An allosteric inhibitor of Mycobacterium tuberculosis ArgJ: Implications to a novel combinatorial therapy

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Abstract

The existing treatment regime against tuberculosis is not adequate, and novel therapeutic interventions are required to target Mycobacterium tuberculosis (Mtb) pathogenesis. We report Pranlukast (PRK) as a novel allosteric inhibitor of Mtb's arginine biosynthetic enzyme, Ornithine acetyltransferase (MtArgJ). PRK treatment remarkably abates the survival of free as well as macrophage-internalized Mtb, and shows enhanced efficacy in combination with standard-of-care drugs. Notably, PRK also reduces the 5-lipoxygenase (5-LO) signaling in the infected macrophages, thereby surmounting an enhanced response against intracellular pathogen. Further, treatment with PRK alone or with rifampicin leads to significant decrease in Mtb burden and tubercular granulomas in Mtb-infected mice lungs. Taken together, this study demonstrates a novel allosteric inhibitor of MtArgJ, which acts as a dual-edged sword, by targeting the intracellular bacteria as well as the bacterial pro-survival signaling in the host. PRK is highly effective against in vitro and in vivo survival of Mtb and being an FDA-approved drug, it shows a potential for development of advanced combinatorial therapy against tuberculosis.

Keywords combinatorial therapy; drug repurposing; infectious disease; Mycobacterium tuberculosis; Ornithine acetyltransferase

Subject Categories Microbiology, Virology & Host Pathogen Interaction; Pharmacology & Drug Discovery

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Introduction

Tuberculosis (TB) accounts for 1.5 million deaths worldwide every year, with a high percentage of individuals primarily from developing nations (WHO, 2012). Emergence of drug-resistant strains has further increased the Mycobacterium tuberculosis (Mt)-associated lethality. In last decade, itself there have been enormous attempts toward the development of novel therapeutic approaches to combat multi-drug-resistant (MDR) and extensively drug-resistant (XDR) Mtb strains (Makarov et al., 2009, 2014; Koul et al., 2011). Novel therapy regimens endorse strategies wherein the pre-approved drugs for other ailments could be re-purposed for targeting lethal Mtb strains (Zumla et al., 2013). The major challenges in finding a suitable target for anti-Mtb drug discovery is its ever-evolving stride and the conserved nature of the essential proteins (Zuniga et al., 2015). Although the past decade has seen major developments in the TB drug discovery pipeline, still there is a constant need for improved therapeutic interventions (Zumla et al., 2013).

Traditionally, the replication machinery has been at the heart of drug discovery and the processes associated with logarithmic growth phase are exploited for drug targeting. However, targeting these vital cellular components may cause serious non-specific effects to the host. On the other hand, the intricate network of metabolic pathways provide novel avenues for specific targeting of pathogens (Boshoff et al., 2004; Tran et al., 2017). Among these, arginine biosynthesis pathway is essential for the survival and pathogenesis of Mtb. Despite the acknowledged significance of arginine biosynthesis in Mtb, inhibitors to target this pathway remain to be discovered. The enzymes involved in this pathway are promising targets for anti-TB drug development (Gordhan, 2002; Mdluli & Spigelman, 2006). Moreover, inhibitors of this pathway may provide novel insights to the significance of arginine biosynthesis in Mt-associated stress responses and persistence.

One of the crucial enzyme from this pathway, Ornithine acetyltransferase (ArgJ) from Mtb, has been implicated as essential gene for the survival and virulence of the pathogen (Sassetti & Rubin, 2003; Sassetti et al., 2003). MtArgJ catalyzes the transfer of acetyl moiety from N-acetyl Ornithine to glutamate, thereby producing Ornithine and N-acetyl glutamate for next round of arginine biosynthesis (Xu et al., 2007; Appendix Fig S1A). Significantly, the absence of a homologous protein in human genome makes MtArgJ an exciting target for drug development. Mycobacterium tuberculosis argJ gene is encoded by the ORF Rv1653 and belongs to the N-terminal...
nucleophile fold family of enzymes (Cole et al., 1998; Xu et al., 2007). The crystal structure of MtArgJ in native form and in complex with Ornithine has been determined at 1.7 and 2.4 Å, respectively (Sankaranarayanan et al., 2010). ArgJ in Mtb is a monofunctional enzyme as it facilitates the transfer of acetyl group to glutamate exclusively from N-acetyl Ornithine. However, ArgJ is reported to be bi-functional in some bacterial species like Neisseria gonorrhoeae, B. subtilis, Geobacillus steaothermophilus, and T. neapolitana, wherein it can utilize both N-acetyl Ornithine and acetyl-CoA for the transfer of acetyl moiety (Xu et al., 2007).

In the present study, we identified a previously unknown allosteric site on MtArgJ and report the discovery of a novel inhibitor that binds and impedes the catalytic efficiency of MtArgJ. The selectivity and specificity of this inhibitor lies in its ability to allosterically modulate the substrate-binding interface. Through a series of in silico, biochemical and biological approaches, we conclude the potency of this compound as a drug candidate against Mtb survival and pathogenesis (Fig 1A). Our data demonstrate the exquisite potency of this inhibitor against arginine biosynthesis in Mtb, thereby abating the pathogen survival, in both in vitro and in vivo infection models. We show that it also targets the pathogen pro-survival pathways in the host, thereby causing an enhanced reduction in the intracellular Mtb survival. Significant effect of this inhibitor in combination with the standard-of-care therapeutic regimen attests to its promise for inclusion in our armamentarium against tuberculosis. Taken together, this study identifies a novel metabolic inhibitor of Mtb, and its potential for improved combinatorial therapy against tuberculosis.

