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REVIEW

Natural products as probes in pharmaceutical research

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Abstract From the start of the pharmaceutical research natural products played a key role in drug discovery and development. Over time many discoveries of fundamental new biology were triggered by the unique biological activity of natural products. Unprecedented chemical structures, novel chemotypes, often pave the way to investigate new biology and to explore new pathways and targets. This review summarizes the recent results in the area with a focus on research done in the laboratories of Novartis Institutes for BioMedical Research. We aim to put the technological advances in target identification techniques in the context to the current revival of phenotypic screening and the increasingly complex biological questions related to drug discovery.

Keywords Natural products · Target identification · Chemical proteomics · Genetic screens · Cancer · Antibiotic · Malaria

Introduction

The discovery and commercialization of natural product-based drugs have shaped the history of the pharmaceutical industry over 200 years. Pharmaceutical research at Sandoz, one of the predecessor companies of Novartis, started in 1917 with the investigation of the ergot alkaloids from the plant pathogenic fungus *Claviceps purpurea* motivated by its use as traditional medicine [45]. Pure ergotamine was isolated in 1918 and brought to the market (Gynergen®) in 1921 to control postpartum bleeding and later migraine. At that time, neither the chemical structure of ergotamine was known, nor was it investigated for its pharmacological properties. The following decades yielded more examples of novel natural products or derivatives, which were approved for human therapy. The resumption of ergot alkaloid research in the 1930s through Albert Hofmann resulted in 3 new drugs as well as the famous lysergic acid derivative LSD-25, which during the following decades was used as a tool to understand the role of the neurotransmitters serotonin and dopamine [45]. The discovery of the cyclic peptide cyclosporine from the fungus *Tolypocladium inflatum* in the 1970s revolutionized the field of transplantation medicine (Sandimmun®) and allowed for the investigation of the cellular function of cyclophilins, a family of proteins binding cyclosporine with high affinity. Apart from the immunosuppressive activity via interrupted signaling in T cells, cyclosporine-related compounds are also under clinical investigation as antiviral and cardioprotective agents via their potent inhibition of cyclophilin A and D, respectively [21, 62]. Another natural product-derived agent, FTY720 (fingolimod, Gilenya®), excels as first in class compound for the treatment of the autoimmune disease, multiple sclerosis. Fingolimod is inspired by the natural product myriocin, a compound originally isolated from an extract of the fruiting bodies of the fungus *Isaria sinclairii*. It mediates its immune-suppressive effect by the modulation of sphingosine 1-phosphate (S1P) receptors, thereby regulating the migration of selected lymphocyte subsets into the central nervous system. The study of the therapeutic mechanism of fingolimod has largely expanded the understanding of the biology of S1P receptors [3]. Last but not least,

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Table 1 Compilation of natural product–target pairs including information on target identification technologies

Compound name	Target	Target identified by	Depicted in figure	References
Arctigenin	ETC	Expression profiling	5, other	[32]
Argyrin B	mEFG1	Resistance profiling	2, anticancer	[43]
Cladosporin	P.f. LysRS	HIP, resistance profiling	4, antiparasitic	[24]
Cyclomarin A	M.t. ClpC1	Chemical proteomics	3, antibacterial	[54]
Cyclomarin A	P.f. Ap3Aase	Chemical proteomics	4, antiparasitic	[5]
Decatransin	SEC61	HIP, resistance profiling	5, other	[27]
Englerin A	TRPC4	Resistance profiling	2, anticancer	[7]
Euphohelioscopin A	PKC	Biochemical	5, other	[11]
GE2270A	S.a. EF-Tu	Resistance profiling	3, antibacterial	[41]
KAE609	P.f.ATP4	Resistance profiling	4, antiparasitic	[56]
Lipiarmycin A3	RNA pol	Resistance profiling	3, antibacterial	[31]
Manassantin B	ETC	Expression profiling	5, other	[32]
Nannocystin A	eEF1A	Chemical proteomics, HIP, RP	2, anticancer	[30]
Novolactone	HSP70	HIP, resistance profiling	2, anticancer	[19]
OSW-1	OSBP, OPR4L	Chemical proteomics	2, anticancer	[6]
Rocaglamide	eIF4A	HIP, resistance profiling	2, anticancer	[49]
Sesquicillin	ETC	Expression profiling	5, other	[32]
Silvestrol	eIF4A	HIP, resistance profiling	2, anticancer	[49]
Thiomuracin A	S.a. EF-Tu	Resistance profiling	3, antibacterial	[41]

ETC, mitochondrial electron transport chain; RP, resistance profiling; M.t., *Mycobacterium tuberculosis*; P.f., *Plasmodium falciparum*; S.a., *Staphylococcus aureus*

the rapamycin derivative everolimus (Afinitor®) has to be mentioned. The therapeutic potential of inhibitors of the mammalian target of rapamycin (mTOR) pathway is still growing. Initially developed for kidney transplantation, its clinical use was extended to heart and liver transplantation [10, 22, 58]. The central role of mTOR for cellular proliferation led to the exploration of everolimus as an anticancer agent [20]. Recently, it has been reported that rapamycin extends lifespan in genetically heterogeneous mice and that everolimus enhances the response to vaccination of aging humans [18, 40]. However, Goldberg et al. performed a detailed study in old mice indicating deleterious consequences of rapamycin to the aging immune system and host survival after infection [17].

The above examples indicate the double role of natural products as drugs and as starting points to explore the underlying biochemistry/physiology in mode of action studies. Today's technologies allow a more rapid elucidation of potential targets and signaling cascades. The review will focus on our work at Novartis Institutes for BioMedical Research in the area of target identification with natural products, covering publications from the last 7 years (period 2008–2015). The examples are grouped according to the main biological activity of the compounds and thus the relevant therapeutic areas. As a further introduction into the topic, a brief overview of commonly employed methods for target identification of bioactive molecules is given in the next section.

Methods for target identification

Before proteins could be purified with relative ease and biochemical assays were miniaturized to formats amenable to sensitive screens, phenotypic screening (i.e., testing the response of an entire cell, tissue or organism to compound treatment) was the standard in pharmaceutical industry over decades. Despite the current sophisticated biochemical assays and readout systems, phenotypic screening is presently seeing a revival at Novartis and other drug discovery companies. Phenotypic screens have demonstrated to deliver more first in class medicines than other strategies [57], but come with the caveat that the modulated target and pathways yet need to be identified. Most of the compounds presented in this review indeed have been identified in phenotypic, cell-based assays and the target was subsequently determined. Table 1 summarizes all compounds mentioned throughout this article and provides information about applied technologies as well as a reference to the figure, which depicts the chemical structure.

Target identification methodologies can be grouped into two classes: biochemical and genetic assays. This article provides examples for both approaches. The biochemical assay used in house for unbiased target discovery is referred to as chemical proteomics [52]. This strategy is based on the immobilization of a bioactive analog of the lead compound to affinity-capture cellular proteins

