Synopsis of the thesis

Title: Pranlukast as an allosteric inhibitor of *M. tuberculosis* Ornithine acetyltransferase: Implication towards novel combinatorial therapy

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Tuberculosis (TB) is a deadly disease responsible for the death of approximately 1.5 million people each year, with the highest being from developing nations. Tuberculosis affects mostly the lungs, and other parts of the body like nerves, bones and liver. *Mycobacterium tuberculosis (Mtb)* is the causative agent of TB in humans. The onset of infection is *via* the deposition of aerosol droplets containing the pathogen, *M. tuberculosis,* onto the lung alveolar surfaces. About one third of the world's population asymptomatically harbors latent *M. tuberculosis* bacterium with a constant risk of disease activation. Due to the emergence of drug-resistant strains and the evolution through multi-drug resistance (MDR) to extensive drug resistance (XDR), the fight against TB has become extremely challenging. Standard treatment for TB comprises four first-line antimicrobials: isoniazid, rifampicin, pyrazinamide and ethambutol. However, resistance to all of these drugs has been observed in several MDR strains of *Mtb*. Despite the recent advances in target identification and drug discovery, there is a relentless need for novel inhibitors against vital pathways of *Mtb.* The novel drug-development regimens endorse strategies wherein the pre-approved drugs for other ailments could be re-purposed, thereby cutting down the cost and time associated with the process of drug discovery. Also, the target selection strategy requires to aim at the key enzymes from the essential biosynthetic pathways, keeping an eye on their underlying dissimilarities when compared to human host.

The challenges in finding a suitable target for anti-*Mtb* drug discovery is it's ever evolving stride and the conserved nature of the essential proteins. Many novel small molecule inhibitors of *Mtb* are undermined, during the course of studies, by cross reactivity with homologs proteins in the host. Traditionally, the replication machinery has been at the heart of drug discovery and the processes associated with

logarithmic growth phase are vastly exploited for drug targeting. However, targeting these vital cellular components may result in some serious non-specific effects to the host. On the other hand, the intricate network of metabolic pathways provides novel avenues for specific targeting of pathogens, precisely for three main reasons:

1. There is an acute shortage of cellular nutrients due to the constant competition between the pathogen and the host, throughout the course of infection.
2. Infectious cycles often lead to the disruption of metabolic pathways, again leading to nutrient scarcity.
3. Survival of the pathogen within the hostile niche and under oxygen starvation conditions further potentiate the demands of crucial metabolites (amino acids, nitrogen bases, carbohydrates etc.) that are used as the building blocks for cellular machinery.
4. Metabolic pathways have evolved with time, to provide the much-required specificity for exclusive targeting of the pathogen, thereby limiting the cross-reactivity with the host pathways.

In order to persist and efficiently replicate in host cells, intracellular pathogens must adapt their metabolism to the available nutrients and physical conditions (mainly pH, oxygen availability and osmotic pressure) in the host. Among the major metabolic, amino acid metabolism holds great importance; as they not only serve to meet the nutritional needs of the pathogen but also play a key role in the strategies employed during pathogenesis. Although the host and the pathogen compete for many metabolites, three amino acids, Arginine, Asparagine and Tryptophan seems to be a focus of this competition because the availability of these amino acids or their derivatives influence both pathogen behavior and the immune response.

Arginine constitutes a major proportion of the total proteins in the cell and arginine and its precursor ornithine are used for the biosynthesis of the most common polyamines, putrescine and spermidine. These molecules are required for optimal growth of the organism and are involved in several physiological processes. Apart from being a critical amino acid for the synthesis of cellular proteins, arginine can also be used as a nitrogen source, under conditions of nitrogen starvation, hence crucial for pathogenesis. The glutamate and glutamine are the key metabolites in the central nitrogen metabolism; both serve as endogenous nitrogen acceptor as well as nitrogen donor. However, reports demonstrate that *Mtb* utilizes arginine and asparagine as the key sources of nitrogen during infection in mice models of tuberculosis. Therefore, our

study focuses on the process of Arginine biosynthesis in *M. tuberculosis*, wherein it is essential for the survival and pathogenesis. Since the arginine metabolism is essential for both the host and the pathogen, and competition for arginine may shift the balance, and thus determine the outcome of the infection. The enzymes involved in this pathway will be a promising target for anti-TB drug development. Despite the acknowledged significance of Arginine biosynthesis in the pathogens like *M. tuberculosis,* inhibitors to target this pathway remain to be discovered. Moreover, inhibitors of this pathway may provide novel insights to the significance of arginine biosynthesis in *Mtb* associated stress responses and persistence.

