Italy.

[51]Dipartimento di Farmacia, University of Salerno, Fisciano (SA), Italy.

[52]Energy Metabolism Laboratory, Institute of Translational Medicine, Swiss Federal Institute of Technology (ETH) Zurich, Schwerzenbach, Switzerland.

[53]Institute of Food Sciences, National Research Council, Avellino, Italy.

[54]National Institute for Agricultural and Veterinary Research (INIAV), Vila do Conde, Portugal.

[55]Center for Study in Animal Science (CECA), ICETA, University of Porto, Oporto, Portugal.

[56]Department of Pharmaceutical and Medicinal Chemistry, Institute of Pharmacy, Paracelsus Medcical University Salzburg, Austria.

[57]Institute of Pharmacy / Pharmaceutical Chemistry and Center for Molecular Biosciences Innsbruck (CMBI), University of Innsbruck, Austria.

[58]School of Pharmacy and Pharmaceutical Sciences, Trinity College Dublin, Ireland.

[59]Department of Pharmacognosy with Medicinal Plant Unit, Medical University of Lublin, Lublin, Poland.

[60]Department of Pharmacognosy and Natural Products Chemistry, Faculty of Pharmacy, National and Kapodistrian University of Athens, Panepistimioupolis Zografou, Athens, Greece.

[61]Laboratory of Pharmaceutical Chemistry, Faculty of Pharmacy, University of Santiago de Compostela, Santiago de Compostela, Spain.

[62]Instituto de Investigación e Innovación en Salud, Facultad de Ciencias de la Salud, Universidad Central de Chile, Santiago, Chile.

[63]Helmholtz-Center for Infection Research, Department of Microbial Drugs, Braunschweig, Germany.

[64]Institute of Pharmacy/Pharmacognosy, Center for Molecular Biosciences Innsbruck (CMBI), University of Innsbruck, Innsbruck, Austria.

[65]Research Group on Community Nutrition and Oxidative Stress, Department of Fundamental Biology and Health Sciences, University of Balearic Islands, Palma de Mallorca, Spain.

[66]CIBER: CB12/03/30038 Fisiopatología de la Obesidad la Nutrición, CIBEROBN, Instituto de Salud Carlos III (ISCIII), University of Balearic Islands, Palma de Mallorca, Spain.

[67]Institute of Molecular Biology "Roumen Tsanev"", Department of Biochemical Pharmacology and Drug Design, Bulgarian Academy of Sciences, Sofia, Bulgaria.

[68]Pharmaceutical Institute, University of Bonn, Bonn, Germany.

[69]Institute of Biomembranes, Bioenergetics and Molecular Biotechnologies, Italian National Council of Research, Bari, Italy.

[70]Natural Products Laboratory, Institute of Biology, Leiden University, Leiden, The Netherlands.

[71]Department of Ecogenomics and Systems Biology, University of Vienna, Vienna, Austria.

[72]Vienna Metabolomics Center, University of Vienna, Vienna, Austria.

[73]Institute of Pharmacy and Molecular Biotechnology, Heidelberg University, Heidelberg, Germany.

[74]School of Pharmaceutical Sciences, University of Geneva, University of Lausanne, Geneva, Switzerland.

[75]Institute of Food Safety and Nutrition, Jinan University, Guangzhou 510632, China.

[76]Oral and Maxillofacial Radiology, Applied Oral Sciences, Faculty of Dentistry, The University of Hong Kong, Hong Kong, China.

[77]Team Bio-PeroxIL, 'Biochemistry of the Peroxisome, Inflammation and LipidMetabolism' (EA7270)/University Bourgogne Franche-Comté/Inserm, Dijon, France.

[78]Bionorica SE, Neumarkt/Oberpfalz, Germany.

[79]UCB Pharma Ltd, Slough SL1 3WE, UK.

[80]Sami Labs Limited, 19/1, 19/2, First Main, Second Phase, Peenya Industrial Area, Bangalore 560 058, Karnataka, India.

[81]Sabinsa Corporation, 20 Lake Drive, East Windsor, NJ 08520, USA.

[82]Sabinsa Corporation, 750 Innovation Circle, Payson, UT 84651, USA.

[83]Johnson & Johnson Pharmaceutical Research & Development, San Diego, CA, USA.

[84]Evotec (UK) Ltd., Oxford, UK.

[85]Università degli Studi di Firenze, NEUROFARBA Dept., Sezione di Scienze Farmaceutiche, Florence, Italy.

Abstract

Natural products and their derivatives have historically made a major contribution to pharmacotherapy, especially for cancer and infectious diseases. Nevertheless, natural products also present challenges for drug discovery, such as technical barriers for screening, isolation, characterization and optimization, which contributed to a decline in the investigation of natural products by the pharmaceutical industry from the 1990s onwards. In recent years, however, several technological and scientific developments — spanning analytical tools, "omics" techniques, microbial culturing, computational methods and novel screening models — are addressing such challenges and opening up new opportunities. Consequently, interest in natural products as a source of drug leads is being revitalized, particularly for tackling antibiotic resistance. In this review, we summarize recent technological developments that are enabling natural product-based drug discovery, highlight selected applications and discuss key opportunities for the field.

Introduction

Historically, natural products (NPs) have played a key role in drug discovery, especially for cancer and infectious diseases[1,2]. Furthermore, NPs or NP analogues have also been a source of major drugs in other therapeutic areas, including statins for cardiovascular disease and oral therapies for multiple sclerosis[3–5].

NPs offer special features and differences in comparison to conventional synthetic molecules – and these differences confer some advantages but also challenges from the perspective of the drug discovery process (**Figure 1**). NPs are characterized by higher scaffold diversity and structural complexity. They typically have higher molecular weights, a larger number of sp^3 carbon atoms and less nitrogen and halogen atoms, but more oxygen atoms, higher numbers of H-bond acceptors and donors, lower calculated octanol–water partition coefficients (cLogP values, indicating higher hydrophilicity) and greater molecular rigidity compared to synthetic compound libraries[1,6–9]. These differences can be advantageous; for example, the higher rigidity of NPs can be valuable in drug discovery tackling protein–protein interactions[10]. Indeed, NPs are a major source of oral drugs "beyond Lipinski's rule of five"[11], and the increasing significance of drugs not conforming to this rule is illustrated by the increase in molecular weights of approved oral drugs over the last 20 years[12]. NP structures are considered to be "optimized" through evolution to serve particular biological functions[1], including regulation of endogenous defense processes and interaction (often competition) with other organisms, which explains their high relevance for infectious diseases and cancer. Furthermore, in some cases, there may be useful insights available regarding efficacy and safety from their use in traditional medicines. Overall, the NP pool can be regarded as being enriched with "bio-active" compounds covering wider areas of the chemical space compared with typical small-molecule libraries[13].

Despite these advantages and multiple successful drug discovery examples, several drawbacks of working with NPs have led pharmaceutical companies to reduce their activities in NP-based drug discovery. The traditional starting point for NP screens is a library of extracts from natural sources, which may not be compatible with target-based assays that have become common in pharmaceutical industry[14]. Identifying the active compound (or compounds) in extracts that show the bioactivity of interest can be challenging, and re-identification of known

compounds is a major issue. It may also be difficult to sustainably access sufficient biological material to isolate the bioactive molecule from the extract[15]. Furthermore, intellectual property (IP) rights for (unmodified) NPs exhibiting relevant bioactivities are a concern, since naturally occurring compounds in their original form cannot be patented (legal frameworks vary among countries and are evolving[16]), although simple derivatives can be (**Box 1**). An additional layer of complexity relates to regulations defining the need for benefit sharing with countries of origin of the biological material, framed in the United Nations 1992 Convention on Biological Diversity, and the 2014 Nagoya Protocol[17].

Although the complexity of NP structures can be advantageous, the generation of structural analogues to explore structure–activity relationships and to optimize NP leads can be challenging, particularly if synthetic routes to these analogues are not straightforward. Another difficulty is that NP-based drug leads are often identified by phenotypic assays, which necessitates the identification of their molecular mechanism of action, also a time-consuming challenging endeavour[18]. Finally, many NPs have multiple targets, which is usually considered as a disadvantage. However, many successful drugs have more than one molecular target[19], and multi-targeting can be viewed as an advantage if diverse pathways with therapeutic potential are simultaneously affected, as illustrated by the KEAP1/NRF2 pathway (**Box 1**).

This review will discuss recent technological and scientific developments that may help to overcome challenges in NP drug discovery (**Figure 1; Table 1**), and then highlight selected key opportunities in NP-based drug discovery.

Advances relevant to NP-based drug R&D

Application of analytical and separation techniques in NP-based drug discovery. Classical NP-based drug research starts with the biological screening of "crude" extracts to identify a "hit" extract with a specific bioactivity (**Figure 2**). This extract is further fractionated with the aim to identify and isolate the NPs responsible for the activity of the extract. Due to the need for multiple fractionation and bioactivity evaluation cycles, traditional bioactivity-guided isolation is a

laborious process with a number of limitations, but various strategies and technologies can be used to address some of them (**Figure 2**).

First, pre-fractionation strategies are now well-established to address limitations connected to the isolation of bioactive NPs from extracts, as well as to improve hit rates. In the pre-fractionation step, the initial "crude" extract is separated into sub-fractions that are enriched with compounds that have physicochemical properties that are more suitable for automated liquid handling systems used in high-throughput screening equipment and/or more drug-like properties (typically moderate hydrophilicity). These fractions are then used for the bioactivity screening (sometimes together with the initial crude extract, for comparison). Moreover, the removal of less relevant compounds reduces the complexity of the pre-fractionated extracts, leading to faster detection of "false-positive" effects due to the presence of already known active compounds and to the reduction of the number of cycles of bioassay-guided fractionation needed to obtain the bioactive constituents. For example, a study applying thorough analysis with several pharmacological targets of pre-fractionated library of microbial NPs produced by Wyeth revealed a better suitability for high-throughput screening and a four-fold increase in the number of active samples as compared to the approach using the respective crude extracts only[20].

In recent years, the structure elucidation of NPs has also been improved through advances with analytical techniques, including the introduction of mass spectrometry with increased resolution and accuracy, and high-field and ultra-high-field NMR instruments with increased sensitivity[21], which is important as the quantities of an NP of interest may be small. Hyphenation of several analytic modules even allows for simultaneous bioactivity evaluation and identification of compounds present in small amounts (analytical scale) directly from complex compound mixtures[22]. For example, triple high-resolution profiling of radical scavenging and α-glucosidase and α-amylase inhibition coupled with high-performance liquid chromatography–photodiode-array detection–high-resolution mass spectrometry–solid-phase extraction–nuclear magnetic resonance spectroscopy (HR-bioassay/HPLC-PDA-HRMS-SPE-NMR) was recently used to profile *Dendrobium officinale* Kimura & Migo (a plant used in traditional Chinese medicine) extract, leading to the identification of a range of NPs with diverse

bioactivities, including two new NPs, 3,4,4′-trihydroxy-5-methoxybibenzyl and 3,4-dihydroxy-3′,4′,5-trimethoxybibenzyl[23].

Such technologies also allow the efficient application of 'omics' strategies in NP-based drug discovery[24–26]. In particular, metabolomics can be used to detect differences between metabolite compositions in various physiological states and to generate hypotheses that may explain these differences. It can also provide extensive metabolite profiles that can help to underpin phenotypic characterization at the molecular level[27]. The increasing amount of accurate information on secondary metabolite compositions that can be obtained before a NP is prioritized for isolation is expected to improve both the identification of previously known constituents (known as dereplication[28,29]) and the annotation of unknown analogues and new NP scaffolds, in part by harnessing computational approaches that are able to generate plausible biosynthetic structures and their respective simulated spectra[30].

For metabolite profiling, natural extracts are analysed by either direct methods such as nuclear magnetic resonance (NMR) spectroscopy or high-resolution mass spectrometry (HRMS), or by hyphenated methods, mainly LC-HRMS. NMR is used for the profiling of main constituents[31,32]. It is simple, reproducible and provides direct quantitative information. Although this method enables the characterisation of only tens to hundreds of constituents due to its inherent low sensitivity, it nevertheless generates detailed structural information. HRMS is the gold standard for qualitative metabolite profiling, and it can be used in the direct infusion mode (DIMS) where samples are directly profiled by MS without a chromatography step, or by MS imaging (MSI)[32]. MSI enables simultaneous detection and spatial localization of NPs of interest and their metabolites directly in tissues, which also makes it very useful for studying metabolism, distribution, and toxicity of drug-candidate NPs[33]. Microsensor technologies for metabolite detection are also of great importance, since in combination with microfluidic platforms they are instrumental in the development of new 3D cell assay systems such as organ-on-chip platforms[34].

The increased resolution and sensitivity of analytical equipment is of particular importance for coping with problems associated with "residual complexity" of

isolated NPs, which include small impurities of structurally related metabolites or conformers. Contamination of an isolated NP with a highly potent impurity can lead to an incorrect assignment of structure to activity. The significance of "residual complexity" is highlighted by a study showing that the paclitaxel´s T-conformation, the preferred conformer binding to tubulin, accounts for less than 5% of paclitaxel in solution[35], and recent work by Pauli and colleagues, who demonstrated that the initially described antimicrobial effect of a new diketopiperazine, rufomyazine, was in fact due to a very minor contamination (0.24% [m/m]) with a highly active analogue of the known antibiotic rufomycin[36].

For detailed secondary metabolite profiling, LC-MS is by far the most frequently used method. Chromatography is often required to deconvolute the numerous isomers present in NP extracts prior to MS detection[31]. The introduction of ultra-high pressure liquid chromatography (UHPLC) with sub-2µm-packed columns has led to shorter analysis times (up to 9 times) and increased peak capacity (up to 3 times) compared to conventional HPLC[37]. On the MS side, high-resolution mass spectrometers enable the routine acquisition of accurate molecular mass information (< 5 ppm and even < 1 ppm mass accuracy). High mass accuracies, together with appropriate heuristic filtering, can provide unambiguous molecular formula assignment of hundreds to thousands of metabolites within a single extract over a dynamic range that may exceed 5 orders of magnitude[37]. However, challenges remain in data mining and in the unambiguous identification of the metabolites using various workflows relying on open web-based tools[38].

For dereplication/annotation procedures of secondary metabolites from bioactive extracts, the first step is often based on molecular formula (MF) determination and cross searching in the literature or structural NP databases with taxonomic information (*e.g.*, the occurrence of a given MF in a given species or in a given genus). This taxonomic information allows a marked reduction in the number of hits and greatly helps the annotation process. Such metadata are however difficult to query in the literature and are gathered in proprietary databases, such as the Dictionary of Natural Products (DNP), which encompasses all NP structures reported with links to their biological sources (see **Dictionary of Natural Products** in Related links). A comprehensive experimental MS/MS database of all NPs reported to date, however, does not exist and a search of experimental spectra

across various platforms is hindered by the lack of standardised collision energy conditions for fragmentation in LC-MS/MS. In this respect, the Global Natural Products Social (GNPS) molecular networking (MN) platform developed in the Dorrestein lab represents a major breakthrough[39]. The MN organizes thousands of sets of MS/MS data recorded from a given set of extracts and visualizes the relationship of the analytes in the form of clusters of structurally related molecules. This provides a way to propagate annotation information and multiply the efficiency of the dereplication process by annotation analogues from a given metabolite in a cluster[40]. This process generates putative structures and their corresponding *in silico*-generated MS/MS spectra, which are obtained with available open source software and can be searched against the recorded experimental spectra. For this, vast theoretical spectral NP databases have been recently created[41]. One issue with *in silico* annotation is the uncertainty of structural assignment among possible predicted candidates. To improve the annotation efficiency, network consensus of re-ranked structural candidates using the MN topology and structural similarity has recently been developed and integrated into the GNPS platform[42]. The annotation process requires improvement and for this orthogonal information from retention time prediction and ultimately from ion mobility MS collision cross section (CCS) simulation must be implemented. Such approaches are currently being evaluated for given sets of compounds at the expense of important computational requirements[43,44]. Further contextualization by overlaying MN of large NP extract libraries with taxonomic information can help improve the dereplication and such efforts have to be pursued. Integration of genomic and epigenomic data that can today be obtained in large volumes has yet to be linked to metabolomic data. This represents a very promising aspect that can be readily implemented in microbial studies[45].

Other useful platform for dereplication of known and discovery of unknown metabolites is METLIN[46]. This platform includes high-resolution tandem mass spectrometry (MS/MS) database (with data collected in both positive and negative ionization modes at multiple collision energies), with fragment similarity search function particularly useful for identification of unknown compounds. The fragment similarity search is done in METLIN independently of the precursor mass, relying only on the fragment ions, and stable isotope data also facilitate analysis by coupling the output of similarity searches with the isotopic m/ z shifts[46]. A range

of other databases and *in silico* tools are also available that can be used for searching of available fragment ion spectra, as well as for the computer-based generation of predicted spectra of fragment ions not present in the currently available databases (*e.g.*, Compound Structure Identification (CSI):FingerID and Input Output Kernel Regression (IOKR)), and for further details the readers are referred to the excellent recent review by Aksenov *et al.*[47].

To accelerate the identification of bioactive natural products, metabolomics data from extracts (*e.g.*, different plant species) can be matched with the biological activities of these extracts. Various chemometric methods (such as multivariate data analysis) can be used to correlate the measured activity with signals in the spectra or chromatograms. This approach can be used to trace the active compound(s) with no need for further bioassays. A recent example used proton NMR (^{1}H-NMR) profiling to identify secondary metabolites with an anti-proliferative activity against a panel of human colorectal cancer cell lines with diverse mutation profiles[48]. These NPs were derived from fourteen Fabaceae species of Mediterranean vegetation. The study also used high-resolution 2D NMR spectroscopy to identify the bioactive constituents that were finally isolated *via* fractionation of the individual plant extracts[48].

Particularly promising research direction for NP discovery and mechanistic characterization represents the integration of metabolomics with data obtained with other "omics" techniques, such as transcriptomics and proteomics. An example of such multi-omics approach is the recent work by Acharya *et al.*, which integrated metabolomics, transcriptomics, and proteomics to mechanistically characterize the effect of interspecies interactions in the modulation of the activity of the keyicin producing kyc cluster[49]. Studying cocultures of kyc-bearing *Micromonospora* sp. and a *Rhodococcus* sp., the authors of this work found that the coculture-dependent changes in keyicin production can be attributed to small molecule signaling consistent with a quorum sensing pathway[49].

Of great potential to accelerate NP-based drug discovery also are analytical advances enabling profiling responses to bioactive molecules on single-cell level. An excellent example of this potential is evident in the work of Irish, Bachmann,

and colleagues, who developed activity metabolomics platform termed multiplexed activity metabolomics (MAM), representing a high-throughput screening-compatible system for single-cell metabolome-scale profiling of bioactivity using human cells directly from primary tissue biopsies[50]. In more detail, MAM integrates phospho-specific flow cytometry (phospho-flow), single-cell chemical biology, and cellular barcoding with metabolomic arrays (characterized chromatographic microtiter arrays originating from biological extracts, e.g., of microbial or plant origin). Using this platform, the authors studied the single-cell responses of acute myeloid leukemia (AML) patient bone marrow biopsy samples (containing both cancerous and non-cancerous cells) exposed to microbial metabolomic arrays obtained from extracts of biosynthetically prolific bacteria, which enabled the identification of previously unreported leukemia blast-targeting anthracycline (specumycin B1, identified from the actinomycete *Streptomyces specus* originating from Blue Springs cave in Sparta, Tennessee) and polyene macrolactams (ciromicin A and its isomer ciromicin A, identified from the soil actinobacterium *Nocardiopsis* sp. FU40) alternating between targeting blasts or non-cancer cells through light-triggered photochemical isomerization[50].

Genome mining and engineering. Two characteristics enable the identification of genes coding for NP biosynthesis in the genome sequence of the producing organism. First, these genes are clustered in the genomes of bacteria and filamentous fungi. Second, many NPs are based on polyketide or peptide cores, and the biosynthetic pathways for these scaffolds involve enzymes — polyketide synthases and non-ribosomal peptide synthetases, respectively — that are encoded by large genes with highly conserved modules[51]. Consequently, 'genome mining' based on searches for genes that are likely to encode the biosynthesis of scaffold structures can be used to identify novel NP biosynthetic gene clusters. A recent example of the power of genome mining is the identification of novel NP scaffolds produced by the myxobacterium *Chondromyces crocatus*[52,53]. This work identified several cryptic megasynthetase gene clusters by computational examination of the *C. crocatus* genome. Application of gene inactivation followed by metabolomics led to the identification of new-scaffold NPs (crocadepsins-depsipeptides) and the elucidation of their biosynthesis pathway[53].

Studies of the phylogeny of already known groups of talented secondary metabolite producers can also enable the discovery of novel NPs. Recently, a major study comparing secondary metabolite profiles and phylogenetic data in Myxobacteria has demonstrated a strong correlation between the taxonomic distance and the production of distinct secondary metabolite families[54]. In filamentous fungi, it was likewise shown that secondary metabolite profiles are closely correlated to their phylogeny[55]. These organisms are rich in secondary metabolites as demonstrated by LC-MS studies of their extracts under laboratory conditions. Concurrent genomic and phylogenomic approaches have demonstrated that even the genomes of well-studied organism groups harbor many gene clusters encoding proteins involved in secondary metabolite biosynthesis with as yet unknown functions[56].

