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DRUG DISCOVERY AND RESISTANCE

Genetic and chemical validation identifies *Mycobacterium tuberculosis* topoisomerase I as an attractive anti-tubercular target

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SUMMARY

DNA topoisomerases perform the essential function of maintaining DNA topology in prokaryotes. DNA gyrase, an essential enzyme that introduces negative supercoils, is a clinically validated target. However, topoisomerase I (Topo I), an enzyme responsible for DNA relaxation has received less attention as an antibacterial target, probably due to the ambiguity over its essentiality in many organisms. The *Mycobacterium tuberculosis* genome harbors a single *topA* gene with no obvious redundancy in its function suggesting an essential role. The *topA* gene could be inactivated only in the presence of a complementing copy of the gene in *M. tuberculosis*. Furthermore, down-regulation of *topA* in a genetically engineered strain of *M. tuberculosis* resulted in loss of bacterial viability which correlated with a concomitant depletion of intracellular Topo I levels. The *topA* knockdown strain of *M. tuberculosis* failed to establish infection in a murine model of TB and was cleared from lungs in two months post infection. Phenotypic screening of a Topo I overexpression strain led to the identification of an inhibitor, thereby providing chemical validation of this target. Thus, our work confirms the attractiveness of Topo I as an anti-mycobacterial target.

1. Introduction

Tuberculosis (TB) is a leading cause of mortality worldwide and accounts for nearly two million deaths each year [1]. The treatment options to control tuberculosis are dwindling because of the increase in the incidence of drug resistant TB cases [2]. Poor patient compliance, and co-morbidities like HIV, diabetes and immunosuppressive treatments, have increased the complexity of TB treatment. In recent years, several new anti-mycobacterial agents have been discovered and are undergoing clinical trials for the treatment of TB [3]. Additionally, new combinations of anti-TB drugs are being developed to reduce the duration of therapy [4]. Given the global burden of multi drug resistant tuberculosis (MDR-TB), it is important to avoid cross-resistance due to existing mechanisms. One approach to overcome this challenge is to develop new drugs with a novel mechanism of action, inhibiting growth via a new, unexploited target or pathway.

Bacterial DNA topoisomerases regulate the level of DNA topological states in the cell. Both DNA supercoiling and relaxation activities, catalyzed by DNA gyrase and Topo I respectively, are essential for the cells to perform transcription, replication, DNA damage repair and recombination [5]. While the type I topoisomerases catalyze their reaction by cleaving and rejoining a single strand of DNA, the type II topoisomerases act on both strands simultaneously [5]. Both enzymes make transient covalent complexes with the cleaved DNA, termed ‘cleavable complex’, during the process of strand passage. Inhibitors like fluoroquinolones and camptothecins, which bind to such complexes irreversibly, result in accumulation of damaged DNA in the cell, thereby, culminating in the induction of the SOS response and cell death [6,7]. The clinical success of fluoroquinolones has unequivocally proven DNA gyrase

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as a valid antibacterial target [8]. Although, several inhibitors of human Topo I are available for the treatment of cancer, none of them inhibit bacterial Topo I, probably owing to structural and functional differences between the two enzymes [9].

There has been limited exploration of bacterial Topo I as an antibacterial drug target [10,11]. This could be due to the ambiguity over its essentiality in some Gram negative bacterial species [12–14]. The *in vitro* essentiality of *topA* in *Escherichia coli* has been contentious with reports suggesting that *topA* could only be inactivated in the presence of suppressor mutations [15,16] or when Topo III (the other type I topoisomerase) is over-expressed [17,18]. However, in the Gram-positive pathogen *Streptococcus pneumoniae,* inhibitors of Topo I have been shown to suppress bacterial growth suggesting Topo I to be an essential enzyme in *S. pneumoniae* [11].

Genomic and biochemical evidence suggests that mycobacterial Topo I is similar in structure and in overall function to other bacterial type 1A topoisomerases, and is different from its human homolog, type 1B topoisomerase [19,20]. The *Mycobacterium tuberculosis* genome encodes a single *topA* gene and the bioinformatics analysis suggests no obvious redundancy in DNA relaxation function, making it a potential target for the development of antimycobacterial agents. Annamalai et al. and Godbole et al. have described the purification of *M. tuberculosis* Topo I and characterization of its enzymatic activity [21,22]. Transposon mutagenesis studies in *M. tuberculosis* H37Rv [23,24] and knockdown (down regulation of expression) studies in *M. tuberculosis* H37Ra [25] have indicated the essentiality of this gene in *M. tuberculosis*. However, essentiality information for this gene using a gene knockout and/or a gene knockdown in *M. tuberculosis* H37Rv, the causative agent of tuberculosis is lacking. While the data from gene knockout experiments provide a reliable proof regarding the essentiality of a gene, target depletion experiments using conditional expression or knockdown (KD) strain enables study of the effect of lowering target protein levels on cellular viability both *in vitro* and *in vivo* [26–33]. Knockdown strains also offer the flexibility of performing such studies under a variety of physiological conditions *in vitro* which mimic the physiological state of *M. tuberculosis* during its course of infection in humans.

