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Strategies for Designing Novel Anti-Tubercular Drugs with Special Reference to Mycobacterial MelF (Rv1936) as a Target

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Abstract

Tuberculosis (TB) is a major public health problem throughout the world. There is an immediate need to device novel anti-tubercular drugs (ATDs). Whole cell screening (WCS) and target based screening (TBS) approaches are widely used to devise new ATDs. WCS is considered relatively a favourable strategy but it lacks in the precise target knowledge, whereas the target based hit compounds may not ensure the drugability. In this manuscript, we have discussed the potent inhibitors designed against the mycobacterial MelF (Rv1936) by using the TBS as well as virtual ligand screening, which also revealed synergistic effect with the first-line bactericidal drugs, *i.e.*, isoniazid and rifampicin.

Keywords: Tuberculosis, WCS, TBS, VLS, MelF, *M. tuberculosis, M. marinum*

Introduction

Tuberculosis (TB) ranks above the human immunodeficiency virus (HIV) infection as the top most killers worldwide among the infectious diseases. There were about 10.4 million new TB cases reported in 2016 worldwide with approximately 1.3 million TB deaths among HIV-negative people and 0.374 million deaths among HIV-positive individuals [1]. Generally, it takes 6 months to cure drug-sensitive TB (DS-TB) and a minimum of 18 months to treat multiple drug resistant TB (MDR-TB). Notably, ~ 5 to 10% of the MDR-TB cases comprise of extensively drug-resistant (XDR)-TB and there are reports when patients are resistant to all the available anti-TB drugs (ATDs), *i.e.* totally drug resistance (TDR)-TB. The U.S Food and Drug Administration has recommended the use of new drugs, *i.e.* bedaquiline and delamanid for individuals diagnosed with MDR-TB, XDR-TB and TDR-TB [2]. However, there is an urgent need to device new ATDs, which shall be effective against all forms of TB, in all individuals and in all locations of the world.

In the post-genomic era, gene sequencing and functional knowledge of various proteins has produced a number of potential drug targets. Favourable drug targets are those that are considered essential for the survival of mycobacteria inside the host cells, but have little or no sequence homology to their human counterparts. The TB structural genomics consortium (TBSGC) is an international alliance of researchers from 15 different countries and 93 research centres, whose main goal is to resolve the 3D structures of *Mycobacterium tuberculosis* proteins by using modern tools for gene cloning, protein expression and structural elucidation [3]. The atomic resolution description of proteins along with the associated substrates or cofactors and the protein-protein interaction networks thus formed can help other researchers in rational structure-based ATDs designing. In addition, the TBSGC actively develops bioinformatics tools, which can help in data mining and that would further complement structural information [3]. The protein structures recently resolved by TBSGC group include chorismate-utilizing enzymes, arginine biosynthesis enzymes, urease and the toxin-antitoxin gene pairs [3].

Computational biology in drug designing

To provide a rapid, cost-effective and advanced drug discovery process, computer aided drug designing (CADD) has been used [6]. Virtual ligand screening (VLS) is an effective tool for *in silico* screening of large compound libraries for obtaining potential leads. Docking algorithms are economical VLS tools (e.g. DOCK, AUTODOCK and Vina), where ligand molecules are docked into the target protein's pockets and this strategy has been successfully used in identifying hits for several mycobacterial targets such as PknG (Rv1827), InhA (Rv1484), MtCM (Rv1885c), MurE (Rv2158c), DHQase (Rv2537c), L-AlaDH (Rv2780), UMPK (Rv2883c), EthR (Rv3855) [3,6,7] etc. Molecular dynamics simulations can be used after VLS for fine-tuning of docked complexes and to obtain binding affinity information [6]. In PA-824 (Pretomanid) development, binding affinities obtained from molecular docking were examined against *M. tuberculosis* bactericidal activity. Docking has also been used to gain insights into key binding interactions within the active site [8]. In fact, PA-824 has recently entered Phase III clinical trials to evaluate the efficacy, safety, tolerability and pharmacokinetics (http://www.newtbdrugs.org). Moreover, the incorporation of drug-like filters can significantly narrow the size of the library to be selected [9]. The CADD approach is supervised by the amount of structural information available followed by biochemical assays to confirm the active hit molecules [10]. Furthermore, the ability to prioritize targets is also important in drug discovery. Recently, a software program known as 'AssessDrugTarget' has been developed [11], which ranks potential drug targets as per indispensability, drugability, epidemiology, and distinct *M. tuberculosis* metabolic signatures, while also taking into consideration the sequence and structural similarity with the other mycobacteria and humans.

Whole-cell /target based screening

Whole-cell screening (WCS) has been shown to be quite successful in giving possible hits, as target based screening (TBS) does not take into consideration the poor penetration and efflux problems. It is suggested that the WCS shall be initially performed to detect the potential hits, followed by TBS to differentiate the mechanism of action of a specific drug candidate [2]. The most successful drug candidate designed through this method is TMC-207 [4]. However, a number of hit compounds of malate synthase inhibitors [5] have been identified through TBS. These compounds were further modified through structure-based drug design and chemical modification. InhA (Rv1484) inhibitors (thiadiazoles) and LeuRS (Rv0041) inhibitors (oxaboroles) have been discovered through biochemical TBS against *M. tuberculosis* (http://www.newtbdrugs.org). Although the method of WCS works well against the growing bacilli, it is not useful for screening non-replicating persisters. In persisting bacteria, there is also no correlation found between the low minimum inhibitory concentration (MIC) and the sterilizing activity e.g. isoniazid (INH), which is highly active against growing bacteria but inactive against the persisters. In contrast, pyrazinamide, which is poorly active against the growing bacteria, but has excellent sterilizing ability against the non-replicating bacteria, would have been missed if the screening had been carried based on MIC values [2]. Owing to the different sub-populations (replicating/non-replicating/persisters) and different *in vivo* growth environments of TB infection, it is important that screens shall be performed against both replicating and non-replicating bacteria and under different growth conditions.