Biosynthetic gene clusters for NPs can be cloned and transferred to other organisms that are well-characterized and easier to culture and genetically manipulate (such as *Streptomyces coelicolor*, *Escherichia coli* and *Saccharomyces cerevisiae*) for heterologous expression[57]. The heterologous expression aims to achieve higher production titers in the heterologous hosts as compared to wild-type strains, helping to overcome the bottleneck of availability of lead compounds more quickly[57–59]. While plasmid constructs were initially developed and used as vectors for the heterologous expression usually of a single gene products (usually carrying DNA inserts with average size below 10 kb), for the identification of complete operons and biosynthetic clusters of genes vectors capable of carrying larger DNA inserts are needed. To meet this need, cosmids (vectors capable of carrying DNA inserts of about 30–40 kb), fosmids (capable of carrying DNA inserts of about 40–50 kb), and more recently bacterial artificial chromosomes (BACs; capable of carrying DNA inserts of 100 kb to more than 300 kb) were developed[60]. For the cloning and expression of large fungal gene clusters, self-replicating fungal artificial chromosomes (FACs) were also developed, which are containing both *Aspergillus* autonomously replicating sequence (AMA1) and *E. coli* F replicon, and were demonstrated to be efficient vectors for fungal DNA insert greater than 100 kb[61]. FACs in combination with metabolomic scoring (MS) were applied to develop a scalable platform, FAC-MS, allowing the characterization of fungal biosynthetic gene clusters and the NPs produced by them at unprecedented scale[62]. The application of FAC-MS for the screening of 56 biosynthetic gene clusters from different fungal species yielded the simultaneous discovery of 15 new metabolites,

including a new macrolactone, valactamide A[62]. Plant cell cultures can also be used for large-scale production of NPs[63], with the commercial production (Phyton Biotech) of paclitaxel as an example of how the use of plant cell cultures in bioreactors can solve the problem of sourcing of rare compounds.

Importantly, it has also become apparent that many biosynthetic gene clusters for NPs may not be expressed under conventional culture conditions, and these cryptic clusters could represent a large untapped source of NPs with valuable characteristics[64]. Several approaches can be pursued to identify such NPs. One approach is sequencing, bioinformatics analysis and heterologous expression of cryptic biosynthetic gene clusters, which has led to the discovery of several new NP scaffolds from cultivable strains[65]. For example, cryptic NPs from *Photorhabdus luminescens* called luminmycins A, B and C were identified by heterologous expression of 18 kb cryptic biosynthetic gene cluster from *P. luminescens* in *Escherichia coli*[66]. Direct cloning and heterologous expression was also used to discover the new antibiotic taromycin A, which was identified upon the transfer of a cryptic 67 kb nonribosomal peptide synthetase biosynthetic gene cluster from *Saccharomonospora sp.* CNQ-490 into *Streptomyces coelicolor*[67]. While heterologous expression is a powerful approach for the activation of biosynthetic gene clusters, it is associated with some limitations such as the need to sometimes clone and manipulate very large genome areas (occupied by some biosynthetic pathways) and the difficulty of selecting an optimal heterologous expression hosts that would provide all regulatory, metabolic and enzymatic conditions necessary for the appropriate production of the encoded NPs of interest. These limitations can be circumvented by approaches relying on activation of biosynthetic gene clusters directly in the native microorganism with the use of targeted genetic manipulations, generally involving the insertion of activating genetic elements or deletion of inhibitory elements (e.g., transcription factors or transcription factor binding sites). A derepression strategy was for example used in the work of Sidda *et al.*, which resulted in the discovery of gaburedins upon the rational deletion of *gbnR*, an *arpA*-like putative transcription repressor in *Streptomyces venezuelae* ATCC 10712[68]. An example for activator overexpression strategy represents the constitutive expression of the *samR0484* gene (encoding a protein that is similar to Large ATP binding of the LuxR (LAL)) in *Streptomyces ambofaciens* ATCC23877, which led to the discovery of stambomycins[69]. Alternatively, it has recently been

shown that <mark>cryptic</mark> biosynthetic gene clusters can be activated using transcription factor decoys[70], which have the same sequence as the binding sites for the transcription factors that are repressing the expression of the clusters. When these decoys are introduced into the bacteria, they sequester the respective inhibitory transcription factors and the "endogenous" binding sites in the genome become unoccupied, leading to de-repression of the previously silent biosynthetic genes and production of the resultant NPs. This approach has been successfully applied to activate eight <mark>cryptic</mark> biosynthetic gene clusters in multiple streptomycetes, and led to the characterization of a novel NP, oxazolepoxidomycin A[70]. The transcription factor decoy strategy is simpler, easier, and faster to perform than deletion of regulatory factor genes. However, it has the same limitation as the other approaches that rely on the introduction of recombinant DNA molecules in cells: it is necessary to develop protocols for efficient insertion of DNA into the targeted host strain. Therefore, improved technologies for microbial genetic manipulation are of great importance, and along this line the recent development of CRISPR-Cas9-based tools can be viewed as a very important advancement. The promise of this technique is exemplified in a recent work by Zhang *et al.*, which demonstrated that CRISPR-Cas9-mediated targeted promotor introduction is efficient for activating diverse biosynthetic gene clusters in multiple *Streptomyces* species, leading to the synthesis of a range of unique metabolites, including a novel pentangular type II polyketide in *Streptomyces viridochromogenes*[71].

Analogous approaches relying on sequencing, bioinformatics, and cloning for heterologous expression can enable novel NPs to be identified from the many strains of bacteria that have not yet been cultivated. For example, a new class of antibiotics known as malacidins has recently been identified *via* a culture-independent approach involving the heterologous expression of biosynthetic gene clusters captured on bacterial DNA extracted from environmental samples[72]. In this study, the research team focused on sequencing and bioinformatic analysis of the metagenomes of 2000 soil samples in the search of biosynthetic gene clusters encoding for calcium-binding motifs, and malacidins were discovered upon the heterologous expression of a 72 kb biosynthetic gene cluster from a desert soil sample (DFD0097) into *Streptomyces albus* host strain[72]. Importantly, this approach can be readily adapted for environmental samples other than soil and biosynthetic gene clusters for diverse NPs. Similarly, Chu *et al.* developed a human

microbiome-based approach involving bioinformatics prediction of NP structures from primary sequences and direct production of the predicted structures by chemical synthesis, which led to the identification of nonribosomal linear hepta-peptides called humimycins as novel antibiotics active against methicillin-resistant *Staphylococcus aureus* (MRSA)[73]. These "synthetic-bioinformatic" NP-like compounds were predicted and chemically synthesized based on the bioinformatic analysis of gene clusters found in human commensal bacterial sequence data. The gene clusters encoding for humimycins were found in particular in the genomes of *Rhodococcus equi* and *R. erythropolis*, and were silent under laboratory fermentation conditions[73]. A major strength of this innovative approach is that it is entirely independent of microbial culture and gene expression, although there are limitations related to the accuracy of computational chemical structure predictions and the feasibility for total chemical synthesis of molecules with more complex structures. **Figure 3** outlines the two alternative strategies for genome mining-driven culture-independent discovery of NPs/ NP-like compounds from environment/microbiota samples, involving heterologous expression or direct synthesis as exemplified from the discovery of malacidins and humimycins, respectively.

Genome mining can also be used for the discovery of bioactive NPs from more complex multicellular organism such as plants or animals. A genome mining approach was recently used by Kersten and Weng to discover the precursor gene of lyciumins, a class of branched cyclic ribosomal peptides with hypotensive action, from *de novo* transcriptome of the root tissue from *Lycium barbarum* (Chinese wolfberry; popularly known as "Goji")[74]. Further genome mining of 116 plant genomes by the authors yielded the discovery of a range of diverse novel lyciumins chemotypes in seven other plants, including important crops such as *Glycine max* (soybean), *Amaranthus hypochondriacus* (amaranth), *Beta vulgaris* (beet), *Chenopodium quinoa* (quinoa), and *Solanum melongena* (eggplant)[74]. An example of the use of genome mining for NP drug discovery from the animal kingdom, is the work of Dutertre *et al.*, which used integrated transcriptomics and proteomics approach for the discovery of novel venom peptides from *Conus marmoreus* snails[75]. A total of 105 conopeptide precursor sequences were identified by the authors from the venom gland transcriptome of *C. marmoreus*, which were identified to belong to 13 gene superfamilies, including seven gene superfamilies

not previously identified in this species, as well as five novel superfamilies. Following proteomics analysis identified thousands of peptides in the venom of *C. marmoreus*, revealing that a limited set of genes and their transcripts were yielding the vast majority of the conopeptide diversity through post-translational processing[75]. In the context of identification of animal-derived NPs, it is also important to consider that some bioactive compounds initially isolated from marine organisms might actually be products of symbiont organisms and genome mining approaches can facilitate the characterization of such NPs. Important illustrative example is provided by the work of Mori *et al.*, which extended on previous studies identifying a range of bioactive compounds from the sponge *Theonella swinhoei* chemotype Y to be actually produced by uncultivated single symbiont, "*Candidatus* Entotheonella factor"[76]. Using these previous findings as a starting point, the authors used single-cell genomics to characterize another phylotype, "*Candidatus* Entotheonella serta*," present in the sponge *T. swinhoei* WA chemotype, which led to the discovery of gene clusters for misakinolide and theonellamide biosynthesis in "*Candidatus* Entotheonella serta" (both groups of compounds were previously known to be produced by the *T. swinhoei* WA sponge)[76]. Another example of a relevant NP from marine invertebrate that was consequently demonstrated to be produced by bacterial symbiont is ET-743 (Yondelis) from the tunicate *Ecteinascidia turbinate*[77]. Meta-omic approach developed by Rath *et al.* revealed that the actual producer of this clinically used anticancer agent is the bacterial symbiont *Candidatus Endoecteinascidia frumentensis*[77]. Similarly to the case with marine organism-associated microbes, the plant microbioms also represent a large reservoir for identification of novel bioactive NPs (*e.g.*, the antitumor agents maytansine, taxol, and camptothecin, which were initially isolated from plants were later shown to be produced by microbial endophytes)[78], and that reservoir could be tapped by genome mining approaches. Illustrative example for this potential is presented in the recent work by Helfrich *et al.*, which involved genome mining of 224 bacterial strains isolated from the *Arabidopsis thaliana* leafs, leading to the identification of more than 1,000 predicted NP biosynthetic gene clusters, hundreds of which were unknown compared to the MIBiG database of characterized biosynthetic gene clusters[79]. Further functional screening for antibiotic activity and following bioactivity-guided fractionation with the identified as most promising *Brevibacillus* sp. Leaf182 revealed three distinct NP scaffolds contributing to the antibiotic activity, while a genome mining-based strategy targeting this species led

Genome/metagenome mining combined with heterologous expression as a strategy for the discovery of new NP therapeutics has been explored by several biotech companies; for example, with Lodo Therapeutics aiming to exploit the human microbiome and Warp Drive Bio (recently acquired by Revolution Medicines Inc.) focusing on actinomycetes[80]. This approach has substantial potential to identify novel NPs given that only a small fraction of the microorganisms existing in nature has been cultured, and also that even for microorganisms that can be cultured, many gene clusters encoding NP biosynthesis remain silent under typical conditions. However, the choice of an optimal heterologous expression strategy is not straightforward, and further progress in this respect would be valuable in making genome/metagenome mining more widely applicable in NP drug discovery.

Targeted gene engineering of NP biosynthetic gene clusters can be valuable if the producing organism is difficult to cultivate or the yield of a NP is too low to allow sufficient NP characterization. As example, rational genetic engineering and heterologous expression was applied to upscale by several orders of magnitude the production of vioprolides, a class of anticancer and antifungal NPs from the myxobacterium *Cystobacter violaceus* Cb vi35, and nonnatural vioprolide derivatives were also generated in the process[81]. Similarly, promotor engineering and heterologous expression of biosynthetic gene clusters was reported to result in 7-fold increase in the production of the cytotoxic NP disorazol[82], and 328-fold increase in the production of the insecticidal macrolide spinosad[83].

Targeted gene manipulation is also a feasible way to alter the biosynthetic pathway in a predictable manner to produce a new NP analogue with improved pharmacological properties, such as higher specific activity, lower toxicity, and better pharmacokinetics. Such approaches, sometimes referred to as "biosynthetic engineering", depend on a solid understanding of the biosynthetic pathway leading to a particular NP, access to the genes specifying this pathway, and the ability to manipulate them either in the original or a heterologous host. Examples of biosynthetic engineering include the generation of analogues of the antitumor agents mithramycin[84] and bleomycin[85], as well as of the antifungal nystatin[86].

Expanding briefly on the example of nystatin, this is a glycosylated polyene macrolide that has been used to treat superficial fungal infections since the 1960s. Engineering of enzymes involved in its biosynthesis in the producing organism, *Streptomyces noursei*, resulted in several analogues[86]. An analogue known as BSG005 demonstrated higher activity and lower toxicity in a murine model of disseminated candidiasis when compared to amphotericin B (the only polyene macrolide currently used to treat systemic fungal infections)[86], and is currently listed as being in preclinical testing by the company Biosergen.

It should be noted that biosynthetic engineering has limitations regarding which parts of the NP molecule can be targeted for modifications, and which chemical groups can be introduced or removed. Considering the complexity of many NPs, however, total synthesis may be cost-prohibitive, and a combined approach of biosynthetic engineering and chemical modification can provide a viable alternative for identifying improved drug candidates. Even if biosynthetic engineering cannot provide a chemical group beneficial for the pharmacological properties of a molecule, it may create a "handle" for addition of a beneficial chemical group by synthetic chemistry. This approach has been demonstrated for biosynthetically engineered analogues of nystatin mentioned above; further synthetic chemistry modifications resulted in compounds with improved *in vivo* pharmacotherapeutic characteristics compared to amphotericin B[87,88].

Advances in microbial culturing systems. The complex regulation of NP production in response to the environment means that the conditions under which producing organisms are studied can have a major influence on the potential to identify novel NPs produced by them[65]. Several strategies have been developed to improve the likelihood of identifying novel NPs compared with monoculturing under standard laboratory conditions and to make "uncultured" microorganisms to grow in a simulated natural environment[89].

One strategy to promote the identification of novel NPs is through modifications of the culture conditions, such as the "One Strain Many Compounds" (OSMAC) approach[90]. The OSMAC approach relies on the ability of microorganisms to adjust their metabolism under different growth conditions (*e.g.*, temperature, pH, nutrient sources), which are associated with the activation of alternative gene sets

and might result in the production of different NPs. In the recent literature, the development of the OSMAC strategy is often attributed to a 1999 work by Schiewe and Zeeck[91], probably because this is the oldest publication identifiable at this moment it the PubMed database upon a search using "OSMAC" as a keyword. However, the underlying concepts were actually first introduced in the academic literature in a work by Zähner in 1977[92], and this approach was already before routinely used in industrial microbiology since 1960s-1970s[93], with different industry-employed scientists using different growth condition variations, although due to the culture of secrecy these initial industry-led investigations were not published in scientific journals. This multiple media approach has been entitled OSMAC (one strain/many compounds) and is often ascribed to work reported in 1999 by Schiewe and Zeek 34. This attribution is incorrect, although it has entered the literature since the process was first described from an academic aspect by Zahner in 1977 35 and had been in general use in the antibiotic discovery programs in the pharmaceutical industry for at least 17 years prior to the Zahner paper. Since industry generally did not publish their techniques (the author was using such systems before 1970, and they had been in general use since the early 1960s), such information is not in the general literature, though the NCI contract conditions that led to their microbial collection were written by a retired microbiologist from Squibb and published in the mid-1980s specified just such an approach.

In a recent example of the use of OSMAC approach, Hussain *et al.* examined the actinomycete *Lentzea violacea* strain AS08 (isolated from north western Himalayas) under different growth medium conditions, which resulted in the identification of new eudesmane sesquiterpenoid with antibacterial and cytotoxic properties as well as a new homologue of virginiae butanolide E showing a moderate antibacterial effects[94]. Similarly, Hemphill *et al.* studied the fungal endophyte *Fusarium tricinctum* upon cultivation on solid rice media supplemented with fruit and vegetable juices, resulting in 80-fold increase in the production of the new cytotoxic NP fusarielin J, as well as in the identification of two new NPs, fusarielin K and fusarielin L, which were not detected when the fungus was grown on rice media lacking either fruit or vegetable juice[95].

While the OSMAC approach is still widely used today for the identification of new bioactive compounds, this approach has limited capacity to mimic the complexities of the natural habitats and it is difficult to predict the combination of cues (which might also involve metabolites secreted by other members of the microbial community) for which the microorganism has evolved to respond by switching metabolic programs. To account for such kind of interactions, co-culturing using "helper" strains can be applied[96]. In a recent example of the use of this approach, co-culture of the endophytic fungus *Fusarium tricinctum* together with *Streptomyces lividans* on solid rice medium resulted in the production of four new naphthoquinone dimers, fusatricinones A–D, and a new lateropyrone derivative, dihydrolateropyrone, that were not present in the axenic fungal culture controls[97]. Similarly, new polyoxygenated steroid with antimicrobial and cytotoxic properties, penicisteroid C, was found upon co-cultivation of *Streptomyces piomogenus* AS63D and *Aspergillus niger* using solid-state fermentation on rice medium[98]. The potential of exploiting microbial interactions for the discovery of new bioactive NPs is very well illustrated in the work of Derewacz *et al.*, who analyzed via multivariate statistical and self-organizing map (SOM) approach the metabolome of genomically characterized *Nocardiopsis* strain, upon co-culture with low inoculum exposure to *Escherichia*, *Bacillus*, *Tsukamurella*, and *Rhodococcus*[99]. Importantly, it was found that around 14% of the metabolomic features in co-cultures were undetectable in monoculture conditions and many features were unique to specific co-culture genera. Moreover, one set of SOM-identified responding features was isolated and structurally characterized by multidimensional NMR, and revealed to comprise previously unreported polyketides, ciromicin A and B, which possess an unusual pyrrolidinol substructure and displayed moderate and selective cytotoxicity[99]. Importantly, study of the molecular mechanisms that are underlying the ability of "helper" strains to increase the cultivability of previously uncultured microbes can lead to the identification of specific growth factors allowing expansion of the range of species that can be successfully cultured. This strategy was used by D'Onofrio *et al.*, for the identification of new acyl-desferrioxamine siderophores as growth factors produced from "helper" strains promoting the growth of previously uncultured isolates from marine sediment biofilm[89,100]. The siderophore-based growth is based on the property of these factors to serve as a soluble source of iron for microbes unable to autonomously produce siderophores, and the

application of this approach led to the culturing of a range of microbes only distantly related to previously cultured organisms[100].

A technology platform for NP discovery dubbed iChip moves even closer to the mimicking the natural environment by harnessing *in situ* incubation in the environment from which the microorganism is sampled. In the iChip, diluted soil samples are seeded in multiple small chambers separated from the environment with a semipermeable membrane[101]. After seeding, the iChip is placed back into the soil from which the sample was taken for *in situ* incubation period, allowing the cultured microorganisms to be exposed to influences from their native environment. The power of this culturing approach was clearly demonstrated by the recent discovery of a new antibiotic teixobactin from a previously uncultured soil microorganism[102,103]. This platform could therefore be of great significance for NP drug discovery, given that it has been estimated that only 1% of soil organisms have so far been successfully cultured by using traditional culturing techniques[104]. Noteworthy, the iChip technology is in part a conceptual follower of some of the approaches used more than 20 years ago by the biotech companies Diversa and even earlier OneCell, which allowed the growth of some previously uncultivated microbes from different environments based on diluting out and suspending in a single drop of medium[93]. Relevant outcomes of the application of OneCell System technologies are described in a 2002 work by Zengler *et al.*, outlining a universal method providing access to previously uncultured microbes from different environments based on cell encapsulation in gel microdroplets for parallel massive microbial cultivation under low-nutrient flux conditions, followed by detection of microdroplets containing microcolonies with flow cytometry[105].

Greater access to microbial and NP diversity can also be achieved by extraction of organisms and/or their NP products *in situ*. Microbes can be "hoovered" up over coral reefs to increase access to microbial diversity, and NPs produced by them can be accessed through the development of culturing methods[106] or through metagenomics and heterologous expression of biosynthetic gene clusters[107], as described above. Extending this approach to directly gain compounds produced in the natural marine environment (which may be missed otherwise), resin capture technology can be used to "hoover" multiple metric tonnes of water to capture compounds on inert sorbent supports ready to be desorbed, analysed and tested

for biological activity. This approach has been recently validated by the work of Ouazzani and colleagues[108], with the development of a solid-phase extraction device (known as a self-operating marine trapping extractor), which was used to identify several new NPs produced in aquarium conditions by *Crambe crambe* in the presence of *Anemonia sulcata*. Sustainable approaches for *in situ* extraction, using green solvents, such as glycerol or natural deep eutectic and ionic solvents (NADES), could be used directly during field work[109,110]. To improve dereplication capabilities, analytical equipment miniaturization is also facilitating *in situ* analysis; examples include the introduction of devices for physicochemical data analysis, such as micro MS (*e.g.*, MALDI) and portable near infrared (NIR) spectroscopy[111,112].