Results

Cloning, expression, purification, and characterization of MtArgJ

To target the arginine biosynthesis in Mtb, we selected Ornithine acetyltransferase (MtOAT/MtArgJ), a crucial enzyme involved in the arginine biosynthesis pathway. MtArgJ catalyzes the transfer of acetyl moiety from N-acetyl Ornithine to glutamate, thereby producing Ornithine for arginine production (Appendix Fig S1A). MtArgJ belongs to the N-terminal nucleophile (Ntn) fold class of enzymes, synthesized as a 404-amino acid long protein, which undergoes an auto-proteolysis event between the Ala199 and Thr200. This auto-proteolysis generates two fragments of approximately equal size (20–21 kDa), which then associate to form a protomeric unit (AB—heterodimer; A2B2 tetramer—dimer of the heterodimer; Sankaranarayanan et al., 2010). The affinity-purified His-tagged MtArgJ thus yields three distinct bands on SDS–PAGE, one full-length and two cleaved products (20 and 21 kDa each; Fig 1B; Marc et al., 2001). Since we got a fraction of uncleaved (inactive) protein also during purification, for calculating the concentration of active MtArgJ in solution, we used densitometric analysis of SDS–PAGE profile (details in Appendix Materials and Methods section). The enzymatic activity of MtArgJ thus obtained was ascertained by a TLC-based assay wherein the enzyme was found to be functionally active (Appendix Fig S1B). The protein was assayed at varying substrate concentration, that is, N-acetyl Ornithine, and the reaction velocity was plotted against the corresponding substrate concentrations. The $K_m$ for N-acetyl Ornithine-mediated synthesis of Ornithine was determined to be 91.8 μM (Fig 1C and D). These results confirm the integrity and functional activity of MtArgJ employed in this study.

Identification of a potential ligand binding pocket on MtArgJ surface

We started our quest to discover a small molecule inhibitor of MtArgJ by investigating the protein surface. MtArgJ structure (PDB ID: 3T6) was probed for surface cavity predictions (by MetaPocket server and CastP analysis) and a well-defined pocket of area 2,019.7 Å² and volume 3,104.8 Å³ located in-between the two active sites was discovered (Fig 2A). This pocket comprises of 48% hydrophobic amino acid residues and 24% and 28% polar and charged residues, respectively (Fig 2A inset, Appendix Fig S1D and E). This large pocket comprised of four loops, four helices, and one β strand contributed by each protomer (AB) of A2B2 tetramer. However, we noticed two flanking loops positioned at the interface of the substrate-binding pockets on either side. To experimentally validate the hydrophobicity of this pocket, we used a fluorescent dye, 8-anilinonaphthalene sulfonate (ANS). It has specificity for the hydrophobic regions of a protein and shows a characteristic fluorescence at 470 nm upon binding (Cardamone & Puri, 1992). We utilized this property of ANS to probe the novel pocket on MtArgJ. As shown in Fig 2B, ANS showed a concentration-dependent increase in binding to MtArgJ, manifested as a steady rise in fluorescence intensity. The kinetic analysis determined a $K_d$ of 937 μM (ANS binding to MtArgJ; Fig 2C). Also, in our blind docking experiment with whole molecule of MtArgJ, 2,000 final conformations were generated and we observed that all the conformers of ANS were sitting and interacting exclusively with this novel pocket (data not shown). In concordance with MetaPocket analysis, these results further validate the presence of a major cavity on MtArgJ surface, which is considerably hydrophobic in nature. We proceeded with characterization of this pocket and as shown in Fig 2D, addition of ANS leads to a decrease in the catalytic activity of MtArgJ. However, the effect was only marginal, which could be attributed to the relatively smaller size of ANS with respect to the binding pocket. Based on our initial results, we hypothesized that a suitable ligand bound to this pocket may cause inhibition of the enzymatic activity of the protein. Moreover, targeting this novel pocket instead of “substrate-binding site” was deemed by us to be more rewarding, since substrate for MtArgJ are small molecules involved in multiple cellular process. Therefore, conventional substrate analogs (as inhibitors) may cross-react with other cellular proteins with similar ligands, giving rise to unwanted side effects. Our initial results gave us a lead to further probe this pocket for inhibitor development against MtArgJ.

In silico screen of FDA-approved drug library against MtArgJ

We performed a high-throughput in silico screen of a small molecule drug library to determine their binding to the major pocket on MtArgJ. Importantly, chosen library was FDA-approved therefore already cleared for cytotoxicity tests and poised for therapeutic repurposing. From the dataset of 1,556 FDA-approved drugs, a total of 1,417 compounds were prepared and sourced into the virtual screening pipeline along with the reference molecule ANS. Initially flexible docking filtered 1,340 compounds based on their internal
Figure 1. Screening methodology and characterization of MtArgJ target enzyme.

A Screening methodology employed in the study.
B SDS-PAGE profile of MtArgJ purified by His-tag affinity chromatography.
C MtArgJ activity assay where product (Ornithine) formation is monitored by a TLC-based assay, and TLC image is shown with increasing substrate (N-acetyl Ornithine) concentration; lower spots represent the product Ornithine, and upper spots represent the other substrate glutamate (kept constant).
D Saturation curve fit using Michaelis–Menten plot for MtArgJ activity as measured by TLC assay. Reaction velocity on y-axis represents amount of product formed per unit time, while x-axis denotes substrate concentrations. Mean and standard error (SE) determined from three experimental replicates (n = 3) and two technical duplicates.

