Strategies for Drug Target Identification in *Mycobacterium leprae*

Marta Acebrón-García-de-Eulate[1,*], Tom L. Blundell[1] and Sundeep Chaitanya Vedithi[1,*]

[1]Department of Biochemistry,
University of Cambridge,
Tennis Court Road,
Cambridge CB2 1GA, UK.

*Corresponding authors:
Marta Acebrón García de Eulate
Email:ma789@cam.ac.uk
Tel: +441223 333628

Sundeep Chaitanya Vedithi
Email:scv26@cam.ac.uk
Tel: +441223 333628

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Teaser: How to identify new therapeutic targets for Leprosy disease with limited structural data for *Mycobacterium leprae*? Step-by-step approach with a combination of *in silico* and *in vitro* experiments.

Abstract

Hansen's disease, or leprosy, continues to be endemic in many parts of the world. Although, multi-drug therapy is successful in curing a large number of patients, some of them abandon it because it is a long-term treatment. Therefore, identification of new drug targets in *Mycobacterium lepra*e is considered of high importance. Here, we introduce an overview of *in silico* and *in vitro* studies that may be of help in this endeavour. Essentiality of *M. leprae* proteins is reviewed with discussion of flux balance analysis, gene expression and knockout. Finally, druggability techniques are proposed for validation of new *M. leprae* protein targets.

Introduction

Although Gerhard Armauer Hansen's discovery of *Mycobacterium leprae* in 1874 was the first bacterial pathogen identified in man [1], Hansen's disease (HD), or leprosy, continues to be endemic in many parts of the world. According to the World Health Organization (WHO) in 2018, 208613 new cases of HD were diagnosed in 152 countries around the world which include 42 in Africa, 32 in the Americas, 11 in South-East Asia, 18 in the Eastern Mediterranean, 23 in Europe and 26 in the Western Pacific Region. *M. leprae* is an obligate intracellular parasite [2] with affinity for keratinocytes, macrophages and histiocytes in the skin [3], [4], but showing fundamental tropism for both myelinated and non-myelinated Schwann cells [5]. In the skin, *M. leprae* bacilli produce dermatological manifestations. Furthermore, the Schwann cell infection produces nerve dysfunction, generating sensory loss, resulting in disability and deformity [6], [7], leading to social stigma. Although the main reason for the HD prevalence is human-to-human transmission [8], it is necessary to be in continuous contact and only 20% of exposed people will develop the disease [9]. In addition, zoonotic transmission of HD has been described for armadillos, red squirrels, ticks and reduviid bugs [10]–[14].

In the past few decades, multi-drug therapy (MDT) and anti-inflammatory therapies have led to improvements in long-term health outcomes for individuals diagnosed with HD. Dapsone, rifampin, and clofazimine are the first-line drugs in MDT, while the second line includes minocycline, clarithromycin, and ofloxacin/levofloxacin [15], [16]. Although millions of people have been cured with this treatment, a large number still suffer long-term complications including irreversible nerve function impairment and disability. Unfortunately, drug-resistant cases, including those with dapsone, rifampin and ofloxacin-resistant *M. leprae*, have been reported in several parts of the world [5], [17]. Furthermore, the MDT is bactericidal to *M. leprae* but does not reverse the existing nerve damage and in some cases produces acute inflammatory responses (called Type 1 and Type II reactions) that increase the nerve degeneration. Our understanding of the molecular basis of the different diseases caused by *mycobacterial* species has improved in the past decade, which has allowed novel therapeutic opportunities [18], [19]. However, in the case of *M. leprae,* unlike other species, this knowledge has been scarce due to the difficulty of growing the bacilli *in vitro* and the fact that only animal models are currently viable [5]. The full genome of *M. leprae* [20]–[23] was published 3 years later than that of *M. tuberculosis,* the closest species phylogenetically, and the number of

3D structures deposited in Protein Data Bank (PDB) is 12, compared to ~1200 for *M. tuberculosis* .

Here, we present an overview of *in silico* and *in vitro* methods to identify new HD drug targets.