Applications of cheminformatics in NP-based drug discovery. The number of identified molecules from nature and the extent of 'chemical space' that they occupy is constantly increasing through the discovery of new NP sources and the exploitation of existing materials[113]. From this large quantity of data, dozens of virtual and physical NP libraries are available for chemoinformatic studies[114]. These libraries can be harnessed to enable predictions for a novel NP's putative ligand–target interactions, biological activities and properties, including metabolism, toxicology and pharmacokinetics.

In silico tools can be particularly helpful in accelerating the identification of macromolecular targets for bioactive NPs identified through phenotypic screening, as well as for unveiling target promiscuity of individual compounds[115,116]. For example, Gong *et al.* applied a reverse docking approach for swinhoeisterols A and B, two novel sterols with an unprecedented 6/6/5/7 ring system, which were isolated from the sponge *Theonella swinhoei*[117]. Since the two compounds exhibited cytotoxicity toward the cancer cell lines A549 and MG-63, this initially observed phenotypic bioactivity was followed by inverse virtual screening against 211 cancer targets related to phenotypic cytotoxic effects. The ten best ranked targets were experimentally tested and led to the discovery of novel h(p300) inhibitors[117]. Reker *et al.* showcased a ligand-based target prediction method which does not require three-dimensional target models but instead dissects the studied compounds into fragments and predicts potential pharmacological targets by comparing the fragments to reference drugs with established targets[118]. The

application of this strategy to the macrolide drug archazolid A, a sub-nanomolar inhibitor of human and murine cancer cell growth which is derived from the myxobacterium *Archangium gephyra*, revealed a complex poly-pharmacology action, involving farnesoid X receptor (FXR) activation as the strongest identified effect, but also inhibition of 5-lipoxygenase (5-LO) and microsomal prostaglandin E2 synthase 1 (mPGES-1), and partial agonism of peroxisome proliferator-activated receptor subtype gamma (PPARγ)[118].

Computational approaches have also proven value in the identification of NP-like inhibitors of protein-protein interactions, which are generally considered to be difficult to target with small molecules. In a prove-of-principle study, Liu *et al.* used structure-based virtual screening of a database of over 90000 NPs and NP-like compounds aiming to identify STAT3 dimerization inhibitors[119]. The virtual screening conducted by the authors initially yielded 14 hits, from which one compound was especially promising and was further demonstrated to inhibit *in vitro* STAT3 DNA-binding activity and to attenuate in cells STAT3-dependent transcription, exhibiting selectivity over STAT1. Cellular STAT3 but not STAT1 dimerization was inhibited by the identified compound and it also selectively decreased STAT3 but not STAT1 phosphorylation, and displayed selective anti-proliferative effect against several cancer cell lines[119].

Moreover, computational approaches are of major value in NP-based drug design[120]. As one example, Merk *et al.* recently computationally identified three novel RXR-targeting NPs, using virtual screening of the DNP database with three different approaches: first, probability of RXR agonism predicted with the software SPiDER that considers pharmacophoric features and molecular properties; second, chemically advanced template search (CATS) descriptors based on pharmacophore feature distributions; and third, a partial-charge- and shape-based molecular representation[121]. The three identified novel RXR-targeting NPs were then used as templates for automated, ligand-based *de novo* design of easily accessible mimetics that inherited the biological activities of their natural templates[121].

Advances in phenotypic screening. Important recent advances have been made with phenotypic screening for novel drugs in general[122]. While these advances are of benefit for screening different kinds of molecules, they may be

particularly relevant for NP-based drug discovery, given that the phenotypic approach has proved to be especially successful in the area of NP testing, with most NP-based drugs having been discovered this way, e.g., in the traditionally strong anti-microbial and anti-cancer areas for NP drug discovery (both traditionally relying on phenotypic assays for microbial/cancer cell cytotoxicity), and that NPs represent evolutionary optimized privileged structures expected to create more hits in phenotypic assays. In part, such recent advances in phenotypic screening have been catalyzed by the recognition that phenotypic approaches have been valuable in the discovery of first-in-class drugs[123], and also by the expectation that phenotypic screens more closely recapitulating human disease may identify molecules having a higher chance of progression in drug development. Several complementary technologies are enabling the development of improved phenotypic screening assays. These include induced pluripotent stem cells, which can be used to create novel (potentially patient-specific) disease models[124,125], gene-editing technologies such as CRISPR to create cells with desired characteristics[126,127], 3-dimensional cell culture technologies (such as organoids and organs-on-a-chip)[128–130] to more closely mimic the appropriate physiological context and cellular imaging technologies to evaluate the effects of compounds in screens[131,132]. In addition to cell-based phenotypic screens[133], screening models using whole (small) animals, such as the nematode *Caenorhabditis elegans*, zebrafish (*Danio rerio*), brine shrimp (*Artemia salina*) and fruit fly (*Drosophila melanogaster*), have also advanced in recent years[134–136].

An example of the application of an untargeted phenotypic approach is the work by Schulze *et al.* providing insight into the mode of action of NPs by cytological profiling[132]. In this study, the cellular phenotypic profiles induced by marine NP prefractions were measured by automated image analysis, generating a score for 250 cellular features and compared against a known set of compounds with established modes of action. The used approach allowed the prediction of modes of action for specific compounds in the prefractions and led to the identification of the mode of action of unique iron siderophores[132]. A similar but more advanced approach, integrating image-based phenotypic screening with high-resolution untargeted metabolomics analysis, was used by Kurita *et al.* who developed the Compound Activity Mapping (CAM) platform for direct prediction of identities and mechanisms of action of the constituents from complex NP extract libraries[131]. The

CAM approach allows dereplication and identification of new compounds by integrating biological (image-based cytological profiling) and chemical (metabolomics) data that are organized into clusters. These clusters might contain known compounds (compounds with known mass spectrum and phenotypic profile) or might indicate the presence of structurally new compounds inducing a relevant phenotype. Moreover, clustering the cytological profiles of the unknown compounds with those of a reference library of pure compounds with known mechanisms of action can facilitate mode of action elucidation, and as a proof of principle Kurita *et al.* discovered quinocinnolinomycins as a new family of NPs with a unique carbon skeleton causing endoplasmic reticulum stress[131].

An example of phenotypic assay tailored to address the NP drug discovery challenge presented by low concentration of the active compounds in extracts is the whole cell-based antisense testing model developed by Ondeyka *et al*.[137]. This antibacterial assay has been designed to screen for NP inhibitors of FabH/F of the type II fatty acid synthesis pathway in *Staphylococcus aureus*. Since the *S. aureus* cells carrying the FabH/F antisense are much more sensitive to the presence of compounds inhibiting the type II fatty acid synthesis pathway, the application of this assay to *Phoma* sp. extracts allowed the identification of phomallenic acids A-C as novel inhibitors of bacterial fatty acid synthase, which would have been missed due to low abundance in the starting extracts if wild type *S. aureus* would have been used[137].

Aside from being a valuable tool for the discovery of novel bioactive compounds, the use of phenotypic models offers the possibility to identify new mechanisms of action of NPs. Phenotypic models might even result in the identification of new therapeutic targets, since phenotypic models are "unbiased" and do not rely on previous knowledge of the molecular targets that mediate the manifestation of the respective phenotypes[122]. An illustrative recent example relevant to NP-based drug discovery is the work of Eliasen and colleagues[138]. It was known that the two NPs promote regeneration in animal models of spinal cord injury and corneal transplant, but inhibition of their initially described target (semaphorin 3A) with other compounds could not fully account for their observed *in vivo* phenotypic effects. By chemical synthesis, Eliasen *et al.* gained sufficient quantities of the two NPs to allow their bioactivity profiling against a panel of 165 G-protein coupled

receptors, ultimately identifying that vinaxanthone and xanthofulvin act as positive allosteric modulators of the succinate receptor 1 (SUCNR1), suggesting this receptor as a new therapeutic target for promoting nerve repair[138].

Knockout, knockdown and overexpression studies are especially valuable in mechanism of action since they enable the exploration of potential changes in small-molecule effects upon selective modulation of single proteins or cellular pathways. Moreover, "unbiased" parallel genome-wide knockdown and knockout screens are also possible; for example, by the use of high-coverage shRNA and CRISPR/Cas9 libraries[139]. An example of the latter approach that is of relevance for NP-based drug discovery is the recent work of Estoppey *et al.*, who used a genome-wide CRISPR/Cas9-based screen to identify the molecular target of the anti-flaviviral NP cavinafungin, a recently reported linear lipopeptide from the fungus *Colispora cavincola*[140], which they found to have inhibitory activity against dengue- and Zika-virus-infected cells[141]. CRISPR/Cas9-based profiling in human cells, followed by orthogonal profiling in yeast cells, revealed that cavinafungin targets the host signal peptidase SEC11, which is involved in the cleavage of viral structural proteins and viral particle assembly.

Other techniques to aid the identification of molecular targets of NPs identified in phenotypic screens include the nematic protein organisation technique (NPOT), drug affinity responsive target stability (DARTS), stable isotope labeling with amino acids in cell culture and pulse proteolysis (SILAC-PP), the cellular thermal shift assay (CETSA) and an extension known as thermal proteome profiling (TPP), stability of proteins from rates of oxidation (SPROX), similarity ensemble approach (SEA), and bioinformatics-based analysis of connectivity (connectivity map, CMAP)[25,142–145].

NPOT is a recently developed label-free technique based on the Kirkwood-Buff theory of molecular crowding and aggregation that makes use of direct binding of small molecules to proteins to facilitate target identification[25]. This technique was used to identify the molecular target underlying the macrophage cholesterol efflux-enhancing properties of evodiamine, a NP derived from the fruits of the traditional Chinese medicinal plant *Evodia rutaecarpa* (Juss.) Benth (wu-zhu-yu in Chinese). The application of NPOT revealed evodiamine to directly bind the

ATP-binding cassette transporter A1 (ABCA1), a membrane transporter contributing to cholesterol efflux, resulting in increased ABCA1 stability and half-life, elevated cellular ABCA1 protein levels, and ultimately increased macrophage cholesterol efflux[25]. DARTS, another label-free approach, is a technique based on the phenomenon that binding of small molecules to protein targets results in increased resistance to proteolytic digestion. This technique was recently applied for characterization of the major cellular target of laurifolioside, a new lathyrane diterpene isolated from the aerial parts of *Euphorbia laurifolia* Juss ex Lam.[146]. In this study, DARTS indicated that laurifolioside directly binds to clathrin heavy chain 1, a protein involved in the cellular uptake of a variety of macromolecules, and the binding was further studied through microscopy, molecular docking, and molecular dynamics studies, overall corroborating this NP as a novel modulator of cellular trafficking pathways[146]. Another approach that also relies in part on proteolytic digestion and allows genome-wide profiling of ligand-protein interactions is SILAC-PP[144]. Particular strength of this approach is that enables the calculation of protein-ligand binding energies based on quantification of protein denaturation profiles. The SILAC-PP approach was validated by the authors who developed the techniques with two different ligands, ATP (ubiquitous biological ligand), and the NP cyclosporine A (well known drug). Consequently, with the first ligand a range of known and some unknown proteins interacting with ATP were detected, and the second ligand (cyclosporine A) displayed as expected a robust interaction with its well-known molecular target protein cyclophilin A[144].

CETSA is a cell-based method[147] making use of the well-known phenomenon that the thermal stability of proteins is altered upon ligand binding[148]. By combining CETSA with multiplexed quantitative mass spectrometry, TPP has been recently developed as approach to monitor proteome-wide changes in protein thermal stabilities in intact cells[149], which was validated using the NP staurosporine and GSK3182571.

Finally, CMAP is a computational algorithm pattern-matching the gene expression profile of a studied molecule against a database of profiles of compounds with known modes of action. This technique was recently used to study the molecular targets of tanshinone IIA, an antitumor NP derived from the

traditional Chinese medicinal plant *Salvia miltiorrhiza* Bunge, which revealed that the anti-tumor effect of tanshinone IIA is linked to inhibition of PKC, PI3K/Akt/mTOR and Ras/MAPK pathways[150]. Another computational approach for mechanism of action elucidation is the similarity ensemble approach (SEA), which relies on comparison of topological fingerprints of the query molecule with the fingerprints of a database with ligands with established molecular targets[145]. This approach was recently used by Gregori-Puigjané *et al.* to discover molecular targets of FDA approved drugs with previously unknown mechanism of action. As a result of this effort, computationally predicted and experimentally validated were the molecular targets of the antitussives clemastine, cloperastine, and nepinalone; the muscle relaxant cyclobenzaprine; the antiemetic benzquinamide; the analgesic nefopam; and the immunomodulator lobenzarit[145].

Understanding molecular modes of action is not just critical for rationalization of bioactivities of NPs, but can also facilitate the discovery of new NPs. An example for the later represents the target-directed genome mining approach developed by Tang *et al.*, which allows prediction of the biological functions of NPs produced by uncharacterized microbial biosynthetic gene clusters based on the presence of antibiotic resistance genes that encode for modified molecular target proteins[151]. To validate this approach, the authors conducted a query of 86 *Salinispora* genomes for duplicated house-keeping genes colocalizing with NP NP biosynthetic gene clusters, which led to the identification of a specific biosynthetic gene cluster containing a putative fatty acid synthase resistance gene. Consequently, this cluster was prioritized for characterization and upon heterologous expression in *Streptomyces* hosts it was found that in line with the initial prediction it produces a group of thiotetronic acid NPs, including the known fatty acid synthase inhibitor thiolactomycin[151].

While the discussed new methods for mode-of-action elucidation are generally applicable to all kind of molecules (also synthetic), they might be of especial importance for NP drug discovery because of its traditional strong reliance on phenotypic assays often yielding hits with certain biological activity (*e.g.*, antimicrobial or cytotoxic) but unknown molecular targets. Consequently, determination of molecular mode of action has been viewed as one of the grand challenges on the way of the movement of NP leads towards clinical application[18],

and the recently developed methods for molecular target elucidation can help to accelerate this process. Moreover, the application of mechanism of action-based phenotypic methods such as CAM early in the NP drug discovery process might support "mode-of-action dereplication" in order to prioritize for further work NPs with modes of action different to previously known drugs.

Finally, it should be noted that while we discuss here in detail advances in phenotypic test methods, there are also many recent examples for highly successful application of target-directed biochemical assays for screening purified NP or extracts. One advantage of this kind of assays is that the molecular target of action is known already at the start, which eliminates the challenging need for mode of action determination. Several success stories that can be briefly mentioned as illustrative examples are: (i) the Eli Lily's discovery of chloroeremomycin, the precursor to oritavancin through the use of biochemical screen (enzyme-linked immunosorbent assay) of NP extracts[152]; the discovery also by Eli Lily of the arylomycin class of NP inhibitors of signal peptidase I by the use of enzyme inhibition screen of 50,000 NP fractions[153] (the first structures of compounds from this class were firstly reported shortly before by researchers from the University of Tübingen, Germany[154]); and the recent discovery by Daiichi-Sankyo researchers of pedopeptins as novel inhibitors of the binding of lipopolysaccharide to CD14 through the use of cell-free lipopolysaccharide-CD14 binding assay for screening of microbial secondary metabolites library[155].

Outlook

As discussed previously, from a drug discovery perspective, the chemical space covered by NPs possesses unique features that make NPs an attractive alternative to conventional synthetic small molecules, and the outlined technological and scientific advances have the potential to invigorate the application of NPs in different disease areas.

Effective strategies to counteract antimicrobial resistance are urgently needed[156], and in this therapeutic area NPs continue to be a preeminent source of new drugs. The development of novel approaches for culturing previously uncultured

microorganisms is of major importance in the search for new antibiotics, with the iChip technology discussed above being one prominent example. The application of iChip has already resulted in discovery of a new promising antibiotic, teixobactin, which is highly active against multi-resistant Gram-positive bacteria in animal models, inhibiting bacterial cell wall synthesis by targeting lipid II, without detectable resistance exhibited by any of the tested *Staphylococcus aureus* or *Mycobacterium tuberculosis* strains[102,103]. The establishment of culture-independent approaches for the identification and production of novel antibiotics — enabled by the technological advances in sequencing, bioinformatics, and synthetic biology discussed above — is also of great significance. The power of this approach is shown by the recent discoveries of malacidins (calcium-dependent antibiotics identified by soil metagenomics-based approach, with activity against multidrug-resistant Gram-positive pathogens)[72] and humimycins (lipid II flippase-inhibiting MRSA-active antibiotics identified by a culture-independent approach from the human microbiome)[73]. Another example of a promising new antibiotic isolated from human microbiota is lugdunin, which was identified from nasal *Staphylococcus lugdunensis* strains, and displays a good activity in animal models of infection, without development of resistance in *S. aureus*[157]. Another promising new group of antibiotics active against Gram-positive bacteria in animal models are lysocins from *Lysobacter* sp.; whether they bind menaquinone or lipid II is currently under debate[136,158]. Novel classes of NP antibiotics with broad spectrum Gram-negative activity include albicidins identified from *Xanthomonas* sp., which were found to *p*-aminobenzoic acid containing peptide-type polymers that act as gyrase inhibitors and exhibit little or no cross-resistance with fluoroquinolones[159,160], and cystobactamids derived from myxobacteria[161,162], which inhibit bacterial type IIa topoisomerases. In the context of Gram-negative targeting, great impact is also expected to be made by the recently elucidated rules for accumulation of small molecules into Gram-negative cells, which were derived through experimental and computational analysis of over 180 compounds, and their application allowed to convert deoxynybomycin, a NP with Gram-positive activity, into an antibiotic also targeting Gram-negative pathogens[163]. As well as searching for new NP antibiotics, there are ongoing efforts to further develop and optimize already known NP antibiotic classes, making use of advances in genetic engineering, total synthesis or semi-synthetic strategies. In a genetic engineering approach, the atypical tetracycline amidochelocardin was generated by combining

tetracycline and chelocardin biosynthetic genes, yielding a novel compound exhibiting a broad spectrum anti-Gram-negative activity and no cross-resistance with known antibiotics[164]. Illustrating a total synthesis strategy, analogues of the known Actinomycete-derived compound, griselimycin, were optimized to improve oral bioavailability and efficacy in murine tuberculosis models. It was demonstrated that these NPs target the sliding clamp of DNA polymerase and inhibit a protein-protein interaction to prevent DNA repair and polymerization, explaining the lack of cross-resistance with other antibiotics[165]. A recent example of NP property optimization to combat antimicrobial resistance is plazomicin (ACHN-490), a semisynthetic aminoglycoside derived from the NP sisomicin, which was isolated from *Micromonospora inyoensis*[166]. The use of sisomicin has been limited due to its susceptibility to many aminoglycoside modifying enzymes, and plazomicin was engineered to evade such modifications and exhibit activity against a broad spectrum of multidrug-resistant strains[167]. The development of plazomicin gives an example on how recent scientific advances can translate in new opportunities for NP-based drug discovery, since its development required synthetic and analytical chemistry techniques that were not available in the 1960s and 1970s when aminoglycoside antibiotic discovery was at its peak. Recent phase III trials have shown excellent efficacy of plazomicin for infections caused by carbapenem-resistant Enterobacteriaceae[168] and for complicated urinary tract infections caused by Enterobacteriaceae (including multidrug-resistant strains)[169]. In June 2018, plazomicin approval was issued by FDA (trade name ZEMDRI, by Achaogen) for the treatment in adults of complicated urinary tract infections, including pyelonephritis, caused by *Escherichia coli*, *Klebsiella pneumoniae*, *Proteus mirabilis*, and *Enterobacter cloacae* (see **FDA** in Related links). An overview of other advanced (phase III) NP/NP-derived antibiotics under development is presented in **Table 2**.

Although the search for new scaffolds and mechanisms targeting bacterial growth remains a primary source of new anti-infective drugs, anti-virulence strategies represent alternative approach as conventional antibiotics become ineffective against a number of pathogenic bacteria[170,171]. A range of diverse NPs have recently been shown to exhibit anti-virulence action through different mechanisms, and anti-virulence NP-based drug discovery is an area with a promise for further exploration in the future. Thus, disulfide analogues obtained from *Allium stipitatum*

Regel were shown to be inhibitors of mycobacterial efflux and biofilm formation with a selectivity towards *Mycobacterium tuberculosis* H37Rv[172]. It was also demonstrated that biofilms of *Acinetobacter baumannii*, a nosocomial pathogen of major concern forming biofilms on medical devices, could be inhibited by peptides known as cahuitamycins derived from the marine strain *Streptomyces gandocaensis*. Increased potency was shown for cahuitamycins D and E, which were obtained *via* mutasynthesis and a series of unnatural starter units[173]. Applying bacteriocins, including lantibiotics, and other antimicrobial peptides as single active ingredients or in combination with existing antimicrobial drugs revealed higher potency in inhibiting biofilm formation compared to tackling existing biofilms[174]. A ring-distortion strategy using the marine sesquiterpenes ilimaquinone and 5-*epi*-ilimaquinone led to the creation of a small library, and promising inhibitors of *Vibrio harveyi* quorum sensing could be found among cyclopentendiones[175]. Diverse other NPs have also been shown to interfere with virulence factors, and such compounds require further study in rigorous preclinical studies and clinical trials to ascertain their therapeutic potential[170]. Promising antivirulence therapies that are currently in development were recently reviewed elsewhere[176]; examples that are NP-derived or NP-inspired include halogenated furanones (produced by the marine red alga *Delisea pulchra* (Greville) Montagne), mimetics of *N*-acyl homoserine lactones, and derivatives of 8-hydroxyquinoline (present in roots of *Centaurea diffusa* Lam.).