Target characterization
In-silico screen
In-vitro validation
Pre-clinical testing

Km = 91.8 µM
A

Large pocket

Active site

Hydrophobic residues (48%)
Polar residues (24%)
Charged residues (28%)

| Site          | Area  | Volume   |
|---------------|-------|----------|
| Allosteric site | 2019.7 Å² | 3104.8 Å³ |
| Active site 1  | 801.8 Å²  | 1002 Å³   |
| Active site 2  | 625.3 Å²  | 805.5 Å³   |

B

C

K_d = 937 μM

[ANS] mM

D

E

Figure 2.
To inhibit MtArgJ, we have also performed a negative validation of our in silico screening strategy by testing 10 compounds from the non-selected group (filtered out), and none of them could inhibit the MtArgJ activity in vitro (Appendix Table S4).

PRK and SRB bind to a novel allosteric pocket discovered on the surface of MtArgJ

To experimentally determine the binding site of PRK and SRB, we designed an ANS-based fluorescence titration assay (Iyer et al., 2016). The MtArgJ, saturated with ANS, gives a characteristically high fluorescence intensity at 470 nm, and any molecule that competes for ANS binding site should result in a dose-dependent decrease in fluorescence. As shown in Fig 3G and H, addition of PRK/SRB leads to diminution in fluorescence intensity at 470 nm in a dose-dependent manner. The data indicate binding of PRK and SRB to the allosteric pocket by competitive displacement of ANS from the MtArgJ complex. Both PRK and SRB were spectroscopically inert in this region. Further, net change in the relative fluorescence unit (RFU) was plotted as a function of ligand concentration to calculate binding constants. The dissociation constant (Kd) for PRK-induced displacement of ANS from MtArgJ was calculated to be 115 μM, whereas that of SRB was 312 μM (Fig 3G and H: inset). The Kd values thus obtained for both the compounds are about 10 times lesser than that for ANS, which establishes their significant binding to the region. These results demonstrate the suggestive affinity and specificity of PRK and SRB for the allosteric pocket discovered here, at the surface of MtArgJ.

PRK and SRB impart thermal stability to MtArgJ in a concentration-dependent manner

To further establish the binding affinity of PRK and SRB to MtArgJ, we used thermal shift assay (TSA), a method orthologous to isothermal titration calorimetry (ITC; Niesen et al., 2007; Iyer et al., 2016) and is being productively used for drug discovery (Renaud et al., 2016). MtArgJ with varying concentration of PRK/SRB was subjected to gradually increasing temperature, and the shift in melting temperature (Tm) was calculated. The extent of change in Tm is indicative of the ligand’s affinity for protein. As shown in Fig 3I and J, thermal stability of MtArgJ demonstrates positive correlation with increasing concentration of both the inhibitors (PRK and SRB). However, the increase in Tm was relatively higher in case of PRK, consistent with its higher affinity for the protein. The apparent dissociation constant (Kd) for PRK and SRB, calculated by plotting net change in Tm versus inhibitor concentration, was 126 and 281 μM, respectively (Fig 3I and J: inset). These results are consistent with our rationale of probing the allosteric site for inhibition of MtArgJ.
Figure 3.
consistent with PRK to have higher affinity for MtArgJ than SRB, thereby imparting enhanced thermal stability to the protein. Importantly, $K_d$ values determined by TSA agreed with the enzyme kinetics and fluorescence spectroscopy data (Figs 3C–H). These results validated the significant affinity of both the inhibitors for MtArgJ. However, PRK induces a more positive shift in thermal stability as compared to SRB at same concentrations. Based on the data so far, it was evident that PRK is a better inhibitor of MtArgJ and has higher affinity for the protein, than SRB. We next asked for the potential residues involved in this allosteric mode of inhibition.

**MD simulation results decipher a proposed mode of PRK/SRB binding to the allosteric pocket on MtArgJ**

Based on the promising results obtained through our biochemical analysis, we sought to determine the molecular basis of PRK- and SRB-mediated inhibition of MtArgJ through computational approaches. We undertook molecular dynamic (MD) simulation to examine the possible mode of PRK/SRB-mediated allosteric inhibition of MtArgJ. Hydrogen bonds contribute to the directionality and stabilization of protein–ligand complexes. Hence, the occurrence of hydrogen bonds between substrate-bound MtArgJ and PRK/SRB was examined (Appendix Tables S5 and S6). While nestled in the allosteric pocket of MtArgJ, the tetrazole ring of PRK interacts with Asp234 and Ser310, respectively, from chain B and chain D while chromene ring showed interactions with Ser310 of chain B and benzamide group nitrogen with Gln305 of chain B on the protein (Fig 4A and B). Sorafenib, on the other hand, showed interactions via amino groups to Gln305 and Arg308 of chain D and carbonyl group to Arg308 of chain B (Fig 4C and D). PRK exhibited more number of interactions with the allosteric pocket than SRB, asserting higher affinity of PRK for MtArgJ. The data show that PRK and SRB both bind to the allosteric pocket on MtArgJ; however, PRK binds with higher affinity than SRB (Fig 3C–J). Details of MD simulation results are represented in Appendix Figs S3–S5 and Appendix Results and Discussion.