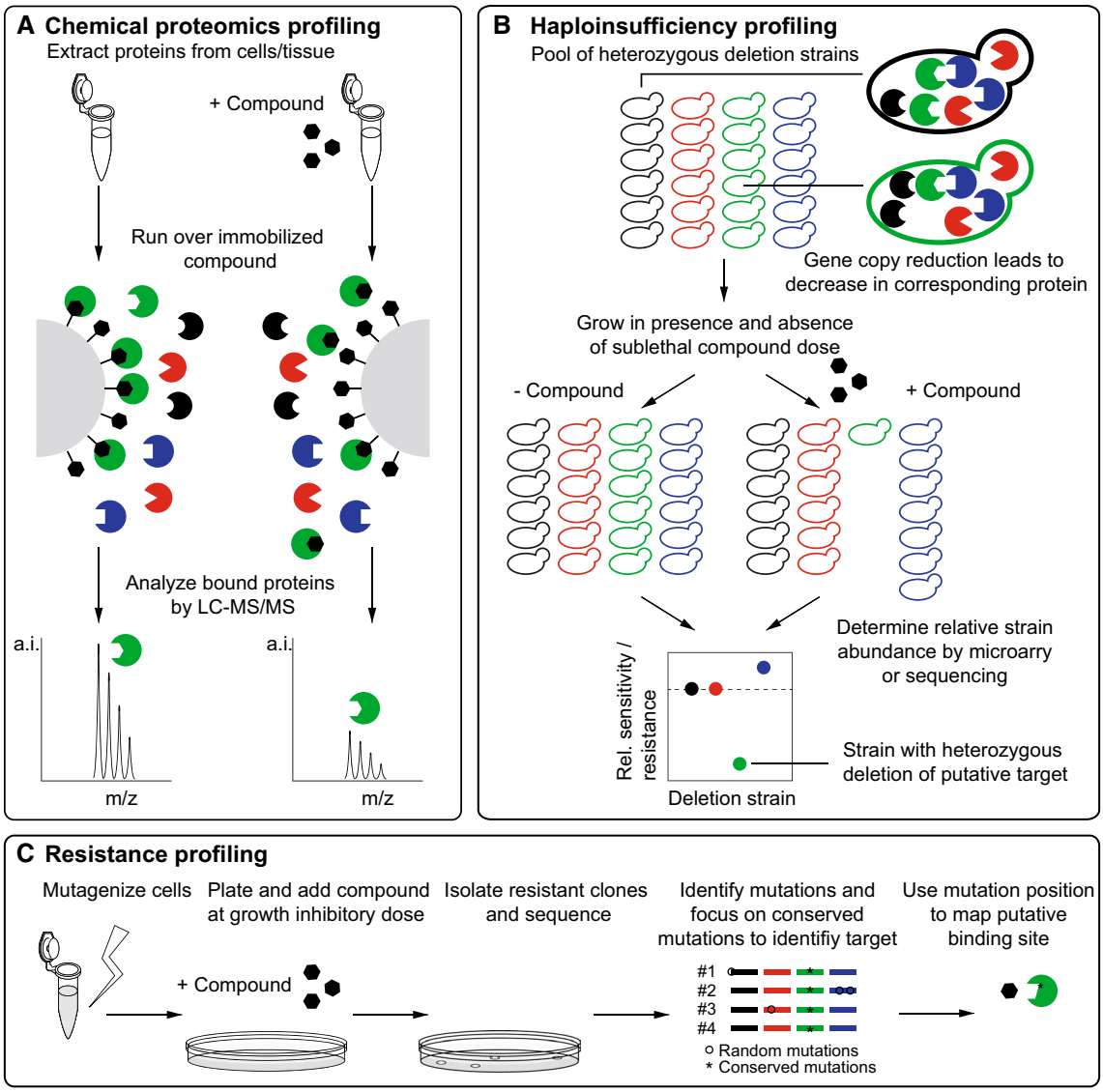

Fig. 1 Target identification technologies. **a** Chemical proteomics using immobilized compound to fish for specific binding proteins in a proteome-wide manner. Competition with extra compound can help to distinguish specific versus non-specific binders. **b** Haploinsufficiency profiling of fungal heterozygous deletion strains in a genome-wide, pooled fashion. Gene-copy reduction in the target gene leads to hypersensitivity and lack of growth. Underrepresentation of distinct mutants in the pool can be identified by microarray or sequencing technologies. **c** Identification of spontaneous or induced resistant mutants due to mutations in the target gene

from cell lysates. The captured proteins are then identified by mass spectrometry (Fig. 1a). A refined version of this experiment adds a dose-dependent competition step of the bound proteins using defined concentrations of unbound compound resulting in fewer false-positive hits. This report presents examples of successful application of this technology (OSW1: [6], cyclomarin A: [5, 54], nannocystin A: [30]). The requirement to immobilize the lead compound using a suitable chemical linker can comprise a resource-intensive task especially in the context of natural compounds that might not be available in large amounts or amenable for simple chemical derivatization. Most genetic assays have the advantage of not requiring such chemical modifications of the test compound. However, these assays do not report direct binding but cellular responses like hypersensitivity, resistance or pathway activations. A powerful assay based on sensitivity is haploinsufficiency profiling (HIP). It has been shown that heterozygous diploid cells that bear a deletion in one copy of a small molecule's target gene show increased sensitivity to that small molecule relative to those strains that have two copies of the gene [16]. This is a likely consequence of decreased expression of the target in the heterozygous diploid cell. The increased sensitivity then results in slower replication

rates and underrepresentation of the affected mutants that can be detected by microarray or sequencing methodologies (Fig. 1b) [23]. Obviously, best applicability of this assay is in the area of antifungal research but due to significant biological conservation the HIP methodology supported target identification of several targets also in other species including *Plasmodium* and human as presented in this study by cladosporin [24], novolactone [19] and rocaglamide [49]. Additional examples are shown in the study by Hoepfner et al. [23]. As the availability of genome-wide heterozygous deletion collections is restricted to few fungal species [47] an alternative and more widely applicable approach is to subject target cells to spontaneous or induced random mutagenesis and then score for resistance against the compound in question. The underlying principle is that mutations in the target protein that affect compound binding but retain the biological activity render cells resistant. Sequencing the resistant mutant and comparing its genomic information to the sensitive wild type thus not only identifies the target but frequently also allows mapping of the compound binding site as presented for GE2270A [41], argyrin B [43], novolactone [19], and rocaglamide [49]. Increased sequencing power paralleled by decreased costs bypass the cumbersome approach to subclone the resistance-conferring mutations through generation of genomic libraries and enable the sequencing of even large mammalian genomes as shown for decatransin [27]. Beyond unbiased approaches, for both, genetic and biochemical concepts a plethora of targeted methodologies exist that allow validation of target hypotheses in a focused fashion. For example, methodologies like CRISPR-based genome editing allow directed changes in gene function and expression in most organisms [39].

Cancer

During the last decade, cancer therapy has evolved from being nonselective to selective and targeted [50]. The deeper understanding of oncogenes, tumor suppressor genes and cancer-causing pathways allowed the development of these targeted therapies. Tamoxifen, binding to the estrogen receptor in estrogen receptor-positive breast cancer, was the first targeted cancer therapy introduced to the market 1977. The field gained momentum through the discovery and development of imatinib (Gleevec®), a selective inhibitor of BCR-Abl tyrosine kinase [59]. In recent years, mainly targeted therapies have been approved as novel cancer treatments; exemplified by an impressive number of 31 new targeted cancer medicines, which have entered the market between 2012 and 2014 [50]. Examples for natural product-based targeted therapies are everolimus (semi-synthetic Rapamycin derivative) targeting mTOR kinase for renal cell carcinomas, romidepsin targeting HDAC in cutaneous T cell lymphoma or the antibody–drug conjugate Trastuzumab emtansine using DM1 (maytansine) as payload. Targeted therapies typically have fewer side effects and most importantly allow for a genetic stratification of patients, largely improving cancer management. Thus, knowing the protein target of an anti-proliferative natural product is a crucial step and the gate keeper before any in-depth analysis of its therapeutic potential.

The plant-derived steroid OSW-1 (see Fig. 2) showed an interesting activity profile in the cancer cell line panel of the National Cancer Institute. The observed sensitivity pattern was also found for the structurally unrelated natural products cephalostatin 1, ritterazine B and schweinfurthin A. For the target identification of the natural product OSW-1 chemical proteomics were used to explore its action. In the pull down experiment, the oxysterol-binding protein (OSBP) and its closest paralog, OSBP-related protein 4L (OPR4L), showed the largest competition by OSW-1. OSBP and ORPs are an evolutionarily conserved protein superfamily involved in signal transduction, lipid transport and lipid metabolism. It was not known that these proteins are involved in cancer cell survival. It was also shown that cephalostatin 1, ritterazine B and schweinfurthin A are targeting also the same protein [6].