In the area of new anti-infective drugs, the search for novel antimycotics and fungicides is also very important because of the increasing resistance of fungal pathogens like *Candida*, *Aspergillus* and *Cryptococcus* spp. against marketed drugs[177]. Notable previous success stories of NP drugs in the area of fungal infections include amphotericin B, which has a long history in systemic antifungal therapy, and more recently, the NP-derived echinocandins (e.g., caspofungin, micafungin and anidulafungin)[178]. Nikkomycin Z is a new "first in class" antifungal NP currently under clinical development, which however was first identified by Bayer pharmaceutical Company in the 1970s and was being actively investigated by that time together with its analogues[179–181]. Therefore, nikkomycin Z also represents an example of an "old molecule" which development has only recently been renewed. An overview of NP/NP-derived antifungals under clinical development is presented in **Table 3**.

With the development of regulatory guidance for complex mixtures of NPs, as well as improved technologies for characterizing such mixtures (some of which are highlighted above), it has become more feasible to develop such mixtures as therapies, rather than seek to identify and purify a single active ingredient[182,183]. For example, to support organizations developing new botanical drugs — defined as products devoted to ameliorating (treatment or prevention) of human diseases, consisting of a combination of vegetable materials, including plant components, algae and fungi — the US FDA released its "Botanical Drug Development Guidance for Industry" in 2016 (see Related links), updating its initial guidance from 2004. One of the potential advantages with botanical drugs could be synergistic therapeutic effects of the mixture compared to a single compound. Metabolomics is a valuable tool in systems biology approaches to identify possible synergistic effects[184]. As example, metabolic profiling paralleled with bioactivity evaluation was applied for the identification of compounds with synergistic action from goldenseal (*Hydrastis canadensis*)[185]. Three synergistically acting compounds were identified in this work (sideroxylin, 8-desmethyl-sideroxylin, and 6-desmethyl-sideroxylin), which were able to enhance the antimicrobial activity of berberine against *Staphylococcus aureus*[185]. Importantly, to prove the existence of synergism, standardized mathematical models for drug interactions must be used, such as the calculation of a combination index based on dose-effect relations observed upon the application of the single compounds and their mixtures[186,187]. Unfortunately, such calculations are not always easy, especially in the presence of a mixture of a high number of potentially bioactive compounds. Moreover, it should be noted that a mixture of bioactive compounds may also present a drawback and not an advantage. For example, the simultaneous administration of two or more NPs may generate differences in metabolism and interference in the complex processes regulating their intake and bioavailability, *e.g.*, through altering the enzymatic reactions of deconjugation and reconjugation[188]. Such altered metabolism might have positive or negative effect on the efficacy and safety of the affected compounds. Bioactive compounds induce specific metabolome profiles in exposed cells depending from their mechanism of action[189], and the thorough analysis of such metabolome signatures can be used for prediction of efficacy and potential side effects of compound combinations, as exemplified by the recent work

of Campos and Zampieri, who used this strategy to predict combination antimicrobial therapies[190]. The variability of NP composition in the starting plant material is also a significant challenge for the development of plant NP mixture-based drugs. This variability is affected by multiple factors such as location of collection (influenced by environmental factors, including local climate, soil composition, or interaction with other organisms in the habitat), subspecies identity (genetic variations), and season and time of collection[1]. Due to such variations, drug development necessitates standardization of the NP mixtures, during which acceptable ranges of key bioactive NPs are defined in order to achieve optimal efficacy and safety. Therefore, in order to perform rational and meaningful standardization, optimally the identities of NPs involved in the bioactivity mechanisms of the extract should be known, as well as their interactions (both beneficial and potentially harmful synergistic effects). In respect to the later aspect, comprehensive analysis of synergistic therapeutic as well as toxic effects is an important direction for future work that should be considered both by developers and regulatory authorities. Moreover, in order to achieve consistent quality, of high importance are: correct botanical identification and prevention of product adulteration by other plant species, practices for maintenance of genetic uniformity (for example, by clonal propagation), and controlled cultivation practices in order to minimize the effects of environmental conditions[182].

FDA-approved botanical drugs are sinecatechins (marketed as Veregen by Fougera Pharmaceuticals), an extract of the leaves of green tea (*Camellia sinensis*), was approved for the treatment of warts outside of the genitals and the anus in 2006, and crofelemer (marketed as Mytesi by Napo Pharmaceuticals), an oligomeric proanthocyanidin-enriched extract from the latex of the Dragon's blood tree (*Croton lechleri*), was approved for HIV/AIDS-related diarrhea in 2012[191–194]. Another botanical mixture that received in 2017 a premarket 510(k) approval by FDA is Curasite™ (Izun Pharmaceuticals), a topical wound-care hydrogel-embedded combination of extracts from *Centella asiatica*, *Echinacea purpurea* and *Sambucus nigra* (see **FDA** in Related links). Earlier approval of compound mixture in the Western medicine represents Curaderm®, which is a mixture of solamargines and was approved in in Australia in 1989 for treatment of skin cancer[195]. Another example of a botanical drug approved for use outside the USA is a standardized extract of cannabis (*Cannabis sativa*), which is marketed as Sativex for the

treatment of spasticity (muscle stiffness/spasm) due to multiple sclerosis (see **GW Pharmaceuticals** in Related links). Notably, the FDA recently approved a cannabidiol oral solution (marketed as Epidiolex) as the first drug comprised of an active ingredient directly derived from *C. sativa*, for the treatment of rare, severe forms of epilepsy (see **FDA** in Related links). Noteworthy, another compound contained in cannabis, dronabinol (produced by chemical synthesis) was approved for use by FDA already in 1985. Cannabidiol is the major non-psychoactive ingredient of *C. sativa*, and it does not have the euphoria-inducing action of tetrahydrocannabinol (the main psychoactive ingredient of cannabis). Interestingly, the use of cannabis to treat epilepsy was not a new finding, but was already referenced in a book about epilepsy published in 1881[196], providing another example of the value that traditional medicine knowledge might bring to modern drug discovery. Importantly, the regulations for botanical drug approvals are different across countries. As example, in the European Union, the conventional legislation on pharmaceutical products for human use also applies to herbal medicinal products, and the Committee for Herbal Medicinal Products (HMPC) was established at the European Medicines Agency (EMA) with the task to propose a list of herbal products that have been in human use for more than 30 years and, therefore, could be considered to be safe for use (see **EMA** in Related links). As an outlook for botanical drugs in late-stage clinical development, **Table 4** presents Phase III trials that are currently in the participant recruiting stage.

An emerging opportunity for NP-based drug discovery arises from the increasingly recognized significance of gut microbiota in health and disease modulation[197–199], in combination with the established potential of NPs to significantly affect the gut microbiome composition[200–203]. However, drug discovery efforts in this area are mainly in early stages, with many open questions remaining; these include lack of clarity of whether gut microbiome-modulating agents will prove suitable for the development of future prescription drugs or would predominantly be applied as dietary supplements, with the latter approach already being widely applied[204]. Nevertheless, modulation of gut microbiota by NP represents a highly promising drug discovery direction, which will likely yield in the near future innovative and effective therapeutics with new modes of action directly targeting the microbiota and not the host cells. The promise of this new emerging field is exemplified by the recently gained new understanding of the mode of action of metformin, first-

in-line antidiabetic drug, the development of which could be traced back to the NP guanidine, ingredient of the traditional European herbal remedy *Galega officinalis* (goat's rue, French lilac)[205]. While the anti-diabetic action of metformin is traditionally considered to manifest in inhibition of liver gluconeogenesis, accumulating evidence indicate metformin´s action in the gut to be implied in the therapeutic effects of this drug[206]. Align with increasing the levels of the glucose-lowering gut-derived incretin hormone glucagon-like peptide-1 (GLP-1)[207], metformin intake was recently also demonstrated to reshape the gut microbiome by enriching it with microbial species exhibiting more favorable metabolic phenotypes such as production of beneficial short-chain fatty acids and alterations of intestinal bile acid metabolism resulting in antagonism of signaling mediated through the bile acid receptor farnesoid X receptor (FXR)[208–210].

Could natural compounds play a role in the immune-mediated destruction of cancer cells? Beyond the already mentioned therapeutic uses of phytochemical compounds, innovative research adapted toxic natural compounds to cancer-specific antibodies to form antibody-drug conjugates (ADCs)[211]. Compounds like maytansine, a macrocyclic compound extracted from the Ethiopian plant, *Maytenus serrata*, monomethyl auristatin E (vedotin) derived from dolastatin 10 from the marine mollusk, *Dolabella auricularia* and calicheamicin from the actinomycete *Micromonospora echinospora* act as warheads to target specific forms of cancer yet reducing systemic toxicity[212]. In 2017 the FDA approved Gemtuzumab ozogamicin (GO), a humanized anti-CD33 antibody tethered to the DNA damaging agent calicheamicin derivative (*N*-acetyl gamma calicheamicin)[213]. This approach would allow the targeted use of highly toxic natural compounds devoid of selective toxicity when used as a single agent.

Another interesting opportunity in the field of NP drug development is based on the capacity of selected natural compounds with an intrinsic ability to trigger a selective yet potent host immune reaction against cancer cells. Such drugs increase the immunogenicity of stressed and dying cancer cells by triggering immunogenic cell death (ICD), characterized by the release of damaged-associated molecular patterns (DAMPs) including high mobility group box 1 (HMGB1) release, calreticulin (CRT) exposure or ATP secretion. Natural compound ICD inducers activate DAMP signaling that promotes adjuvanticity and antigenicity of otherwise non-immunogenic cancer types. ICD-inducing phytochemicals are able to provide

long-term clinical benefits of anticancer treatments and the properties of immune memory that can protect cancer patients against relapse altogether opening new avenues for drug discovery or repurposing[214–216]. Best described examples include anthracycline glycosides daunorubicin and doxorubicin or adriamycin[217] from *Streptomyces peucetius*. Daunorubicin treatment of AML cells triggers CRT exposure and release of heat shock protein (HSP) 70 and HSP90[218]. Moreover mitoxantrone, an anthracenedione anthracycline derivative, stimulated the production of CXCL8 involved in the translocation of CALR to the outer plasma membrane whereas knockdown of CXCL8/CXCL2 receptors reduced mitoxantrone-induced CALR exposure in both human and murine cancer cells, as well as the capacity to trigger an immune response *in vivo*. Physical stimuli like ionizing or UV irradiation and photodynamic therapy (PDT) with hypericin activate anti-cancer immunity *via* ICD[219]. Interestingly hypericin is a cytotoxic anthraquinone derivative that was extracted from Saint John's wort, *Hypericum perforatum*[220,221]. Hypericin-mediated photodynamic therapy (HPDT) induces ICD[222]. In addition, cardiac glycosides (CGs) are a well-known class of pharmacologically active compounds essentially because of their use against obstructive heart diseases. As an inhibitor of the essential Na/K ATPase, this compound category was also described to have potent anti-cancer potential[223–225] *via* induction of apoptosis or necrosis accompanied by induction of autophagy/mitophagy[226] in solid tumors. In 2012, Menger *et al.* demonstrated that CGs digoxin, digitoxin, ouabain and lanatoside C augmented the anti-cancer potential of DNA-damaging agents in immunocompetent mice. A combination of chemotherapy and CGs led to a vaccination of syngeneic mice against injection of the same cancer cell[227]. Sukkurwala *et al.* used the collection of the National Cancer Institute (NCI) Mechanistic Diversity Set with 800 compounds to reveal ICD hallmarks in vitro. Again, CGs scillaren A and proscillaridin, lanatoside, and digitoxigenin were the most potent ICD inducers[228]. In the future, the ICD-inducing potential of natural compounds needs to be better investigated and their use to be assessed whether as single agents or in combination with otherwise tolerogenic cell death modality-inducing anti-cancer agents to allow immunogenic adjuvancy.

Recent advances in onco-immunology led to the development of checkpoint inhibitors. These therapeutic approaches were so far essentially based on the development of specific antibodies. In the future, a small molecule–based approach targeting the checkpoint inhibitors could show a number of

advantages[229]. As such compounds like BMS-1001 and BMS-1116[230] were shown to restore anti-CD3–mediated T cell activation. In the future, could natural compounds inhibit the interaction between PD1 and PDL1 like these synthetic molecules? Interestingly, natural compound derivatives were shown to modulate the inhibition of the arginine catabolism. Indeed, immunosuppressive myeloid cells express arginase 1 leading to degradation of arginine into ornithine and urea. As arginine is required for expression of the T cell receptor complex, a reduction of extracellular arginine by arginase 1 in the tumor microenvironment would negatively affect T cell effector function. Moreover, generation of ROS and NOS after arginine depletion is also promoting immune suppression. It was shown that the nitrooxy aspirin analog NCX-4016 improved the immune response to tumors as this compound was for example able to block both arginase 1 and nitric oxide synthase[231]. In the future, natural compound libraries should be screened for such modulatory functions in the immune microenvironment for a future therapeutic use alone or in combination.

Other well-established opportunities for NP-based drug discovery and application for which there is continued interest are only mentioned very briefly here, and have been reviewed extensively elsewhere. These include the broad field of anticancer drug discovery[232]; better tissue targeting of NP drugs through nanoparticles and other new delivery systems[233,234] or by conjugation with antibodies[212,235] or aptamers[236]; and the use of NP peptides derived from animal venoms, particularly for central nervous system indications such as pain[237]. Further opportunities for NP-based drug discovery will also emerge from the application of new synthetic chemistry approaches that allow both enrichment of screening compound libraries with NPs, NP analogues and NP-inspired molecules, as well as superior structure functionalization approaches (including late-stage functionalization) for optimization of NP leads[238].

Finally, a key challenge in NP-based drug discovery that has not already been mentioned is that the required innovative scientific and technological expertise is often scattered through different academic institutions and industrial entities, and focused efforts are needed to help translate research in academia to the stage where candidate products could progress into clinical development. In some respects, this has become more challenging in recent years given the decline in

the number of large companies actively engaged in NP research. Several approaches have been adopted to address the challenges of translating academic research in NP drug discovery. A conventional solution for the need of interdisciplinary knowhow and academy-industry interaction is to focus the relevant scientific and industrial expertise "under one umbrella" and in close spatial proximity. For example, the Phytovalley Tirol, centered in Innsbruck, Austria, brings together several research institutions and companies (among others, the Austrian Drug Screening Institute (ADSI), Michael Popp Research Institute for New Phyto-Entities, Bionorica Research GmbH, and Biocrates Life Sciences AG), with the aim of accelerating NP-based drug discovery. Academy-industry collaborative projects focusing on NP drug discovery are especially meaningful because the patenting constraints are linked to lower expected profitability for NP-based drugs, which translates to lower willingness of pharmaceutical companies to supply full-scale R&D funding. NP drug discovery therefore offers a unique niche for diverse forms of academy-industry collaborations and unconventional approaches to acquisition of research funding, including use of crowdsourcing, charitable contributions, government grants, and joint ventures. Several interdisciplinary consortia aiming to tackle challenges in NP-based drug discovery are shortly presented in **Box 2**.

In conclusion, there is a growing global need for novel therapeutics to address major unmet clinical needs in therapeutic areas in which NPs have traditionally been valuable — particularly infectious diseases, as highlighted above. The structural diversity of NP scaffolds also offers a variety of complex building blocks for drug discovery in general, which by themselves have therapeutic value, as well as providing starting points for medicinal chemistry optimisation. While drug development overall continues to be challenged by high attrition rates, and there are further challenges for NPs because of issues such as accessibility, supply sustainability, and intellectual property constraints (**Figure 1**), we believe that the scientific and technological advances discussed in this article will provide a strong basis for NP-based drug discovery to continue making major contributions to medicine.

Competing interests

A.G.A. is founder and executive director of the International Natural Product Sciences Taskforce (INPST).

Maciej Banach has served on the speakers bureau of Abbott/Mylan, Abbott Vascular, Actavis, Akcea, Amgen, Biofarm, KRKA, MSD, Sanofi-Aventis, Servier and Valeant, and has served as a consultant to Abbott Vascular, Akcea, Amgen, Daichii Sankyo, Esperion, Lilly, MSD, Polfarmex, Resverlogix, Sanofi-Aventis; grants from Sanofi and Valeant.

G.K.B. is a board member of Bionorica SE., Germany.

Maria Daglia has received consultancy honorarium from Pfizer Italia and Mylan for training courses for Chemists. She is member of the International Natural Product Sciences Taskforce (INPST) Board of Directors.

A.T.D.K. is a member of the Scientific and Medical Advisory Board of Evgen Pharma plc.

I.E.O. is Dean of Faculty of Pharmacy, Gazi University, Ankara, Turkey, member of Traditional Chinese Medicine Experts Group in European Pharmacopeia, and member of Scientific Board of Austrian Drug Screening Institute (ADSI).

B.L.F. is a member of the International Natural Product Sciences Taskforce (INPST) Board of Directors. BLF received research funding from Dr. Willmar Schwabe GmbH&Co KG, Karlsruhe, Germany.

K.M.G. has received reimbursement for speaking at conferences sponsored by companies selling nutritional products, and is part of an academic consortium that has received research funding from Abbott Nutrition, Nestec and Danone.

C.W.G. is chairman of the scientific advisory board of Cyxone AB, SE.

M.H.'s research group has received charitable donations from Fa. Willmar Schwabe, DE and recently completed a research project sponsored by Fa. Pukka Herb, UK.

Kaiviti Consulting (M.J.S.M.) is a consulting business for the nutraceutical & Biotech industries focusing on clinical trials, product development and consumer education.

F. N. is cofounder and shareholder of OncoNox and Aura Biopharm.

G.P. is on the board of Neurotez and Neurotrope, and serves as advisor for Phoenix Biotech.

M.R. serves as an advisor for the Nestlé Institute of Health Sciences.

G.L.R. is included in the board of director of the International Natural Product Sciences Taskforce (INPST) and is a member of the governing board of the Italian Society of Human Nutrition (Italy).

N.T.T. is Founder and CEO of NTZ Lab Ltd. and Advisory Board member of INPST.

M.W. collaborates with Finzelberg GmbH and Schwabe GmbH.

J.L.W. collaborates with Nestlé and Firmenich.

M.A.P. is CEO and Owner of Bionorica SE.

J.H. is an employee and hold shares in UCB Pharma Ltd.

M.M. is Founder and Chairman of Sami–Sabinsa Group of Companies.

David S. Bredt is Johnson and Johnson San Diego R&D Site Leader Global Head Discovery Neuroscience.

Michael Bodkin is Vice-President Research Informatics at Evotec (UK) Ltd.

References

1.   Atanasov, A. G. *et al.* Discovery and resupply of pharmacologically active plant-derived natural products: A review. *Biotechnol. Adv.* **33**, 1582–1614 (2015).

2.   Harvey, A. L., Edrada-Ebel, R. & Quinn, R. J. The re-emergence of natural products for drug discovery in the genomics era. *Nat. Rev. Drug Discov.* **14**, 111–29 (2015).

3.   Newman, D. J. & Cragg, G. M. Natural Products as Sources of New Drugs from 1981 to 2014. *J. Nat. Prod.* **79**, 629–61 (2016).

4.   Waltenberger, B., Mocan, A., Šmejkal, K., Heiss, E. H. E. H. & Atanasov, A. A. G. A. G. Natural Products to Counteract the Epidemic of Cardiovascular and Metabolic Disorders. *Molecules* **21**, 807 (2016).

5.   Tintore, M., Vidal-Jordana, A. & Sastre-Garriga, J. Treatment of multiple sclerosis — success from bench to bedside. *Nat. Rev. Neurol.* **15**, 53–58 (2019).

6.   Feher, M. & Schmidt, J. M. Property distributions: differences between

drugs, natural products, and molecules from combinatorial chemistry. *J. Chem. Inf. Comput. Sci.* **43**, 218–27 (2003).

7. Barnes, E. C., Kumar, R. & Davis, R. A. The use of isolated natural products as scaffolds for the generation of chemically diverse screening libraries for drug discovery. **33**, 372–381 (2016).

8. Li, J. W.-H. & Vederas, J. C. Drug Discovery and Natural Products: End of an Era or an Endless Frontier? *Science (80-.).* **325**, 161–165 (2009).

9. Clardy, J. & Walsh, C. Lessons from natural molecules. *Nature* **432**, 829–837 (2004).

10. Lawson, A. D. G., MacCoss, M. & Heer, J. P. Importance of Rigidity in Designing Small Molecule Drugs To Tackle Protein–Protein Interactions (PPIs) through Stabilization of Desired Conformers. *J. Med. Chem.* **61**, 4283–4289 (2018).

