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Published in:

Future Medicinal Chemistry

DOI:

[10.4155/fmc-2017-0273](https://doi.org/10.4155/fmc-2017-0273)

Publication date:

2018

Citation for published version (APA):

Baptista, R., Bhowmick, S., Nash, R. J., Baillie, L., & Mur, L. (2018). Target discovery focused approaches to overcome bottlenecks in the exploitation of antimycobacterial natural products. *Future Medicinal Chemistry*, 10(7). <https://doi.org/10.4155/fmc-2017-0273>

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Target discovery focused approaches to overcome bottlenecks in the
exploitation of anti-mycobacterial natural products

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Abstract

Tuberculosis is a major global health hazard. The search for new anti-mycobacterials has focused on such as screening combinatorial chemistry libraries or designing chemicals to target pre-defined pockets of essential bacterial proteins. The relative inefficiencies of these have led to a reappraisal of natural products for new anti-mycobacterial drug leads. However, progress has been limited, we suggest through a failure in many cases to define the drug target and optimise the hits using this information. We highlight methods of target discovery needed to develop a drug into a candidate for clinical trials. We incorporate these into suggested analysis pipelines which could inform the research strategies to accelerate the development of new drug leads from natural products.

Keywords: Tuberculosis, target discovery, natural products.

1. Natural Products: Moving back to the forefront of anti-mycobacterial target discovery

Tuberculosis (TB) is the world's leading cause of death from infectious disease, causing an estimated 1.4 million deaths in 2015[1]. This effectively represents a reverse of trends seen during the middle years of the 20th Century when antibiotics, developed from the 1940s on, appeared to be effective in controlling the disease. However, over the last two decades TB is again a major public health hazard with the appearance of drug-resistant strains of TB (DR-TB), multidrug-resistant TB (MDR-TB), extensively drug-resistant TB (XDR-TB) and more recently strains resistant to all the anti-tubercular chemotherapies[2]. This situation has arisen for a great extent due to the complex drug therapy regime used for TB which reduces patient compliance and adherence to prescribed medication. Thus, anti-tubercular chemotherapy consists of at least a 6-month drug regimen involving an initial two month phase of four agents (isoniazid, rifampicin, pyrazinamide and ethambutol) followed by an additional four months with isoniazid and rifampicin[3,4].

MDR-TB is resistant to rifampicin and isoniazid, the most effective anti-tuberculous drugs. XDR-TB strains are resistant to at least rifampicin and isoniazid but also fluoroquinolones and to, at least, one of the injectable drugs; capreomycin, kanamycin or amikacin. Treatment of MDR-TB consists of a 2-year therapy with a combination of four to six first- and second-line anti-tubercular drugs[5]. TB control is therefore contingent on the development of new drugs and in the past decade there have been major efforts made in this area. There is also a need for new drugs that act quickly, giving fewer opportunities for the TB-causing organism to develop resistance. Any new drugs also need to be inexpensive to produce, so that it can be widely adopted in countries outside of the first world. Due to renewed research and development efforts, bedaquiline, a diarylquinolone, became the first anti-TB drug approved by the United States Food and Drug Administration (FDA) in more than 40 years.[6] Additionally, innovative methodologies have allowed the development of more effective therapies, the 'revitalization' of old drugs, re-use of drugs in different contexts, and the reduction of drugs rejected due to their toxicity profile. A vitally important aspect of research leading to new drugs or use of existing drugs is a requirement to fully comprehend, at least as far as possible, their

mechanisms of action and, this way, prevent problems with toxicity at the early stages of clinical trials.

In the early part of the last century, research into natural products was prominent in the search for new drug leads. Indeed, the attractiveness of natural products is obvious considering they reflect the outcome of evolutionary pressures acting on genomes to produce bioactives to serve many roles including anti-microbial[7]. Streptomycin, isolated from actino-bacterium *Streptomyces griseus*, is an important example and on first introduction was as a standard treatment against TB. However, with the emergence of DR-TB and its increased prevalence with the onset of human immunodeficiency virus/acquired immune deficiency syndrome (HIV/AIDS), streptomycin has been replaced by other therapies. Streptomycin is also problematic due to its oto- and nephrotoxicity with prolonged therapies.[8] With the advent of combinatorial chemistry and high-through-put screening strategies, drug discovery focusing on natural products became less popular. It was thought that screening vast combinatorial libraries would speed up the development process by eliminating lengthy natural product identification and often complex synthesis programmes; the poor hit rate though of the combinatorial approach and low diversity of the libraries led to most big Pharma companies abandoning this approach and looking for new and better ways to explore natural products. As a result of the combinatorial library focus, very few antibiotics, derived or based on natural products, had been approved in the past two decades by the FDA.