HIP in yeast, as described in the methods section, has also been used to explore the mode of action (MoA) of different anti-proliferative compounds. The following examples of natural products are targeting conserved nodes in cellular pathways, whose inhibition results in growth-inhibitory activities in the lower eukaryote *S. cerevisiae* and in mammalian cells. Novolactone, an unusual tetracyclic compound produced by a fungal strain (see Fig. 2), was broadly profiled to identify a biological activity. Since it scored in a yeast growth assay it was submitted to the HIP assay and turned out to be a potential modulator of the chaperone network. Using a combination of cellular and in vitro approaches, it was confirmed that novolactone targets cytosolic and ER-localized isoforms of Hsp70 through a highly conserved covalent interaction at the interface between the substrate-binding and ATPase domains [19]. Inappropriate Hsp70 activity can disrupt protein homeostasis, an event observed in many cancers. Consequently, affecting Hsp70 function has been observed to phenocopy Hsp90 inhibition and cause extensive tumor-selective apoptosis [46, 53]. Novolactone thus provides a valuable tool to investigate the Hsp70/Hsp90 interplay in a disease context.

The plant compound silvestrol and the related rocaglamide scaffold are targeting the translation initiation (see Fig. 2). Using HIP in yeast, the eukaryotic initiation factor elF4a could be identified as the primary molecular target of this compound family. The target could be confirmed by genome sequencing of resistant yeast clones showing

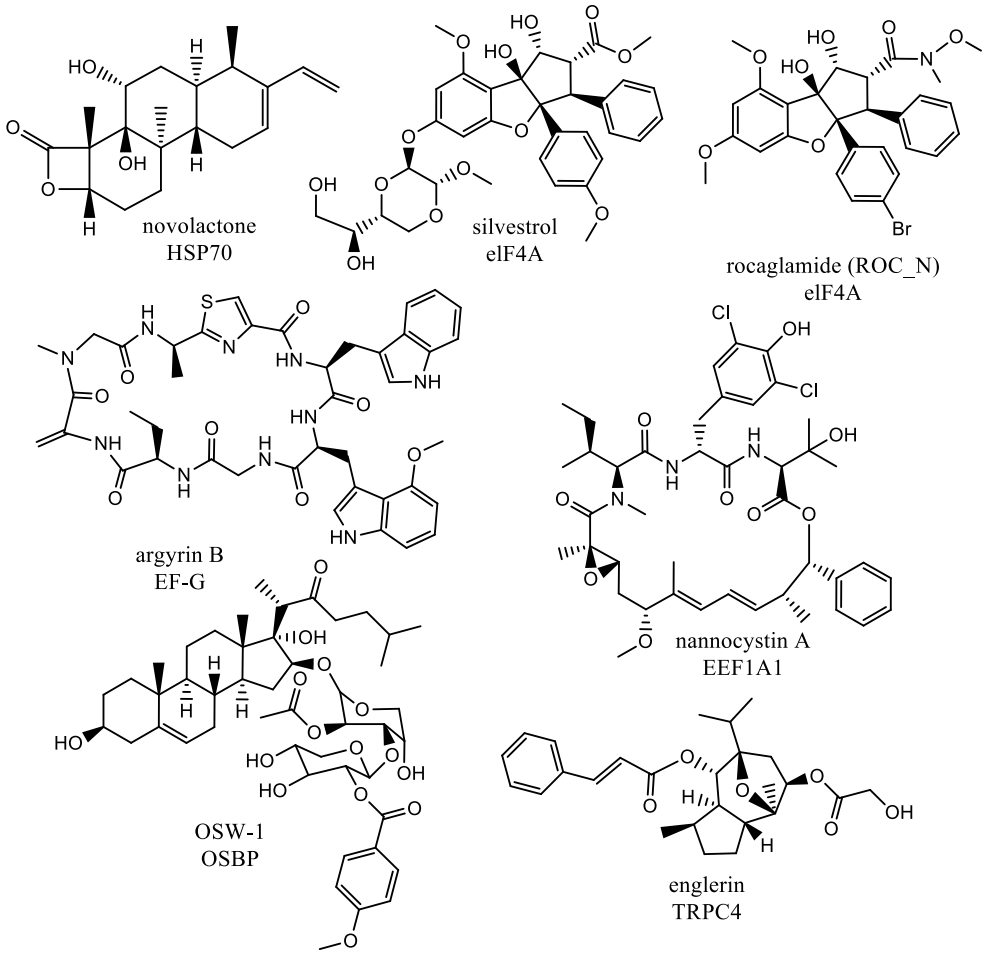

Fig. 2 Chemical structures of potential cancer agents

different mutations in the components of the eIF4F complex. The data support a model, whereby rocaglamide stabilizes an eIF4A–RNA interaction to either alter the level and/or impair the activity of the eIF4F complex [49]. Deregulated signaling pathways in cancers converge to influence protein synthesis. Thus, targeting factors involved in translation initiation is a viable therapeutic strategy that has demonstrated success in pre-clinical cancer settings [8].

The target of the nannocystin class, an unusual macrocyclic compound class isolated from myxobacteria with cytotoxic activity (see Fig. 2) [25, 30], was determined to be the eukaryotic translation elongation factor 1α (EF-1α). The target for this compound was confirmed by the application of different technologies including HIP in yeast, chemical proteomics, profiling in the cell line panel and sequencing of a resistance cancer cell line [30]. Nannocystin A not only shares the same target but also a conserved binding site with didemnin B, a marine natural product that shows promising results in pre-clinical settings for the treatment of cancers [55].

One approach for target identification is based on compound profiling in cellular assays against a large panel of cancer cell lines. For this approach, a cancer cell line encyclopedia (CCLE) was established including 947 well-characterized cancer cell lines allowing the identification of genetic, lineage and gene expression-based predictors of drug sensitivity [2]. For example for the plant compound englerin A (see Fig. 2), which was first isolated in 2008 from the bark of the African plant *Phyllanthus engleri*, it was shown that the block of cell proliferation is correlated with the expression of the TRPC4 cation channel in sensitive cell lines suggesting TRPC4 to be the biologically relevant the target. This could be confirmed by electrophysiology experiments showing that englerin A can induce currents across the membranes of cells in presences of TRPC4, but not in its absence [7]. The same target was found in parallel by Waldmann et al. following a similar approach [1].

The antibacterial- and anti-tumor-active argyrin B, isolated from myxobacteria and actinomycetes (see Fig. 2) [13, 51], showed in the CCLE a selective profile inhibiting cell viability in 18 from 512 cell lines with an IC50 below 1 μM. The profile shows a strong correlation to mitochondrial electron transport inhibitors like rotenone or antimycin A. This correlation led to the assumption, that argyrin A is acting by inhibition of mitochondrial protein synthesis

via mitochondrial elongation factor G1 (mEF-G1), which could be confirmed by lower sensitivity of argyrin against mEFG1 knockout cell lines. For the antibacterial activity of argyrin B the bacterial homolog of mEF-G1 was found to be the target in bacteria via resistant mutant selection, whole genome sequencing and crystallography [43].