11. Doak, B. C., Over, B., Giordanetto, F. & Kihlberg, J. Oral Druggable Space beyond the Rule of 5: Insights from Drugs and Clinical Candidates. *Chem. Biol.* **21**, 1115–1142 (2014).

12. Shultz, M. D. Two Decades under the Influence of the Rule of Five and the Changing Properties of Approved Oral Drugs. *J. Med. Chem.* acs.jmedchem.8b00686 (2018). doi:10.1021/acs.jmedchem.8b00686

13. Lachance, H., Wetzel, S., Kumar, K. & Waldmann, H. Charting, Navigating, and Populating Natural Product Chemical Space for Drug Discovery. *J. Med. Chem.* **55**, 5989–6001 (2012).

14. Henrich, C. J. & Beutler, J. A. Matching the power of high throughput screening to the chemical diversity of natural products. *Nat. Prod. Rep.* **30**, 1284 (2013).

15. Cragg, G. M., Schepartz, S. A., Suffness, M. & Grever, M. R. The taxol supply crisis. New NCI policies for handling the large-scale production of novel natural product anticancer and anti-HIV agents. *J. Nat. Prod.* **56**, 1657–68 (1993).

16. Harrison, C. Patenting natural products just got harder. **32**, 403–404

(2014).

17.   Burton, G. & Evans-Illidge, E. A. Emerging R and D Law: The Nagoya
        Protocol and Its Implications for Researchers. *ACS Chem. Biol.* **9**, 588–591
        (2014).

18.   Corson, T. W. & Crews, C. M. Molecular Understanding and Modern
        Application of Traditional Medicines: Triumphs and Trials. *Cell* **130**, 769–
        774 (2007).

19.   Keiser, M. J. *et al.* Predicting new molecular targets for known drugs.
        Nature **462**, 175–181 (2009).

20.   Wagenaar, M. M. Pre-fractionated microbial samples--the second
        generation natural products library at Wyeth. *Molecules* **13**, 1406–26
        (2008).

21.   Schilling, F. *et al.* Next-generation heteronuclear decoupling for high-field
        biomolecular NMR spectroscopy. *Angew. Chem. Int. Ed. Engl.* **53**, 4475–9
        (2014).

22.   Tahtah, Y. *et al.* High-resolution PTP1B inhibition profiling combined with
        high-performance liquid chromatography–high-resolution mass
        spectrometry–solid-phase extraction–nuclear magnetic resonance
        spectroscopy: Proof-of-concept and antidiabetic constituents in crude
        extract of Eremophila lucida. *Fitoterapia* **110**, 52–58 (2016).

23.   Chu, C. *et al.* Antidiabetic constituents of Dendrobium officinale as
        determined by high-resolution profiling of radical scavenging and α-
        glucosidase and α-amylase inhibition combined with HPLC-PDA-HRMS-SPE-
        NMR analysis. *Phytochem. Lett.* **31**, 47–52 (2019).

24.   Potts, M. B. *et al.* Mode of action and pharmacogenomic biomarkers for
        exceptional responders to didemnin B. *Nat. Chem. Biol.* **11**, 401–408
        (2015).

25.   Wang, L. *et al.* Novel interactomics approach identifies ABCA1 as direct
        target of evodiamine, which increases macrophage cholesterol efflux. *Sci.
        Rep.* **8**, 11061 (2018).

26. Zhang, A. *et al.* Discovery and verification of the potential targets from bioactive molecules by network pharmacology-based target prediction combined with high-throughput metabolomics. *RSC Adv.* **7**, 51069–51078 (2017).

27. Liu, X. & Locasale, J. W. Metabolomics: A Primer. *Trends Biochem. Sci.* **42**, 274–284 (2017).

28. Allard, P.-M. *et al.* Pharmacognosy in the digital era: shifting to contextualized metabolomics. *Curr. Opin. Biotechnol.* **54**, 57–64 (2018).

29. Hubert, J., Nuzillard, J.-M. & Renault, J.-H. Dereplication strategies in natural product research: How many tools and methodologies behind the same concept? *Phytochem. Rev.* **16**, 55–95 (2017).

30. Allard, P.-M., Genta-Jouve, G. & Wolfender, J.-L. Deep metabolome annotation in natural products research: towards a virtuous cycle in metabolite identification. *Curr. Opin. Chem. Biol.* **36**, 40–49 (2017).

31. Wolfender, J.-L., Marti, G., Thomas, A. & Bertrand, S. Current approaches and challenges for the metabolite profiling of complex natural extracts. *J. Chromatogr. A* **1382**, 136–164 (2015).

32. Covington, B. C., McLean, J. A. & Bachmann, B. O. Comparative mass spectrometry-based metabolomics strategies for the investigation of microbial secondary metabolites. *Nat. Prod. Rep.* **34**, 6–24 (2017).

33. Karlsson, O. & Hanrieder, J. Imaging mass spectrometry in drug development and toxicology. *Arch. Toxicol.* **91**, 2283–2294 (2017).

34. Kieninger, J., Weltin, A., Flamm, H. & Urban, G. A. Microsensor systems for cell metabolism - from 2D culture to organ-on-chip. *Lab Chip* **18**, 1274–1291 (2018).

35. Ganesh, T. *et al.* Evaluation of the tubulin-bound paclitaxel conformation: synthesis, biology, and SAR studies of C-4 to C-3' bridged paclitaxel analogues. *J. Med. Chem.* **50**, 713–725 (2007).

36. Choules, M. P. *et al.* Residual complexity does impact organic chemistry and drug discovery: The case of rufomyazine and rufomycin. *J. Org. Chem.*

83, 6664–6672 (2018).

37.   Eugster, P. J. *et al.* Ultra high pressure liquid chromatography for crude
       plant extract profiling. *J. AOAC Int.* **94**, 51–70 (2011).

38.   Kind, T. *et al.* Identification of small molecules using accurate mass MS/MS
       search. *Mass Spectrom. Rev.* **37**, 513–532 (2018).

39.   Wang, M. *et al.* Sharing and community curation of mass spectrometry
       data with Global Natural Products Social Molecular Networking. *Nat.
       Biotechnol.* **34**, 828–837 (2016).

40.   Yang, J. Y. *et al.* Molecular networking as a dereplication strategy. *J. Nat.
       Prod.* **76**, 1686–1699 (2013).

41.   Allard, P.-M. *et al.* Integration of Molecular Networking and In-Silico MS/MS
       Fragmentation for Natural Products Dereplication. *Anal. Chem.* **88**, 3317–
       23 (2016).

42.   da Silva, R. R. *et al.* Propagating annotations of molecular networks using
       in silico fragmentation. *PLoS Comput. Biol.* **14**, e1006089 (2018).

43.   Randazzo, G. M. *et al.* Prediction of retention time in reversed-phase liquid
       chromatography as a tool for steroid identification. *Anal. Chim. Acta* **916**,
       8–16 (2016).

44.   Zhou, Z., Xiong, X. & Zhu, Z.-J. MetCCS predictor: a web server for
       predicting collision cross-section values of metabolites in ion mobility-mass
       spectrometry based metabolomics. *Bioinformatics* **33**, 2235–2237 (2017).

45.   Hautbergue, T., Jamin, E. L., Debrauwer, L., Puel, O. & Oswald, I. P. From
       genomics to metabolomics, moving toward an integrated strategy for the
       discovery of fungal secondary metabolites. *Nat. Prod. Rep.* **35**, 147–173
       (2018).

46.   Guijas, C. *et al.* METLIN: A Technology Platform for Identifying Knowns and
       Unknowns. *Anal. Chem.* **90**, 3156–3164 (2018).

47.   Aksenov, A. A., da Silva, R., Knight, R., Lopes, N. P. & Dorrestein, P. C.
       Global chemical analysis of biology by mass spectrometry. *Nat. Rev. Chem.*

1, 0054 (2017).

48. Graziani, V. *et al.* Metabolomic approach for a rapid identification of natural products with cytotoxic activity against human colorectal cancer cells. *Sci. Rep.* **8**, 5309 (2018).

49. Acharya, D. *et al.* Omics Technologies to Understand Activation of a Biosynthetic Gene Cluster in Micromonospora sp. WMMB235: Deciphering Keyicin Biosynthesis. *ACS Chem. Biol.* **14**, 1260–1270 (2019).

50. Earl, D. C. *et al.* Discovery of human cell selective effector molecules using single cell multiplexed activity metabolomics. *Nat. Commun.* **9**, 39 (2018).

51. Ziemert, N., Alanjary, M. & Weber, T. The evolution of genome mining in microbes - a review. *Nat. Prod. Rep.* **33**, 988–1005 (2016).

52. Viehrig, K. *et al.* Structure and Biosynthesis of Crocagins: Polycyclic Posttranslationally Modified Ribosomal Peptides from <i>Chondromyces crocatus<i/>. *Angew. Chem. Int. Ed. Engl.* **56**, 7407–7410 (2017).

53. Surup, F. *et al.* Crocadepsins-depsipeptides from the myxobacterium <i>Chondromyces crocatus<i/> found by a genome mining approach. *ACS Chem. Biol.* **13**, 267–272 (2018).

54. Hoffmann, T. *et al.* Correlating chemical diversity with taxonomic distance for discovery of natural products in myxobacteria. *Nat. Commun.* **9**, 803 (2018).

55. Helaly, S. E., Thongbai, B. & Stadler, M. Diversity of biologically active secondary metabolites from endophytic and saprotrophic fungi of the ascomycete order Xylariales. *Nat. Prod. Rep.* (2018). doi:10.1039/C8NP00010G

56. Zerikly, M. & Challis, G. L. Strategies for the Discovery of New Natural Products by Genome Mining. *ChemBioChem* **10**, 625–633 (2009).

57. Zhang, H., Boghigian, B. A., Armando, J. & Pfeifer, B. A. Methods and options for the heterologous production of complex natural products. *Nat. Prod. Rep.* **28**, 125–51 (2011).

58. Anyaogu, D. C. & Mortensen, U. H. Heterologous production of fungal secondary metabolites in *Aspergilli*. *Front. Microbiol.* **6**, 77 (2015).

59. Sucipto, H., Pogorevc, D., Luxenburger, E., Wenzel, S. C. & Müller, R. Heterologous production of myxobacterial α-pyrone antibiotics in *Myxococcus xanthus*. *Metab. Eng.* **44**, 160–170 (2017).

60. Nora, L. C. *et al.* The art of vector engineering: towards the construction of next-generation genetic tools. *Microb. Biotechnol.* **12**, 125–147 (2019).

61. Bok, J. W. *et al.* Fungal artificial chromosomes for mining of the fungal secondary metabolome. *BMC Genomics* **16**, 343 (2015).

62. Clevenger, K. D. *et al.* A scalable platform to identify fungal secondary metabolites and their gene clusters. *Nat. Chem. Biol.* **13**, 895–901 (2017).

63. Mustafa, N. R., de Winter, W., van Iren, F. & Verpoorte, R. Initiation, growth and cryopreservation of plant cell suspension cultures. *Nat. Protoc.* **6**, 715–742 (2011).

64. Mao, D., Okada, B. K., Wu, Y., Xu, F. & Seyedsayamdost, M. R. Recent advances in activating silent biosynthetic gene clusters in bacteria. *Curr. Opin. Microbiol.* **45**, 156–163 (2018).

65. Rutledge, P. J. & Challis, G. L. Discovery of microbial natural products by activation of silent biosynthetic gene clusters. *Nat. Rev. Microbiol.* **13**, 509–523 (2015).

66. Bian, X., Plaza, A., Zhang, Y. & Müller, R. Luminmycins A-C, cryptic natural products from *Photorhabdus luminescens* identified by heterologous expression in *Escherichia coli*. *J. Nat. Prod.* **75**, 1652–5 (2012).

67. Yamanaka, K. *et al.* Direct cloning and refactoring of a silent lipopeptide biosynthetic gene cluster yields the antibiotic taromycin A. *Proc. Natl. Acad. Sci.* **111**, 1957–1962 (2014).

68. Sidda, J. D. *et al.* Discovery of a family of γ-aminobutyrate ureas via rational derepression of a silent bacterial gene cluster. *Chem. Sci.* **5**, 86–89 (2014).

69. Laureti, L. *et al.* Identification of a bioactive 51-membered macrolide complex by activation of a silent polyketide synthase in Streptomyces ambofaciens. *Proc. Natl. Acad. Sci. U. S. A.* **108**, 6258–63 (2011).

70. Wang, B., Guo, F., Dong, S.-H. & Zhao, H. Activation of silent biosynthetic gene clusters using transcription factor decoys. *Nat. Chem. Biol.* **15**, 111–114 (2019).

71. Zhang, M. M. *et al.* CRISPR–Cas9 strategy for activation of silent Streptomyces biosynthetic gene clusters. *Nat. Chem. Biol.* **13**, 607–609 (2017).

72. Hover, B. M. *et al.* Culture-independent discovery of the malacidins as calcium-dependent antibiotics with activity against multidrug-resistant Gram-positive pathogens. *Nat. Microbiol.* **3**, 415–422 (2018).

73. Chu, J. *et al.* Discovery of MRSA active antibiotics using primary sequence from the human microbiome. *Nat. Chem. Biol.* **12**, 1004–1006 (2016).

74. Kersten, R. D. & Weng, J.-K. Gene-guided discovery and engineering of branched cyclic peptides in plants. *Proc. Natl. Acad. Sci. U. S. A.* **115**, E10961–E10969 (2018).

75. Dutertre, S. *et al.* Deep venomics reveals the mechanism for expanded peptide diversity in cone snail venom. *Mol. Cell. Proteomics* **12**, 312–29 (2013).

76. Mori, T. *et al.* Single-bacterial genomics validates rich and varied specialized metabolism of uncultivated *Entotheonella* sponge symbionts. *Proc. Natl. Acad. Sci.* **115**, 1718–1723 (2018).

77. Rath, C. M. *et al.* Meta-omic Characterization of the Marine Invertebrate Microbial Consortium That Produces the Chemotherapeutic Natural Product ET-743. *ACS Chem. Biol.* **6**, 1244–1256 (2011).

78. Newman, D. J. Are Microbial Endophytes the 'Actual' Producers of Bioactive Antitumor Agents? *Trends in Cancer* **4**, 662–670 (2018).

79. Helfrich, E. J. N. *et al.* Bipartite interactions, antibiotic production and biosynthetic potential of the Arabidopsis leaf microbiome. *Nat. Microbiol.* **3**,

909–919 (2018).

80. Cully, M. Deal watch: Roche taps potential antibiotics mine with Warp Drive Bio. *Nat. Rev. Drug Discov.* **17**, 8–9 (2017).

81. Yan, F. *et al.* Biosynthesis and Heterologous Production of Vioprolides: Rational Biosynthetic Engineering and Unprecedented 4-Methylazetidinecarboxylic Acid Formation. *Angew. Chemie Int. Ed.* **57**, 8754–8759 (2018).

82. Tu, Q. *et al.* Genetic engineering and heterologous expression of the disorazol biosynthetic gene cluster <i>via<i/> Red/ET recombineering. *Sci. Rep.* **6**, 21066 (2016).

83. Song, C. *et al.* Enhanced Heterologous Spinosad Production from a 79-kb Synthetic Multioperon Assembly. *ACS Synth. Biol.* **8**, 137–147 (2019).

84. Méndez, C., González-Sabín, J., Morís, F. & Salas, J. A. Expanding the Chemical Diversity of the Antitumoral Compound Mithramycin by Combinatorial Biosynthesis and Biocatalysis: The Quest for Mithralogs with Improved Therapeutic Window. *Planta Med.* **81**, 1326–38 (2015).

85. Hindra *et al.* Genome Mining of *Streptomyces mobaraensis* DSM40847 as a Bleomycin Producer Providing a Biotechnology Platform To Engineer Designer Bleomycin Analogues. *Org. Lett.* **19**, 1386–1389 (2017).

86. Brautaset, T. *et al.* Improved antifungal polyene macrolides via engineering of the nystatin biosynthetic genes in Streptomyces noursei. *Chem. Biol.* **15**, 1198–206 (2008).

87. Preobrazhenskaya, M. N. *et al.* Synthesis and study of the antifungal activity of new mono- and disubstituted derivatives of a genetically engineered polyene antibiotic 28,29-didehydronystatin A1 (S44HP). *J. Antibiot. (Tokyo).* **63**, 55–64 (2010).

88. Tevyashova, A. N. *et al.* Structure-antifungal activity relationships of polyene antibiotics of the amphotericin B group. *Antimicrob. Agents Chemother.* **57**, 3815–22 (2013).

89. Lewis, K., Epstein, S., D'Onofrio, A. & Ling, L. L. Uncultured

microorganisms as a source of secondary metabolites. *J. Antibiot. (Tokyo).* **63**, 468–476 (2010).

90.   Bode, H. B., Bethe, B., Höfs, R. & Zeeck, A. Big Effects from Small Changes: Possible Ways to Explore Nature's Chemical Diversity. *ChemBioChem* **3**, 619 (2002).

91.   SCHIEWE, H.-J. & ZEECK, A. Cineromycins, .GAMMA.-Butyrolactones and Ansamycins by Analysis of the Secondary Metabolite Pattern Created by a Single Strain of Strepomycest. *J. Antibiot. (Tokyo).* **52**, 635–642 (1999).

92.   Zähner, H. Some Aspects of Antibiotics Research. *Angew. Chemie Int. Ed. English* **16**, 687–694 (1977).

93.   Newman, D. Screening and identification of novel biologically active natural compounds. *F1000Research* **6**, 783 (2017).

94.   Hussain, A. *et al.* Novel bioactive molecules from Lentzea violacea strain AS 08 using one strain-many compounds (OSMAC) approach. *Bioorg. Med. Chem. Lett.* **27**, 2579–2582 (2017).

95.   Hemphill, C. F. P. *et al.* OSMAC approach leads to new fusarielin metabolites from Fusarium tricinctum. *J. Antibiot. (Tokyo).* **70**, 726–732 (2017).

96.   Vartoukian, S. R., Palmer, R. M. & Wade, W. G. Strategies for culture of 'unculturable' bacteria. *FEMS Microbiol. Lett.* **309**, no-no (2010).

97.   Moussa, M. *et al.* Co-culture of the fungus *Fusarium tricinctum* with *Streptomyces lividans* induces production of cryptic naphthoquinone dimers. *RSC Adv.* **9**, 1491–1500 (2019).

98.   Abdel-Razek, A. S., Hamed, A., Frese, M., Sewald, N. & Shaaban, M. Penicisteroid C: New polyoxygenated steroid produced by co-culturing of Streptomyces piomogenus with Aspergillus niger. *Steroids* **138**, 21–25 (2018).

99.   Derewacz, D. K., Covington, B. C., McLean, J. A. & Bachmann, B. O. Mapping Microbial Response Metabolomes for Induced Natural Product Discovery. *ACS Chem. Biol.* **10**, 1998–2006 (2015).

100. D'Onofrio, A. *et al.* Siderophores from Neighboring Organisms Promote the Growth of Uncultured Bacteria. *Chem. Biol.* **17**, 254–264 (2010).

101. Nichols, D. *et al.* Use of ichip for high-throughput in situ cultivation of 'uncultivable' microbial species. *Appl. Environ. Microbiol.* **76**, 2445–50 (2010).

102. Ling, L. L. *et al.* A new antibiotic kills pathogens without detectable resistance. *Nature* **517**, 455–459 (2015).

103. Homma, T. *et al.* Dual Targeting of Cell Wall Precursors by Teixobactin Leads to Cell Lysis. *Antimicrob. Agents Chemother.* **60**, 6510–6517 (2016).

104. Pham, V. H. T. & Kim, J. Cultivation of unculturable soil bacteria. *Trends Biotechnol.* **30**, 475–484 (2012).

105. Zengler, K. *et al.* Cultivating the uncultured. *Proc. Natl. Acad. Sci.* **99**, 15681–15686 (2002).

106. Timmermans, M. L., Picott, K. J., Ucciferri, L. & Ross, A. C. Culturing marine bacteria from the genus *Pseudoalteromonas* on a cotton scaffold alters secondary metabolite production. *Microbiologyopen* e00724 (2018). doi:10.1002/mbo3.724

107. Ross, A. C., Gulland, L. E. S., Dorrestein, P. C. & Moore, B. S. Targeted Capture and Heterologous Expression of the *Pseudoalteromonas* Alterochromide Gene Cluster in *Escherichia coli* Represents a Promising Natural Product Exploratory Platform. *ACS Synth. Biol.* **4**, 414–420 (2015).

108. Vlachou, P. *et al.* Innovative Approach to Sustainable Marine Invertebrate Chemistry and a Scale-Up Technology for Open Marine Ecosystems. *Mar. Drugs* **16**, 152 (2018).

109. Zainal-Abidin, M. H., Hayyan, M., Hayyan, A. & Jayakumar, N. S. New horizons in the extraction of bioactive compounds using deep eutectic solvents: A review. *Anal. Chim. Acta* **979**, 1–23 (2017).

110. Dai, Y., van Spronsen, J., Witkamp, G.-J., Verpoorte, R. & Choi, Y. H. Ionic liquids and deep eutectic solvents in natural products research: mixtures of solids as extraction solvents. *J. Nat. Prod.* **76**, 2162–73 (2013).