The importance of a good anti-mycobacterial therapy does not reside only on drug potency and safety, but also in some key features that targets should display. Kana et al. defines the “ideal” target as a macromolecule that 1) acts in the growth, survival and latency of the bacteria, 2) regulates crucial checkpoints of bacterial metabolism, 3) has a low tolerance for mutation and 4) is found at targetable locations within the cell. Additionally, the drug target must not show such similarity with human homologues enough to compromise the drug safety[9]. To meet such requirements many groups favour an approach where drugs are derived to known mycobacterial proteins. Besides avoiding the possibility of new, unexpected targets being defined, this approach could be flawed since it does not

consider the cell environment as essential to the drugs mode of action. Thus, several details are not evaluated; for example the pharmacokinetics of drug internalization by the mycobacteria, innate resistance mechanisms (e.g. efflux pumps) and the physico-chemical properties of both bacteria and compound. Consequently, potent inhibitors of a specific target can display unsatisfactory values of *in vitro* minimum inhibitory concentration (MIC) against *Mycobacterium* species[10]. One example of target-based drug discovery that has recently failed is isocitrate lyase, a key enzyme within the glyoxylate shunt pathway. This enzyme was suggested as a suitable target since it is essential to the growth and virulence of *M. tuberculosis* in both acute and chronic phases of infection.[11] This work was discontinued most likely to the target active site being “shallow” and lack of hydrophobic patches that could be targets for inhibitors[12]. None of the drugs that are currently in clinical trials have arisen following a target-to-drug approach instead these have tended to follow drug-to-target strategies based on whole cell-based screenings[13].

It is against this background that natural product discovery based on whole-cell screenings have become a viable strategy to provide druggable molecules. However, this strategy should be combined with innovative ‘-omics’ technologies, chemical biology and genetics which have made possible the discovery of new drug and target leads[14]. This is especially useful in the search for new agents against *M. tuberculosis* where natural products have assumed a prominent role[15]. The potential of natural products as anti-mycobacterial drug leads lies in their intrinsic cell permeability and structural, functional and stereochemical diversity, that provide unique scaffolds for further drug optimisation towards increased potency and selectivity[16,17]. Moreover, new natural products could be a useful tool to unveil the role of several proteins which are transcribed from the *M. tuberculosis* genome but whose functions are unknown[18,19].

These points notwithstanding, the recent failures to take forward natural products or their derived synthetics needs to be recognised and the underlying causes identified. We suggest that these arise through incomplete workflow methodologies which massively affect further development of an anti-tubercular drug. A cursory glance at the literature will show many examples of anti-mycobacterial

activity being detected in a natural product but these observations are not further characterised and can be thought of as effectively useless from the perspective of defining a new drug lead. In particular, the unequivocal identification of the biological target coupled with a clear mechanism of action of the bioactive is often not done[20]. This is essential to allow the reduction in the number of microbial “off-targets” and toxicity to human systems and so leading to the development of potent and safe drugs against *M. tuberculosis*.

This review will focus on anti-mycobacterial target discovery methodologies and highlight examples of “best practice” where natural product research has included optimisation of target identification and validation steps [21] (Table 1). In doing so, it will identify potential pipelines for target discovery and validation which could inform research strategies based on natural products. For readers wishing to consider only the discovery of anti-mycobacterial natural products we recommend the following review articles[22–24].

Table 1: Methods used for the targeting and optimisation of anti-mycobacterial drug leads derived from natural products.

Natural product	MIC (mM)	Target	Target discovery strategy						Drug optimisation	
			Biochemical approaches	Whole-cell based screening	Mutant generation and WGS	Affinity probes and proteomics	Genomic library screenings	Crystal structure	Analogues synthesis and SAR	Docking studies
Agelasine D	1.98 a)	BCG3185c					X			
Agrimophol	12.5	Rv3852		X		X				
Berberine	74.3	Ftsz	X					X	X	
Brunsvicamides	b)	MptpB	X	X						
Cyclomarin A	0.30	ClpC1		X		X				
Ecumicin	0.14	ClpC1		X	X	X		X		
Halicyclamine A	13.5	DedA					X			
Lassomycin	0.41 - 1.65	ClpC1	X	X					X	
Pyridomycin	0.72	InhA		X	X			X		
Thiolactomycin	118.9	KasA, KasB	X	X				X	X	
Trichoderin	0.10	ATP synthase					X			

a) Against *M. smegmatis*.

b) IC₅₀ against MptpB was 7.3-8 µM.