Antibiotics

The growing medical need for new antibiotic and antiviral treatments led Novartis to re-enter anti-infective research in 2002. From the beginning the main focus was on phenotypic screens and a close collaboration between the anti-infective group and the natural product group was initiated. Early on, a screen for growth inhibition of *Staphylococcus aureus* was performed using both the pure natural product library and the extract library. Taking full advantage of technological progress in screening and NP-related methods [36], it was possible to identify new chemical starting points for drug discovery. New members of the class of molecules known as thiopeptides could be discovered from the extract screening alongside previously described compounds. The thiomuracins are produced by a rare-actinomycetes bacterium typed as a *Nonomuraea* species (see Fig. 3) [41]. The thiopeptides are chromosomally encoded, ribosomally synthesized proteins, and isolation of gene clusters for production of thiomuracin and the related thiopeptide GE2270A revealed the post-translational machinery required for maturation. The main metabolite of the strain, thiomuracin A, shows minimal inhibitory concentrations (MIC) of 0.25 and 0.5 µg/ml against *Enterococcus faecalis* and *S. aureus*. As a means to identify the target of the thiomuracins, spontaneously resistant mutants of *S. aureus* were selected. Mutants harbored single nucleotide changes in *tuf*A (G826A/C), the gene encoding Elongation Factor Tu (EF-Tu), resulting in amino acid substitutions within the codon for glycine 275. Residue 275 is located within EF-Tu domain II, the binding site for the thiopeptide antibiotic GE2270A [41]. A lead optimization program was initiated using the thiopeptides GE2270A and thiomuracin as starting points [33, 34]. Like GE2270 A and thiomuracin, the clinical candidate LFF571 has antimicrobial activity against a range of Gram-positive bacteria, including *Clostridium difficile* with MIC_{90} values between 0.125 and 0.5 µg/ml [35]. Spontaneous mutants with reduced susceptibility to LFF571 were analyzed for changes in EF-Tu. *C. difficile* possesses two identical copies of the gene encoding EF-Tu, *tuf*A and *tuf*B. All mutants exhibited *tuf*B mutation resulting in amino acid substitution G260E, only one mutant harbored the change in both *tuf*A and *tuf*B. Thus, through genetic analysis of spontaneous mutants in *S. aureus* and *C. difficile* the target of thiomuracins and the clinical candidate LFF571 could be identified as EF-Tu. In addition to potently inhibiting a target that is unexploited by marketed human therapeutics, the thiomuracins and LFF571 have a low propensity for selecting for antibiotic resistance and confer no measurable cross-resistance. In clinical trials, LFF571 proved to be safe and efficacious for the treatment of *C. difficile* infections in patients [42].

Anti-infective research with a focus on tropical or neglected diseases is the aim of the Novartis Institute for Tropical Diseases (NITD), which was inaugurated in Singapore in 2002. As part of the efforts to identify new anti-tuberculosis agents, the pure natural product library was screened in a growth inhibition screen using *Mycobacterium bovis* BCG strain. Hits were profiled for their activity against *M. tuberculosis* (MTB). The goal of the project was to identify natural products which are active against replicating mycobacteria as well as against the dormant stages. Starting with a library of approximately 10,000 pure natural products and yielding 173 primary hits, only 4 compounds could be validated using the criteria stated above (see Fig. 3). Two compounds belonged to the class of thiopeptides and were not pursued further. Lipiarmycin A3 and related macrocyclic polyketides purified from a *Catellatospora* strain of the actinomycetes group exhibited excellent inhibitory activity against multidrug-resistant strains of MTB with MIC values below 0.1 µg/ml. Sequence analysis of the *rpo*B and *rpo*C genes from spontaneous lipiarmycin-resistant mutants of MTB revealed that missense mutations in these genes caused resistance to lipiarmycin [31]. Although both lipiarmycin and rifampicin are known to inhibit the bacterial RNA polymerase, the sites of mutation in the *rpo*B gene were found to be different in MTB strains resistant to these inhibitors.

Finally, cyclomarin A, a cyclic peptide produced by actinomycetes of the genus *Streptomyces*, proved to be bactericidal against replicating MTB and dormant forms in macrophages (see Fig. 3) [54]. The cidal concentration was determined as 0.3 and 2.5 µM, respectively. In addition, cyclomarin A was active against a panel of multidrug-resistant clinical isolates of MTB thus indicating that it acts through a novel mechanism. No bactericidal activity was found in a panel of Gram-positive and Gram-negative organisms. Since, no spontaneous mutants with a decreased susceptibility for cyclomarin A could be obtained, a chemical proteomic approach via affinity chromatography was performed and the target in MTB was identified as ClpC1. ClpC proteins function as regulatory subunits of the caseinolytic Clp protease. ClpC is a chaperone that unfolds protein substrates in an ATP-dependent manner before channeling them to the ClpP protease chamber of the complex. In bacteria, the Clp protease plays a crucial role in protein quality control by removing unfolded proteins from the cell and by specifically controlling the turnover

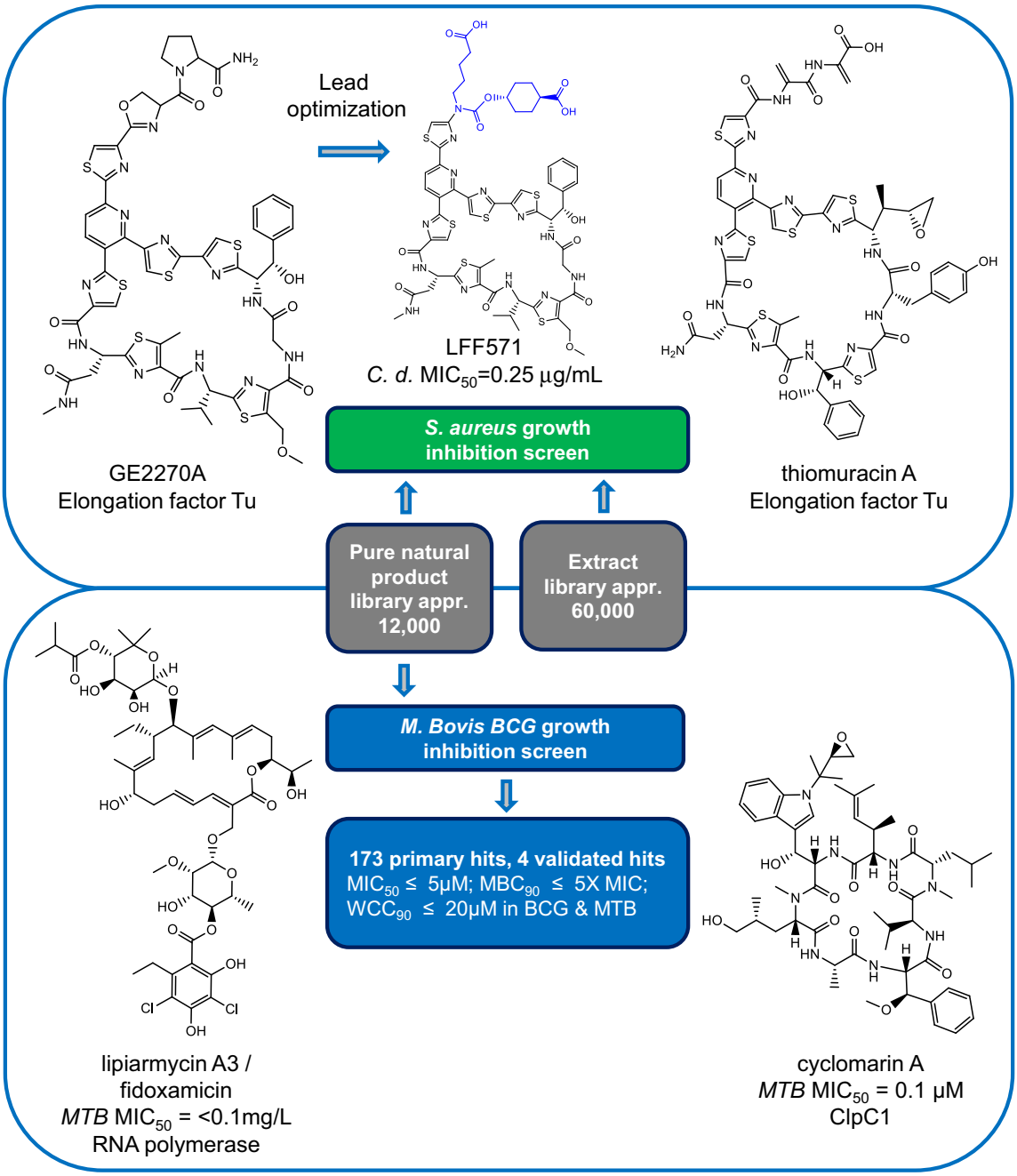

Fig. 3 Chemical structures of antibacterial compounds and flowchart of *Staphylococcus aureus* and *Mycobacterium bovis* BCG screens

of regulatory proteins [4]. Cyclomarin A was shown to increase the proteolytic activity in mycobacteria cells, thus it acts as an activator of the Clp protease [54]. Recently, another cyclic peptide from a *Nonomuraea* sp. strain, ecumicin, was also described to target ClpC in MTB [15]. Target identification for this tridecapeptide was achieved via genomic analysis of spontaneous mutants and verified using the in vitro protein–compound binding technology DART [14].