111. Nemes, P. & Vertes, A. Ambient mass spectrometry for in vivo local analysis and in situ molecular tissue imaging. *TrAC Trends Anal. Chem.* **34**, 22–34 (2012).

112. Pasquini, C. Near infrared spectroscopy: A mature analytical technique with new perspectives – A review. *Anal. Chim. Acta* **1026**, 8–36 (2018).

113. Pye, C. R., Bertin, M. J., Lokey, R. S., Gerwick, W. H. & Linington, R. G. Retrospective analysis of natural products provides insights for future discovery trends. *Proc. Natl. Acad. Sci. U. S. A.* **114**, 5601–5606 (2017).

114. Chen, Y., de Bruyn Kops, C. & Kirchmair, J. Data Resources for the Computer-Guided Discovery of Bioactive Natural Products. *J. Chem. Inf. Model.* **57**, 2099–2111 (2017).

115. Kaserer, T., Schuster, D. & Rollinger, J. M. Chemoinformatics in Natural Product Research. in *Applied Chemoinformatics* 207–236 (Wiley-VCH Verlag GmbH & Co. KGaA, 2018). doi:10.1002/9783527806539.ch6c

116. Schneider, P. & Schneider, G. A Computational Method for Unveiling the Target Promiscuity of Pharmacologically Active Compounds. *Angew. Chem. Int. Ed. Engl.* **56**, 11520–11524 (2017).

117. Gong, J. *et al.* New steroids with a rearranged skeleton as (h)P300 inhibitors from the sponge Theonella swinhoei. *Org. Lett.* **16**, 2224–7 (2014).

118. Reker, D. *et al.* Revealing the macromolecular targets of complex natural products. *Nat. Chem.* 1072–1078 (2014). doi:10.1038/nchem.2095

119. Liu, L.-J. *et al.* Identification of a natural product-like STAT3 dimerization inhibitor by structure-based virtual screening. *Cell Death Dis.* **5**, e1293–e1293 (2014).

120. Yu, W. & MacKerell, A. D. Computer-Aided Drug Design Methods. in *Methods in Molecular Biology (Clifton, N.J.)* **1520**, 85–106 (2017).

121. Merk, D., Grisoni, F., Friedrich, L., Gelzinyte, E. & Schneider, G. Computer-Assisted Discovery of Retinoid X Receptor Modulating Natural Products and Isofunctional Mimetics. *J. Med. Chem.* **61**, 5442–5447 (2018).

122. Moffat, J. G., Vincent, F., Lee, J. A., Eder, J. & Prunotto, M. Opportunities and challenges in phenotypic drug discovery: an industry perspective. *Nat. Rev. Drug Discov.* **16**, 531–543 (2017).

123. Swinney, D. C. & Anthony, J. How were new medicines discovered? *Nat. Rev. Drug Discov.* **10**, 507–519 (2011).

124. Shi, Y., Inoue, H., Wu, J. C. & Yamanaka, S. Induced pluripotent stem cell technology: a decade of progress. *Nat. Rev. Drug Discov.* **16**, 115–130 (2017).

125. McNulty, J. *et al.* iPSC Neuronal Assay Identifies Amaryllidaceae Pharmacophore with Multiple Effects against Herpesvirus Infections. *ACS Med. Chem. Lett.* **7**, 46–50 (2016).

126. Fellmann, C., Gowen, B. G., Lin, P.-C., Doudna, J. A. & Corn, J. E. Cornerstones of CRISPR–Cas in drug discovery and therapy. *Nat. Rev. Drug Discov.* **16**, 89–100 (2017).

127. Premsrirut, P. *et al.* RNAi and CRISPR/Cas9 Based In Vivo Models for Drug Discovery. *Blood* **130**, (2017).

128. Esch, E. W., Bahinski, A. & Huh, D. Organs-on-chips at the frontiers of drug discovery. *Nat. Rev. Drug Discov.* **14**, 248–60 (2015).

129. Fang, Y. & Eglen, R. M. Three-Dimensional Cell Cultures in Drug Discovery and Development. *SLAS Discov. Adv. Life Sci. R&D* **22**, 456–472 (2017).

130. Gunasekara, D. B. *et al.* Development of Arrayed Colonic Organoids for Screening of Secretagogues Associated with Enterotoxins. *Anal. Chem.* **90**, 1941–1950 (2018).

131. Kurita, K. L., Glassey, E. & Linington, R. G. Integration of high-content screening and untargeted metabolomics for comprehensive functional annotation of natural product libraries. *Proc. Natl. Acad. Sci. U. S. A.* **112**, 11999–2004 (2015).

132. Schulze, C. J. *et al.* 'Function-first' lead discovery: mode of action profiling of natural product libraries using image-based screening. *Chem. Biol.* **20**, 285–95 (2013).

133. Wagner, B. K. The resurgence of phenotypic screening in drug discovery and development. *Expert Opin. Drug Discov.* **11**, 121–125 (2016).

134. Szabo, M. *et al.* Cell and small animal models for phenotypic drug discovery. *Drug Des. Devel. Ther.* **11**, 1957–1967 (2017).

135. Brillatz, T. *et al.* Zebrafish-based identification of the antiseizure nucleoside inosine from the marine diatom Skeletonema marinoi. *PLoS One* **13**, e0196195 (2018).

136. Hamamoto, H. *et al.* Lysocin E is a new antibiotic that targets menaquinone in the bacterial membrane. *Nat. Chem. Biol.* **11**, 127–33 (2015).

137. Ondeyka, J. G. *et al.* Discovery of Bacterial Fatty Acid Synthase Inhibitors from a *Phoma* Species as Antimicrobial Agents Using a New Antisense-Based Strategy ⊥. *J. Nat. Prod.* **69**, 377–380 (2006).

138. Eliasen, A. M., Chin, M. R., Axelrod, A. J., Abagyan, R. & Siegel, D. Cascade reactions leading to the mechanism of action of vinaxanthone and xanthofulvin, natural products that drive nerve repair. *Tetrahedron* **74**, 3238–3245 (2018).

139. Deans, R. M. *et al.* Parallel shRNA and CRISPR-Cas9 screens enable antiviral drug target identification. *Nat. Chem. Biol.* **12**, 361–366 (2016).

140. Ortíz-López, F. J. *et al.* Cyclic Colisporifungin and Linear Cavinafungins, Antifungal Lipopeptides Isolated from *Colispora cavincola*. *J. Nat. Prod.* **78**, 468–475 (2015).

141. Estoppey, D. *et al.* The Natural Product Cavinafungin Selectively Interferes with Zika and Dengue Virus Replication by Inhibition of the Host Signal Peptidase. *Cell Rep.* **19**, 451–460 (2017).

142. Chang, J., Kim, Y. & Kwon, H. J. Advances in identification and validation of protein targets of natural products without chemical modification. *Nat. Prod. Rep.* **33**, 719–30 (2016).

143. Schirle, M. & Jenkins, J. L. Identifying compound efficacy targets in phenotypic drug discovery. *Drug Discov. Today* **21**, 82–89 (2016).

144. Adhikari, J. & Fitzgerald, M. C. SILAC-Pulse Proteolysis: A Mass Spectrometry-Based Method for Discovery and Cross-Validation in Proteome-Wide Studies of Ligand Binding. *J. Am. Soc. Mass Spectrom.* **25**, 2073–2083 (2014).

145. Gregori-Puigjane, E. *et al.* Identifying mechanism-of-action targets for drugs and probes. *Proc. Natl. Acad. Sci.* **109**, 11178–11183 (2012).

146. Dal Piaz, F. *et al.* Drug Affinity Responsive Target Stability (DARTS) Identifies Laurifolioside as a New Clathrin Heavy Chain Modulator. *J. Nat. Prod.* **79**, 2681–2692 (2016).

147. Molina, D. M. *et al.* Monitoring Drug Target Engagement in Cells and Tissues Using the Cellular Thermal Shift Assay. *Science (80-.).* **341**, 84–87 (2013).

148. Vedadi, M. *et al.* Chemical screening methods to identify ligands that promote protein stability, protein crystallization, and structure determination. *Proc. Natl. Acad. Sci.* **103**, 15835–15840 (2006).

149. Savitski, M. M. *et al.* Tracking cancer drugs in living cells by thermal profiling of the proteome. *Science (80-.).* **346**, 1255784–1255784 (2014).

150. Lv, C. *et al.* The antitumor natural product tanshinone IIA inhibits protein kinase C and acts synergistically with 17-AAG. *Cell Death Dis.* **9**, 165 (2018).

151. Tang, X. *et al.* Identification of Thiotetronic Acid Antibiotic Biosynthetic Pathways by Target-directed Genome Mining. *ACS Chem. Biol.* **10**, 2841–2849 (2015).

152. Allen, N. From Vancomycin to Oritavancin: The Discovery and Development of a Novel Lipoglycopeptide Antibiotic. *Antiinfect. Agents Med. Chem.* **9**, 23–47 (2010).

153. Kulanthaivel, P. *et al.* Novel Lipoglycopeptides as Inhibitors of Bacterial Signal Peptidase I. *J. Biol. Chem.* **279**, 36250–36258 (2004).

154. HÖLTZEL, A. *et al.* Arylomycins A and B, New Biaryl-bridged Lipopeptide Antibiotics Produced by Streptomyces sp. Tue 6075. II. Structure

Elucidation. *J. Antibiot. (Tokyo).* **55**, 571–577 (2002).

155. Kozuma, S. *et al.* Screening and biological activities of pedopeptins, novel inhibitors of LPS produced by soil bacteria. *J. Antibiot. (Tokyo).* **67**, 237–242 (2014).

156. WHO. United Nations meeting on antimicrobial resistance. *Bull. World Health Organ.* **94**, 638–639 (2016).

157. Zipperer, A. *et al.* Human commensals producing a novel antibiotic impair pathogen colonization. *Nature* **535**, 511–516 (2016).

158. Santiago, M. *et al.* Genome-wide mutant profiling predicts the mechanism of a Lipid II binding antibiotic. *Nat. Chem. Biol.* **14**, 601–608 (2018).

159. Cociancich, S. *et al.* The gyrase inhibitor albicidin consists of p-aminobenzoic acids and cyanoalanine. *Nat. Chem. Biol.* **11**, 195–7 (2015).

160. Kretz, J. *et al.* Total synthesis of albicidin: a lead structure from Xanthomonas albilineans for potent antibacterial gyrase inhibitors. *Angew. Chem. Int. Ed. Engl.* **54**, 1969–73 (2015).

161. Baumann, S. *et al.* Cystobactamids: myxobacterial topoisomerase inhibitors exhibiting potent antibacterial activity. *Angew. Chem. Int. Ed. Engl.* **53**, 14605–9 (2014).

162. Hüttel, S. *et al.* Discovery and Total Synthesis of Natural Cystobactamid Derivatives with Superior Activity against Gram-Negative Pathogens. *Angew. Chem. Int. Ed. Engl.* **56**, 12760–12764 (2017).

163. Richter, M. F. *et al.* Predictive compound accumulation rules yield a broad-spectrum antibiotic. *Nature* **545**, 299–304 (2017).

164. Lešnik, U. *et al.* Construction of a new class of tetracycline lead structures with potent antibacterial activity through biosynthetic engineering. *Angew. Chem. Int. Ed. Engl.* **54**, 3937–40 (2015).

165. Kling, A. *et al.* Antibiotics. Targeting DnaN for tuberculosis therapy using novel griselimycins. *Science* **348**, 1106–12 (2015).

166. Shaeer, K. M., Zmarlicka, M. T., Chahine, E. B., Piccicacco, N. & Cho, J. C.

Plazomicin: A Next-Generation Aminoglycoside. *Pharmacother. J. Hum. Pharmacol. Drug Ther.* **39**, 77–93 (2019).

167. Endimiani, A. *et al.* ACHN-490, a Neoglycoside with Potent In Vitro Activity Against Multidrug-Resistant Klebsiella pneumoniae Isolates. *Antimicrob. Agents Chemother.* **53**, 4504–7 (2009).

168. McKinnell, J. A. *et al.* Plazomicin for Infections Caused by Carbapenem-Resistant Enterobacteriaceae. *N. Engl. J. Med.* **380**, 791–793 (2019).

169. Wagenlehner, F. M. E. *et al.* Once-Daily Plazomicin for Complicated Urinary Tract Infections. *N. Engl. J. Med.* **380**, 729–740 (2019).

170. Silva, L. N., Zimmer, K. R., Macedo, A. J. & Trentin, D. S. Plant Natural Products Targeting Bacterial Virulence Factors. *Chem. Rev.* **116**, 9162–9236 (2016).

171. Tay, S. B. & Yew, W. S. Development of quorum-based anti-virulence therapeutics targeting Gram-negative bacterial pathogens. *Int. J. Mol. Sci.* **14**, 16570–99 (2013).

172. Danquah, C. A. *et al.* Analogues of Disulfides from Allium stipitatum Demonstrate Potent Anti-tubercular Activities through Drug Efflux Pump and Biofilm Inhibition. *Sci. Rep.* **8**, 1150 (2018).

173. Park, S. R. *et al.* Discovery of cahuitamycins as biofilm inhibitors derived from a convergent biosynthetic pathway. *Nat. Commun.* **7**, 10710 (2016).

174. Mathur, H. *et al.* Fighting biofilms with lantibiotics and other groups of bacteriocins. *NPJ biofilms microbiomes* **4**, 9 (2018).

175. Evanno, L. *et al.* A Ring-Distortion Strategy from Marine Natural Product Ilimaquinone Leads to Quorum Sensing Modulators. *European J. Org. Chem.* **2018**, 2486–2497 (2018).

176. Dickey, S. W., Cheung, G. Y. C. & Otto, M. Different drugs for bad bugs: antivirulence strategies in the age of antibiotic resistance. *Nat. Rev. Drug Discov.* **16**, 457–471 (2017).

177. Denning, D. W. & Bromley, M. J. How to bolster the antifungal pipeline.

Science (80-.). **347**, 1414–1416 (2015).

178. Patil, A. & Majumdar, S. Echinocandins in antifungal pharmacotherapy. *J. Pharm. Pharmacol.* **69**, 1635–1660 (2017).

179. Ostrosky-Zeichner, L., Casadevall, A., Galgiani, J. N., Odds, F. C. & Rex, J. H. An insight into the antifungal pipeline: selected new molecules and beyond. *Nat. Rev. Drug Discov.* **9**, 719–727 (2010).

180. Dähn, U. *et al.* Stoffwechselprodukte von mikroorganismen. 154. Mitteilung. Nikkomycin, ein neuer hemmstoff der chitinsynthese bei pilzen. *Arch. Microbiol.* **107**, 143–60 (1976).

181. DELZER, J. *et al.* New nikkomycins by mutasynthesis and directed fermentation. *J. Antibiot. (Tokyo).* **37**, 80–82 (1984).

182. Schmidt, B. M., Ribnicky, D. M., Lipsky, P. E. & Raskin, I. Revisiting the ancient concept of botanical therapeutics. *Nat. Chem. Biol.* **3**, 360–366 (2007).

183. Schmidt, B. *et al.* A natural history of botanical therapeutics. *Metabolism* **57**, S3–S9 (2008).

184. Wang, M. *et al.* Metabolomics in the context of systems biology: bridging traditional Chinese medicine and molecular pharmacology. *Phytother. Res.* **19**, 173–82 (2005).

185. Junio, H. A. *et al.* Synergy-Directed Fractionation of Botanical Medicines: A Case Study with Goldenseal (*Hydrastis canadensis*). *J. Nat. Prod.* **74**, 1621–1629 (2011).

186. Tallarida, R. J. Drug synergism: its detection and applications. *J. Pharmacol. Exp. Ther.* **298**, 865–72 (2001).

187. Kurin, E. *et al.* Synergy study of the inhibitory potential of red wine polyphenols on vascular smooth muscle cell proliferation. *Planta Med.* **78**, 772–8 (2012).

188. Scalbert, A. & Williamson, G. Dietary Intake and Bioavailability of Polyphenols. *J. Nutr.* **130**, 2073S-2085S (2000).

189. Zampieri, M. *et al.* High-throughput metabolomic analysis predicts mode of action of uncharacterized antimicrobial compounds. *Sci. Transl. Med.* **10**, eaal3973 (2018).

190. Campos, A. I. & Zampieri, M. Metabolomics-Driven Exploration of the Chemical Drug Space to Predict Combination Antimicrobial Therapies. *Mol. Cell* **74**, 1291-1303.e6 (2019).

191. Hsu, S. Green tea and the skin. *J. Am. Acad. Dermatol.* **52**, 1049–1059 (2005).

192. Jones, K. Review of *Sangre de Drago* (*Croton lechleri*) - A South American Tree Sap in the Treatment of Diarrhea, Inflammation, Insect Bites, Viral Infections, and Wounds: Traditional Uses to Clinical Research. *J. Altern. Complement. Med.* **9**, 877–896 (2003).

193. Chen, S. T. *et al.* New therapies from old medicines. *Nat. Biotechnol.* **26**, 1077–1083 (2008).

194. Crutchley, R. D., Miller, J. & Garey, K. W. Crofelemer, a Novel Agent for Treatment of Secretory Diarrhea. *Ann. Pharmacother.* **44**, 878–884 (2010).

195. Newman, D. J. & Cragg, G. M. Natural products as sources of new drugs over the 30 years from 1981 to 2010. *Journal of Natural Products* **75**, (2012).

196. Rubin, R. The Path to the First FDA-Approved Cannabis-Derived Treatment and What Comes Next. *JAMA* **320**, 1227–1229 (2018).

197. Marchesi, J. R. *et al.* The gut microbiota and host health: a new clinical frontier. *Gut* **65**, 330–339 (2016).

198. Abdollahi-Roodsaz, S., Abramson, S. B. & Scher, J. U. The metabolic role of the gut microbiota in health and rheumatic disease: mechanisms and interventions. *Nat. Rev. Rheumatol.* **12**, 446–455 (2016).

199. Lynch, S. V. & Pedersen, O. The Human Intestinal Microbiome in Health and Disease. *N. Engl. J. Med.* **375**, 2369–2379 (2016).

200. Scherlach, K. & Hertweck, C. Mediators of mutualistic microbe-microbe

interactions. *Nat. Prod. Rep.* **35**, 303–308 (2018).

201. Modi, S. R., Collins, J. J. & Relman, D. A. Antibiotics and the gut microbiota. *J. Clin. Invest.* **124**, 4212–4218 (2014).

202. Peterson, C. T. *et al.* Effects of Turmeric and Curcumin Dietary Supplementation on Human Gut Microbiota: A Double-Blind, Randomized, Placebo-Controlled Pilot Study. *J. evidence-based Integr. Med.* **23**, 2515690X18790725 (2018).

203. Eid, H. M. *et al.* Significance of microbiota in obesity and metabolic diseases and the modulatory potential by medicinal plant and food ingredients. *Front. Pharmacol.* **8**, (2017).

204. Valencia, P. M., Richard, M., Brock, J. & Boglioli, E. The human microbiome: opportunity or hype? *Nat. Rev. Drug Discov.* **16**, 823–824 (2017).

205. Bailey, C. J. Metformin: historical overview. *Diabetologia* **60**, 1566–1576 (2017).

206. Pascale, A., Marchesi, N., Govoni, S., Coppola, A. & Gazzaruso, C. The role of gut microbiota in obesity, diabetes mellitus, and effect of metformin: new insights into old diseases. *Curr. Opin. Pharmacol.* **49**, 1–5 (2019).

207. Bahne, E. *et al.* Involvement of glucagon-like peptide-1 in the glucose-lowering effect of metformin. *Diabetes, Obes. Metab.* **18**, 955–961 (2016).

208. Wu, H. *et al.* Metformin alters the gut microbiome of individuals with treatment-naive type 2 diabetes, contributing to the therapeutic effects of the drug. *Nat. Med.* **23**, 850–858 (2017).

209. de la Cuesta-Zuluaga, J. *et al.* Metformin Is Associated With Higher Relative Abundance of Mucin-Degrading Akkermansia muciniphila and Several Short-Chain Fatty Acid-Producing Microbiota in the Gut. *Diabetes Care* **40**, 54–62 (2017).

210. Sun, L. *et al.* Gut microbiota and intestinal FXR mediate the clinical benefits of metformin. *Nat. Med.* **24**, 1919–1929 (2018).

211. Rodrigues, T. & Bernardes, G. J. L. Development of Antibody-Directed

Therapies: Quo Vadis? *Angew. Chem. Int. Ed. Engl.* **57**, 2032–2034 (2018).

212. Beck, A., Goetsch, L., Dumontet, C. & Corvaïa, N. Strategies and challenges for the next generation of antibody–drug conjugates. *Nat. Rev. Drug Discov.* **16**, 315–337 (2017).

213. Khan, N. *et al.* Expression of CD33 is a predictive factor for effect of gemtuzumab ozogamicin at different doses in adult acute myeloid leukaemia. *Leukemia* **31**, 1059–1068 (2017).

214. Galluzzi, L., Buqué, A., Kepp, O., Zitvogel, L. & Kroemer, G. Immunogenic cell death in cancer and infectious disease. *Nat. Rev. Immunol.* **17**, 97–111 (2017).