To validate the results obtained from MD simulations, we performed site-directed mutagenesis of the allosteric pocket. The potential inhibitor-interacting residues identified by the MD simulation data, viz. Gln305, Ser310 and Asp234, were mutated into alanine one by one. Also, we made double mutants of Gln305/Ser310 and Asp234/Gln305. All these mutants exhibit drastic decrease in the enzymatic activity of MtArgJ, the double mutants being totally inactive (Appendix Fig S6b). These results attest to the allosteric nature of this pocket and support the results obtained from biochemical assays performed earlier (Fig 3A–J). Hence, we hypothesized that both PRK and SRB could be a potential drug candidate for targeting MtArgJ activity in Mtb. This prompted us to test the dose-dependent effect of PRK and SRB on the survival of pathogenic Mtb.

**PRK and SRB significantly compromises the in vitro survival of pathogenic Mtb**

So far, we characterized the affinity parameters and the possible mechanism involved in the PRK/SRB-based inhibition of MtArgJ. We then proceed to determine the efficacy of these inhibitors on pathogenic strain of Mtb (H37Rv) and the two multiple drug-resistant (MDR) strains (Jal2261 and Jal2287). H37Rv cells were exposed to varying concentrations of PRK and SRB. The microplate Alamar blue assay (MABA) was employed to determine the MIC$_{90}$ (minimum inhibitory concentration- 90% inhibition in cell survival) of the inhibitors. Alamar blue (AB) is an oxidation–reduction indicator dye that has been widely used to measure the sensitivity of mycobacteria to anti-TB drugs (Franzblau, 2000). A color transition from non-fluorescent blue to fluorescent pink indicating reduction in AB dye occurs during mycobacterial growth. Inhibitor-mediated depletion in growth interferes with AB reduction and subsequent color development. Administering pathogenic Mtb (H37Rv) with increasing concentration of PRK or SRB resulted in decreased fluorescence intensity (Fig 5A). Rifampicin (Rif) was taken as a positive control. The minimum inhibitory concentration (MIC$_{90}$) was calculated by plotting cell viability (%) against inhibitor concentration. Based on the MABA assay, the calculated MIC$_{90}$ for PRK and SRB against Mtb H37Rv are 5 and 10 µg/ml, respectively (Fig 5B and C). Next, the Hill’s plot analysis of fluorescence intensity versus inhibitor concentration revealed the IC$_{50}$ of PRK- and SRB-mediated inhibition of Mtb survival to be 3.02 and 6.7 µg/ml, respectively (Fig 5D and E). The results suggest the potential anti-tubercular properties of lead compounds. However, it also indicated the superiority of PRK over SRB in inhibiting the growth and survival of Mtb. We also tested the efficacy of PRK and SRB on two MDR strains: Jal2261 and Jal2287. PRK showed an MIC$_{90}$ of 15 and 25 µg/ml for both Jal2261 and Jal2287 strains, respectively (Appendix Fig S7A and B). However, SRB demonstrated comparatively higher MIC (Appendix Fig S7C and D) for both the strains. The results showed the promising effect of PRK on pathogenic Mtb including MDR strains.

**PRK and SRB showed marked reduction in Mtb survival in combination with standard-of-care anti-TB drugs**

After establishing the efficacy of PRK and SRB as potent inhibitor of mycobacterial growth, we tested their efficacy in combination with the standard-of-care anti-TB drugs (rifampicin (R), isoniazid (H),...
and ethambutol (E). 

*Mtb* H37Rv cells were seeded in a 96-well plate for 48 h at 37°C and then treated with combination of inhibitors. The inhibitory properties of RHE combination was compared with novel combination of RH + PRK, wherein ethambutol was replaced with PRK. This enabled us to compare the efficacy of our inhibitor against a standard-of-care metabolic drug of *Mtb*, that is, ethambutol. A combination of rifampicin (40 ng/ml), isoniazid (30 ng/ml), and ethambutol (1.5 μg/ml) was used as a reference control to compare the efficacy of novel combination of rifampicin and isoniazid with PRK/SRB. The results indicate a 10-fold decrease in CFU upon RH + PRK treatment at the end of 24 h (Fig 5F). On the other hand, RH + SRB showed only a modest decrease of 0.2 log unit CFU (Fig 5G). These results demonstrate that PRK effectively inhibits *Mtb* survival and works very efficiently in combination with

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**Figure 4.** Binding interactions of PRK and SRB with the allosteric pocket of *MtArgJ*.  
A PRK (orange) interacting with the Asp234 and Gln305 of B chain, Ser310 of B and D chains in the allosteric pocket of the *MtArgJ*.  
B LIGPLOT for the interaction of PRK with the allosteric pocket of *MtArgJ*.  
C SRB (magenta) interacting with Gln305 of D chain and Arg308 of B and D chains in the allosteric pocket of the *MtArgJ*.  
D LIGPLOT for the interaction of SRB with the allosteric pocket of *MtArgJ*.  

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Combinatorial therapy:
- Rifampicin (R)
- Isoniazid (H)
- Ethambutol (E)
- PRK/SRB

Figure 5.
the standard-of-care anti-TB drugs (rifampicin and isoniazid). This new combination of Rif, Inh and PRK is significantly effective against Mtb survival than the pre-existing RHE combination and holds a potential for improved therapy regimen. Therefore, we further tested the efficacy of PRK on macrophage infection model of Mtb.