2. Methodological options for target discovery

2.1. Biochemical approaches

The “classical” approach to target discovery and validation aims to assess biochemical differences arising following treatment with the natural product. Such change could arise from the perturbation of enzymatic activities and these approaches can give valuable insights of the mode of action and target. Putative mycobacterial targets that have been explored using this strategy can be classified according to the pathway they affect. Thus, notable biochemical targets have proven to be in peptidoglycan biosynthesis, arabinogalactan and lipoarabinomanan biosynthesis, mycolic acids biosynthesis, shikimate pathway, pantothenate biosynthesis, biotine pathway, glyoxylate shunt pathway and DNA metabolism[25,26].

Scientists following such biochemical approaches have provided some essential insights to a robust, multifaceted pipeline that can yield some potentially druggable anti-mycobacterials. Thiolactomycin (TLM) (Fig.1) was isolated from *Nocardia sp.*, and displays a MIC of 25 µg/mL against *M.*

tuberculosis[27]. Despite the quite high MIC, TLM's physicochemical properties, its low cytotoxicity in *ex vivo* macrophage models and high *in vivo* availability, are features that encouraged its further development[27–29]. Several studies have revealed that both β -ketoacyl-ACP synthases I / II and acetyl coenzyme-A (CoA) were targets of TLM in *E. coli*[30]. Based on the incorporation of radiolabelled isotopes into fatty acids and mycolic acid metabolism, it was concluded that TLM reversibly inhibits the synthesis of both fatty acids and mycolic acids with a high selectivity (at 0.75 $\mu\text{g/mL}$) for Fas-II β -ketoacyl-ACP synthase[31]. Kremer et al. over-expressed KasA and KasB, two synthases belonging to the Fas-II family, to validate these as targets of TLM[32]. Several analogues of TLM, either in racemic mixtures or as pure enantiomers, have been synthesised and tested *in vitro* against *M. tuberculosis*[33]. When considered together, these TLM analogues will contribute to structure-activity relationship studies, vital for TLM optimisation. Thus, a dataset of 40 TLM analogues, β -ketoacyl-ACP synthase III (mtFabH) inhibitors were subjected to a 3D-quantitative structure-activity relationship (3D-QSAR) analysis. The derived QSAR model and associated docking studies is informing our understanding of the mechanism of TLM as a mtFabH inhibitor[34].

Brunsvicamides are cyclic hexapeptides that were first isolated from the cyanobacterium *Tryphonema*. These, especially Brunsvicamide B and C (Fig. 1), were shown to target protein tyrosine phosphatase B (MtpB) which is an essential virulence factor of mycobacterial species. The activity of these compounds were successfully assessed against a range of protein phosphatases where MtpB showed an IC_{50} of 7.3 μM [35]. The characterisation of MtpB as the target directly led to the identification of novel synthetic inhibitors of this protein using high throughput screens[36]. These, along with diverse natural variants could provide sufficient chemical diversity to perform SAR studies of MtpB and therefore new treatments for tuberculosis[37].

Another example is berberine (Fig. 1), a natural benzylisoquinoline alkaloid obtained from the plant genus *Berberis*. This was found to be active against *M. smegmatis* and MDR-TB at 25 $\mu\text{g/mL}$ [38]. Studies which included *in silico* molecular docking analysis revealed the target to be a protein called FtsZ[39]. This protein forms a ring to aid in the formation of the septum between dividing bacterial

cells. Additionally proper ordering of the septum is dependent on FtsZ GTPase activity. Berberine was found to inhibit FtsZ ring assembly in a dose-dependent manner by a real-time light-scattering assay and GTPase activity using a GTP hydrolysis assay. The binding between FtsZ and berberine were confirmed by isothermal titration calorimetry. The clear identification of this protein as the berberine target led directly to the derivation of berberine analogues aimed at a structure-activity relationship analysis using 2D- and 3D-QSAR[40,41]. The relative success of these analyses will be an important determinant in the optimisation of berberine as a potential drug.