Leprosy genes identified by gene ontology

The first completed *M. leprae* genome, from a strain originally isolated in Tamil Nadu, India and designated TN, was published in 2001 [20]. TN strain genome has 3,268,203 base pairs, comprising only 1614 open reading frames (49.6%), but also 1,310 pseudogenes or noncoding regions (41%). In the other three consensual strains (Thai-53, NHDP63 and Br4923), the number of genes is conserved and the sequence identity between the four strains is higher than 99% [24]. Therefore, *M. leprae* has the largest known pseudogene content among all prokaryotes [25]. Several studies comparing orthologues of different *Mycobacterium* species were performed in order to understand which *M. leprae* genes were silenced. Seventy-five percent of the *M. leprae* pseudogenes were identified in other mycobacterium genomes and were associated with adaptation to a specific environment. *M. leprae* has lost 9 of the 13 σ factors present in the *Mycobacterium* genus, which activate gene transcription under different environmental stresses [26], and alternative carbon and energy pathways (several transferases, oxidoreductases and hydrolases proteins) [20], [27]–[29]. In conclusion the enormous genome reduction is explained by the high evolutionary specification to unique niches, the Schwann cells and macrophages.

A more interesting outcome from the orthologue studies, comparing *M. leprae* with other *Mycobacterium* species, is that 86 *M. leprae* proteins are not present in other *Mycobacterium* genomes. Further genomic ontology function studies in comparison with human genome have been performed and some of these proteins were identified as transmembrane proteins, although their functions are still largely unknown. ML2177c was identified as a probable uridine nucleoside phosphorylase, ML1724 as a putative enoyl-CoA hydratase/isomerase and ML1727 as a putative phosphoserine phosphatase [29]. In summary, the functions of 83 specific *M. leprae* proteins are unknown at present.

Target selection by gene essentiality

Gene essentiality for growth and/or survival is one of the main criteria used for target selection in novel antibacterial drug discovery. *M. leprae* gene essentiality is based on essentiality of homologs, mainly from gene knockout experiments in *M. tuberculosis* [30]–[33]. Moreover, in *M. tuberculosis* high-density transposon mutagenesis libraries are explored in order to identify essential genes [33]. In fact, the *M. leprae* genome has high conservation of saturated transposon binding sites of *M. tuberculosis.* Borah Khushboo *et al.* [34] constructed a genome-scale metabolic *in silico* model of *M. leprae* using flux balance analysis (FBA). They compared reactions that theoretically maximised the biomass production with RNA-seq dataset from *ex vivo M. leprae* grown in mouse footpads. In conclusion, their model projected that *M. leprae* is an obligate intracellular parasite and is not able to grow without nutrients that are obtained from the host.

As a result of these studies, 65% of the total *M. leprae* genes were demonstrated to be essential. The percentage total genome essentiality is higher in *M. leprae* than in other *Mycobacterium* species because of the evolutionary loss of non-essential genes by pseudogenization. *M. leprae* conserves essentiality in the biosynthesis of essential amino acids, except methionine [35], DNA replication, protein synthesis and central carbon and energy pathways. The remaining essential proteins play roles in peptidoglycan biosynthesis, fatty acid biosynthesis, LPS lipid A biosynthesis and extracellular polysaccharide biosynthesis.

Avoiding side effects & off-target activity of novel drugs:

In order to avoid side effects in patients, chosen drug targets should not have homology with either human or human microbiota proteins. In this context the unique mycobacterial cell wall, formed by peptidoglycan, arabinogalactan and mycolic acids, is of interest. Enzymes involved in these pathways do not have homologues in human, therefore drugs that target the pathways have proved successful antibiotics [36]–[38]. Peptidoglycan is part of the cell wall of all gram-negative bacteria, nevertheless arabinogalactan and mycolic acids are specific to *Mycobacteria*. Inhibitors of arabinan synthesis, as such as ethambutol [36], and of the mycolic acid pathway, such as isoniazid and ethionamide [37], [38], are efficient anti-mycobacterial drug therapeutics. A further cell wall component worthy of comment is the phenolic glycolipid-1 (PGL-I) whose sugar

composition is specific to *M. leprae*. In fact, PGL-I is the antigen used for diagnosis in Hansen's disease. PGL-I covers a high percentage of *M. leprae* cell wall and has a very important role in the bacterial virulence as mediator of Schwann cell invasion [39] and evasion of host immune system [7]. Therefore, targeting PGL-I biosynthesis would be specific to *M. leprae*. In fact, the PGL pathway has been targeted in other *Mycobacterial* species [40] and *M. leprae* antibiotics have proven able to give rise to loss of PGL-I production [41].