PRK significantly inhibits the survival of Mtb in macrophage infection model without affecting the host cell

THP-1 cells, a human monocytic cell line, stimulated for differentiation by PMA treatment were infected with pathogenic Mtb (H37Rv). The Mtb-infected THP-1 cells were treated with varying inhibitor (PRK or SRB) concentrations or DMSO (control) at different time points. Once treated, the cells were lysed at desired time points (0, 12, 24, and 48 h) and plated for colony formation assays. The CFU (colony-forming unit) is calculated and plotted against time at three different inhibitor concentrations. As shown in Fig 6A, treatment of Mtb-infected THP-1 cells with PRK (5 μg/ml) leads to 100-fold reduction in CFU within 48 h. Moreover, identical assay with mouse macrophages (Raw264.7) treated with PRK leads to about 200-fold decrease in CFU (Fig 6B). In comparison, SRB was much less potent and shown about 10- to 30-fold decrease in CFU for human and mouse macrophage cell lines, respectively (Fig 6A and B). These results demonstrate the superiority of PRK over SRB in reducing the mycobacterial burden from the infected macrophages.

Next, we investigated the efficacy of PRK and SRB in combination with the standard-of-care anti-TB drugs. The Mtb-infected macrophages were treated with either pre-existing RHE combination or novel R/H/PRK and R/H/SRB cocktails. Interestingly, treatment with RH + PRK combination exhibited almost 40- to 50-fold decrease in CFU from that of parent combination (RHE) in both human and murine macrophage cell lines (Fig 6C and D). However, RH + SRB leads to only 0.2–0.3 log unit decrease in CFU signifying its reduced efficiency toward combination therapy (Fig 6C and D). These results demonstrate the efficacy of PRK in reducing the Mtb burden from host macrophages and its enhanced efficiency in combination with standard-of-care drugs.

We further demonstrate the active effect of PRK treatment on macrophage-internalized Mtb and its possible side effects on the host cell survival. Macrophages were infected with GFP-tagged Mtb H37Rv (Mtb-GFP henceforth) followed by treatment with PRK at varying time points. Cells were harvested and flow-sorted based on GFP expression followed by assessment of macrophage viability by PI (propidium iodide) staining. The PRK treatment leads to diminished GFP intensity with time, suggesting reduced Mtb burden in infected macrophages (Fig 6E and F). However, host cell (macrophage) viability remains unaffected as determined by PI staining (Fig 6G). Detailed scatter plots for the FACS experiments are shown in Appendix Fig S8A and B. These results demonstrate the potency of Pranulakst (PRK) as a promising anti-tubercular molecule with no deleterious effect on the host cell survival. Also, the enhanced effect of PRK alone and in combination with the therapy drugs, on the macrophage-internalized Mtb, is interesting and beneficial from the host’s perspective.

PRK treatment reduces the infection-associated apoptosis in the host

It has been reported that during the early phase of Mtb infection, macrophages undergo apoptosis as an innate defense mechanism, thereby increasing the levels of pro-apoptotic proteins like caspases 3 and 8 (Duan et al., 2002; Derrick & Morris, 2007; Behar et al., 2011; Aguilo et al., 2014). Hence, we explored the effect of PRK on infection-associated apoptosis in the host. To examine this, we choose to monitor caspase 3-dependent apoptosis in Mtb-infected macrophages by monitoring active caspase 3 levels in supernatant media of Mtb-infected THP1 and Raw264.7 macrophages in presence and absence of PRK (1 and 10 μM).

Relative decrease in chemiluminescence as a measure of extracellular caspase 3 levels upon PRK treatment.

Data information: All the experiments were performed in triplicate and confirmed with biological duplicates at least. Statistical significance between experimental groups was determined by two-tailed, unpaired Student’s t-test (****p < 0.0001, ***p < 0.001, **p < 0.01, *p < 0.1).
Combinatorial therapy:
- Rifampicin (R)
- Isoniazid (H)
- Ethambutol (E)
- PRK/SRB

**Figure 6.**
macrophages. As shown in Fig 6H–J, treatment with increasing concentrations of PRK leads to reduced extracellular caspase 3 level in the Mtb-infected human as well as murine macrophages (THP-1 and Raw 264.7, respectively). However, uninfected macrophages showed no increase in caspase 3 levels while the infected macrophages showed a gradual increase in caspase 3 with time (up to 48 h). The results demonstrate decrease in Mtb-associated macrophage apoptosis upon PRK treatment in both human and mouse macrophage cell lines. The above results suggest that PRK not only targets the macrophage-associated Mtb but also limits the infection-induced host cell apoptosis. These results instigated us to further test the in vivo efficacy of PRK in mouse models of infection.

PRK-mediated killing of Mtb is rescued upon arginine supplementation

Our in vitro studies showed that PRK efficiently inhibits the activity of MtaArgJ, a crucial enzyme in the arginine biosynthesis pathway of the pathogen. So, we hypothesized that the in vivo (cell-based) effect is due to the reduction in arginine levels within the pathogen. Therefore, we aimed to confirm the mechanism of action of PRK-mediated killing of Mtb H37Rv. To examine this, we supplemented the Mtb minimal media cultures with arginine alongside PRK treatment and calculated the CFU post-treatment. As shown in Fig 7A, the Mtb cultures treated with PRK (1 μg/ml) had significant decrease in the cell survival. However, upon supplementation with arginine (1 mM), the effect of PRK-mediated cell death is rescued. In the samples treated with rifampicin (0.4 μg/ml), arginine supplementation had no effect on the cell death. This shows that PRK induces its bactericidal effect on Mtb by reducing the arginine levels in the pathogen, while no such effect was observed when a totally different antibiotic (the one against bacterial RNA pol) was used instead. The inability of Mtb to survive under arginine deficit created by PRK is also consistent with the suitability of MtaArgJ as a target against Mtb survival.