215. Diederich, M. Natural compound inducers of immunogenic cell death. *Arch. Pharm. Res.* (2019). doi:10.1007/s12272-019-01150-z

216. Radogna, F., Dicato, M. & Diederich, M. Natural modulators of the hallmarks of immunogenic cell death. *Biochem. Pharmacol.* **162**, 55–70 (2019).

217. Arcamone, F.-M. Fifty years of chemical research at Farmitalia. *Chemistry* **15**, 7774–91 (2009).

218. Fredly, H., Ersvær, E., Gjertsen, B.-T. & Bruserud, O. Immunogenic apoptosis in human acute myeloid leukemia (AML): primary human AML cells expose calreticulin and release heat shock protein (HSP) 70 and HSP90 during apoptosis. *Oncol. Rep.* **25**, 1549–56 (2011).

219. Adkins, I., Fucikova, J., Garg, A. D., Agostinis, P. & Špíšek, R. Physical modalities inducing immunogenic tumor cell death for cancer immunotherapy. *Oncoimmunology* **3**, e968434 (2014).

220. Kubin, A., Wierrani, F., Burner, U., Alth, G. & Grünberger, W. Hypericin-- the facts about a controversial agent. *Curr. Pharm. Des.* **11**, 233–53 (2005).

221. Davids, L. M., Kleemann, B., Kacerovská, D., Pizinger, K. & Kidson, S. H. Hypericin phototoxicity induces different modes of cell death in melanoma

and human skin cells. *J. Photochem. Photobiol. B.* **91**, 67–76 (2008).

222. Garg, A. D., Krysko, D. V., Vandenabeele, P. & Agostinis, P. Hypericin-based photodynamic therapy induces surface exposure of damage-associated molecular patterns like HSP70 and calreticulin. *Cancer Immunol. Immunother.* **61**, 215–221 (2012).

223. Schneider, N. F. Z., Cerella, C., Simões, C. M. O. & Diederich, M. Anticancer and Immunogenic Properties of Cardiac Glycosides. *Molecules* **22**, 1932 (2017).

224. Diederich, M., Muller, F. & Cerella, C. Cardiac glycosides: From molecular targets to immunogenic cell death. *Biochem. Pharmacol.* **125**, 1–11 (2017).

225. Slingerland, M., Cerella, C., Guchelaar, H. J., Diederich, M. & Gelderblom, H. Cardiac glycosides in cancer therapy: from preclinical investigations towards clinical trials. *Invest. New Drugs* **31**, 1087–94 (2013).

226. Radogna, F. *et al.* Cell type-dependent ROS and mitophagy response leads to apoptosis or necroptosis in neuroblastoma. *Oncogene* **35**, 3839–3853 (2016).

227. Menger, L. *et al.* Cardiac glycosides exert anticancer effects by inducing immunogenic cell death. *Sci. Transl. Med.* **4**, 143ra99 (2012).

228. Sukkurwala, A. Q. *et al.* Screening of novel immunogenic cell death inducers within the NCI Mechanistic Diversity Set. *Oncoimmunology* **3**, e28473 (2014).

229. Konstantinidou, M., Zarganes-Tzitzikas, T., Magiera-Mularz, K., Holak, T. A. & Dömling, A. Immune Checkpoint PD-1/PD-L1: Is There Life Beyond Antibodies? *Angew. Chemie Int. Ed.* **57**, 4840–4848 (2018).

230. Kerr, W. G. & Chisholm, J. D. The Next Generation of Immunotherapy for Cancer: Small Molecules Could Make Big Waves. *J. Immunol.* **202**, 11–19 (2019).

231. De Santo, C. *et al.* Nitroaspirin corrects immune dysfunction in tumor-bearing hosts and promotes tumor eradication by cancer vaccination. *Proc.*

Natl. Acad. Sci. U. S. A. **102**, 4185–90 (2005).

232. Mann, J. Natural products in cancer chemotherapy: past, present and future. *Nat. Rev. Cancer* **2**, 143–8 (2002).

233. Watkins, R., Wu, L., Zhang, C., Davis, R. M. & Xu, B. Natural product-based nanomedicine: recent advances and issues. *Int. J. Nanomedicine* **10**, 6055–74 (2015).

234. Chowdhury, M. R. *et al.* Ionic-Liquid-Based Paclitaxel Preparation: A New Potential Formulation for Cancer Treatment. *Mol. Pharm.* **15**, 2484–2488 (2018).

235. Butler, M. S., Robertson, A. A. B. & Cooper, M. A. Natural product and natural product derived drugs in clinical trials. *Nat. Prod. Rep.* **31**, 1612–61 (2014).

236. Li, F. *et al.* A water-soluble nucleolin aptamer-paclitaxel conjugate for tumor-specific targeting in ovarian cancer. *Nat. Commun.* **8**, 1390 (2017).

237. Wulff, H., Christophersen, P., Colussi, P., Chandy, K. G. & Yarov-Yarovoy, V. Antibodies and venom peptides: new modalities for ion channels. *Nat. Rev. Drug Discov.* (2019). doi:10.1038/s41573-019-0013-8

238. Boström, J., Brown, D. G., Young, R. J. & Keserü, G. M. Expanding the medicinal chemistry synthetic toolbox. *Nat. Rev. Drug Discov.* **17**, 709–727 (2018).

239. Dayalan Naidu, S., Kostov, R. V & Dinkova-Kostova, A. T. Transcription factors Hsf1 and Nrf2 engage in crosstalk for cytoprotection. *Trends Pharmacol. Sci.* **36**, 6–14 (2015).

240. Hayes, J. D. & Dinkova-Kostova, A. T. The Nrf2 regulatory network provides an interface between redox and intermediary metabolism. *Trends Biochem. Sci.* **39**, 199–218 (2014).

241. Mills, E. L. *et al.* Itaconate is an anti-inflammatory metabolite that activates Nrf2 via alkylation of KEAP1. *Nature* **556**, 113–117 (2018).

242. Cuadrado, A. *et al.* Therapeutic targeting of the NRF2 and KEAP1

partnership in chronic diseases. *Nat. Rev. Drug Discov.* **18**, 295–317 (2019).

243. Linker, R. A. *et al.* Fumaric acid esters exert neuroprotective effects in neuroinflammation via activation of the Nrf2 antioxidant pathway. *Brain* **134**, 678–92 (2011).

244. Singh, K. *et al.* Sulforaphane treatment of autism spectrum disorder (ASD). *Proc. Natl. Acad. Sci.* **111**, 15550–15555 (2014).

245. Spencer, S. R., Wilczak, C. A. & Talalay, P. Induction of glutathione transferases and NAD(P)H:quinone reductase by fumaric acid derivatives in rodent cells and tissues. *Cancer Res.* **50**, 7871–5 (1990).

246. Soušek, J. *et al.* Alkaloids and organic acids content of eightFumaria species. *Phytochem. Anal.* **10**, 6–11 (1999).

247. Linker, R. A. & Haghikia, A. Dimethyl fumarate in multiple sclerosis: latest developments, evidence and place in therapy. *Ther. Adv. Chronic Dis.* **7**, 198–207 (2016).

248. Schimrigk, S. *et al.* Oral fumaric acid esters for the treatment of active multiple sclerosis: an open-label, baseline-controlled pilot study. *Eur. J. Neurol.* **13**, 604–610 (2006).

249. Kappos, L. *et al.* Efficacy and safety of oral fumarate in patients with relapsing-remitting multiple sclerosis: a multicentre, randomised, double-blind, placebo-controlled phase IIb study. *Lancet* **372**, 1463–1472 (2008).

250. Fox, R. J. *et al.* Efficacy and Tolerability of Delayed-release Dimethyl Fumarate in Black, Hispanic, and Asian Patients with Relapsing-Remitting Multiple Sclerosis: Post Hoc Integrated Analysis of DEFINE and CONFIRM. *Neurol. Ther.* **6**, 175–187 (2017).

251. Fernández, Ó. *et al.* Efficacy and Safety of Delayed-release Dimethyl Fumarate for Relapsing-remitting Multiple Sclerosis in Prior Interferon Users: An Integrated Analysis of DEFINE and CONFIRM. *Clin. Ther.* **39**, 1671–1679 (2017).

252. Zhang, Y., Talalay, P., Cho, C. G. & Posner, G. H. A major inducer of

anticarcinogenic protective enzymes from broccoli: isolation and elucidation of structure. *Proc. Natl. Acad. Sci. U. S. A.* **89**, 2399–403 (1992).

253. Dinkova-Kostova, A. T. *et al.* Direct evidence that sulfhydryl groups of Keap1 are the sensors regulating induction of phase 2 enzymes that protect against carcinogens and oxidants. *Proc. Natl. Acad. Sci. U. S. A.* **99**, 11908–13 (2002).

254. Morroni, F. *et al.* Neuroprotective effect of sulforaphane in 6-hydroxydopamine-lesioned mouse model of Parkinson's disease. *Neurotoxicology* **36**, 63–71 (2013).

255. Liu, Y. *et al.* Sulforaphane enhances proteasomal and autophagic activities in mice and is a potential therapeutic reagent for Huntington's disease. *J. Neurochem.* **129**, 539–547 (2014).

256. Kim, H. V. *et al.* Amelioration of Alzheimer's disease by neuroprotective effect of sulforaphane in animal model. *Amyloid* **20**, 7–12 (2013).

257. Zhao, J., Moore, A. N., Clifton, G. L. & Dash, P. K. Sulforaphane enhances aquaporin-4 expression and decreases cerebral edema following traumatic brain injury. *J. Neurosci. Res.* **82**, 499–506 (2005).

258. Benedict, A. L. *et al.* Neuroprotective effects of sulforaphane after contusive spinal cord injury. *J. Neurotrauma* **29**, 2576–86 (2012).

259. Alfieri, A. *et al.* Sulforaphane preconditioning of the Nrf2/HO-1 defense pathway protects the cerebral vasculature against blood-brain barrier disruption and neurological deficits in stroke. *Free Radic. Biol. Med.* **65**, 1012–1022 (2013).

260. Wu, S. *et al.* Sulforaphane produces antidepressant- and anxiolytic-like effects in adult mice. *Behav. Brain Res.* **301**, 55–62 (2016).

261. Li, B. *et al.* Sulforaphane ameliorates the development of experimental autoimmune encephalomyelitis by antagonizing oxidative stress and Th17-related inflammation in mice. *Exp. Neurol.* **250**, 239–49 (2013).

262. Egner, P. A. *et al.* Rapid and sustainable detoxication of airborne pollutants by broccoli sprout beverage: results of a randomized clinical trial in China.

Cancer Prev. Res. (Phila). **7**, 813–823 (2014).

263. Dinkova-Kostova, A. T. *et al.* Extremely potent triterpenoid inducers of the phase 2 response: correlations of protection against oxidant and inflammatory stress. *Proc. Natl. Acad. Sci. U. S. A.* **102**, 4584–9 (2005).

264. Liby, K. T. & Sporn, M. B. Synthetic Oleanane Triterpenoids: Multifunctional Drugs with a Broad Range of Applications for Prevention and Treatment of Chronic Disease. *Pharmacol. Rev.* **64**, 972–1003 (2012).

265. Camarinha-Matos, L. M. & Afsarmanesh, H. Collaborative networks: a new scientific discipline. *J. Intell. Manuf.* **16**, 439–452 (2005).

266. *Virtual Organizations*. (Kluwer Academic Publishers, 2005). doi:10.1007/b102339

267. Tahtah, Y. *et al.* High-resolution PTP1B inhibition profiling combined with high-performance liquid chromatography-high-resolution mass spectrometry-solid-phase extraction-nuclear magnetic resonance spectroscopy: Proof-of-concept and antidiabetic constituents in crude extra. *Fitoterapia* **110**, 52–58 (2016).

268. Liu, R. *et al.* Metabolomics Strategy Using High Resolution Mass Spectrometry Reveals Novel Biomarkers and Pain-Relief Effect of Traditional Chinese Medicine Prescription Wu-Zhu-Yu Decoction Acting on Headache Modelling Rats. *Molecules* **22**, 2110 (2017).

269. Weber, W. & Fussenegger, M. The impact of synthetic biology on drug discovery. *Drug Discov. Today* **14**, 956–963 (2009).

270. Trosset, J.-Y. & Carbonell, P. Synthetic biology for pharmaceutical drug discovery. *Drug Des. Devel. Ther.* **9**, 6285 (2015).

271. Miettinen, K. *et al.* The seco-iridoid pathway from Catharanthus roseus. *Nat. Commun.* **5**, 3606 (2014).

272. Goodwin, S., McPherson, J. D. & McCombie, W. R. Coming of age: ten years of next-generation sequencing technologies. *Nat. Rev. Genet.* **17**, 333–51 (2016).

273. Martinez-Klimova, E. & Rodríguez-Peña, K. Endophytes as sources of antibiotics. *Biochem. Pharmacol.* **134**, 1–17 (2017).

274. Xie, L., Draizen, E. J. & Bourne, P. E. Harnessing Big Data for Systems Pharmacology. *Annu. Rev. Pharmacol. Toxicol.* **57**, 245–262 (2017).

275. Butler, K. T., Davies, D. W., Cartwright, H., Isayev, O. & Walsh, A. Machine learning for molecular and materials science. *Nature* **559**, 547–555 (2018).

276. Fleming, N. How artificial intelligence is changing drug discovery. *Nature* **557**, S55–S57 (2018).

277. Luechtefeld, T., Marsh, D., Rowlands, C. & Hartung, T. Machine Learning of Toxicological Big Data Enables Read-Across Structure Activity Relationships (RASAR) Outperforming Animal Test Reproducibility. *Toxicol. Sci.* **165**, 198–212 (2018).

278. Fakhrudin, N. *et al.* Computer-aided discovery, validation, and mechanistic characterization of novel neolignan activators of peroxisome proliferator-activated receptor gamma. *Mol. Pharmacol.* **77**, 559–66 (2010).

279. Koehbach, J. *et al.* Oxytocic plant cyclotides as templates for peptide G protein-coupled receptor ligand design. *Proc. Natl. Acad. Sci. U. S. A.* **110**, 21183–8 (2013).

Text boxes

Box <mark>1</mark>. **Impact on complex diseases: NP activators of the KEAP1/NRF2 pathway**

An example of a pathway affected by diverse multi-targeting NPs is the KEAP1/NRF2 pathway. This pathway regulates the expression of networks of genes encoding proteins with versatile cytoprotective functions, and has essential roles in the maintenance of redox and protein homeostasis, and the resolution of inflammation[239,240,241].

Activation of this pathway can protect against damage by most types of oxidants and pro-inflammatory agents, and it restores redox and protein homeostasis[242]. The pathway has therefore attracted attention for the development of drugs for the prevention and treatment of complex diseases, including neurological conditions such as relapsing-remitting multiple sclerosis[243] and autism spectrum disorder[244].

Dimethyl fumarate, the methyl ester of the NP fumarate <mark>(tricarboxylic acid (TCA) cycle intermediate)</mark>, which is found in both animals and plants (*e.g.*, in *Fumaria officinalis*. and *Capsella bursa-pastoris*), is one of the earliest discovered inducers of the KEAP1/NRF2 pathway[245,246]. <mark>The origins of the development of the fumarate ester, dimethyl fumarate (DMF) as a drug date back to the use in traditional medicine of the plant *Fumaria officinalis*, from which fumaric acid derived its name. Initially, fumaric acid derivatives were used for the treatment of psoriasis as it was thought that psoriasis is caused by a metabolic deficiency in the TCA cycle, which could be compensated for by repletion of fumarate[247]. Despite this erroneous assumption, DMF is effective in treating psoriasis, both topically and orally, and is the active principle of Fumaderm® for the treatment of moderate to severe psoriasis.</mark> Dimethyl fumarate has been used clinically for the treatment of plaque psoriasis for several decades in Germany, and more recently <mark>based on the knowledge of the immunopathology of psoriasis and the safety profile of fumaric acid esters, it has been tested in other immunological conditions.</mark> The initial

repurposing development of dimethyl fumarate first demonstrated effectiveness of oral fumaric acid esters (Fumaderm)[248] and dimethyl fumarate (BG00012)[249] formulations in pilot clinical trials. Then, murine models of chronic multiple sclerosis (myelin oligodendrocyte glycoprotein induced experimental autoimmune encephalomyelitis) showed that the neuroprotective effect of fumaric acid esters in neuroinflammation is mediated through activation of the KEAP1/NRF2 pathway[243]. Dimethyl fumarate has also demonstrated a favorable bioactivity profile in later larger phase III trials[250,251] and has rapidly became a very successful drug for relapsing-remitting multiple sclerosis, approved for the treatment of this disease by the US FDA and EMA in 2013. This astonishingly simple molecule, now marketed as Tecfidera®, has a net present value of $5.6bn and its producer Biogen a market capitalisation of $40.6bn (https://www.evaluate.com/node/14519/pdf).

The isothiocyanate sulforaphane, isolated from broccoli (*Brassica oleracea*)[252], is among the most potent naturally-occurring inducers of the KEAP1/NRF2 pathway[253], and has protective effects in animal models of Parkinson's[254], Huntington's[255] and Alzheimer's[256] diseases, traumatic brain injury[257], spinal cord contusion injury[258], stroke[259], depression[260], and multiple sclerosis[261]. Sulforaphane-rich broccoli extract preparations are being developed as preventive intervention in areas of the world with unavoidable exposures to environmental pollutants, such as China; the initial results of a randomized clinical trial showed rapid and sustained, statistically significant increases in the levels of excretion of the glutathione-derived conjugates of benzene and acrolein[262] and are currently being extended in a follow-up dose-response trial (NCT02656420). In a placebo-controlled, double-blind, randomized clinical trial in young individuals (aged 13-27) with autism spectrum disorder, sulforaphane reversed many of the clinical abnormalities[244]; these encouraging findings led to an ongoing clinical trial in children (aged 3-12) (NCT02561481). An alpha-cyclodextrin complex of sulforaphane known as SFX-01 (developed by Evgen Pharma), is also currently undergoing clinical trials in patients with breast cancer (NCT02055716, NCT02970682) and subarachnoid haemorrhage (NCT02614742).

Finally, the pentacyclic triterpenoids bardoxolone methyl (known also as RTA 402) and omaveloxolone (RTA 408), which semi-synthetic derivatives of the NP oleanolic acid, are the most potent (active at nanomolar concentrations) activators of the

KEAP1/NRF2 pathway known to date[263]. These compounds have shown protective effects in numerous animal models of chronic disease[264], and are currently in clinical trials for a wide range of indications, such as chronic kidney disease in type 2 diabetes, pulmonary arterial hypertension, melanoma, radiation dermatitis, ocular inflammation, and Friedreich's ataxia[242].