Antiparasitic

In 2006, a public–private partnership including several Novartis Research Institutes as well as Wellcome Trust and Medicines for Malaria Venture was initiated with the goal to find a one-dose cure for malaria. One of the approaches for the identification of new malaria drugs was a phenotypic screen for growth inhibition of *Plasmodium falciparum*. The screen was conducted with approximately 12,000

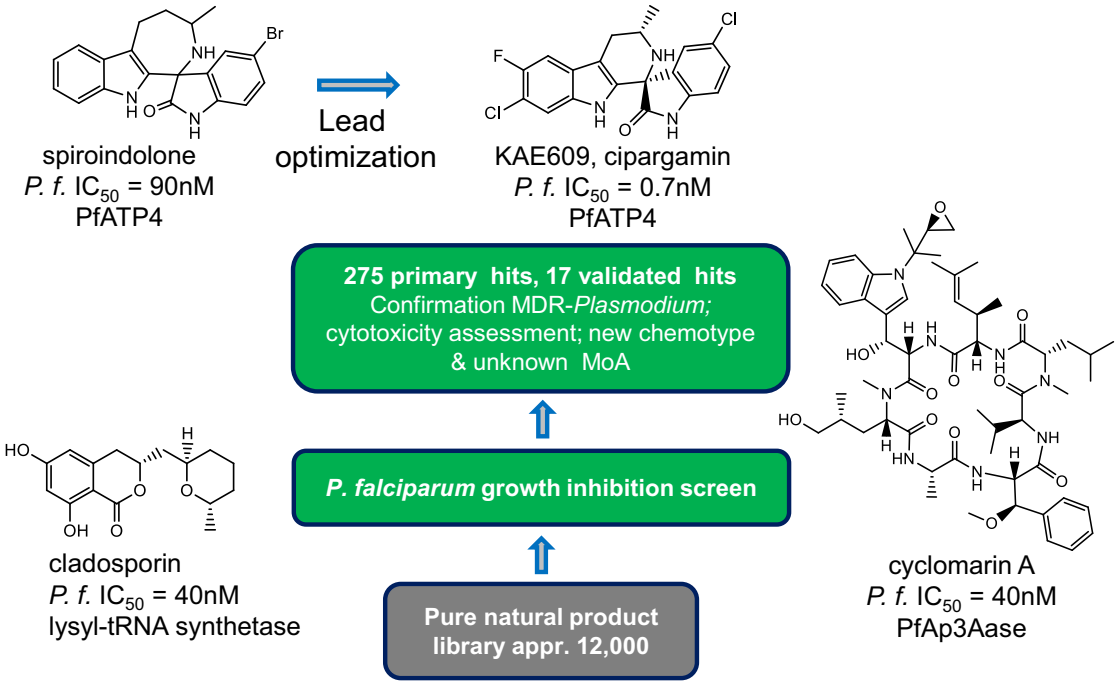

Fig. 4 Chemical structures of antiparasitic natural products and flowchart of *Plasmodium* screen

compounds from the pure natural product library, which also included natural product-like compounds. The resulting 275 primary hits with submicromolar activity against *Plasmodium falciparum* were further profiled against multidrug-resistant parasites and mammalian cells (see Fig. 4). Pharmacokinetic and physical properties were then determined for the remaining 17 compounds. A synthetic compound from the spiroindolone class was chosen as starting point for a medicinal chemistry lead optimization effort. Synthesis and evaluation of about 200 derivatives yielded the optimized compound KAE609 [48, 61]. To gain insight into the mechanism of action in vitro selection of resistance was applied. After 3–4 months of constant drug pressure, the IC_{50} values had increased 7- to 24-fold and resistant *Plasmodium* clones were subjected to genomic analysis. A number of mutations and copy number variation events could be localized to locus *pfatp4* encoding P-type cation-transporter ATPase4 (PfATP4), a membrane transport protein that regulates sodium homeostasis and thus the osmoregularity of the parasite [56]. It is estimated that about 5 % of the ~30,000 anti-malarial compounds identified through phenotypic screening also target PfATP4 [9]. KAE609 showed rapid clearance of parasites from the blood of patients in clinical trials and a favorable safety profile [37, 60].

Two structurally interesting validated hits from the above-mentioned phenotypic screening campaign were chosen for target identification studies, namely cladosporin and cyclomarin A (see Fig. 4). Cladosporin is a fungal isocoumarin and was previously reported with a range of biological activities, but not as an antiprotozoal agent. The simple structure of the compound together with the potent inhibition of *Plasmodium falciparum* blood and liver stages prompted further investigation of the mode of action. Cladosporin showed some, although weak, activity against yeast and consequently was profiled using the HIPHOP approach. The resulting HIP profile was unique among the >2000 compounds tested so far and suggested that cladosporin specifically inhibits protein synthesis by directly targeting *P. falciparum* cytosolic lysyl-tRNA synthetase (PfKRS). Expression and analysis of human and *Plasmodium* homologs in yeast demonstrated the specificity of cladosporin for the plasmodial enzyme. The natural product is >100-fold more potent against parasite lysyl-tRNA synthetase relative to the human enzyme [24]. A crystal structure of ternary PfKRS–lysine–cladosporin complex reveals cladosporin's remarkable ability to mimic the natural substrate adenosine [29]. Interestingly, the plant-derived natural product febrifugine was recently characterized as an inhibitor of prolyl tRNA synthetase in *Plasmodium* [26, 28]. Thus, through the target identification activities with the two natural products cladosporin and febrifugine compelling evidence was generated that tRNA synthetases are a promising target class for anti-malarial drug discovery and development activities.

Surprisingly, cyclomarin A, which we identified as a potent inhibitor of mycobacterial growth previously, also exhibited potent activity against blood stage *Plasmodium*

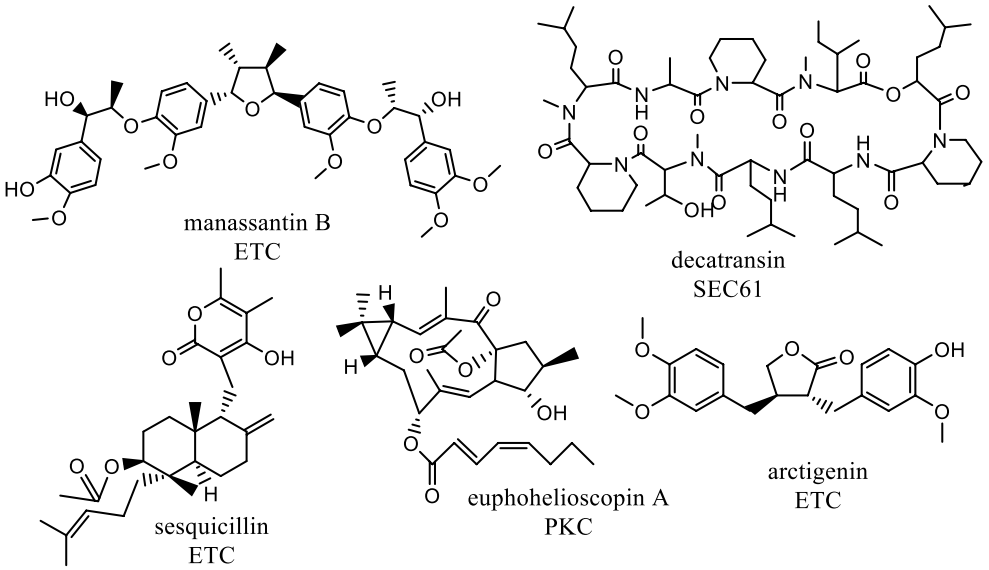

Fig. 5 Chemical structures of biologically active compounds

falciparum. Unfortunately, the derivative of cyclomarin used as a tool compound in the chemical proteomic experiments with *Mycobacterium* protein lysate did not show an activity against *Plasmodium*. Further chemistry efforts yielded an active derivative and through affinity chromatography it could be demonstrated that the enzyme PfAp3Aase specifically binds to cyclomarin. PfAp3Aase cleaves the signaling molecule adenosine-triphosphate into ADP and AMP. Cyclomarin is an nM inhibitor of the plasmodial enzyme but not of the closest human homolog hFHIT [5]. A co-crystal revealed the binding mode of cyclomarin A to dimeric PfAp3Aase in an unusual stoichiometry. One molecule of cyclomarin A binds the dimer at the interface of the two monomers and thereby blocks binding of two substrate molecules. Cyclomarin is a highly intriguing example of how natural products with interesting biological activities can help to elucidate novel targets and novel biology. To the best of our knowledge, a natural product which hits two distinct targets in two important human pathogens is unprecedented.