We then sought to confirm the decline in arginine levels in PRK-treated samples through mass spectrometry. We treated the Mtb cultures with PRK or DMSO, and the whole-cell metabolites were isolated and subjected to ESI-MS analysis. The area and intensity of the arginine peak (m/z 174) was determined, and the arginine levels were estimated based on a standard arginine plot. A heavy isotope-labeled arginine (13C labeled arginine: m/z = 180) was used as an internal standard (Appendix Fig S9A and B). As shown in the Fig 7B, the levels of arginine in the Mtb H37Rv (wild type) cells are calculated to be 1.7 μg/ml. However, in the PRK-treated samples, there was a significant decline in arginine levels, as low as 150 ng/ml, at higher inhibitor concentration (0.5–5 μg/ml). We got similar results by estimating the levels through either peak area or peak intensity (Appendix Fig S9C). These results demonstrate that PRK treatment actively reduces the arginine levels in Mtb cells, thereby attenuating the cell growth and survival of the pathogen.

PRK also targets the host macrophage leukotriene signaling to limit the intracellular Mtb growth

The above results showed that PRK is potent in reducing the Mtb growth by inhibiting the pathogen’s arginine biosynthesis pathway and is highly effective on the macrophage models of infection as well. However, the enhanced effect of PRK on the macrophage-internalized Mtb suggests for an additional target within macrophages, aiding to potent reduction of pathogen survival within the host. PRK is a known inhibitor of cysteinyl leukotriene receptor-1 (CysLTR1), on the mammalian cells and is used for treatment of asthma (Barnes & Pujet, 1997). Macrophages also express CysLTR1 in response to various inflammatory stimuli, including pathogenic bacterial colonization. Also, studies have shown that in macrophages and dendritic cells, PRK acts through a yet another mechanism, wherein it targets the leukotriene and prostaglandin (eicosanoids) biosynthesis, which are ligands for CysLTRs (Theron et al, 2014). Phospholipase-A2 converts membrane phospholipids to arachidonic acid, which is used as a precursor to synthesize leukotrienes and prostaglandins by the enzymes like 5-LO (5-lipoxygenase), FLAP (5-lipoxygenase-activating protein), and COX-2 (cyclooxygenase 2; Drazen, 1998; Mayer-Barber et al, 2014). Divangahi et al have shown that Mtb infection activates the 5-lipoxygenase pathway, which facilitates the host cell necrosis, thereby helping the pathogen dissemination. This also prevents the cross-antigen presentation by dendritic cells, thereby inhibiting the induction of T-cell immunity (Behar et al, 2010; Divangahi et al, 2010).

We have shown here that CysLTR1 and 5-lipoxygenase (5-LO) genes are upregulated by 4.5-fold and 3.5-fold, respectively, in the macrophages infected with Mtb (Fig 7C). Along with that, FLAP and COX-2 were also significantly upregulated by Mtb infection (Fig 7C). Notably, upon PRK treatment, we observed a remarkable decline in the transcript levels of 5-LO (4.5-fold), Cox-2 (8.5-fold), and FLAP (2.5-fold; Fig 7D–F). This shows that PRK downregulates the 5-lipoxygenase pathway, thereby reducing the Mtb survival and dissemination within the macrophages. Also, PRK treatment causes a notable decline in the CysLTR1 transcript levels in the macrophages, along with downregulation in the MCP-1 levels, a
**Figure 7.**

Archita Mishra et al. *Targeting arginine biosynthesis in Mtb*.

(A) H37Rv CFU (log₁₀)

(B) Arginine conc in samples based on peak area

(C) Relative fold expression

(D) 5-LO

(E) Cox-2

(F) FLAP

(G) CysLTR1

(H) MCP1
downstream effector of CysLTR1 signaling (Ichiyama et al., 2005, 2009) (Fig 7G and H). The decrease in the CysLTR1 gene expression could be due to two reasons: (i) since the leukotriene biosynthesis is inhibited, CysLTR1 is downregulated in the absence of its ligand (leukotrienes), (ii) since CysLTR1 levels are upregulated upon Mtb infection, PRK-mediated reduction in Mtb within macrophages will lead to reduction in the CysLTR1 levels as well. Taken together, these results demonstrate the enhanced effect of PRK on the macrophage-infected Mtb. In addition to targeting the arginine biosynthesis in Mtb, PRK also targets the host 5-lipoxygenase signaling, thereby potentiating its bactericidal effect in the macrophage model of infection. This study also highlights the importance of 5-LO and leukotriene signaling in the Mtb pathogenesis.