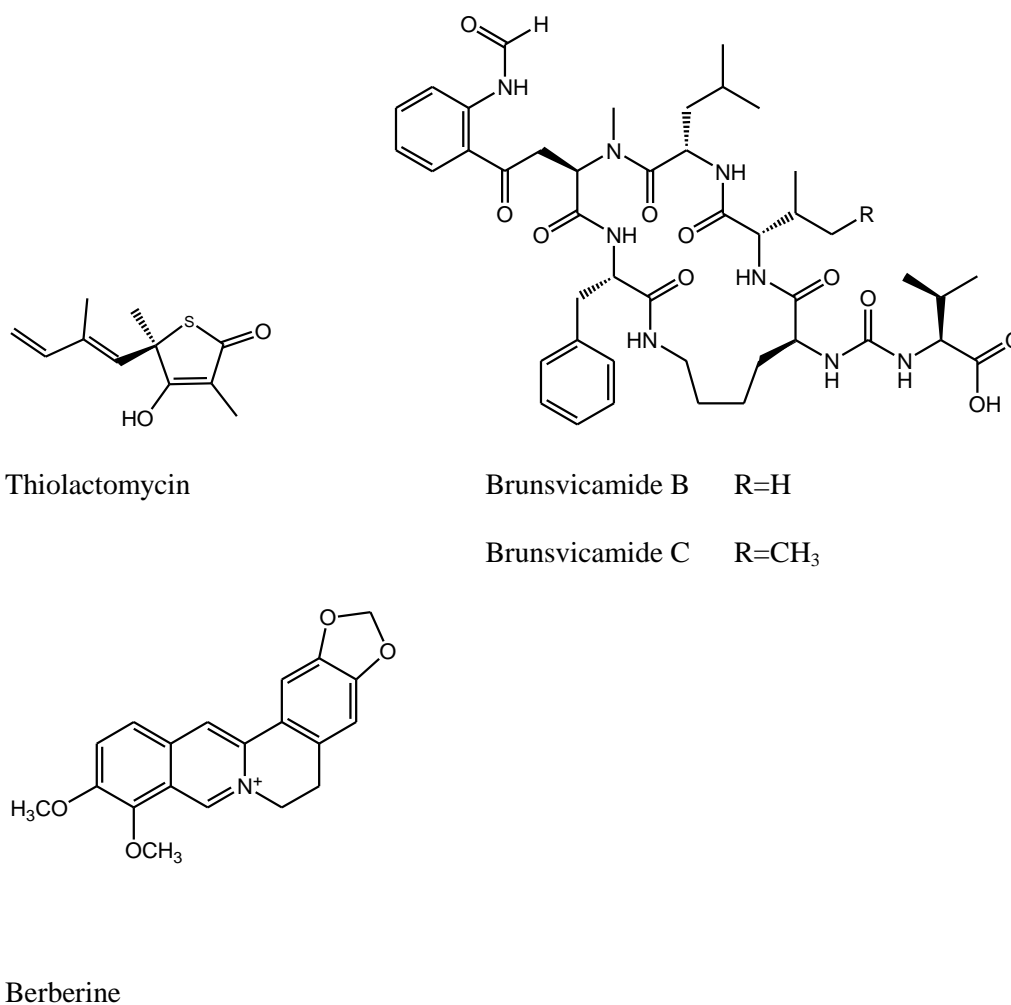


Fig. 1. Chemical structures of thiolactomycin, brunsvicamide B and C and berberine.

2.2. Genomic-based approaches

Some of the more recent approaches used in drug target discovery take advantage of the great advances in genome sequencing capabilities. These encompass a wide variety of approaches including transcriptomic analyses and DNA-protein binding studies based on chromatin immunoprecipitation (ChIP). In this approach antibodies are used to enrich for particular chromatin components and the relative amount of associated DNA sequences can reveal epigenetic control points[42].

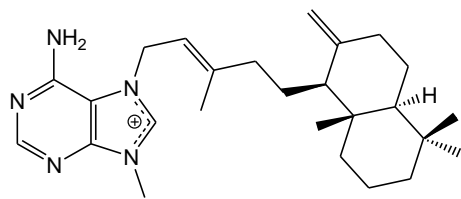
Considering genomic approaches to search for anti-mycobacterials targets, some are based on screening genomic libraries for clones that could confer tolerance when expressed in heterologous hosts. This methodology was used to identify the targets for halicyclamine A, trichoderin A and agelasine D, three natural products derived from the marine sponges *Haliclona* sp. 05A08, *Trichoderma* sp. and the genus *Agelas*, respectively (Fig. 2)[43–45]. Genomic cosmid libraries were constructed from strains of *M. smegmatis* and *M. bovis* BCG which exhibited spontaneous resistance to one of the above compounds and each cosmid was introduced into a susceptible strain. Cosmids with derived alleles that are mutated in the target(s) could increase the minimum inhibitory concentration (MIC) required to affect the recipient mycobacterial strain. By following this strategy, DedA was identified as the target of halicyclamine A and BCG3185c as the target for agelasine D[46,47], but unfortunately, these results were not validated by other approaches. In contrast, cosmid screening suggested that trichoderin A targeted ATP synthase, which was validated by measurements of the ATP content in *M. bovis* BCG[48]. The first total synthesis of trichoderin A has recently been described and this will be essential in structure-activity relationship (SAR) studies[49].