Enzymes that are able to adapt to low oxygen environments are also those not encoded by human genome, such as *glcB* [42], *oxyR* [43] and *mshC* [44], which are activated for the intracellular survival of *M. leprae*. Ongoing with adaptation to the *in vivo* environment, this characteristic bacterial stringent response is critical for *M. tuberculosis* and *M. leprae* survival, which is regulated by the enzyme RelA (Bifunctional (p)ppGpp synthase/hydrolase RelA) [45]. Another mechanism that is conserved in humans or *E. coli* but not in Mycobacteria is protein synthesis. Gene expression and protein biosynthesis are classic targets for several anti-mycobacterial drugs such as fluoroquinolones [46] and rifampicin [47]. However, possible new targets within these metabolic pathways continue to be explored, for example LeuRS [48] and GyrB [49]. A further pathway, menaquinone biosynthesis, is essential due to it being the only quinone in *M. tuberculosis* and *M. leprae*, while human beings obtain menaquinone from their diet or microbiome [50]. In our group we have performed a comparative chokepoint analyses of *M. tuberculosis, M. abscessus* and *M. leprae* and excluded the chokepoint reactions found in human [51]. Chokepoint enzymes catalyse reactions that have a unique substrate or produce a unique product of the full metabolic network [52]. Inhibiting chokepoint enzymes results in the accumulation of a specific substrate or product, giving rise to cellular toxicity. In a study performed in our group [51] we identified 188 chokepoint enzymes with less than 40% identity to human homologues and demonstrated that 72% of the chokepoint enzymes are essential proteins (Table 1) and 78% of the chokepoint enzymes are present in the three *Mycobacterium* species.

Table 1.

Chokepoint enzymes that are essential (essentiality inferred from homology) [52]	
Metabolic Pathway	**Target genes**
Biosynthesis of essential amino acids	argH, hisA, hisE, hisI, hisS, ilvB, ilvC, ilvD, ilvE, leuB, leuS, lysS, thrC
DNA replication	dnaN, dnaZX, nrdE
Protein synthesis	gatA, gatB
Central carbon and energy pathways	aroA, aroB, aroD, aroF, dfp, gmk, hemE, hemH, menD, nadA, nadC, panC, purB, purH, ribF, rmlB, rmlC, rmlD, thiL
Peptidoglycan biosynthesis	murB, murC, murD, murG
Fatty acid biosynthesis	fabG1, inhA
LPS lipid A biosynthesis	glmU
Extracellular polysaccharide biosynthetic	rfbA

Druggability

Target druggability is an important aspect to consider before starting a new drug design project. A druggable target is a protein known to be capable or predicted to be able to bind with high affinity to a drug. For targets that are successful in other species and have a homologue in *M. leprae*, comparison of sequence identity and a structural analysis of the drug-binding site can provide valuable information on the suitability of the target for drug discovery. On the other hand, for new targets, *in-silico* approaches can help to identify potential sites on the protein where a drugs can bind. Computational analyses can provide information on surface area and volume of pockets and cavities where the drug can interact, and determine whether the drug will displace solvent molecules, providing an entropic contribution to the binding free energy to compensate for the loss of rotational

and translational entropy on binding the drug candidate [56]. An example of such an analysis in *M. leprae* is the exhaustive study to calculate the differences in the Dapsone cavity between dihydropteroate synthase mutants [53]. The impact of the cavity/pocket identification can be improved by incorporating fragment hotspot maps analysis [54]. The hotspot maps program provides user-friendly affinity maps that identify potential drug interacting regions (hydrophobic, donor and acceptor regions) within the protein. An example of a target identification and druggability study that is performed on the arginine biosynthetic pathway, where ArgD and ArgH showed interesting pockets with hotspot regions in *M. leprae* and *M. abscessus* [51].