**PRK treatment reduces the Mtb burden and tubercular granulomas from the lungs of Mtb-infected mice**

Chronic Mtb infection is characterized by the formation of lung-associated granulomas, an organized aggregate of immune cells with infected macrophages at the core (Ramakrishnan, 2012). To investigate the effect of PRK on chronic Mtb exposure, we used BALB/c mice and infected them with Mtb H37Rv strain through aerosol (Fig 8A). One month after successful establishment of infection, we treated the mice with PRK (40 mg/kg body weight), Rif (10 mg/kg body weight), or combination of PRK with Rif. Next, these mice were sacrificed at three time points (0, 12, and 24 days) and lung-associated granulomas were analyzed (Fig 8B–E). We observed a marked reduction in the tubercular granulomas after 12 and 24 days of treatment with PRK in comparison with PBS (phosphate-buffered saline)-treated mice (Fig 8B and C). Notably, Rif + PRK combination had practically no visible granulomas in the mice lungs, after 24 days of treatment (Fig 8D and E). To determine the lung-associated bacterial burden, we homogenized the mice lungs and plated at different dilutions on 7H11 solid media supplemented with OADC and PANTA. The plates were incubated for 21 days, and colonies were analyzed for CFU count. We observed significant reduction in Mtb burden, with a 0.5 log unit decrease in CFU, in PRK-treated mice as compared to PBS control (Fig 8F). Notably, PRK in combination with standard-of-care anti-TB drug, Rif, showed improved results with maximum diminution of lung-associated Mtb burden and a decrease in CFU by 1 log unit as compared to treatment with Rif alone (Fig 8G). The number of granulomas per tissue section of the mice lungs was calculated by the H&E staining analysis of the lung slides (Fig 8H and I). We observed that there was a significant decrease in the tubercular granulomas in the PRK-treated mice. However, PRK showed a more remarkable effect in combination with rifampicin. Moreover, there was no splenic or hepatic cytotoxicity at the administered dosages, as shown in the detailed histopathological H&E staining images, analyzed by expert pathologists (Appendix Figs S10 and S11). The results demonstrate the potency of PRK in combating Mtb infection and its improved efficiency in combination with Rif, thereby proving its in vivo efficacy on the pre-clinical model of tuberculosis.

**Discussion**

The importance of arginine biosynthesis pathway in the survival and pathogenesis of *Mycobacterium tuberculosis* is well-established. However, there are no attempts to target these metabolic enzymes for anti-TB drug discovery. One of the key enzymes from this pathway is Ornithine acetyltransferase (OAT/MtArgJ) that recycles the acetyl group during the process of arginine biosynthesis. Targeting MtArgJ for anti-TB drug development is advantageous for two main reasons: (i) It is an essential gene for the survival and virulence of the pathogen (Sassetti & Rubin, 2003; Sassetti et al., 2003), and (ii) it lacks a homologous protein in human (host) genome. The later imparts specificity and subsequently minimizes the potential of cross-reactivity. Although there are reported inhibitors for various metabolic pathways of Mtb, inhibitors that target arginine biosynthesis, more specifically the enzyme Ornithine acetyltransferase (MtArgJ) remains unexplored (Neres et al., 2012; Capodagli et al., 2014; Palde et al., 2016). The substrates for arginine biosynthesis are small molecules common to many other cellular pathways, including those in the host. Hence, designing a substrate analog as an inhibitor is often accompanied with severe off-target effects in such systems of host-pathogen interaction. On the other hand, allostERIC sites are evolutionarily less conserved and hence provide selectivity and specificity for drug targeting with minimum side effects (Wenthur et al., 2014). Therefore, we rationalized that an inhibitor targeting allostERIC site may unlock the way for inhibiting arginine biosynthesis pathway and thus the survival of Mtb, while minimizing the side effects.
A. Infection (30 days incubation) → Drug Treatment → Harvesting lungs to assess Mtb burden.

B. Control

C. PRK

D. Rif

E. Rif + PRK

F. Graph showing CFU per gm lung tissue (log10) for different treatments.

G. Graph showing CFU per gm lung tissue (log10) for different treatments.

H. Bar graph showing granuloma per tissue section (lungs) for Control, PRK, Rif, PRK+Rif.

I. Images showing (i) Control, (ii) PRK treated, (iii) Rif treated, (iv) Rif+PRK treated.

Figure 8.
In the present study, we employed structure-based in silico and functional in vitro strategies to characterize mycobacterial ArgJ for drug targeting. We first characterized a novel allosteric pocket on MtArgJ and established its relevance in modulating enzyme activity. The fluorescence spectroscopic studies validated the partial hydrophobic nature of this pocket and its potential as an inhibitor-binding site. The results lead to the identification of two lead compounds, Pranlukast (PRK) and Sorafenib (SRB) from the library of 1,556 (FDA-approved) drugs. Pranlukast (PRK) is an FDA-approved small molecule antagonist of human cysteinyl leukotriene receptor-1 (hCysLTR1) and is recommended for the treatment of chronic bronchial asthma (Barnes & Pujet, 1997), Whereas Sorafenib (SRB) is an imatinib derivative, used for the inhibition of tyrosine kinases implicated in advanced renal-cell carcinomas (Escudier et al., 2007). In our study, we extensively characterized both the inhibitors (PRK and SRB) for their biochemical and biological properties. We showed that both the compounds bind to the novel pocket on MtArgJ, thereby allosterically modulating the substrate-binding and subsequent enzymatic activity. Our activity-based assay demonstrates PRK and SRB to inhibit MtArgJ activity in a non-competitive manner. PRK showed higher affinity for MtArgJ in both fluorescence-based and thermal shift assays and was efficiently inhibiting the catalysis at lower concentrations than SRB.