Targeting the mycobacterial MelF protein (Rv1936)

The existence of a *lux*-like operon, *mel2* (Rv1936-1941), in non-bioluminescent human pathogenic mycobacterial species, *i.e.*, *M. tuberculosis* and *M. marinum* suggests that it exerts an important role in mycobacterial pathogenesis [12,13]. Interestingly, this *mel2* locus is absent in *M. bovis*, *M. leprae*, *M. avium*, etc. and the *melF* mutant of *mel2* locus has been found to be more susceptible to reactive oxygen species (ROS) and reactive nitrogen species (RNS) generating compounds in the laboratory medium, thus suggesting its role to resist ROS and RNS stress response [14]. The mutation in *melF* also reveals a polar effect on the downstream genes of *mel2* locus, *i.e. melG*, *melH* and *melK*, etc. [15], as the increased susceptibility of *melF* mutant to ROS/RNS could be partially retrieved by *melF* alone and fully by the entire *mel2* locus. Notably, an *M. marinum melF* mutant was found to be defective for growth in interferon-γ-plus-lipopolysaccharide activated murine macrophages [15]. A deletion of *melF* of *mel2* locus in *M. tuberculosis* also resulted in increased susceptibility to ROS [13] and a slow dissemination of the *mel2* mutants was observed in the infected C57BL/6J mice thus demonstrating that *mel2* plays a critical role in bacterial persistence and dissemination [13].

We recently identified potential inhibitors against *M. marinum* and *M. tuberculosis* targeting *M. marinum* MelF protein of the *mel2* locus [6] so that the ability of *M. marinum* to survive ROS/RNS stress could be reduced. Such inhibitors could significantly reduce the MelF flavin oxidoreductase activity as well as bacterial growth *in vitro* system. A library of over a million compounds was screened using *in silico* VLS to identify inhibitors against the modeled structure of MelF protein. The 3D structure of MelF protein was generated and its geometry-optimized surface scrutiny by multiple methods revealed a deep pocket with very high druggability index. A series of compounds were predicted to dock into the active site and the shortlisted compounds met the Lipinski's rule of 5 for drug-likeliness [9]. The top ranked 1000 inhibitors were further screened to identify 178 inhibitors to maximize the scaffold diversity by manually evaluating the interaction of each compound with the target site. *M. marinum melF* was cloned, expressed and purified as maltose binding protein-tagged recombinant protein in *Escherichia coli*. After establishing the flavin dependent oxidoreductase activity of MelF (~ 82 kDa), the inhibitors were screened for the diminished enzyme activity of whole cell lysate as well as the purified MelF. Amongst these, 16 inhibitors could significantly diminish the enzyme activity of purified MelF. For the six best inhibitors, the MIC was determined to be 3.4-19.4 μM and 13.5-38.8 μM for *M. marinum* and *M. tuberculosis,* respectively. Similarly, the minimal bactericidal concentration was determined to be 6.8-38.8 μM and 27-38.8 μM against *M. marinum* and *M. tuberculosis*, respectively. One inhibitor each in combination with INH also showed synergistic effect against *M. marinum* and *M. tuberculosis*, respectively with no cytotoxicity in HeLa cells. Similarly, two inhibitors showed an enhanced bactericidal effect for *M. tuberculosis* in combination with rifampicin (RIF), with no cytotoxicity in HeLa cells [6]. The mechanism for such synergistic inhibitory effects could be the activation of ROS/RNS by INH/RIF, and that presumably becomes acute with the simultaneous blocking of MelF protein.

Strikingly, mycobacterial dezaflavin-dependent nitroreducase (Ddn, Rv3547) and its homologues Rv1261c and Rv1558 are also delineated to induce protection from the oxidative stress and the bactericidal agents [16], similar to *M. marinum* MelF. Also, the mycobacterial F420-deficient Ddn mutant, defective in the formation of dezaflavin has been reported to be hypersensitive to INH, moxifloxacin and clofazimine, hence suggesting that the inhibitors that reduce Ddn or dezaflavin biosynthesis could synergize with the present ATDs, as seen with MelF inhibitors [6]. Moreover, mycobacterial peroxynitrite redutase/peroxidase function to resist RNS generated by the host cells [17], thus suggesting them to be potential drug targets. In fact, it is postulated that all the bactericidal antibiotics kill bacteria by generating ROS/RNS, and that has been demonstrated for delamanid, PA-824 and clofazimine for their bactericidal effects against *M. tuberculosis* [18,19]. The oxidative stress thus produced increases the susceptibility of mycobacterial cells to existing anti-TB drugs. In a similar manner, Bulatovic and coworkers [20], earlier documented that the oxidative stress could increase the susceptibility of *M. tuberculosis* to INH, which supported our findings that the inhibitors designed against MelF target could synergize with INH [6]. In brief, this study indicates for designing new inhibitors with the synergistic effect by targeting mycobacterial MelF/DDn and that may enhance the competence of present drugs.

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