Natural products in drug discovery: advances and opportunities

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Abstract | Natural products and their structural analogues 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 their pursuit by the pharmaceutical industry from the 1990s onwards. In recent years, several technological and scientific developments — including improved analytical tools, genome mining and engineering strategies, and microbial culturing advances — are addressing such challenges and opening up new opportunities. Consequently, interest in natural products as drug leads is being revitalized, particularly for tackling antimicrobial resistance. Here, we summarize recent technological developments that are enabling natural product-based drug discovery, highlight selected applications and discuss key opportunities.

[H1] Introduction

Historically, natural products (NPs) have played a key role in drug discovery, especially for cancer and infectious diseases,[1,2] but also in other therapeutic areas, including cardiovascular diseases (statins) and multiple sclerosis (fingolimod)[3–5].

NPs offer special features in comparison to conventional synthetic molecules, which confer both advantages and challenges for the drug discovery process. NPs are characterized by an enormous scaffold diversity and structural complexity. They typically have a higher molecular mass, a larger number of sp^3 carbon atoms and oxygen atoms but fewer nitrogen and halogen 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] The increasing significance of drugs not conforming to this rule is illustrated by the increase in molecular

masses of approved oral drugs over the last 20 years[12]. NPs are structurally 'optimized' by evolution to serve particular biological functions[1], including the regulation of endogenous defence mechanisms and the interaction (often competition) with other organisms, which explains their high relevance for infectious diseases and cancer. Furthermore, their use in traditional medicine may provide insights regarding efficacy and safety. Overall, the NP pool is enriched with 'bioactive' compounds covering a wider area of the chemical space compared with typical synthetic small-molecule libraries[13].

Despite these advantages and multiple successful drug discovery examples, several drawbacks of NPs have led pharmaceutical companies to reduce NP-based drug discovery programs. NP screens typically involve a library of extracts from natural sources (**Figure 1**), which may not be compatible with traditional target-based assays[14]. Identifying the bioactive compound(s) of interest can be challenging, and dereplication tools have to be applied to avoid re-discovery of known compounds. Accessing sufficient biological material to isolate and characterize a bioactive NP may also be challenging[15]. Furthermore, gaining intellectual property (IP) rights for (unmodified) NPs exhibiting relevant bioactivities can be a hurdle, since naturally occurring compounds in their original form may not always be patented (legal frameworks vary among countries and are evolving[16]), although simple derivatives can be patent-protected (see **Box 1** for examples). An additional layer of complexity relates to the 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 Nagoya Protocol, which entered into force in 2014[17], as well as recent developments concerning benefit sharing linked to utilization of marine genetic resources[18].

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 are difficult. Also, NP-based drug leads are often identified by phenotypic assays, and deconvolution of their molecular mechanisms of action can be time-consuming[19]. Fortunately, there have been substantial advances[20] both in the development of screening assays (for example, harnessing the potential of induced pluripotent stem cells

and gene editing technologies) and in strategies to identify the modes of action of active compounds (see REFS. [21–23] for reviews).

Here, we discuss recent technological and scientific advances that may help to overcome challenges in NP-based drug discovery, with an emphasis on three areas: analytical techniques, genome mining and engineering, and cultivation systems. In the concluding section, we highlight promising future directions for NP drug discovery.

[H1] Application of analytical techniques

Classical NP-based drug research starts with biological screening of 'crude' extracts to identify a bioactive 'hit' extract, which is further fractionated to isolate the active NP(s). 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**). For example, to create libraries that are compatible with high-throughput screening, crude extracts can be pre-fractionated into sub-fractions that are more suitable for automated liquid handling systems. In addition, fractionation methods can be adjusted such that sub-fractions preferentially contain compounds with drug-like properties (typically moderate hydrophilicity). Such approaches can increase the number of hits compared to using crude extracts, as well as enabling more efficient follow-up of promising hits[24].

Metabolomics was developed as an approach to simultaneously analyse multiple metabolites in biological samples. Enabled by technological developments in chromatography and spectrometry, metabolomics was historically applied first in other research fields, such as biomedical and agricultural sciences[2]. Advances in the analytical instrumentation used in NP research[25,26], coupled with computational approaches that can generate plausible NP analogue structures and their respective simulated spectra[27], has also enabled application of 'omics' approaches such as metabolomics in NP-based drug discovery. Metabolomics can provide accurate information on the metabolite composition in NP extracts, thus helping to prioritize NPs for isolation, to accelerate dereplication[28,29], and to annotate unknown analogues and new NP scaffolds. Moreover, metabolomics can

detect differences between metabolite compositions in various physiological states of producing organisms and enable the generation of hypotheses to explain them, and can also provide extensive metabolite profiles to underpin phenotypic characterization at the molecular level[30]. Both options are very useful in understanding the molecular mechanisms of action of NPs.

For metabolite profiling, NP extracts are analysed by nuclear magnetic resonance (NMR) spectroscopy or high-resolution mass spectrometry (HRMS), or respective hyphenated methods involving upstream liquid chromatography (LC)[31,32], such as LC-HRMS, which can separate numerous isomers present in NP extracts[33]. Moreover, such hyphenated methods might integrate both HRMS and NMR, allowing the simultaneous use of the advantages of both techiques[34,35]. NMR analysis of NP extracts is simple and reproducible, and provides direct quantitative information and detailed structural information, although it has relatively low sensitivity, meaning that it generally only enables profiling of major constituents[32]. The applications of NMR in NP research are versatile[37] and the technique is used both directly for metabolomics of unfractionated NP extracts as well as for structural characterization of compounds and fractions obtained with appropriate separation methods, most often LC. HRMS is the gold standard for qualitative and quantitative metabolite profiling[33], and is most commonly applied in combination with LC. HRMS can also be used in the direct infusion mode (DIMS)[38], where samples are directly profiled by MS without a chromatography step, or in MS imaging (MSI)[36], which enables determination of spatial distribution of NPs within living organisms. HRMS enables routine acquisition of accurate molecular mass information, which together with appropriate heuristic filtering, can provide unambiguous molecular formulae assignment for hundreds to thousands of metabolites within a single extract over a dynamic range that may exceed 5 orders of magnitude[31,39]. However, challenges remain in data mining and in the unambiguous identification of the metabolites using various workflows relying on open web-based tools[40].