Cellular processes

After a phenotypic screen, rapid target identification allows for informed hit triaging to prioritize compounds, which can be followed by focused investigations into the modulated biology and the therapeutic potential of the compounds. The knowledge of the target, however, does not always support an obvious therapeutic avenue. More often it raises questions concerning safety or identifies weaknesses of the primary screen by enriching for hit compounds modulating unwanted processes. For the scientific community such compounds still comprise nuggets as they provide high-affinity chemical tweezers to investigate biological processes. And they provide an excellent testing ground to benchmark and improve target identification technologies and map out blind spots. Euphohelioscopin A, a potent hit of a hematopoietic stem cell differentiation screen was subsequently identified to act on the protein kinase C (PKC) and exert its pro-monocytic effect by PKC activation (see Fig. 5) [11]. Due to the tumorigenic potential of PKC the scope of therapeutic applications for euphohelioscopin A remains to be defined. However, the study by Lichtervelde et al., showed that the PKC activation property of euphohelioscopin A is distinct compared to previously known PKC modulators and thus adds to the toolbox to investigate such a central node in biology. Another example is decatransin that was identified in a screen investigating B cell biology. HIP followed by mutagenesis identified the ER translocon SEC61 as the efficacy target [27]. In the secretory B cell context, the modulation of the central gate through which secretory proteins have to pass through made sense. However, this target is essential for many other cellular processes beyond B cell biology raising questions about its therapeutic value. But interestingly, in addition to decatransin there is a previously identified class of natural products, the cotransins, which are chemically distinct but inhibit the same target. Conserved resistance-conferring residues suggest a similar binding site for both compound classes despite no obvious structural similarities [38]. Thus, it will be interesting to investigate the detailed mechanism how these compounds modulate the translocon machinery and add to our understanding of

signal sequence recognition and protein translocation. A third example comes from a phenotypic screen with natural products on whole zebrafish animals. One observed phenotype was developmental arrest without necrosis, a specific phenotype that was not detected among a panel of known cytotoxic compounds. In-depth profiling of these hits including manassantin B, sesquicillin and arctigenin identified these compounds as mitochondrial electron transport chain (ETC) inhibitors [32]. Even though this mechanism of action did not provide novel insight into zebrafish biology, the study demonstrated the usability of multiplexed transcription profiling for target identification. Uncoupling activities do not necessarily require interaction with a target protein; consequently other methodologies like chemical proteomics or screening for resistant mutants did not yield results for these compounds. This highlights the importance of using a multi-pronged target identification approach to unveil the complexity of nature's treasures.

Conclusions

Following the era of high-throughput screening using massive compound libraries in combination with biochemical or standardized cellular reporter gene assays, in the last 5 years an increasing popularity of complex phenotypic screens using induced pluripotent stem cells and primary cells from diverse organs can be observed. Phenotypic screening in current drug discovery environment is regarded as a new challenge rather than a neoclassical approach [12]. The complexity of the assay and the availability of the investigated biological material often limit the number of compounds to be screened. At Novartis the pure natural product library consisting of approximately 15,000 natural products and derivatives is generally screened in these setups. Understanding the mode of actions of active principles in complex biological systems significantly helps the drug discovery process and increases chances for success. Thus, as a consequence of the above-mentioned trends in drug discovery the need for target identification activities is increasing. See Table 1 for an overview of the natural product–target pairs mentioned in the article and the deployed target identification methods.

In the anti-infective field phenotypic screening reemerged already 8–10 years ago, following an era of largely unsuccessful target-based drug discovery using biochemical screens [44]. The examples provided in the paper highlight the power of phenotypic screening in the anti-infective field and specifically the fruitful combination of phenotypic screens with natural product libraries. In fact, many of the compounds targeting anticancer or other cellular mechanisms could also be identified with first biological activities in a simple yeast growth inhibition screen. For example, this holds true for the HSP70 inhibitor novolactone and the SEC61 inhibitor decatransin.

The examples provided also emphasize the opportunity natural products offer for the exploration of novel biological space in drug discovery. Here, the case of the cyclic peptide cyclomarin is standing out. Following up on activity in phenotypic screens, we could show that cyclomarin surprisingly has two distinct and completely unrelated targets in *Mycobacterium tuberculosis* and *Plasmodium falciparum*. Both targets, ClpC for mycobacteria and Ap3pAse for protozoal diseases, can now be considered as novel and druggable starting points for drug discovery. The studies on target and pathway identification of new natural products rationalize their valuable role in modern drug discovery by exploiting so far less systematically investigated facets of their biochemical functions.

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References

1. Akbulut Y, Gaunt HJ, Muraki K, Ludlow MJ, Amer MS, Bruns A, Vasudev NS, Radtke L, Willot M, Hahn S et al (2015) (−)-Englerin A is a potent and selective activator of TRPC4 and TRPC5 calcium channels. Angew Chem Int Ed Engl 54:3787–3791. doi:10.1002/anie.201411511
2. Barretina J, Caponigro G, Stransky N, Venkatesan K, Margolin AA, Kim S, Wilson CJ, Lehar J, Kryukov GV, Sonkin D et al (2012) The Cancer Cell Line Encyclopedia enables predictive modelling of anticancer drug sensitivity. Nature 483:603–607. doi:10.1038/nature11003
3. Brinkmann V, Billich A, Baumruker T, Heining P, Schmouder R, Francis G, Aradhye S, Burtin P (2010) Fingolimod (FTY720): discovery and development of an oral drug to treat multiple sclerosis. Nat Rev Drug Discov 9:883–897. doi:10.1038/nrd3248
4. Brotz-Oesterhelt H, Sass P (2014) Bacterial caseinolytic proteases as novel targets for antibacterial treatment. Int J Med Microbiol 304:23–30. doi:10.1016/j.ijmm.2013.09.001
5. Buerstner N, Roggo S, Ostermann N, Blank J, Delmas C, Freuler F, Gerhartz B, Hinniger A, Hoepfner D, Liechty B et al (2015) Gift from nature: cyclomarin A kills mycobacteria and malaria parasites using distinct modes of action. ChemBioChem. doi:10.1002/cbic.201500472
6. Burgett AW, Poulsen TB, Wangkanont K, Anderson DR, Kikuchi C, Shimada K, Okubo S, Fortner KC, Mimaki Y, Kuroda M et al (2011) Natural products reveal cancer cell dependence on oxysterol-binding proteins. Nat Chem Biol 7:639–647. doi:10.1038/nchembio.625
7. Carson C, Raman P, Tullai J, Xu L, Henault M, Thomas E, Yeola S, Lao J, McPate M, Verkuyl JM et al (2015) Englerin A agonizes the TRPC4/C5 cation channels to inhibit tumor cell line proliferation. PLoS ONE 10:e0127498. doi:10.1371/journal.pone.0127498
8. Chu J, Pelletier J (2014) Targeting the eIF4A RNA helicase as an anti-neoplastic approach. Biochim Biophys Acta. doi:10.1016/j.bbagrm.2014.09.006