Moving beyond cosmid library screening, whole-genome sequencing (WGS) analyses are assuming a very significant role in target discovery by (for example) identifying distinctive genes present only within *Mycobacterium*. However, this powerful approach needs to be carefully used as suggested targets can be intractable, non-essential or undrugable[50]. Thus, we suggest that the genomic information should be used within a combined methodology pipeline complemented by validation techniques based on target discovery on tuberculosis[13]. This pipeline could be as follows. Firstly,

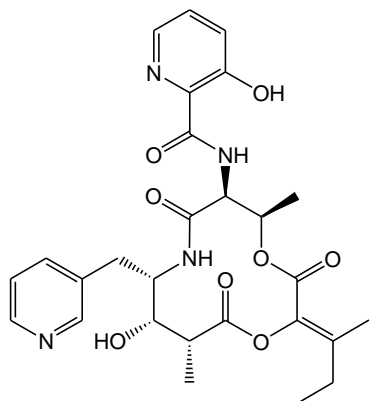
the MIC must be confirmed using whole mycobacterial cell based *in vitro* screens. These will inform what concentrations of natural target to use in suggestive rounds of culturing with the aim of deriving variants which exhibit resistance. Such resistance could arise from target over-expression or deletion, transcriptional or translational disruption or mutation of a key motif. Bacterial DNA from mutants that are resistant to high concentrations of the specific molecule is extracted and sequenced, aiming at confirmation of the mutational site[51].

This seemingly straight-forward strategy may be confounded by the up-regulation of bacterial defence mechanisms such as efflux pumps. Indeed, increased activity of efflux pumps may be a bacterial response to the xenometabolite, rather than direct genetic alteration of operons encoding mutations in the efflux pumps. In this context, it is relevant that antibiotic resistance clinical isolates do not show any mutations in the genes and regulators of bacterial efflux systems. As a result the efflux pumps can provide “false-positives” i.e. strains where there have been no mutations events linked to the bioactive target. Thus, Ioerger et al. suggested the inclusion of an efflux-pump inhibitor then screening for mycobacterial resistance to any bioactive[51]. Suitable efflux-pump inhibitors could be natural products such as reserpine or verapamil[52].

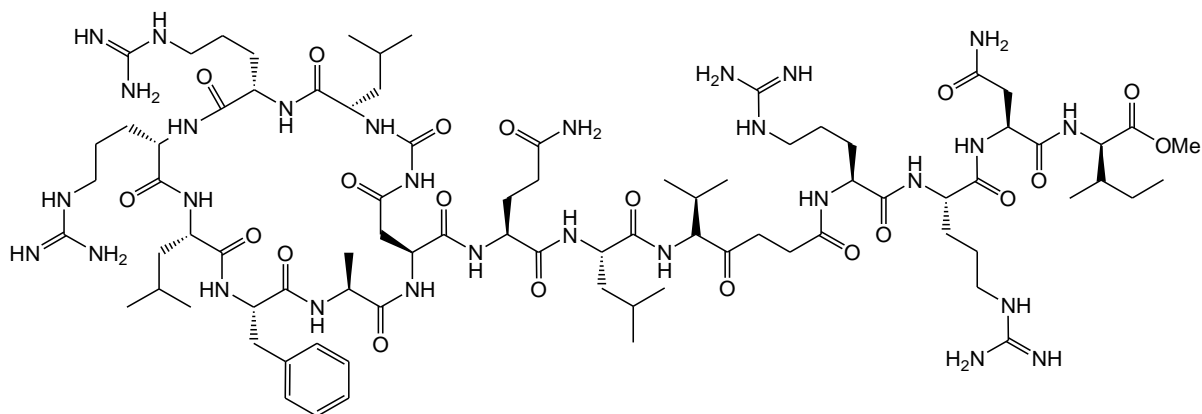
The discovery of the recent natural product lead against TB, pyridomycin (Fig. 2), is an example of a complete and validated target discovery based research, which included genomic analyses. Pyridomycin, was isolated from *Dactylosporangium fulvum* and *Streptomyces pyridomyceticus*, and exhibited a MIC of 0.39 µg/mL against *M. tuberculosis*. Hartkoorn and his colleagues identified InhA, a NADH-dependent enoyl-Acyl-Carrier-Protein as the principal target of pyridomycin following WGS of a resistant mutant colony of *M. tuberculosis*. Although InhA is also the target of isoniazid, pyridomycin behaves as a competitive inhibitor of this protein as shown by its activity against isoniazid-resistant clinical isolates. To validate the target, wild-type *inhA* and the mutant form was over-expressed in *M. tuberculosis* H37Rv and this allowed the steady state kinetics and susceptibility to isoniazid and pyridomycin to be accurately measured[53].



Agelasine D



Pyridomycin



Lassomycin

Fig. 2. Chemical structures of halicyclamine A, trichoderin A, agelasine D, pyridomycin and lassomycin.