Dereplication of secondary metabolites in bioactive extracts includes the determination of molecular mass and formula and cross-searching in the literature or structural NP databases with taxonomic information, which greatly assists the identification process. Such metadata, which are difficult to query in

the literature, are often compiled 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). However, a comprehensive experimental MS/MS database of all NPs reported to date does not exist and a search for experimental spectra across various platforms is hindered by the lack of standardised collision energy conditions for fragmentation in LC-MS/MS[25].

In this respect, the Global Natural Products Social (GNPS) molecular networking platform developed in the Dorrestein laboratory is an important addition to the toolbox[41]. The molecular networking organizes thousands of sets of MS/MS data recorded from a given set of extracts and visualizes the relationship of the analytes as clusters of structurally related molecules. This improves the efficiency of dereplication by enabling annotation of isomers and analogues of a given metabolite in a cluster[42]. The recorded experimental spectra can be searched against putative structures and their corresponding predicted MS/MS spectra generated by tools such as the competitive fragmentation modeling (CFM-ID)[43]. Based on such approaches, vast databases of theoretical NP spectra have been created and applied in dereplication[44]. The GNPS molecular networking approach has limitations, however, such as better applicability to some classes of NPs compared to others and the uncertainty of structural assignment among possible predicted candidates. Efforts to address such issues are ongoing[45,46,47], including overlaying molecular networks of large NP extract libraries with taxonomic information to improve the confidence of annotation[48]. Overall, molecular networking mainly allows better prioritization of the isolation of unknown compounds by strengthening the dereplication process and elucidating relationships between NP analogues, and rigorous structure elucidation for NPs of interest should not be neglected.

Another useful platform for metabolite identification is METLIN[49], which includes a high-resolution tandem mass spectrometry (MS/MS) database with a fragment similarity search function that is useful for identification of unknown compounds. Other databases and *in silico* tools such as Compound Structure Identification (CSI): FingerID and Input Output Kernel Regression (IOKR) can be used to search available fragment ion spectra, as well as generate predicted spectra of

fragment ions not present in current databases[50]. A novel computational platform for predicting the structural identity of metabolites derived from any identified compound has also been recently reported[51], which should increase the searchable chemical space of NPs.

To accelerate the identification of bioactive NPs in extracts, metabolomics data can be matched to the biological activities of these extracts[52]. Various chemometric methods such as multivariate data analysis can correlate the measured activity with signals in the NMR and MS spectra, enabling the active compound(s) to be traced in complex mixtures with no need for further bioassays[53,54,55]. Furthermore, several analytical modules involving different bioassays and detection technologies can be linked to allow simultaneous bioactivity evaluation and identification of compounds present in small amounts (analytical scale) in complex compound mixtures[34,35].

Metabolomics data can be integrated with data obtained by other 'omics' techniques such as transcriptomics and proteomics and/or with imaging-based screens. For example, Acharya *et al.* used this approach to characterize NP-mediated interactions between a *Micromonospora* species and a *Rhodococcus* species[56]. In another interesting example, Kurita *et al.* developed a compound activity mapping (CAM) platform for the prediction of identities and mechanisms of action of constituents from complex NP extract libraries by integrating cytological profiling[57] with untargeted metabolomics data from a library of extracts[58], and identified quinocinnolinomycins as a new family of NPs causing endoplasmic reticulum stress[58].

Analytical advances that enable the profiling of responses to bioactive molecules at the single-cell level can also accelerate NP-based drug discovery. Irish, Bachmann, and colleagues developed a high-throughput platform for metabolomic profiling of bioactivity by integrating phospho-specific flow cytometry, single-cell chemical biology and cellular barcoding with metabolomic arrays (characterized chromatographic microtiter arrays originating from biological extracts)[59]. Using this platform, the authors studied the single-cell responses of bone marrow biopsy samples from patients with acute myeloid leukemia following exposure to microbial metabolomic arrays

obtained from extracts of biosynthetically prolific bacteria, which enabled the identification of new bioactive polyketides[59].

Finally, advances in analytical technologies continue to support the rigorous structure determination of NPs of interest. The progressive development of higher-field NMR instruments and probe technology[60,61] has enabled NP structure determination from very small quantities (below 10 µg)[62,63], which is important as the available quantities of NPs are often limited. In addition, microcrystal electron diffraction (MicroED) has recently emerged as a cryo-electron microscopy-based technique for unambiguous structure determination of small molecules[64], and is already finding important applications in NP research[65]. The increased resolution and sensitivity of analytical equipment can also help address problems associated with "residual complexity" of isolated NPs; that is when biologically potent but unidentified impurities in an isolated NP sample (which could include structurally related metabolites or conformers) lead to an incorrect assignment of structure and/or activity[66,67]. To avoid futile downstream development efforts, Pauli et al. recommended that lead NPs should undergo advanced purity analysis at an early stage using quantitative NMR and LC–MS[67].

[H1] Genome mining and engineering

Advances in knowledge on biosynthetic pathways for NPs and in developing tools for analysing and manipulating genomes are further key drivers for modern NP-based drug discovery. Two key characteristics enable the identification of biosynthetic genes in the genomes of the producing organisms. First, these genes are clustered in the genomes of bacteria and filamentous fungi. Second, many NPs are based on polyketide or peptide cores, and their biosynthetic pathways involve enzymes — polyketide synthases and non-ribosomal peptide synthetases, respectively — that are encoded by large genes with highly conserved modules[68].

"Genome mining" is based on searches for genes that are likely to govern biosynthesis of scaffold structures, and can be used to identify NP biosynthetic gene clusters (for examples, see refs [69–71]). Prioritizing gene clusters for further work is facilitated by advances in biosynthetic knowledge and predictive

bioinformatics tools, which can provide hints about whether the metabolic products of the clusters have chemical scaffolds that are new or known, thereby supporting dereplication[72,73]. Such predictive tools for gene cluster analysis can be applied in combination with spectroscopic techniques to accelerate the identification of NPs[65] and determine the stereochemistry of metabolic products[66]. Furthermore, to extend genome mining from a single genome to entire genera, microbiomes or strain collections, computational tools have been developed, such as BiG-SCAPE, which enables sequence similarity analysis of biosynthetic gene clusters, and CORASON, which uses a phylogenomic approach to elucidate evolutionary relationships between gene clusters[76].