9. Cully M (2014) Trial watch: next-generation antimalarial from phenotypic screen shows clinical promise. Nat Rev Drug Discov 13:717. doi:10.1038/nrd4457
10. Dantal J (2012) Everolimus: preventing organ rejection in adult kidney transplant recipients. Expert Opin Pharmacother 13:767–778. doi:10.1517/14656566.2012.662955
11. de Lichtervelde L, Antal CE, Boitano AE, Wang Y, Krastel P, Petersen F, Newton AC, Cooke MP, Schultz PG (2012) Euphohelioscopin A is a PKC activator capable of inducing macrophage differentiation. Chem Biol 19:994–1000. doi:10.1016/j.chembiol.2012.06.010
12. Eder J, Sedrani R, Wiesmann C (2014) The discovery of first-in-class drugs: origins and evolution. Nat Rev Drug Discov 13:577–587. doi:10.1038/nrd4336
13. Ferrari P, Vekey K, Galimberti M, Gallo GG, Selva E, Zerilli LF (1996) Antibiotics A21459 A and B, new inhibitors of bacterial protein synthesis. II. Structure elucidation. J Antibiot 49:150–154
14. Gao W, Kim JY, Anderson JR, Akopian T, Hong S, Jin YY, Kandror O, Kim JW, Lee IA, Lee SY et al (2015) The cyclic peptide ecumicin targeting ClpC1 is active against *Mycobacterium tuberculosis* in vivo. Antimicrob Agents Chemother 59:880–889. doi:10.1128/AAC.04054-14
15. Gao W, Kim JY, Chen SN, Cho SH, Choi J, Jaki BU, Jin YY, Lankin DC, Lee JE, Lee SY et al (2014) Discovery and characterization of the tuberculosis drug lead ecumicin. Org Lett 16:6044–6047. doi:10.1021/ol5026603
16. Giaever G, Shoemaker DD, Jones TW, Liang H, Winzeler EA, Astromoff A, Davis RW (1999) Genomic profiling of drug sensitivities via induced haploinsufficiency. Nat Genet 21:278–283. doi:10.1038/6791
17. Goldberg EL, Romero-Aleshire MJ, Renkema KR, Ventevogel MS, Chew WM, Uhrlaub JL, Smithey MJ, Limesand KH, Sempowski GD, Brooks HL et al (2015) Lifespan-extending caloric restriction or mTOR inhibition impair adaptive immunity of old mice by distinct mechanisms. Aging Cell 14:130–138. doi:10.1111/acel.12280
18. Harrison DE, Strong R, Sharp ZD, Nelson JF, Astle CM, Flurkey K, Nadon NL, Wilkinson JE, Frenkel K, Carter CS et al (2009) Rapamycin fed late in life extends lifespan in genetically heterogeneous mice. Nature 460:392–395. doi:10.1038/nature08221
19. Hassan AQ, Kirby CA, Zhou W, Schuhmann T, Kityk R, Kipp DR, Baird J, Chen J, Chen Y, Chung F et al (2015) The novolactone natural product disrupts the allosteric regulation of Hsp70. Chem Biol 22:87–97. doi:10.1016/j.chembiol.2014.11.007
20. Hasskarl J (2014) Everolimus. Recent Results Cancer Res 201:373–392. doi:10.1007/978-3-642-54490-3_23
21. Hausenloy DJ, Boston-Griffiths EA, Yellon DM (2012) Cyclosporin A and cardioprotection: from investigative tool to therapeutic agent. Br J Pharmacol 165:1235–1245. doi:10.1111/j.1476-5381.2011.01700.x
22. Hirt SW, Bara C, Barten MJ, Deuse T, Doesch AO, Kaczmarek I, Schulz U, Stypmann J, Haneya A, Lehmkuhl HB (2013) Everolimus in heart transplantation: an update. J Transplant 2013:683964. doi:10.1155/2013/683964
23. Hoepfner D, Helliwell SB, Sadlish H, Schuierer S, Filipuzzi I, Brachat S, Bhullar B, Plikat U, Abraham Y, Altorfer M et al (2014) High-resolution chemical dissection of a model eukaryote reveals targets, pathways and gene functions. Microbiol Res 169:107–120. doi:10.1016/j.micres.2013.11.004
24. Hoepfner D, McNamara CW, Lim CS, Studer C, Riedl R, Aust T, McCormack SL, Plouffe DM, Meister S, Schuierer S et al (2012) Selective and specific inhibition of the *Plasmodium falciparum* lysyl-tRNA synthetase by the fungal secondary metabolite cladosporin. Cell Host Microbe 11:654–663. doi:10.1016/j.chom.2012.04.015
25. Hoffmann H, Kogler H, Heyse W, Matter H, Caspers M, Schummer D, Klemke-Jahn C, Bauer A, Penarier G, Debussche L et al (2015) Discovery, structure elucidation, and biological characterization of nannocystin A, a macrocyclic myxobacterial metabolite with potent antiproliferative properties. Angew Chem Int Ed Engl 54(35):10145–10148. doi:10.1002/anie.201411377
26. Jain V, Yogavel M, Oshima Y, Kikuchi H, Touquet B, Hakimi MA, Sharma A (2015) Structure of prolyl-tRNA synthetase-halofuginone complex provides basis for development of drugs against malaria and toxoplasmosis. Structure 23:819–829. doi:10.1016/j.str.2015.02.011
27. Junne T, Wong J, Studer C, Aust T, Bauer BW, Beibel M, Bhullar B, Bruccoleri R, Eichenberger J, Estoppey D et al (2015) Decatransin, a new natural product inhibiting protein translocation at the Sec61/SecYEG translocon. J Cell Sci 128:1217–1229. doi:10.1242/jcs.165746
28. Keller TL, Zocco D, Sundrud MS, Hendrick M, Edenius M, Yum J, Kim YJ, Lee HK, Cortese JF, Wirth DF et al (2012) Halofuginone and other febrifugine derivatives inhibit prolyl-tRNA synthetase. Nat Chem Biol 8:311–317. doi:10.1038/nchembio.790
29. Khan S, Sharma A, Belrhali H, Yogavel M, Sharma A (2014) Structural basis of malaria parasite lysyl-tRNA synthetase inhibition by cladosporin. J Struct Funct Genomics 15:63–71. doi:10.1007/s10969-014-9182-1
30. Krastel P, Roggo S, Schirle M, Ross NT, Perruccio F, Aspesi P Jr, Aust T, Buntin K, Estoppey D, Liechty B et al (2015) Nannocystin A: an elongation factor 1 inhibitor from myxobacteria with differential anti-cancer properties. Angew Chem Int Ed Engl 54:10149–10154. doi:10.1002/anie.201505069
31. Kurabachew M, Lu SH, Krastel P, Schmitt EK, Suresh BL, Goh A, Knox JE, Ma NL, Jiricek J, Beer D et al (2008) Lipiarmycin targets RNA polymerase and has good activity against multidrug-resistant strains of *Mycobacterium tuberculosis*. J Antimicrob Chemother 62:713–719. doi:10.1093/jac/dkn269
32. Lai K, Selinger DW, Solomon JM, Wu H, Schmitt E, Serluca FC, Curtis D, Benson JD (2013) Integrated compound profiling screens identify the mitochondrial electron transport chain as the molecular target of the natural products manassantin, sesquicillin, and arctigenin. ACS Chem Biol 8:257–267. doi:10.1021/cb300495e
33. LaMarche MJ, Leeds JA, Amaral A, Brewer JT, Bushell SM, Deng G, Dewhurst JM, Ding J, Dzink-Fox J, Gamber G et al (2012) Discovery of LFF571: an investigational agent for *Clostridium difficile* infection. J Med Chem 55:2376–2387. doi:10.1021/jm201685h
34. LaMarche MJ, Leeds JA, Dzink-Fox J, Gangl E, Krastel P, Neckermann G, Palestrant D, Patane MA, Rann EM, Tiamfook S et al (2012) Antibiotic optimization and chemical structure stabilization of thiomuracin A. J Med Chem 55:6934–6941. doi:10.1021/jm300783c
35. Leeds JA, Sachdeva M, Mullin S, Dzink-Fox J, Lamarche MJ (2012) Mechanism of action of and mechanism of reduced susceptibility to the novel anti-*Clostridium difficile* compound LFF571. Antimicrob Agents Chemother 56:4463–4465. doi:10.1128/AAC.06354-11
36. Leeds JA, Schmitt EK, Krastel P (2006) Recent developments in antibacterial drug discovery: microbe-derived natural products–from collection to the clinic. Expert Opin Investig Drugs 15:211–226. doi:10.1517/13543784.15.3.211
37. Leong FJ, Li R, Jain JP, Lefevre G, Magnusson B, Diagana TT, Pertel P (2014) A first-in-human randomized, double-blind, placebo-controlled, single- and multiple-ascending oral dose study of novel antimalarial Spiroindolone KAE609 (Cipargamin) to assess its safety, tolerability, and pharmacokinetics in healthy adult volunteers. Antimicrob Agents Chemother 58:6209–6214. doi:10.1128/AAC.03393-14