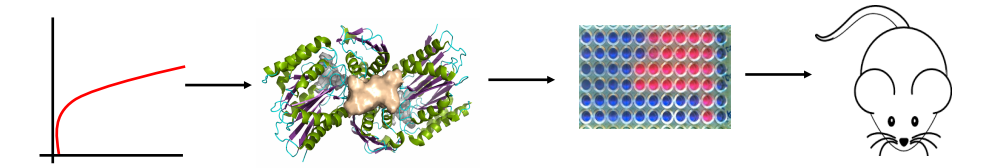
Ornithine acetyltransferase (*Mt*ArgJ), one of the crucial enzymes during the biosynthesis of arginine in *Mtb,* is essential for its survival and pathogenesis. *Mt*ArgJ lacks a homolog in human genome, thereby being a good target against *Mtb.* We hypothesize that a targeted inhibitor against this key player of mycobacterial metabolism has the potential to combat the *Mtb* survival and pathogenesis. In the present thesis, we have characterized the potential of *Mt*ArgJ from *M. tuberculosis* as a valuable target for drug design against tuberculosis. Most importantly, the approach is to specifically target a novel allosteric site identified in this study, on the *Mt*ArgJ surface. Since we are not using the age-old approach of substrate analog as an inhibitor, we hereby further minimize or even eliminate the chances of cross-reactivity with the host cellular proteins. In the later parts, we have identified an allosteric inhibitor of *Mt*ArgJ, that significantly reduces the survival of pathogenic *Mtb* through the pre-clinical models of tuberculosis.

Chapter 1 of this thesis gives a detailed account of the history of Tuberculosis, and its pathogenesis. The chapter further elaborates on the metabolic pathways of *Mycobacterium tuberculosis*, with special emphasis on the arginine biosynthesis pathway. The drug discovery regime and therapeutic challenges associated with the disease are discussed in the later parts of the chapter. All the information discussed in this chapter serves a preface for the work done throughout the thesis, and outlinesthe objectives for rest of the chapters.

Chapter 2 describes the characterization and kinetic analysis of *Mt*ArgH, the last enzyme from the arginine biosynthetic pathway in *M. tuberculosis*. This chapter demonstrates the importance of a c-terminal cysteine residue (Cys^{441}) in the catalysis and thermal stability of the enzyme. We further propose the existence of a product

mediated feed-back inhibition of *Mt*ArgH, wherein fumarate, one of the product of *Mt*ArgH, gradually modifies the Cys^{441} through succination.

Chapter 3 to 5 discuss about the work carried out on the enzyme Ornithine acetyltransferase (*Mt*ArgJ), a crucial enzyme for arginine biosynthesis in *M. tuberculosis.* We have identified a selective allosteric inhibitor against this key player of mycobacterial metabolism, employing the below-mentioned strategy. First step was to characterize the target, followed by a structure based *in-silico* screen. The best hits were subjected to *in-vitro* validation, leading to the *in-vivo* testing of the potential molecule, in the pre-clinical model of tuberculosis.



Chapter 3 starts with the characterization of the *Mt*ArgJ, wherein we identified a novel hydrophobic pocket present on the enzyme surface. We further characterized the potential of this pocket in allosterically modulating the active site. This was then followed by a structure based *in-silico* screen with a library of FDA approved drugs, specifically targeting this novel allosteric pocket on *Mt*ArgJ.

Chapter 4 deals with the *in vitro* validation of the identified compounds from *in-silico* screen. We here identified two lead molecules, Pranlukast (PRK) and Sorafenib (SRB), to have significant affinity for the allosteric site on *Mt*ArgJ, leading to the inhibition of its enzymatic activity. We further propose the key residues involved in this interaction, thereby suggesting a potential molecular mechanism of inhibition.

Chapter 5 leads us to the *in-vitro* and *in-vivo* characterization of these compounds as a potent anti-tubercular agent. We first demonstrate its efficacy in deducing the survival of the pathogenic strains of *Mtb in-vitro* and in the macrophage models of infection. We also tested the efficacy of these compounds in combination with the standard of care TB therapy drugs, and found PRK to work efficiently in such combinations. Finally, we evidence the potency of PRK in compromising the survival and pathogenesis of *Mtb* in mice models of tuberculosis infection. PRK is presently being used as a drug against chronic asthma, therefore its human safety is already

assured. This will facilitate its induction into the direct clinical trials against tuberculosis. Taken together, the work done in this thesis demonstrates a novel metabolic inhibitor of *Mtb* pathogenesis, through the pre-clinical models of infection with the potential for development of advanced combinatorial therapy against tuberculosis.