Phylogenetic studies of known groups of talented secondary metabolite producers can also empower discovery of novel NPs. Recently, a study comparing secondary metabolite profiles and phylogenetic data in myxobacteria demonstrated a correlation between the taxonomic distance and the production of distinct secondary metabolite families[77]. In filamentous fungi, it was likewise shown that secondary metabolite profiles are closely correlated to their phylogeny[78]. These organisms are rich in secondary metabolites, as demonstrated by LC-MS studies of their extracts under laboratory conditions[79]. Concurrent genomic and phylogenomic analyses implied that even the genomes of well-studied organism groups harbor many gene clusters for secondary metabolite biosynthesis with as yet unknown functions[80]. The phylogeny of biosynthetic gene clusters, together with analysis of the absence of known resistance determinants, was recently used to prioritize members of the glycopeptide antibiotic family that could have novel activities. This led to the identification of the known antibiotic complestatin and the newly discovered corbomycin as compounds that act through a previously uncharacterized mechanism involving inhibition of peptidoglycan remodelling[81].

Many microorganisms cannot be cultured or tools for their genetic manipulation are not sufficiently developed, which makes it more challenging to access their NP-producing potential. However, biosynthetic gene clusters for NPs can be cloned and heterologously expressed in organisms that are well-characterized and easier to culture and to genetically manipulate (such as *Streptomyces coelicolor*, *Escherichia coli* and *Saccharomyces cerevisiae*)[82]. The aim is to

achieve higher production titers in the heterologous hosts as compared to wild-type strains, improving the availability of lead compounds[82–84]. Vectors that can carry large DNA inserts are needed for the cloning of complete NP biosynthetic gene clusters. Cosmids (which can have inserts of 30–40 kb), fosmids (which can harbor 40–50 kb) and bacterial artificial chromosomes (BACs; which can have inserts of 100 kb to >300 kb) have been developed[85]. For fungal gene clusters, self-replicating fungal artificial chromosomes (FACs) have been developed, which can have inserts of >100 kb[86]. FACs in combination with metabolomic scoring were used to develop a scalable platform, FAC-MS, allowing the characterization of fungal biosynthetic gene clusters and their respective NPs at unprecedented scale[87]. The application of FAC-MS for the screening of 56 biosynthetic gene clusters from different fungal species yielded the discovery of 15 new metabolites, including a new macrolactone, valactamide A[87].

Even in culturable microorganisms, many biosynthetic gene clusters may not be expressed under conventional culture conditions, and these silent clusters could represent a large untapped source of NPs with drug-like properties[88]. Several approaches can be pursued to identify such NPs. One approach is sequencing, bioinformatic analysis and heterologous expression of silent biosynthetic gene clusters, which has already led to the discovery of several new NP scaffolds from cultivable strains[89]. Direct cloning and heterologous expression was also used to discover the new antibiotic taromycin A, which was identified upon the transfer of a silent 67 kb nonribosomal peptide synthetase biosynthetic gene cluster from *Saccharomonospora sp.* CNQ-490 into *Streptomyces coelicolor*[90]. To transfer a biosynthetic gene cluster of such size, a platform based on transformation-associated recombination (TAR) cloning was developed. This platform enables direct cloning and manipulation of large biosynthetic gene clusters in *Saccharomyces cerevisiae*, maintenance and manipulation of the vector in *E. coli*, and heterologous expression of the cloned gene clusters in actinobacteria (such as *Streptomyces coelicolor*) following chromosomal integration[90], and is an alternative to BACs for heterologous expression of large biosynthetic gene clusters.

Heterologous expression has limitations, such as the need to clone and manipulate very large genome regions occupied by biosynthetic gene clusters

and the difficulty of identifying a suitable host that provides all conditions necessary for the production of the corresponding NPs. These limitations can be circumvented by activating biosynthetic gene clusters directly in the native microorganism through targeted genetic manipulations, generally involving the insertion of activating regulatory elements or deletion of inhibitory elements such as repressors or their binding sites. For example, a de-repression strategy of deleting *gbnR*, a gene for transcriptional repressor in *Streptomyces venezuelae* ATCC 10712 was used by Sidda *et al.* in the discovery of gaburedins, a family of γ-aminobutyrate-derived ureas[91]. An example of the activator-based strategy is the constitutive expression of the *samR0484* gene in *Streptomyces ambofaciens* ATCC 23877, which led to the discovery of stambomycins A-D, 51-membered cytotoxic glycosylated macrolides[72]. Alternatively, silent biosynthetic gene clusters can be activated using repressor decoys[92], which have the same DNA nucleotide sequence as the binding sites for the repressors that prevent the expression of the clusters. When these decoys are introduced into the bacteria, they sequester the respective repressors and the "endogenous" binding sites in the genome remain unoccupied, leading to de-repression of the previously silent biosynthetic genes and production of the corresponding NPs. This approach has been applied to activate eight silent biosynthetic gene clusters in multiple streptomycetes, and led to the characterization of a novel NP, oxazolepoxidomycin A[92]. The repressor decoy strategy is simpler, easier, and faster to perform than the deletion of genes encoding regulatory factors. However, it has the same limitation as other approaches that rely on the introduction of recombinant DNA molecules in cells: it is necessary to develop protocols for efficient introduction of DNA into the targeted host strain, and the decoy must be maintained on a high-copy plasmid to ensure efficient repressor sequestration.

Another approach focused on exchange of regulatory elements is based on the CRISPR-Cas9 technology. The promise of this technique is exemplified in a recent work by Zhang *et al.*, which demonstrated that CRISPR-Cas9-mediated targeted promoter introduction can efficiently activate diverse biosynthetic gene clusters in multiple *Streptomyces* species, leading to the production of unique metabolites, including a novel polyketide in *Streptomyces viridochromogenes*[93]. The CRISPR-Cas9 technology was also used to knockout genes encoding two

well-known and frequently rediscovered antibiotics in several actinomycete strains, which led to the production of different rare and previously unknown variants of antibiotics that were otherwise obscured, including amicetin, thiolactomycin, phenanthroviridin, and 5-chloro-3-formylindole[94].

Approaches relying on sequencing, bioinformatics, and heterologous expression can also enable the identification of novel NPs from bacterial strains that have not yet been cultivated (**Figure 3**). For example, Hover *et al.* searched the metagenomes of 2,000 soil samples for biosynthetic gene clusters for lipopeptides with calcium-binding motifs. This led to the discovery of malacidins, members of the calcium-dependent antibiotic family, via heterologous expression of a 72-kb biosynthetic gene cluster from a desert soil sample in a *Streptomyces albus* host strain[95]. However, in comparison to some of the other above-discussed strategies[72,91,92], this metagenomic-based discovery approach is more suited to finding new members of known NP classes rather than discovery of entirely new classes. In another study, Chu *et al.* developed a human microbiome-based approach that identified nonribosomal linear heptapeptides called humimycins as novel antibiotics active against methicillin-resistant *Staphylococcus aureus* (MRSA)[96]. The structure of the NPs was predicted via bioinformatic analysis of gene clusters found in human commensal bacteria, followed by their chemical synthesis. A major strength of this innovative approach is that it is entirely independent of microbial cultivation and heterologous gene expression. Nevertheless, there are limitations related to the accuracy of computational chemical structure predictions and the feasibility of total chemical synthesis if structures are complex.