Dimethyl fumarate   Sulforaphane   Bardoxolone methyl (RTA 402)   Omaveloxolone (RTA 408)

Advancements in internet technology have also facilitated the formation of virtual organizations, virtual enterprises, and collaborative virtual laboratories[265,266]. Virtual consortia represent national and international collaborative networks that do not have single base but coordinate most of their activities online. An example with a strong focus on NP-based drug discovery is the International Natural Product Sciences Taskforce (INPST) that we have recently established (see **INPST** in Related links). The INPST provides a platform for synergistic integration of expertise, technology and materials provided by the participating academic and industrial entities.
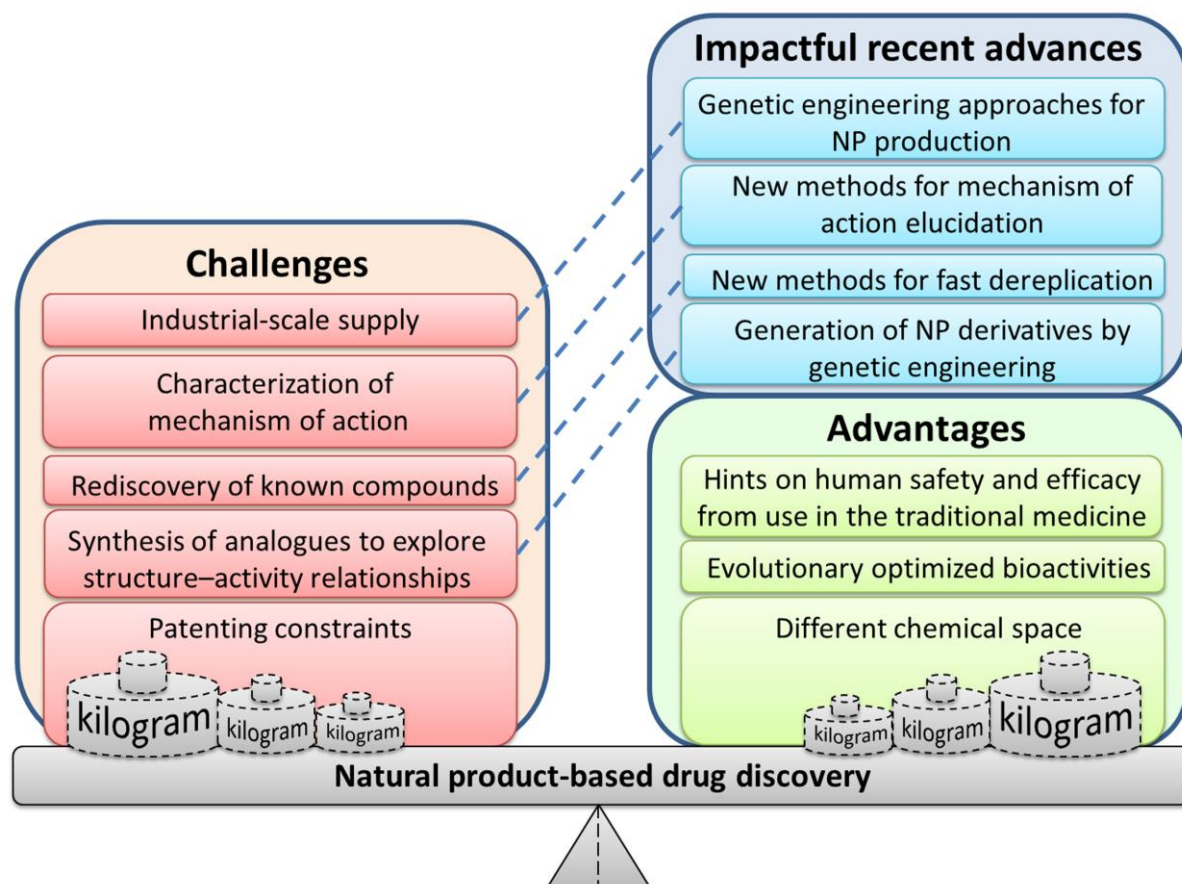
Figures

Figure 1: Challenges, advantages, and impactful recent advances associated with NP-based drug discovery. Advantages, challenges, and impactful recent advances associated with NP-based drug discovery are presented in green, red, and blue boxes, respectively. Dashed lines connect some of the challenges with the recent advances that help to address them, which are discussed in the main text. A continuous challenge for NP-based drug discovery remains patenting: while there are differences in legal frameworks among countries, unmodified NPs overall cannot be patented. A traditional approach to circumvent this limitation is the patenting of NP derivatives that are consciously developed to optimize properties of unmodified NP leads.
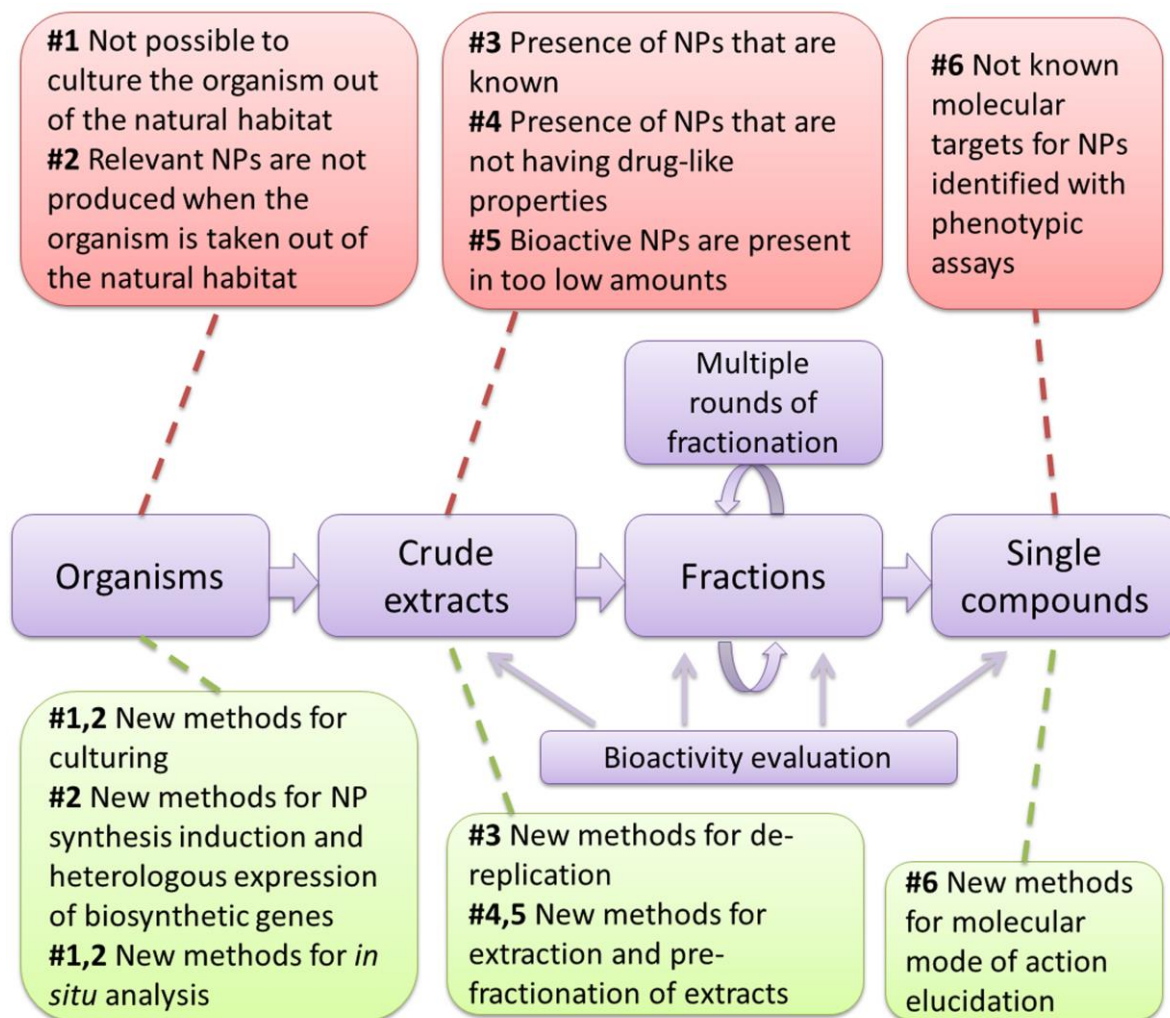
Figure 2: Outline of traditional bioactivity-guided isolation steps in natural product drug discovery. Steps in the process are shown in purple boxes, with associated key limitations shown in red boxes and advances that are helping to address these limitations in modern NP-based drug discovery shown in green boxes. Numbers in bold are used to match up limitations and advances to address them. The process begins with extraction of NPs from organisms such as bacteria. The choice of extraction method determines which compound classes will be present in the extract (for example, the use of more polar solvents will result in a higher abundance of polar compounds in the crude extract). To maximize the

diversity of the extracted NPs, the biological material can be subjected to extraction with several solvents of different polarity. Upon the identification of a crude extract with promising pharmacological activity, the next step is its (often multiple) consecutive bioactivity-guided fractionation until the pure bioactive compound(s) are isolated. A key limitation for the potential of this approach to identify novel NPs is that many potential source organisms cannot be cultured or stop producing relevant NPs when taken out of their natural habitat. These limitations are being addressed through development of new methods for culturing, for *in situ* analysis, for NP synthesis induction, and for heterologous expression of biosynthetic genes. At the crude extracts step, challenges include the presence in the extracts of NPs that are already known, NPs that do not have drug-like properties or insufficient amounts of NPs for characterization. These challenges can be addressed through the development of methods for de-replication, extraction and pre-fractionation of extracts. Finally, at the last stage when bioactive compounds are identified by phenotypic assays, significant time and effort are typically needed to identify the affected molecular targets. This challenge can be addressed by the development of methods for accelerated elucidation of molecular modes of action.
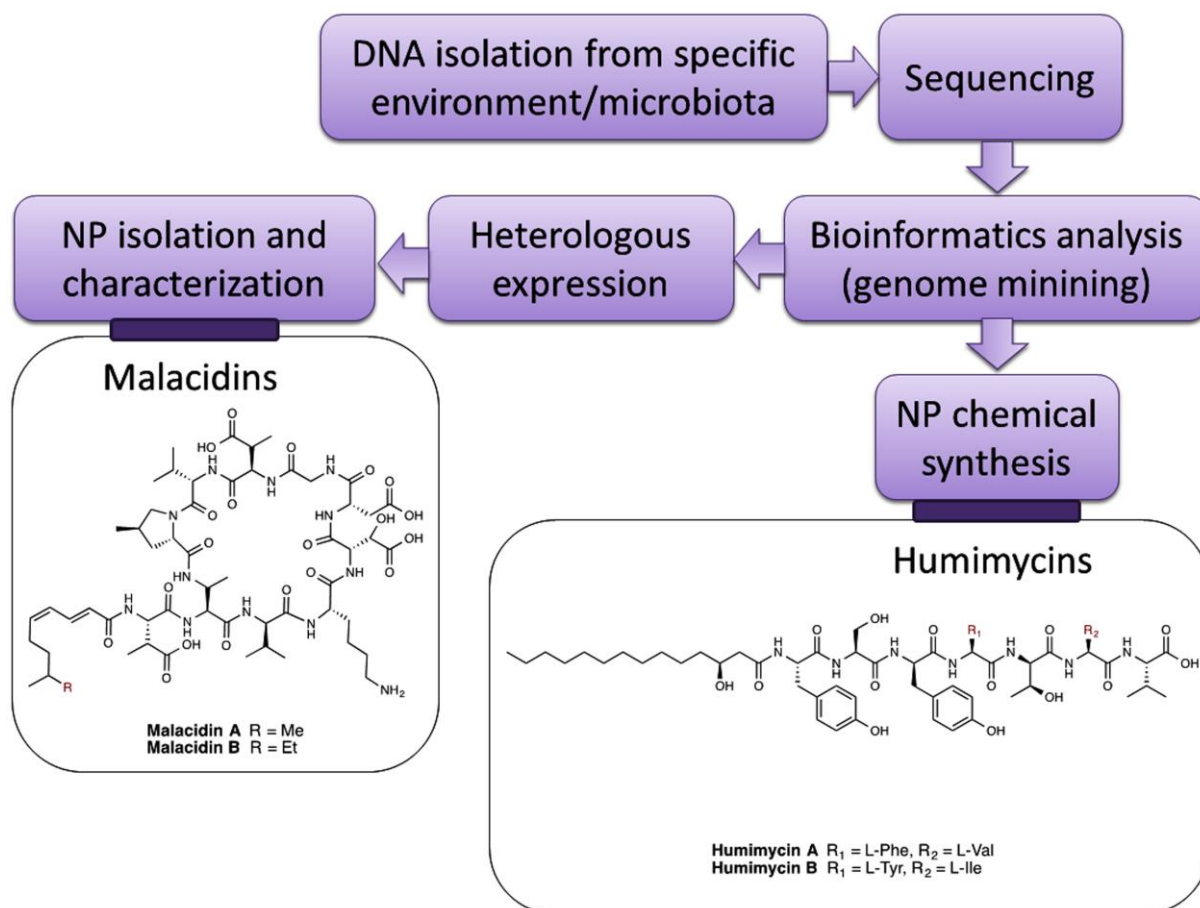
Figure 3: Strategies for genome mining-driven culture-independent discovery of NPs/NP-like compounds from environment/microbiota samples. The vast majority of microbes from different environments and microbiota communities have not been cultured, and their NP-producing capacity was largely inaccessible until recently. Two different genome mining-based approaches to explore the biosynthetic capacity of such uncultured microorganisms now rely on DNA extraction, sequencing, bioinformatics analysis and consequently targeted heterologous expression of biosynthetic gene clusters prioritized as being likely to yield relevant new NPs or alternatively direct chemical synthesis of "synthetic-bioinformatic" NP-like compounds. These two approaches are exemplified by the recent discoveries of malacidins and humimycins, respectively. Malacidins represent a new class of antibiotics with activity against multidrug-resistant Gram-positive pathogens, which were discovered based on bioinformatic analysis of the metagenomes of 2000 soil samples in the search of biosynthetic gene clusters encoding for calcium-binding motifs, and following heterologous expression of a biosynthetic gene cluster from a specific desert soil sample

Tables

Table 1: Recent technological and scientific developments with impact on NP-based drug discovery and development.

Category	Important recent developments	Significance for NP-based drug discovery
Application of analytical and separation techniques in NP-based drug discovery	• Pre-fractionation strategies aiming enrichment with compounds that have physicochemical properties that are more suitable for automated liquid handling systems used in high-throughput screening equipment and/or more drug-like properties. • Mass spectrometry technology with increased resolution and accuracy, such as hybrid MS instruments (Q-TOF, IT-TOF, etc.), high-field FT-ICR-MS or orbitrap, as well as high and ultra-high field NMR instruments with increased sensitivity[21] for compound identification. • Hyphenation approaches allowing direct coupling of analytical chemistry	• Improved rate of identification of bioactive NPs[20]. • Structural characterization of bioactive NPs available in small amounts[23]. • Capacity for fast dereplication and metabolomic-guided isolation of biologically active compounds[41,48]. • Integral information for the action of compounds at the system level with the use of "omics" approaches (including the identification of biomarkers and possible off-target effects)[24,268]. • Aid in the NP "residual complexity" analysis[36]. • Possibility for simultaneous compound identification and bioactivity evaluation,

	techniques with biological activity determination (*e.g.,* coupling of liquid or thin-layer chromatography with bioassays) [267].	applicable for complex compound mixtures[23,267].
Genome mining and engineering	• Advances in the knowledge of metabolic pathways and in the tools and methods for genetic manipulation[60,61,71,269–271]. • Next-generation sequencing (NGS) technologies for transcriptomics, epigenomics and metagenomics[272].	• Identification of new NPs, facilitated by the study of the genes that are responsible for their production [52,53,73–75]. • No need for strain cultivation, possibility for metagenomics-based gene cluster identification and analysis, and direct NP production[72,73]. • Application of genetic engineering to upscale the production of NPs in the native producing organisms or in heterologous expression hosts[81–83]. • Generation of new NP derivatives by manipulation of genes responsible for their synthesis[84,85].
Advances in microbial culturing systems	• Development of innovative microbial culturing techniques, such as iChip (isolation chip)[89,101] or co-culturing using "helper"	• Cultivation of new microbial species by the use of innovative culturing approaches, and consequently the

	strains[96], reflecting advances in the knowledge of the importance of species interactions (*e.g.*, of plants with plant endophytes) for the production of many NPs[273]. • Advances in techniques for *in situ* extraction and chemistry studies (including analytical equipment miniaturization)[108,109,112].	production of novel NPs[102]. • Induction of the production of new NPs by modification of the culturing conditions (including optimization of the culture medium and co-culturing with "helper" strains)[94,95,97]. • Chemistry capture, extraction, and analysis directly in the native habitat[108].
Applications of cheminformatics in NP-based drug discovery	• Exponentially increasing available data with biological and pharmacological significance, including NP virtual libraries available for chemoinformatic studies[114,274]. • Low-cost computation availability and the development of superior machine learning methods[275,276]. • Bioinformatics-guided synthetic approaches[73]. • Predictive approaches that allow for data fusion read-across structure activity relationships (RASAR),	• Identification of NPs interacting with a relevant molecular target by virtual screening[119,121,278]. • Molecular target prediction for NPs by the application of inverse virtual screening[117,118]. • Computer-driven NP-based drug design[121]. • Exclusion from further testing due to predicted toxicity[277].

	which have been demonstrated to be 80-95% accurate across 9 health hazards, outperforming animal test reproducibility[277].	
Advances in phenotypic screening	• Advancement of 3D cell culture technologies, including organoids, spheroids, scaffolds, hydrogels, 3D bioprinting, and organs-on-chips[128–130]. • Advanced methods of gaining and differentiation of stem cells[124]. • Advanced cellular imaging technologies to evaluate the effects of compounds in screens[131,132]. • Development of CRISPR/CAS9 gene editing technology, and RNA interference (RNAi) technologies for gene silencing[126,127]. • Advanced techniques for elucidation of molecular targets of action of bioactive compounds identified by phenotypic models[25,142,143]. • Use of model organisms[134], including	• The possibility of conducting medium- to high-throughput screening for pharmacological effects of NPs in superior *in vitro* systems or in whole-animal models[125,130,131,135–137]. • Aid in the elucidation of the molecular mechanisms of action of NPs (including the possibility to identify novel therapeutic targets)[25,138,141,279].

	the nematode *Caenorhabditis elegans*, zebrafish (*Danio rerio*), brine shrimp (*Artemia salina*) and fruit fly (*Drosophila melanogaster*).	

Table 2 | **Advanced (phase III) NP/NP-derived antibiotics under development.** In order to focus attention on the most recent developments, presented in the table are recent (not completed yet or completed since 2016) trials Phase III, with NP or NP-derived antibiotics.

Name	NP lead of origin	Conditions (NCT number)	Sponsors/collaborators
Cefiderocol	Cephalosporin	Healthcare-associated pneumonia, bloodstream infections, hospital acquired pneumonia, complicated urinary tract infection, sepsis, ventilator associated pneumonia (NCT02714595); healthcare-associated pneumonia, hospital acquired pneumonia, ventilator associated pneumonia (NCT03032380)	Shionogi
Lefamulin	Pleuromutilin	Community acquired pneumonia (NCT02813694); community acquired pneumonia (NCT02559310)	Nabriva Therapeutics
Solithromycin	Erythromycin	Uncomplicated urogenital gonorrhea (NCT02210325); community-acquired bacterial pneumonia (NCT02210325)	Melinta Therapeutics, National Institute of Allergy and Infectious Diseases, Biomedical Advanced Research and Development Authority
SPR994	Carbapenem	Complicated urinary tract infection, acute pyelonephritis (NCT03788967)	Spero Therapeutics
Sulopenem	Carbapenem	Uncomplicated urinary tract infections (NCT03354598); complicated urinary tract infections (NCT03357614); intra abdominal infections (NCT03358576)	Iterum Therapeutics

Table 3 | **NP/NP-derived antifungals under clinical development.**

Name	NP lead of origin	Conditions (Study Phase: NCT number)	Sponsors/collaborators
Nikkomycin Z	NP, first-in-class	Coccidioidomycosis (Phase I /Phase II: NCT00614666)	University of Arizona, FDA Office of Orphan Products Development
Rezafungin	Echinocandin	Candidemia, mycoses, fungal infection, invasive candidiasis (Phase III: NCT03667690)	Cidara Therapeutics
SCY-078	Enfumafungin	Invasive candidiasis, candidemia (Phase III: NCT03363841); invasive candidiasis, mucocutaneous candidiasis (Phase III: NCT03059992); candida vulvovaginitis (Phase III: NCT03734991)	Scynexis
VL-2397	NP, first-in-class	Invasive aspergillosis, invasive pulmonary aspergillosis (Phase II: NCT03327727)	Vical

Table 4 | **Botanical drugs in late-stage clinical development (Phase III trials that are currently in the participant recruiting stage).**

Name	Conditions (NCT number)	Sponsors/collaborators
Ashwagandaha root extract	Stress reaction, sleep disturbance, craving, obesity (NCT03112824)	Loma Linda University
Birch bark extract (Oleogel-S10)	Epidermolysis bullosa (NCT03068780)	Amryt Pharma
Cannabis extract	Drug resistant epilepsy (NCT03808935)	The Epilepsy Research Program of the Ontario Brain Institute, Ontario Brain Institute, University of Toronto, University Health Network Toronto, London Health Sciences Centre, MedReleaf
Cannabis extract (CannTrust CBD Oil)	Amyotrophic lateral sclerosis, motor neuron disease (NCT03690791)	Gold Coast Hospital and Health Service, CannTrust
Cannabis extract (Nabiximols)	Tourette syndrome, tic disorders (NCT03087201)	Hannover Medical School, German Research Foundation
Echinacea purpurea extract	Respiratory tract infections (NCT03812900)	Bioforce, Cantonal Hospital of St. Gallen, Labormedizinisches Zentrum Dr. Risch
Echinacea purpurea extract (Echinaforce)	Viral respiratory tract infection (NCT02971384)	Bioforce
French *Pinus pinaster* bark extract (Pycnogenol)	Attention deficit hyperactivity disorder (NCT02700685)	Nina Hermans, Universiteit Antwerpen
Mastic gum extract (RPh201)	Nonarteritic anterior ischemic optic neuropathy (NCT03547206)	Regenera Pharma
Mistletoe extract (abnobaVISCUM 900)	Superficial bladder cancer (NCT02106572)	Abnoba
Mistletoe extract (Iscador Qu)	Pancreatic cancer (NCT02948309)	Karolinska University Hospital, University of Witten/Herdecke, Karolinska Institutet, Regional Cancer Centre Stockholm Gotland, Stiftelsen Konung Gustaf V:s Jubileumsfond för cancerforskning, Signe & Ane Gyllenbergs Stiftelse, Ekhagastiftelsen, Dagmar Ferbs Minnesfond, Cancerforskningsfonden i Norrland, Immunpathologisches Labor, University Tübingen, Prof. Dr. med. R. Klein
Muscadine grape skin extract (Muscadine Plus)	Adenocarcinoma of the prostate (NCT03535675)	Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins, Greater Washington Community Foundation
Trichilia catigua extract (LABCAT TCJUSS)	Depression (NCT02532660)	Laboratório Catarinense SA, Universidade Federal do Ceara, Financiadora de Estudos e Projetos
Withania somnifera extract (Ashwagandha, Sensoril)	Schizophrenia (NCT03437668)	Chengappa, K.N. Roy, MD, Stanley Medical Research Institute, University of Maryland, University of Pittsburgh