We have applied this *in-silico* approach to identify possible pockets on ketoacyl-ACP reductase (fabG1), which catalyses one of the FAS-II cycle steps involved in mycolic acid biosynthesis. We considered fabG1 as an interesting new target because it is essential, a chokepoint (see Table 1) and it is implicated in PGL-I biosynthesis. Inhibiting PGL-I biosynthesis should have a significant impact on bacterial virulence by restricting Schwann cell invasion by *M. leprae* [40]. MODELLER software [55] was used to model the fabG1 protein of *M. leprae* using PDB id 5ovk as the template (beta-ketoacyl-acyl carrier protein reductase MabA – orthologue in *Mycobacterium smegmatis*). The resultant model is of high-quality with Molprobity Score of 4.74 at 94th Percentile. Fpocket [56] and fragment hotspot maps for fabG1 model were used to explore potential binding pockets/sites (Figure 2). Apparently, fabG1 has very deep pockets but only some with the required juxtaposed hydrophobic, donor and acceptor regions comprising a hotspot where a drug can bind. Interestingly, this year an inhibitor of *M. tuberculosis* FabG1 was published [57]. This paper confirms that following these steps for target identification can effectively guide novel drug discovery projects.

Concluding remarks

Developing a novel drug against *M. leprae* is a significant challenge due the difficulty of growing the bacilli *in vitro* and the fact that only animal models are currently viable [5]. Although multi-drug therapy currently cures a large number of Hansen's disease patients, the treatment is of long duration and adverse reactions during the course of treatment can exacerbate nerve damage. In comparison with other *mycobacterial* species, *M. leprae* is an intracellular parasite with a very specific niche, Schwann cells. Such distinction can help to develop therapies that specifically target *M. leprae*. Leprosy target discovery

requires a combined *in silico* and *in vitro* approach. As the availability of experimental data on structural proteomics of *M. leprae* is scarce, it is necessary to base analyses on data from other *mycobacterial* species, requiring calculation of the gene/protein sequence identity and checking the conserved regions between the different orthologues. The power of bioinformatic methods rely on their ability to screen a vast number of genes and calculate their impacts on bacterial growth and infection. Here, we present a step-by-step target selection for *M. leprae* drug discovery resulting in a table with 45 candidates, some of which are novel mycobacterial targets such as *dnaZX, nrdE, fabG1* and *rfbA*. Most importantly, targets identified will be effective if they are associated with specific features of *M. leprae*, for example, inhibition of enzymes in the PGL-I biosynthesis pathway. Predicting druggability is further recommended in target selection in order to avoid narrow and superficial pockets that would likely not be hotspots and would not bind ligands with high affinity. The application of bioinformatics methods in leprosy target discovery is generating many exciting target leads for further experimental validation.

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Figure legends:

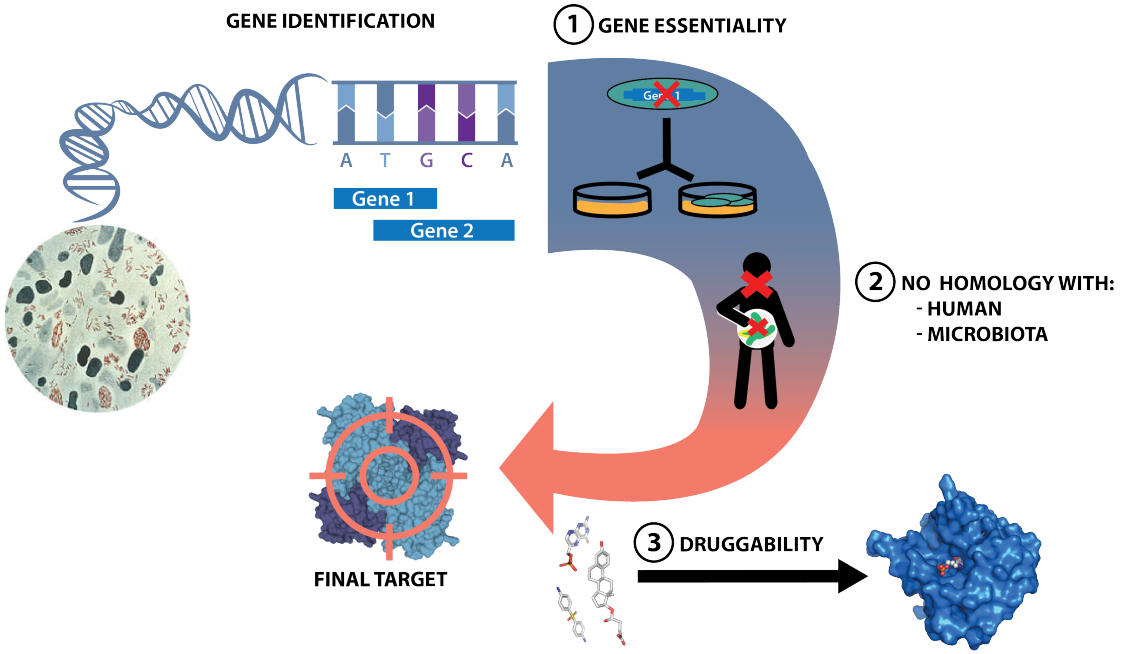

Figure 1. Integration of *in silico* and *in vivo* bioinformatics data contributes to understanding the activity of *Mycobacterium leprae* at a molecular level and identifying novel therapeutic targets. *M. leprae* gene identification by ontology shows that the *M. leprae* genome is different from that of other *mycobacterial* species due to its high genome reduction. Diverse types of data from homolog knockout experiments, flux balance analysis (FBA) and chokepoint analyses can be combined to detect essential genes for the bacteria infection, and to ensure no off-target activity on the host. Various bioinformatics tools can be applied to essential gene products in order to evaluate their druggability and identify new *M. leprae* drug targets.

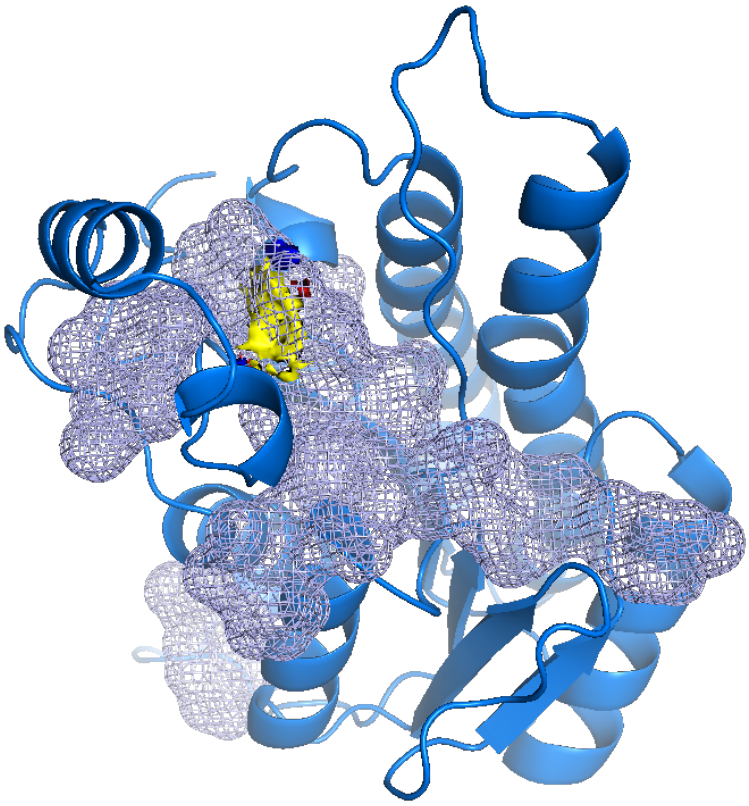

Figure 2. FabG1 Fpocket and hotspot map analysis. The protein binding-site volume as calculated by Fpocket is represented as a light blue mesh. The hydrophobic region of the hotspot is colored yellow, the donor region in blue and that for the acceptor in red.

Author Contributions

SCV carried out the protein modelling of fabG1. MAGDE calculated the cavities and ran the hotspot analyses of candidate proteins. MAGDE wrote the review with contributions of TLB and SCV. All authors have given approval to the final version of the manuscript.

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Abbreviations

fabG1 – β- ketoacyl-ACP reductase

FBA – Flux balance analysis

HD – Hansen's disease

MDT – Multi-drug therapy

PGL-I – Phenolic glycolipid – 1

WHO – World Health Organization