The genomes of plants or animals can also be mined for novel NPs. For example, mining of 116 plant genomes enabled by identification of a precursor gene for the biosynthesis of lyciumins, a class of branched cyclic ribosomal peptides with hypotensive action produced by *Lycium barbarum* (popularly known as goji), identified diverse novel lyciumin chemotypes in 7 other plants, including crops such as soybean, beet, quinoa, and eggplant[97]. Genome mining in the animal kingdom is exemplified by the work of Dutertre *et al.*, which used an integrated transcriptomics and proteomics approach to discover thousands of novel venom peptides from *Conus marmoreus* snails[98]. Proteomics analysis revealed that the

vast majority of the conopeptide diversity was derived from a set of ~100 genes through variable peptide processing[98].

Some bioactive compounds initially isolated from marine organisms might be products of symbionts, and genome mining can facilitate the characterization of such NPs. For example, it has been shown that bioactive compounds from the sponge *Theonella swinhoei* are produced by bacterial symbionts[99], and characterization of the symbiont "*Candidatus* Entotheonella serta" using single-cell genomics led to the discovery of gene clusters for misakinolide and theonellamide biosynthesis[100]. Another example of a marine NP produced by a bacterial symbiont is ET-743 (trabectedin), originally isolated from the tunicate *Ecteinascidia turbinate. A* meta-omics approach developed by Rath *et al.* revealed that the producer of this clinically used anticancer agent is the bacterial symbiont *Candidatus Endoecteinascidia frumentensis*[101].

Similarly, plant microbiomes also represent a large reservoir for the identification of novel bioactive NPs (such as the antitumor agents maytansine, taxol, and camptothecin, which were initially isolated from plants and later shown to be produced by microbial endophytes)[102] that can be tapped by genome mining approaches. An illustrative example is a recent work by Helfrich *et al.*, which identified hundreds of novel biosynthetic gene clusters by genome mining of 224 bacterial strains isolated from *Arabidopsis thaliana* leaves[103]. A combination of bioactivity screening and imaging mass spectrometry was used to select a single species for further genomic analysis and led to the isolation of a NP with an unprecedented structure, the *trans*-acyltransferase polyketide synthase-derived antibiotic macrobrevin[103].

Targeted genetic engineering of NP biosynthetic gene clusters can be of a high value, if the producing organism is difficult to cultivate or the yield of a NP is too low to allow comprehensive NP characterization. Rational genetic engineering and heterologous expression contributed to increase the production of vioprolides, a depsipeptide class of anticancer and antifungal NPs in the myxobacterium *Cystobacter violaceus* Cb vi35, by several orders of magnitude. In addition, non-natural vioprolide analogues were generated by this approach[104]. Similarly, promoter engineering and heterologous expression of biosynthetic gene clusters

was reported to result in a 7-fold increase in the production of the cytotoxic NP disorazol[105], and a 328-fold increase in the production of spinosad, an insecticidal macrolide produced by the bacterium *Saccharopolyspora spinosa*[106].

Besides increasing NP yields, targeted gene manipulation can also be used to alter biosynthetic pathways in a predictable manner to produce new NP analogues with improved pharmacological properties, such as higher specific activity, lower toxicity, and better pharmacokinetics. Such biosynthetic engineering approaches depend on a solid understanding of the biosynthetic pathway leading to a specific NP, access to the genes specifying this pathway, and the ability to manipulate them either in the original or a heterologous host. Recent advances in biosynthetic engineering have enabled faster and more efficient production of NP analogues, including the development of methods for accelerated engineering and recombination of modules of polyketide synthase (PKS) gene clusters[107], non-ribosomal peptide synthetases (NRPSs)[108,109], and NRPS-PKS assembly lines[110], as well as elucidation of mechanisms for polyketide chain release that are contributing to NP structural diversification[111,112]. Examples of biosynthetic engineering applied to several important NPs include the generation of analogues of the immunosuppressant rapamycin[113], the antitumor agents mithramycin[114] and bleomycin[115], and the antifungal nystatin[116].

It should be noted that biosynthetic engineering has limitations regarding the parts of the NP molecule that can be targeted for modifications, and the chemical groups that can be introduced or removed. Considering the complexity of many NPs, however, total synthesis may be prohibitively costly, and a combined approach of biosynthetic engineering and chemical modification can provide a viable alternative for identifying improved drug candidates. For example, biosynthetic engineering may create a "handle" for addition of a beneficial chemical group by synthetic chemistry, as demonstrated for the biosynthetically engineered analogues of nystatin mentioned above; further synthetic chemistry modifications resulted in compounds with improved *in vivo* pharmacotherapeutic characteristics compared to amphotericin B[117,118].

[H1] Advances in microbial culturing systems

The complex regulation of NP biosynthesis in response to the environment means that the conditions under which producing organisms are cultivated can have a major impact on the chance to identify novel NPs[89]. Several strategies have been developed to improve the likelihood of identifying novel NPs compared with monoculture under standard laboratory conditions and to make ”uncultured” microorganisms grow in a simulated natural environment (**Figure 4**)[119].

One well-established approach to promote the identification of novel NPs is the modulation of culture conditions such as temperature, pH and nutrient sources. This strategy may lead to activation of silent gene clusters, thereby promoting production of different NPs. The term “One Strain Many Compounds" (OSMAC) was coined for this approach about 20 years ago[120], but the concept has a longer history[121], with its use being routine in industrial microbiology since the 1960s[122].

While OSMAC is still widely used for the identification of new bioactive compounds (see REFS [123,124] for recent examples), this approach has limited capacity to mimic the complexities of the natural habitats. It is difficult to predict the combination of cues (which might also involve metabolites secreted by other members of the microbial community) to 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[125]. This can enable the production and identification of new NPs, as illustrated by recent studies in which particular fungi were co-cultured with *Streptomcyes* species[126,127].