2.3. Proteomic approaches

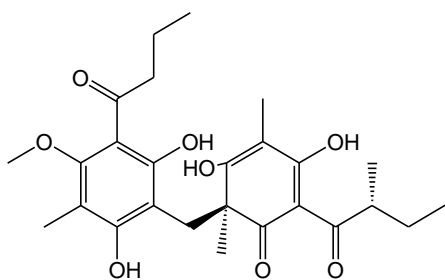
In situ identification of protein-molecule interactions is another valuable tool for the discovery of targets in a native cellular environment. These are based on advances in organic chemistry that have permitted the detection of target and off-target interactions by the modification of key “activatable” groups of active molecules[55,56]. Chemical proteomics is based on the manipulation of a natural product of interest with a tag, normally a terminal alkyne, azide or cyclopropene, which will react via bio-orthogonal ‘click’ chemistry reaction with a fluorophore or affinity label. This reaction will occur after cells, previously incubated with the natural product, are lysed. These terminal groups are small enough to not interfere negatively with the molecular binding between the target and the reactive site on the natural product. Biotin or a fluorophore can be directly attached to the natural product, instead of the tag, if permitted by their reactivity. The result of the reaction between the natural product and the fluorophore or affinity label can be analysed by SDS-PAGE with in-gel fluorescence or LC-MS proteomics[57].

An affinity-based method was used to discover the target of agrimophol (Fig. 3), a phloroglucinol from *Agrimonia pilosa*, which is a plant used in traditional Chinese medicine. Interestingly, this strategy was followed when no agrimophol-resistant mutants were obtained. Whole-cell based screens against *M. tuberculosis* found that agrimophol was able to inhibit pH homeostasis. A click-chemistry reaction was carried out to produce a biotinylated triazole and proteins were fractionated by SDS-PAGE. Following screening with the pre-biotinylated probe a1b, the *M. tuberculosis* protein Rv3852; whose function is unclear, was suggested as the target of agrimophol; a conclusion that was further validated by protein mass fingerprinting[58]. A similar methodology was used to discover the target of cyclomarin A (Fig. 3) produced by a marine bacterium (*Streptomyces* sp.)[59]. This is a cyclic peptide, identified from a whole-cell screening, and has MICs of 0.3 and 2.5 μM against *M. tuberculosis* in culture broth and in human-derived macrophages, respectively[59]. In attempting the identification of the cyclomarin A target in *M. tuberculosis*, no spontaneous resistant mutants could be isolated, Therefore, a proteomic approach appeared to be an appropriate alternative method. Here, cyclomarin A was linked to sepharose beads and co-incubated with a *M. bovis* BCG cell lysate to

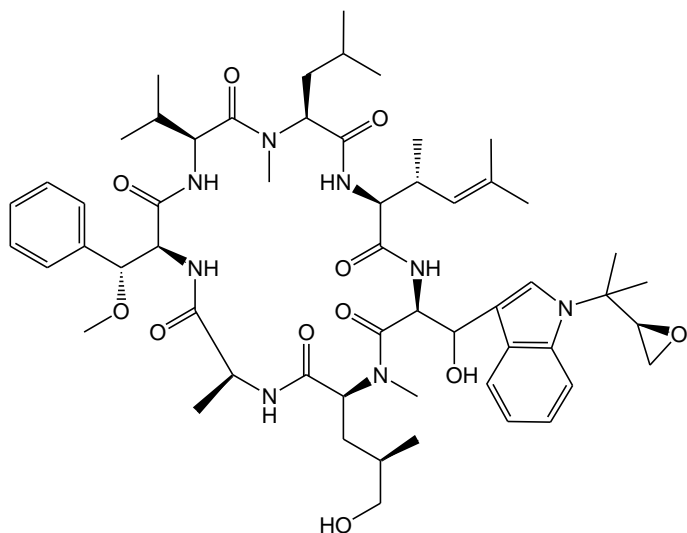
enrich for the interacting target. The mixture was then separated using SDS-PAGE gels to reveal ClpC1 was the major cyclomarin A interactor[59]. With the target now defined follow on studies revealed the co-crystal structure of the complex cyclomarin A-ClpC1. This will be indispensable to the design and synthesis of new anti-mycobacterials that target ClpC1[60].

Another strategy that can be used to identify targets from natural products is DARTS (drug affinity responsive target stability). It is based on the protective action against proteolysis conferred through target-protein interactions. Comparison of the degradation patterns in natural product interacting (“protected”) samples versus “unprotected” controls can be used to suggest protein targets. This technique is known to be fast and avoids any costly structural modifications on the natural product as demanded in the techniques described previously.