38. Mackinnon AL, Paavilainen VO, Sharma A, Hegde RS, Taunton J (2014) An allosteric Sec61 inhibitor traps nascent transmembrane helices at the lateral gate. Elife 3:e01483. doi:10.7554/eLife.01483
39. Mali P, Esvelt KM, Church GM (2013) Cas9 as a versatile tool for engineering biology. Nat Methods 10:957–963. doi:10.1038/nmeth.2649
40. Mannick JB, Del Giudice G, Lattanzi M, Valiante NM, Praestgaard J, Huang B, Lonetto MA, Maecker HT, Kovarik J, Carson S et al (2014) mTOR inhibition improves immune function in the elderly. Sci Transl Med 6:268ra179. doi:10.1126/scitranslmed.3009892
41. Morris RP, Leeds JA, Naegeli HU, Oberer L, Memmert K, Weber E, LaMarche MJ, Parker CN, Burrer N, Esterow S et al (2009) Ribosomally synthesized thiopeptide antibiotics targeting elongation factor Tu. J Am Chem Soc 131:5946–5955. doi:10.1021/ja900488a
42. Mullane K, Lee C, Bressler A, Buitrago M, Weiss K, Dabovic K, Praestgaard J, Leeds JA, Blais J, Pertel P (2015) Multicenter, randomized clinical trial to compare the safety and efficacy of LFF571 and vancomycin for *Clostridium difficile* infections. Antimicrob Agents Chemother 59:1435–1440. doi:10.1128/AAC.04251-14
43. Nyfeler B, Hoepfner D, Palestrant D, Kirby CA, Whitehead L, Yu R, Deng G, Caughlan RE, Woods AL, Jones AK et al (2012) Identification of elongation factor G as the conserved cellular target of argyrin B. PLoS ONE 7:e42657. doi:10.1371/journal.pone.0042657
44. Payne DJ, Gwynn MN, Holmes DJ, Pompliano DL (2007) Drugs for bad bugs: confronting the challenges of antibacterial discovery. Nat Rev Drug Discov 6:29–40. doi:10.1038/nrd2201
45. Petersen F (2006) Natural product research at Novartis Pharmaceuticals - a historical overview. In: Engel G, Herrling P (eds) Exploring the frontiers—in celebration of Albert Hofmann's 100th birthday. Schwabe, Basel, pp 28–73
46. Powers MV, Clarke PA, Workman P (2008) Dual targeting of HSC70 and HSP72 inhibits HSP90 function and induces tumor-specific apoptosis. Cancer Cell 14:250–262. doi:10.1016/j.ccr.2008.08.002
47. Roemer T, Davies J, Giaever G, Nislow C (2012) Bugs, drugs and chemical genomics. Nat Chem Biol 8:46–56. doi:10.1038/nchembio.744
48. Rottmann M, McNamara C, Yeung BK, Lee MC, Zou B, Russell B, Seitz P, Plouffe DM, Dharia NV, Tan J et al (2010) Spiroindolones, a potent compound class for the treatment of malaria. Science 329:1175–1180. doi:10.1126/science.1193225
49. Sadlish H, Galicia-Vazquez G, Paris CG, Aust T, Bhullar B, Chang L, Helliwell SB, Hoepfner D, Knapp B, Riedl R et al (2013) Evidence for a functionally relevant rocaglamide binding site on the eIF4A-RNA complex. ACS Chem Biol 8:1519–1527. doi:10.1021/cb400158t
50. Santhosh S, Kumar P, Ramprasad V, Chaudhuri A (2015) Evolution of targeted therapies in cancer: opportunities and challenges in the clinic. Future Oncol 11:279–293. doi:10.2217/fon.14.198
51. Sasse F, Steinmetz H, Schupp T, Petersen F, Memmert K, Hofmann H, Heusser C, Brinkmann V, von Matt P, Hofle G et al (2002) Argyrins, immunosuppressive cyclic peptides from myxobacteria. I. Production, isolation, physico-chemical and biological properties. J Antibiot 55:543–551
52. Schirle M, Bantscheff M, Kuster B (2012) Mass spectrometry-based proteomics in preclinical drug discovery. Chem Biol 19:72–84. doi:10.1016/j.chembiol.2012.01.002
53. Schlecht R, Scholz SR, Dahmen H, Wegener A, Sirrenberg C, Musil D, Bomke J, Eggenweiler HM, Mayer MP, Bukau B (2013) Functional analysis of Hsp70 inhibitors. PLoS ONE 8:e78443. doi:10.1371/journal.pone.0078443
54. Schmitt EK, Riwanto M, Sambandamurthy V, Roggo S, Miault C, Zwingelstein C, Krastel P, Noble C, Beer D, Rao SP et al (2011) The natural product cyclomarin kills *Mycobacterium tuberculosis* by targeting the ClpC1 subunit of the caseinolytic protease. Angew Chem Int Ed Engl 50:5889–5891. doi:10.1002/anie.201101740
55. Singh R, Sharma M, Joshi P, Rawat DS (2008) Clinical status of anti-cancer agents derived from marine sources. Anticancer Agents Med Chem 8:603–617
56. Spillman NJ, Allen RJ, McNamara CW, Yeung BK, Winzeler EA, Diagana TT, Kirk K (2013) Na(+) regulation in the malaria parasite *Plasmodium falciparum* involves the cation ATPase PfATP4 and is a target of the spiroindolone antimalarials. Cell Host Microbe 13:227–237. doi:10.1016/j.chom.2012.12.006
57. Swinney DC, Anthony J (2011) How were new medicines discovered? Nat Rev Drug Discov 10:507–519. doi:10.1038/nrd3480
58. Trotter JF, Lizardo-Sanchez L (2014) Everolimus in liver transplantation. Curr Opin Organ Transpl 19:578–582. doi:10.1097/MOT.0000000000000127
59. Waller CF (2014) Imatinib mesylate. Recent Results Cancer Res 201:1–25. doi:10.1007/978-3-642-54490-3_1
60. White NJ, Pukrittayakamee S, Phyo AP, Rueangweerayut R, Nosten F, Jittamala P, Jeeyapant A, Jain JP, Lefevre G, Li R et al (2014) Spiroindolone KAE609 for *falciparum* and *vivax* malaria. N Engl J Med 371:403–410. doi:10.1056/NEJMoa1315860
61. Yeung BK, Zou B, Rottmann M, Lakshminarayana SB, Ang SH, Leong SY, Tan J, Wong J, Keller-Maerki S, Fischli C et al (2010) Spirotetrahydro beta-carbolines (spiroindolones): a new class of potent and orally efficacious compounds for the treatment of malaria. J Med Chem 53:5155–5164. doi:10.1021/jm100410f
62. Zeisel MB, Lupberger J, Fofana I, Baumert TF (2013) Host-targeting agents for prevention and treatment of chronic hepatitis C: perspectives and challenges. J Hepatol 58:375–384. doi:10.1016/j.jhep.2012.09.022