Here, we report that *topA* is essential for the survival of *M. tuberculosis* H37Rv both *in vitro* and *in vivo.* Depletion of intracellular protein levels upon down regulation of *topA* expression led to loss of viability of *M. tuberculosis* H37Rv *in vitro* and *in vivo.* Phenotypic screening of a compound library using a Topo I overexpression strain of *M. tuberculosis* resulted in the identification of a Topo I inhibitor. The essential and vulnerable nature of Topo I demonstrated in this study makes this an attractive target for discovering novel drugs against *M. tuberculosis*.

2. Materials and methods

2.1. Bacterial strains, media, chemicals and reagents

Bacterial strains used in this study are described in Table 1. Restriction enzymes, 1 kb DNA ladder were from New England Biolabs, USA; Hygromycin B was obtained from Roche, USA; pristinamycin (pyostacine) from Sanofi-Aventis, France. Hybond membrane and chemiluminiscence Western and Southern blot kits were from GE Healthcare, USA; 0.1 mm Zirconia beads and Minibead-beater were from Biospec products, USA.

2.2. Purification of pristinamycin components

Commercially available 250 mg tablet of pyostacine was crushed using a pestle and agate mortar. The resulting powder was resuspended in methanol and centrifuged at 14000 rpm. The supernatant was used for separation of pristinamycin components, a depsipeptide (P_1) and a macrolide (P_2). The separation was performed using Gilson Auto Purification System 845Z with Agilent PDA on a Sunfire 150 mm × 300 mm, C18, 10 μM column (Waters Corporation) with a gradient of 10 mM Ammonium Acetate in water pH 4.0 (solvent A) and Acetonitrile (solvent B). The pristinamycin components were identified by analyzing the peaks for required mass using UPLCMS (Waters Acquity UPLCMS system with PDA). Pooling and concentrating of P_1 and P_2 fractions resulted in a yield of about 52 mg of polyketide and about 48 mg of polypeptide. The purity of the compounds was determined using HPLC (Agilent 1100 Series with PDA). The structure of pristinamycin components were confirmed by recording ^{1}H NMR using 500 MHz Bruker NMR instrument.

2.3. Culture conditions

LB medium was used for growing *E. coli*. Middlebrook 7H9 broth supplemented with 10% Albumin, Dextrose, NaCl (ADN) supplement (Delta Biologicals, India), 0.2% glycerol (SIGMA) and 0.05% Tween80 (SIGMA) was used for culturing mycobacteria. LB agar supplemented with appropriate antibiotics was used when necessary. 7H11 plates were used to plate mycobacteria with appropriate antibiotics and inducer as needed. The media were supplemented with appropriate antibiotics as necessary. Unless mentioned otherwise, the *topA* knockdown strain was grown in 7H9 broth supplemented with 50 μg/ml hygromycin and 50 ng/ml P_1. Hygromycin was used at a concentration of 150 μg/ml and kanamycin at 50 μg/ml for *E. coli* cultures where necessary.

2.4. Plasmid constructs

Plasmids used in this study are listed in Table 1. Briefly, the recombination plasmid containing a deletion in the *topA* gene of *M. tuberculosis* H37Rv was constructed by sub-cloning the mutant gene with flanking sequences into a suicide vector, pAZI0290 [34] at the NcoI and BglII sites. The resulting plasmid pAZI0298, had a 299 bp deletion in the *topA* gene with 804 bp upstream flanking and 800 bp downstream flanking sequences. The complementation construct pBAN1240 was generated by cloning the full length *topA* gene amplified *from M. tuberculosis* H37Rv genomic DNA into pAZI0333, a non-replicating vector with *attP-int* for integration of the plasmid into the chromosome and Ptrc promoter [35] to drive the expression of cloned gene. pBAN0303, the *M. tuberculosis topA* conditional recombinant plasmid (KD construct) was generated by cloning 690 bp of *topA* gene from its 5′ end, amplified from *M. tuberculosis* H37Rv genomic DNA into NcoI and SphI sites of pAZI9479 [30]. Topo I over-expression strain was prepared by transforming a recombinant plasmid harboring full length *M. tuberculosis topA* gene cloned in pMV261 plasmid [36].

2.5. Generation of *M. tuberculosis topA* gene knockout (KO), knockdown (KD) and overexpression (OE) strains

The recombinant plasmids were electroporated into *M. tuberculosis* H37Rv by the procedure described by Wards and Collins [37]. The gene knockout was performed by using the two-step method as described by Parish and Stoker [38]. At each step, the strains {single cross over (SCO), merodiploid or double cross over (DCO)} were selected in the presence of appropriate antibiotic markers and confirmed by a set of PCRs using the primers listed in Table S1. The allelic exchange substrate for *topA* gene KO on plasmid pAZI0298 consisted of a mutant *topA* gene with a 299 bp markerless deletion (302–600 bp) with 804 bp upstream and 800 bp downstream fragments flanking the deleted region. The SCO