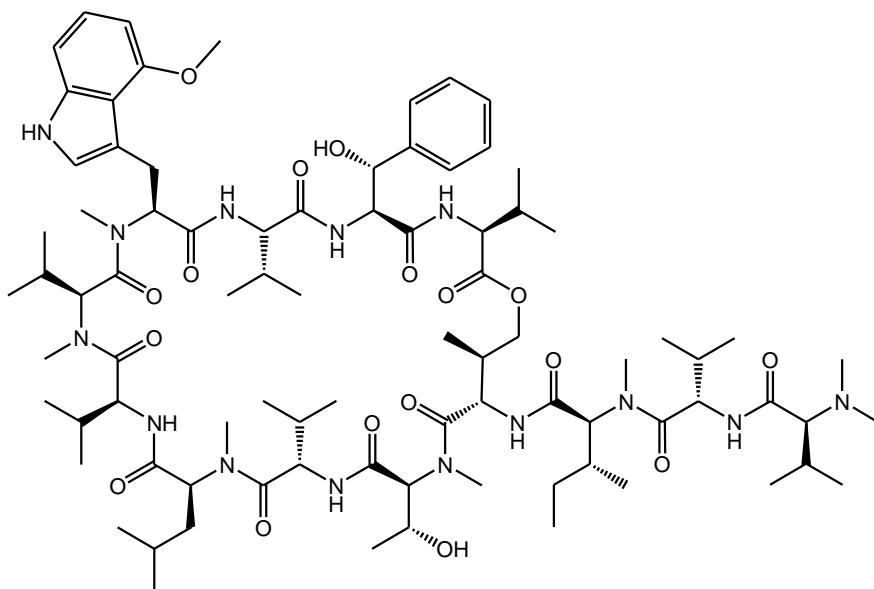
Ecumicin (Fig. 3), a macrocyclic tridecapeptide isolated from *Nonomuraea* sp. MJM5123, was discovered following high-throughput screening from different extracts of several actinomycetes.[61] It was highly active against *M. tuberculosis*, (MIC 0.26 µg/mL) and against strains resistant to streptomycin, rifampicin and cycloserine[61] but its further development required definition of its mode of action within the bacteria. Initially, the genomic DNA of ecumicin-resistant strains of *M. tuberculosis* were sequenced and compared with the corresponding wild-type strain. As with agrimophol ClpC1, was one of the targets found and was further validated using a proteomics-based test for drug affinity responsive target stability (DARTS). Thus, ecumicin was able to protect ClpC1 from nonspecific proteolytic degradation in wild-type *M. tuberculosis* but not in the mutated form. Additional validation was made after LC-MS analysis of the excised band. In a logical, and important further step, Gao et al co-crystallised ecumicin docked with ClpC1 which provided crucial information on the protein pocket required for interaction with the natural product and hence its molecular mechanisms of action. This information has facilitated the informed design of synthetic derivatives of ecumicin that could be more effective antibiotics[62].



Agrimophol



Cyclomarin A



Ecumicin

Fig. 3 Chemical structures of agrimophol, cyclomarin A and ecumicin.

3. Computational chemistry strategies to inform and optimise anti-mycobacterial natural products

X-ray crystallography can provide important insights on specific binding between drug and target through high-resolution 3D structural data. For instance, X-rays were used to confirm that N-terminus of ClpCq bound lassomycin via positively charged guanidium groups. Docking studies showed that the N-terminal Gln17 was the major interacting residue through hydrogen binding. Moreover, lassomycin binding was reduced when Gln17 was mutated to Arg- or His[54].

The widespread use of X-ray crystallography has led to a rapid increase in three-dimensional protein structures suitable to use in *in silico* methods for hit identification and lead optimization. In addition to the computational chemistry methodologies that have been successfully applied on natural products to optimise drugs with antimycobacterial activity, other drug design techniques show considerable promise. Fragment-based *de novo* drug design, for instance, has enabled the re-designing of molecules, in a drug-target environment. This has led to the production of more active synthetic molecules based on the original scaffold and interactions[63]. However, this method has never been used in drug design of natural products against tuberculosis targets[64].

Different techniques in computational chemistry now also play important roles in optimisation of a drug where previous research can be considered to only have provided a scaffold for further development. Once again natural products are often not taken forward to this important stage as no target is identified and validated and so will fail in the advancing the druggability of any identified bioactive. Thus, computerised approaches allow the visualisation and analysis of three-dimensional structures of the bioactive molecule and the target protein and their interactions. Based on these, it is possible to use virtual screening approaches to design derivatives, which allow better interaction between target and drug without altering its physical and chemical properties, assuring the drug is effective and not toxic and making synthesis more efficient. This was successfully applied with the generation and screening of new InhA inhibitors *in silico*[65].