Study of the molecular mechanisms 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 number of species that can be successfully cultured. This strategy was used by D'Onofrio *et al.* for the identification of new acyl-desferrioxamine siderophores (iron-chelating compounds) as growth factors produced by "helper" strains promoting the growth of previously uncultured isolates from marine sediment biofilm[119,128]. The siderophore-assisted growth is based on the property of these compounds to provide iron for microbes unable to autonomously produce siderophores themselves, and the application of this approach led to the isolation of previously uncultivated microorganisms[128]. The development of strategies to cultivate

microbial symbionts that produce NPs only upon interaction with their hosts can promote access to new NPs. Microbial symbionts interacting with insects or other organisms are a highly promising reservoir for the discovery of novel bioactive NPs produced in a unique ecological context [129–132]. To stimulate NP production, culturing strategies can be developed that better mimic the native environment of microbial symbionts of insects, including the use of media either containing lyophilized dead insects[133] or L-proline, a major constituent of insect hemolymph[134].

Strategies to mimic the natural environment even more closely by harnessing *in situ* incubation in the environment from which the microorganism is sampled have been developed, dating back to more than 20 years ago with the biotech companies OneCell and Diversa. They developed platforms that allowed the growth of some previously uncultivated microbes from different environments based on diluting out and suspension in a single drop of medium[122,135]. More recently, such strategies have been highlighted by the development and application of a platform dubbed the iChip, in which diluted soil samples are seeded in multiple small chambers separated from the environment with a semipermeable membrane[136]. After seeding, the iChip is placed back into the soil from which the sample was taken for an *in situ* incubation period, allowing the cultured microorganisms to be exposed to influences from their native environment. The power of this culturing approach was demonstrated by the discovery of a new antibiotic teixobactin produced by a previously uncultured soil bacterium[137,138]. This platform may be of a 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[139].

The "omics" strategies discussed in previous sections can complement efforts to explore NPs produced upon microbial interactions. The application of such strategy is illustrated in the work of Derewacz *et al.*, who analyzed the metabolome of a genome-sequenced *Nocardiopsis* bacterium upon co-culture with bacteria of the genera *Escherichia*, *Bacillus*, *Tsukamurella*, and *Rhodococcus*[140]. Around 14% of the metabolomic features found in co-cultures were undetectable in monocultures, with many of those being unique to specific co-culture genera, and the previously unreported polyketides ciromicin A and B,

which possess an unusual pyrrolidinol substructure and displayed moderate and selective cytotoxicity, were identified[140]. Other examples include a "culturomics" approach combining multiple culture conditions with MS profiling and 16S rRNA-based taxonomy to identify prokaryotic species from the human gut[141], and an ultrahigh-throughput screening platform based on microfluidic droplet single-cell encapsulation and cultivation followed by next-generation sequencing and LC-MS, which allows investigation of pairwise interactions between target microorganisms[142]. The latter approach enabled identification of a slow-growing oral microbiota species inhibiting the growth of *Staphylococcus aureus*[142].

Historically early-adopted microbial culturing approaches led to a bias reflected in the predominant discovery of NPs from microorganisms that are easy to cultivate (such as Streptomycetes and some common filamentous fungi). As a result, a vast number of NPs from such "easy to culture" microbes are already characterised, and conventional screening efforts tend to yield disappointing returns associated with frequent re-discovery of known NPs and their closely related congeners. Therefore, culturing strategies aimed at previously unexplored (or under-investigated) microbial groups, with the potential to produce NPs with entirely new scaffolds and bioactivities (such as *Burkholderia*, *Clostridium* and *Xenorhabdus*) are of high interest[143,144]. Closthioamide, the first secondary metabolite from a strictly anaerobic bacterium, was discovered from *Clostridium cellulolyticum* by this approach[145]. Targeted isolation of such species is important, and a genome-guided approach to achieve this goals has recently been demonstrated for *Burkholderia* strains in environmental samples[146]. Another highly innovative approach to the isolation and cultivation of previously uncultured bacteria was recently reported by Cross *et al.*[147], who used genomic information to engineer antibodies predicted to target selected microorganisms and to specifically capture these microorganisms from complex communities and to isolate them in pure cultures. This approach was validated by isolation and cultivation of previously uncultured bacteria from the human oral cavity[147], and it could be applicable to a wide range of target organisms if suitable cultivation conditions can be identified for the isolated cells.

Despite these advances in culturing strategies, artificial conditions still do not fully represent the complex environment of natural habitats. To circumvent this

problem, microbial and NP diversity can also be accessed via extraction of organisms and/or their NPs *in situ*. To directly gain compounds produced in the natural marine environment (which may be missed otherwise), resin capture technology can be used to capture compounds on inert sorbent supports ready to be desorbed, analysed and tested for biological activity[148]. Sustainable approaches for *in situ* extraction with green solvents, such as glycerol or natural deep eutectic and ionic solvents (NADES), could be used directly during field work[149,150]. To improve dereplication, analytical equipment miniaturization is also facilitating *in situ* analysis; examples include the introduction of devices for physicochemical data analysis, such as micro MS and portable near infrared (NIR) spectroscopy[151,152].

[H1] Outlook for NPs in drug discovery

The technological advances discussed above have the potential to reinvigorate NP-based drug discovery in both established and emerging areas. NPs have long been the key source of new drugs against infectious diseases, especially antibiotics (see REFS [153,154] for reviews). Selected NPs with antimicrobial properties discovered by leveraging advances discussed in the sections above, including strategies to exploit the human microbiome for novel NPs[96,155] are highlighted in **Figure 3** and **Figure 4**. Along with the search for new NPs with antimicrobial activities, researchers are continuing to develop and optimize already known NP classes, making use of advances in biosynthetic engineering (for example, REF. [156]), total synthesis (for example, REF. [157]) or semi-synthetic strategies (for example, REF. [158,159]). In addition, antivirulence strategies could represent an alternative approach to fighting infections,[160] for which NPs targeting bacterial quorum sensing could be of interest (for example, REF. [161]).

NPs also have a successful history as cancer therapeutics, which has been well-covered in other reviews[162–165]. An important new opportunity in this field is the capacity of some NPs to trigger selective yet potent host immune reaction against cancer cells, particularly given the intense interest at present in strategies that could improve response rates to immune checkpoint inhibitors by turning "cold" tumors "hot"[166]. For example, NPs such as cardiac glycosides[167] can 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), which could open new avenues for drug discovery or repurposing[168–170].

Botanical therapies containing complex mixtures of NPs have long attracted interest owing to the potential for synergistic therapeutic effects of components within the mixture[171,172]. However, the variability of the NP composition in the starting plant material owing to factors such as environmental variations in the location the plants were collected at is a major challenge for the development of botanical drugs[1]. With the advances in technology for their characterization such as metabolomics discussed above, as well as development of regulatory guidance for complex mixtures of NPs (see Related links), it is becoming more feasible to develop such mixtures as therapeutics, rather than to identify and purify a single active ingredient[173].

Since gut microbiota are considered to play a major role in health and disease[174–176] and NPs are known to affect the gut microbiome composition[177–180], this area is an emerging opportunity for NP-based drug discovery. However, drug discovery efforts in this area are still in their infancy, with many open questions remaining[181]. A future direction may be the characterization of single microbiota-derived species for particular therapeutic applications, and the above-discussed advances in culturing strategies, genome mining and analytics will be of great importance in this respect.

Many advances discussed above are supported by computational tools including databases (such as genomic, chemical, or spectral analysis data; see REF.[182] for a recent review on NP databases) and tools that enable the analysis of genetic information, the prediction of chemical structures and pharmacological activities[183], the integration of datasets with diverse information (such as tools for multi-omics analysis[184]) and machine learning applications[185].

Although this article focuses on technologies that are enabling the discovery of novel NPs, it is important to acknowledge that unmodified NPs may possess sub-optimal efficacy or ADMET (absorption, distribution, metabolism, excretion, and

toxicity) properties. So, for development of NP hits into leads and ultimately into successful drugs, chemical modification may be required. In addition, bringing a compound into clinical development requires a sustainable and economically viable supply of sufficient quantities of the compound. Total chemical synthesis, semi-synthesis using a NP as a starting point for analogue generation and biosynthetic engineering modifying biosynthetic pathways of the producing organism will be of great importance in this context (**Figure 5**). Recent advances in chemical synthesis and biosynthetic engineering technologies are strongly empowering NP-based drug discovery and development by enabling property optimization of complex NP scaffolds that were previously regarded as inaccessible. This allows the enrichment of screening libraries with NPs, NP-hybrids, NP analogues and NP-inspired molecules, as well as superior structure functionalization approaches (including late-stage functionalization) for optimization of NP leads[96,107–110,186–190].

Finally, although NP-based drug discovery offers a unique niche for diverse forms of academia–industry collaborations, a key challenge is that scientific and technological expertise is often scattered over many academic institutions and companies. Focused efforts are needed to support translational NP research in academia, which has become more difficult in recent years given the decline in the number of large companies actively engaged in NP research. A conventional solution to improve academia-industry interaction is to focus the relevant 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), the Michael Popp Research Institute for New Phyto-Entities, Bionorica Research, and Biocrates Life Sciences AG) with the aim of accelerating NP-based drug discovery. Another solution could be virtual consortia, such the International Natural Product Sciences Taskforce (INPST) that we have recently established (see INPST in Related links), which provides a platform for integration of expertise, technology, and materials from the participating academic and industrial entities.

In conclusion, NPs remain a promising pool for the discovery of scaffolds with high structural diversity and various bioactivities that can be directly developed

or used as starting points for optimisation into novel drugs. While drug development overall continues to be challenged by high attrition rates, there are additional hurdles for NPs due to issues such as accessibility, sustainable supply, and intellectual property constraints. We, however, believe that the scientific and technological advances discussed in this review provide a strong basis for NP-based drug discovery to continue making major contributions to human health and longevity.

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Box 1 | **Natural products that activate the KEAP1/NRF2 pathway**

An example of a pathway affected by diverse 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, mitochondrial biogenesis, and the resolution of inflammation[191–194].

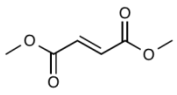
Activation of this pathway can protect against damage by most types of oxidants and pro-inflammatory agents, and it restores redox and protein homeostasis[195]. The pathway has therefore attracted attention for the development of drugs for the prevention and treatment of complex diseases, including neurological conditions such as relapsing-remitting multiple sclerosis[196] and autism spectrum disorder[197].

Dimethyl fumarate (DMF), the methyl ester of the NP fumarate (a tricarboxylic acid cycle intermediate that is found in both animals and plants), is one of the earliest discovered inducers of the KEAP1/NRF2 pathway[198,199]. The origins of the development of DMF as a drug date back to the use in traditional medicine of the plant *Fumaria officinalis*. 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[200]. Despite this erroneous assumption, DMF is effective in treating psoriasis, both topically and orally, and is the active principle of Fumaderm, which has been used clinically for several decades in the treatment of plaque psoriasis in Germany. More recently, a DMF formulation developed by Biogen has been tested in other immunological disorders, with successful phase III trials in multiple sclerosis[201,202] leading to its approval by the FDA and EMA in 2013.

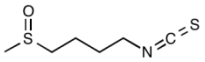
The isothiocyanate sulforaphane, isolated from broccoli (*Brassica oleracea*)[203], is among the most potent naturally-occurring inducers of the KEAP1/NRF2 pathway[204] and has protective effects in animal models of Parkinson's[205], Huntington's[206] and Alzheimer's[207] diseases, traumatic brain injury[208], spinal cord contusion injury[209], stroke[210], depression[211], and multiple sclerosis[212].

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[213] and a follow-up trial (NCT02656420) also demonstrated dose-response dependent benzene detoxication[214]. 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[197]; these encouraging findings led to a recently completed clinical trial in children (aged 3–12) (NCT02561481; results of the trial are not yet publicly available). An alpha-cyclodextrin complex of sulforaphane known as SFX-01 (developed by Evgen Pharma) is being clinically studied for its potential to reverse resistance to endocrine therapies in patients with $ER^{+}HER2^{-}$ metastatic breast cancer (phase II trial completed[215]) and in patients with subarachnoid haemorrhage (phase II trial NCT02614742 recently completed; results are not yet publicly available).

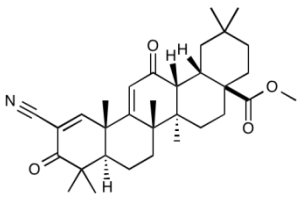
Finally, the pentacyclic triterpenoids bardoxolone methyl (also known as RTA 402) and omaveloxolone (RTA 408), which are semi-synthetic derivatives of the NP oleanolic acid, are the most potent (active at nanomolar concentrations) activators of the KEAP1/NRF2 pathway known to date[216]. These compounds have shown protective effects in numerous animal models of chronic disease[217], 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[195].