Although these approaches are powerful, they seldom can function *ex nihilo* i.e. design a drug based only on knowledge of the protein target pocket. Given this, natural product libraries play an important role in screening as they present a wide-ranging diversity of scaffolds complemented with a range of structural and functional features[66].

4. Future perspective

The examples given above have highlighted several approaches used to exploit natural products as new anti-mycobacterial drugs. Although these present impressive progress, none has yet achieved the status of a commercially available drug. This reflects the relative ease that anti-mycobacterial natural products can be identified but is not matched by strategies where their targets are quickly defined. Without this, the value of a natural product as a drug lead cannot be assessed and, if suitable, further optimised. However, current research has also furnished approaches which can be rationalised into a robust discovery pipeline; often based on the use of the latest genomic and computational platforms (Fig. 4). It should be noted that key steps in the pipeline, target discovery and lead optimisation, encompass many approaches. Particularly with target discovery, adoption of multiple approaches at each step will undoubtedly increase the probability of successful definition of the targets, and not “off-targets” for anti-mycobacterial activity. Therefore, the results of multiple strategies should be complementary and mutually confirmatory. This would also represent an efficient use of resources since the process of obtaining the natural products with their typically low yields, sometimes difficulty in confirming structures and complex/expensive synthetic routes, can impede progress. Given these points, research groups are urged to follow such a strategy to increase the probability of success; i.e. leading to drug candidates. This would allow the power of natural selection acting on natural products to be more effectively exploited in the search for new anti-mycobacterials.

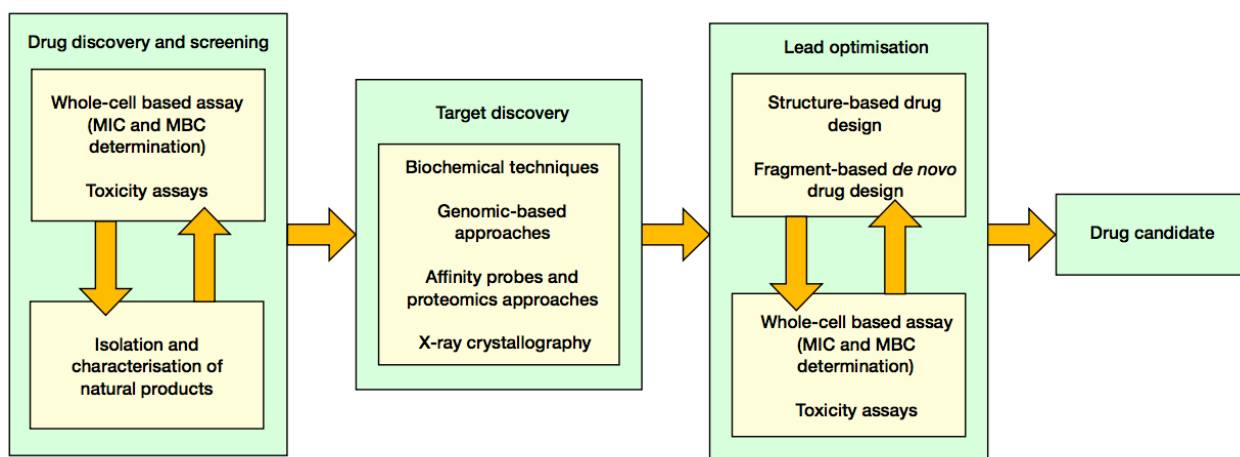


Fig. 4. Strategies for anti-mycobacterial discovery pipeline from natural products. MIC, minimal inhibitory concentration; MBC, minimal bactericidal concentration.

Executive summary

Difficulties of target deconvolution

- High throughput screenings of combinatorial libraries for target discovery have a poor hit rate
- Natural products yield scaffolds that can be used as tools towards novel targets discovery

Target discovery from natural products

- Natural products based research programmes normally do not follow a complete pipeline of target discovery and validation
- Whole-cell based screenings combined with ‘-omics’ technologies make possible the discovery of new targets from new drug leads
- Use of ‘-omics’ allows small amounts of sample to discover of new targets
- Target discovery allows the consequent rational optimisation of compounds or total synthesis *by de novo* techniques

Financial & competing interests disclosure

R.B. would like to acknowledge the Life Sciences Research Network Wales for the PhD Studentship. S.B. is thankful to Aberystwyth University for its AberDoc PhD Scholarship. **IBERS receives strategic funding from the Biotechnology and Biological Sciences Research Council (BBSRC), UK.** R.N. is CEO of Phytoquest

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*of interest; **of considerable interest.

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