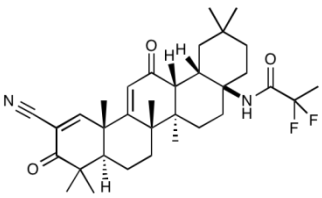
Dimethyl fumarate



Sulforaphane



Bardoxolone methyl (RTA 402)



Omaveloxolone (RTA 408)

Figure legends

Figure 1 | 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. The process begins with extraction of natural products (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, such as XXXXX XXXXXX .

Figure 2 | **Applications of advanced analytical technologies empowering modern natural-product-based drug discovery**. **a** | An illustrative example of the application of LC-HRMS metabolomics in the screening of natural product (NP) extracts is the work of Kurita et al., in which 234 bacterial extracts were subjected to image-based phenotypic bioactivity screening and LC-HRMS metabolomics. Clustering of the resulting data allowed prioritization of promising extracts for further analysis, resulting in the discovery of new NPs, quinocinnolinomycins A–D. **b** | Another illustrative example of LC–HRMS screening of NP extracts is the work of Clevenger et al., who obtained novel NP extracts through heterologous expression of FACs containing uncharacterized biosynthetic gene clusters (BGCs) from diverse fungal species in *Aspergillus nidulans.* Analysis of the LC–HRMS metabolomics data with a FAC-score algorithm directed the simultaneous discovery of 15 new NPs and the characterization of their BGCs.

Figure 3 | Strategies for genome mining-driven discovery of NPs/NP-like compounds. a | Genome mining-based approaches to explore the biosynthetic capacity of microorganisms rely on DNA extraction, sequencing, and bioinformatics analysis. 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. In the case of unculturable microorganisms, the bioinformatics analysis step can be followed by either targeted heterologous expression of biosynthetic gene clusters prioritized as being likely to yield relevant new NPs or direct chemical synthesis of 'synthetic–bioinformatic' NP-like compounds. **b**,**c** | These two approaches are exemplified by the recent discoveries of malacidins (panel **b**) and humimycins (panel **c**), respectively. A major strength of the 'synthetic–bioinformatic' approach is that it is entirely independent of microbial culture and gene expression. Its limitations are the accuracy of computational chemical structure predictions and the feasibility of total chemical synthesis.

Figure 4 | **Application of advanced microbial culturing approaches to identify new natural products**. New strategies for isolating previously uncultured microorganisms can enable access to new natural products (NPs) produced by them. **a** | To recapitulate the effect of complex signals coming from

the native environment, microorganisms can be cultivated directly in the environment from which they were isolated. This concept is used with the iChip platform, in which diluted environmental samples are seeded in multiple small chambers separated from the native environment with a semipermeable membrane. The potential of this approach is illustrated by the recent discovery of teixobactin, a new antibiotic with activity against Gram-positive bacteria. **b** | Another important recent development involves obtaining information from environmental samples using 'omics' techniques such as metagenomics to identify and partially characterize microorganisms present in a specific environment prior to culturing. An approach relying on such preliminary information was recently used to engineer capturing antibodies based on genetic information, which resulted in the successful cultivation of previously uncultured bacteria from the human mouth. This reverse genomics workflow was validated by the isolation and cultivation of three species of Saccharibacteria (TM7) along with their interacting Actinobacteria hosts, as well as SR1 bacteria that are members of a candidate phylum with no previously cultured representatives.

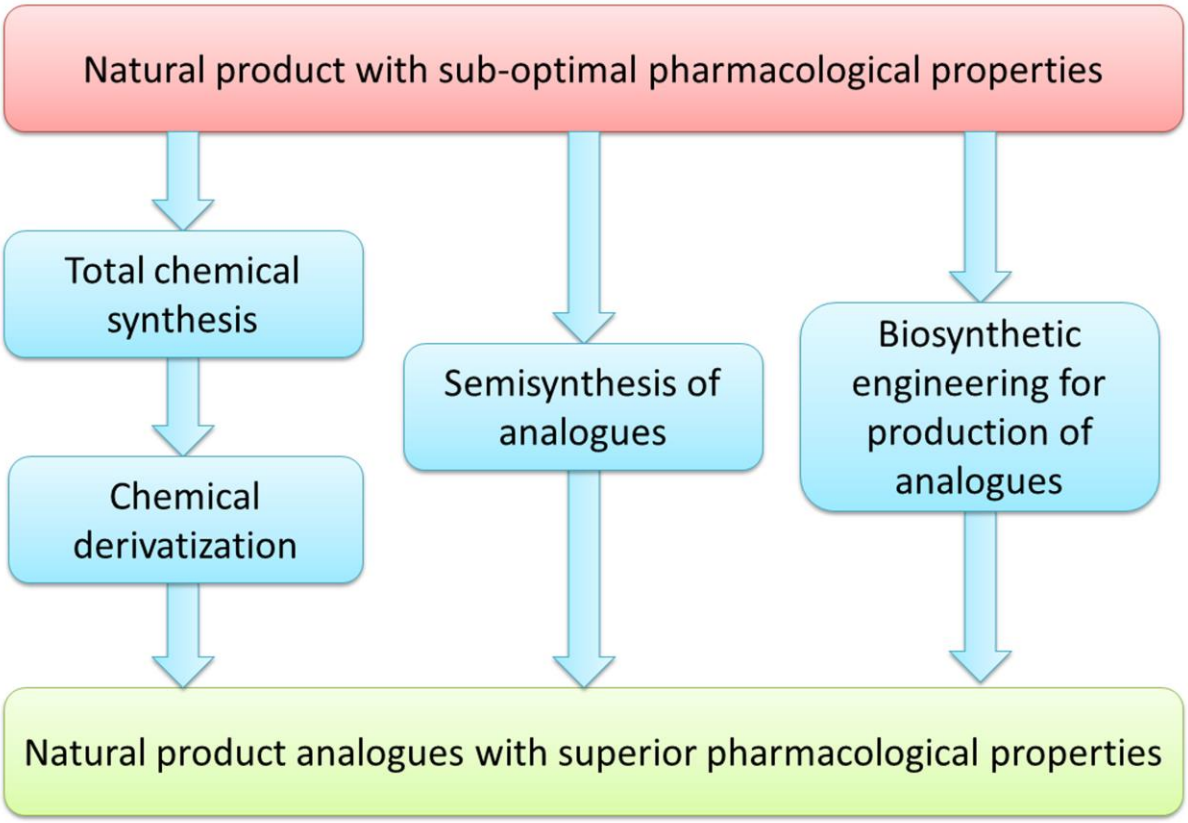


Figure 5 | Strategies to obtain NP analogues with superior properties. Unmodified NPs often possess sub-optimal properties, and superior analogues need to be obtained in order to yield valuable new drugs. NP analogues can be accessed through the development of total chemical synthesis followed by chemical derivatization, through semisynthesis using a NP as a starting point for the introduction of chemical modifications, and through biosynthetic engineering using manipulations of biosynthetic pathways of the producing organism to generate NP analogues. Tetracyclines are an example of NP-derived antibiotics that already yielded several generations of successfully marketed semisynthetic and synthetic derivatives. The first generation of tetracyclines (*e.g.*, chlortetracycline, tetracycline) were unmodified NPs, while the two following generations of analogues with optimized properties were semisynthetic (second-generation, doxycycline, minocycline; third-generation, tigecycline), and the most recently developed fourth generation (eravacycline) are entirely synthetic analogues accessed *via* total synthesis[218,219]. The biosynthetic engineering approach also already reveals its high potential, as demonstrated for example by the generation of analogues of rapamycin[113], bleomycin[115], and nystatin[116].

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EMA:

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FDA Botanical Drug Development Guidance for Industry:

https://www.fda.gov/downloads/Drugs/Guidances/UCM458484.pdf

GARDP:

https://www.gardp.org/

GW Pharmaceuticals:

https://www.gwpharm.com

IMI:

https://www.imi.europa.eu/

INPST:

https://inpst.net/

Acknowledgements

The authors are grateful to Peter Kirkpatrick for his editorial contribution, which resulted in a greatly improved manuscript.

A.G.A. acknowledges the support by the Austrian Science Fund (FWF) project P25971-B23 ('Improved cholesterol efflux by natural products').

V.B. acknowledges the support by grant from Austrian Science Fund (FWF) P27682-B30.

N.B. is recipient of the Australian Research Council DECRA Fellowship.

A.C. and E.I. thank the support by Ministerio de Ciencia, Innovación y Universidades, Spain (Project AGL2017-89417-R).

Marc Diederich is supported by the National Research Foundation (NRF) by the MEST of Korea for Tumor Microenvironment Global Core Research Center (GCRC) grant, [grant number NRF-2011-0030001], by the Creative-Pioneering

Researchers Program through Seoul National University [Funding number: 370C-20160062], by the Brain Korea 21 (BK21) PLUS program, by the "Recherche Cancer et Sang" foundation, by the "Recherches Scientifiques Luxembourg" association, by the "Een Häerz fir kriibskrank Kanner" association, by the Action LIONS "Vaincre le Cancer" association and by Télévie Luxembourg.
Research in the laboratory of A.T.D.K. is funded by Cancer Research UK and Tenovus Scotland.
B. L. F. acknowledges BMBF (TUNGER 036/FUCOFOOD) and AIF (AGEsense) for supporting his research.
M.I.G. acknowledges financial support from the European Union's Horizon 2020 research and innovation programme, project PlantaSYST (SGA-CSA No. 739582 under FPA No. 664620).
K.M.G. is supported by the UK Medical Research Council (MC_UU_12011/4), the National Institute for Health Research (NIHR Senior Investigator (NF-SI-0515-10042) and the NIHR Southampton Biomedical Research Centre), the European Union (Erasmus+ Capacity-Building ENeA SEA Project and Seventh Framework Programme (FP7/2007-2013), projects EarlyNutrition and ODIN (Grant agreements 289346 and 613977), the US National Institute On Aging of the National Institutes of Health (Award No. U24AG047867) and the UK ESRC and BBSRC (Award No. ES/M00919X/1).
Research in the laboratory of C.W.G. is supported by the Austrian Science Fund (FWF) through project I3243 and the Vienna Science and Technology Fund (WWTF) through project LS13-017.
A.K. acknowledges the research grant from project INNOVMAR (reference NORTE-01-0145-FEDER-000035, within Research Line NOVELMAR), supported by North Portugal Regional Operational Programme (NORTE 2020), under the PORTUGAL 2020 Partnership Agreement, through the European Regional Development Fund (ERDF).
A.L. acknowledges HKBU SDF16-0603-P02 for supporting this research.
F.A.M. acknowledges the support by Ministerio de Economia y Competitividad, Spain, (Project AGL2017-88083-R).
A.M. acknowledges the support by a grant of the Romanian Ministry of Research and Innovation, CNCS - UEFISCDI, project number PN-III-P1-1.1-PD-2016-1900 - "PhytoSal", within PNCDI III.
G.P. acknowledges the support by NIH G12-MD007591.

M.R. acknowledges support by the Swiss National Science Foundation (Schweizerischer Nationalfonds, SNF), and by the Horizon 2020 program of the European Union.
J.M.R. acknowledges the support from the Austrian Science Fund (FWF: P24587), the Natvantage grant 2018 and the University of Vienna, Austria.
G.L.R. acknowledges the group of Cellular and Molecular Nutrition (BJ-Lab) at the Institute of Food Sciences, National Research Council, Avellino, Italy.
D.S. acknowledges the support by FWF S10711. D.S. is an Ingeborg Hochmair Professor at the University of Innsbruck.
K.S.W. is supported by the National Centre for Research and Development (4/POLTUR-1/2016) and the National Science Centre (2017/27/B/NZ4/00917) and Medical University of Lublin, Poland.
E.S.S. thanks Universidad Central de Chile, through Proyecto Interno I+D 2016 (CIP16011), for supporting this research.
Hermann Stuppner acknowledges support by the Austrian Research Promotion Agency (FFG), the Austrian Science Fund (FWF) and the Horizon 2020 program of the European Union (RISE, 691158).
Antoni Sureda was granted by Instituto de Salud Carlos III, CIBEROBN (CB12/03/30038) and EU-COST Action (CA16112).
M.W. acknowledges the support by Support from DFG, BMBF, EU, CSC, DAAD, AvH, Land Baden Württemberg.
J.L.W. is grateful to the Swiss National Science Foundation (SNF) for supporting its natural product metabolomics projects (grants nos. 310030E-164289, 31003A_163424 and 316030_164095).
J.X. acknowledges Multi-Year Research Grant of University of Macau (MYRG2018-00169-ICMS) for supporting his research.
S.B.Z. acknowledges the support by University of Vienna, Vienna, Austria.

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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.

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