The MD simulation analysis of MtArgJ-NAO complex with PRK/SRB suggested plausible interactions involved between the inhibitor and the allosteric site. PRK showed four critical hydrogen bond interactions with the allosteric pocket and lead to destabilization of major catalytic residues (viz. Thr200) involved in MtArgJ activity. Similar investigation of MtArgJ_NAO complex bound to SRB showed lesser interactions than PRK. It is important to note that allosteric inhibition is not very common and this report is first of a kind for any Ornithine acetyltransferases (OAT) to our knowledge. Here, we highlight a conceptual advance of harnessing a site topologically distinct from the catalytic one, as an Achilles heel for compromising a target essential to the survival of a pathogen.

Further, treatment of Mtb H37Rv with PRK or SRB showed a marked reduction in mycobacterial survival. It also reduced the mycobacterial burden from infected human and murine macrophage models. In concordance with biochemical data, PRK was more potent than SRB against both extracellular and intracellular Mtb survival. We demonstrate that PRK, while killing the intracellular Mtb, does not have a detrimental effect on the host cell survival. Although, Mtb induces anti-apoptotic signals during the latent phase of infection, there are various reports showing Mtb-infected macrophages to undergo apoptosis as their early defense mechanism against infection, thereby increasing the levels of pro-apoptotic proteins like caspase 1, 3, 5, 7, and 8 (Duan et al., 2002; Derrick &

**Mechanism of action**

**PRK mediated inhibition of Mtb survival (in vivo)**

![Mechanism of action](image.png)

- **Healthy Macrophage**
- **Mtb infection**
- **Mtb infected Macrophage**
- **Heavily infected Macrophage**
  - High CysLTR1
  - High 5-LO
  - Decreased immune response
- **Pralukast**
  - Mtb induced cell death
  - Pathogen dissemination
  - Arginine deficiency in Mtb
  - Macrophages
  - Low 5-LO
  - Low CysLTR1
  - Decreased Mtb survival

**Figure 9.** Pranlukast (PRK): A dual-edge sword for Mycobacterium tuberculosis. A representative schematic for the mechanism of PRK-mediated reduction in Mtb survival and pathogenesis. We show that PRK inhibits the essential arginine biosynthesis in Mtb, which leads to bacterial cell death. Simultaneously, PRK treatment also reduces the pathogen-specific pro-survival pathways (5-LO signaling, eicosanoid biosynthesis, and CysLTR1 signaling) in the macrophage infected with Mtb, thereby enabling efficient intracellular elimination of pathogen.
Morris, 2007; Behar et al., 2011; Aguiló et al., 2014). Our results show that PRK reduces the Mtb-induced apoptosis in macrophages, thereby rescuing them from infection-associated cell death at early time points (up to 48 h post-infection). Notably, PRK shows an additive effect in combination with the standard-of-care anti-TB drugs. In combination with rifampicin and isoniazid, PRK showed significant reduction in Mtb survival and performed better than a pre-existing drug—ethambutol. Further, we demonstrated that PRK treatment leads to an active decline in the arginine levels in pathogenic Mtb. Supplementation of the Mtb culture with arginine rescues the PRK-mediated killing of the pathogen. This proves that the mechanism of action of PRK-mediated killing of Mtb is via inhibition of arginine biosynthesis.

Eicosanoid (leukotrienes, prostaglandins, etc.) signaling has been long associated with the bacterial infections and inflammation, also in the case of Mtb. Separate studies have shown that CysLTR1 inhibitor Pranlukast also inhibits the lipoxygenase signaling in the macrophages and dendritic cells. In this study, we show that PRK acts as a dual-edge sword, wherein it targets arginine biosynthesis in the pathogen and 5-lipoxygenase (5-LO) signaling in the host, which is known to facilitate Mtb survival in the macrophages. By reducing the expression of eicosanoid biosynthetic enzymes (5-LO, FLAP, and COX-2), PRK treatment effectively clears out Mtb burden from the infected macrophages. This way, PRKs exhibit an enhanced effect on the macrophage-internalized Mtb, by directly targeting the intracellular bacteria as well as enabling the host cell to combat the pathogenic attack, as represented in the schematic Fig 9.

Tubercular granulomas are a major hallmark of the successful infection and dissemination of Mtb in the infected lungs (Ramakrishnan, 2012). PRK showed a marked decrease in the tubercular granulomas in the lungs of Mtb-infected mice models, when administered alone or in combination with Rif. We observed significant reduction in Mtb burden from the lungs of infected mice upon PRK treatment, as determined by CFU analysis of infected lung tissues. Also, we observed notable decline in the lung-associated tubercular granulomas in the infected mice, upon PRK treatment, both alone and in combination with rifampicin. These results thus highlight the enormous potential of direct repurposing of PRK toward novel anti-TB therapeutics.

Our results unveil the potential of an ab-initio approach for repurposing pre-approved drugs for novel ailments. Since PRK is being administered as drug against chronic bronchial asthma, its safety toward human is already approved. Therefore, it has the potential for subjection into direct clinical trials on tuberculosis patients in near future. The compelling advantage of PRK in combination with standard-of-care anti-TB drugs and its efficacy in preclinical model of tuberculosis upholds its promising as an anti-TB drug. A continued effort toward clinical translation of PRK is required for development of a novel and efficient combinatorial therapy against tuberculosis.

Materials and Methods

In vitro biochemical assay for MArgJ activity

The dialyzed MArgJ (100 nM) was incubated with different concentrations of N-acetyl Ornithine (10 nM to 10 mM) and glutamate (0.5 mM) in 10 mM Tris–NaCl buffer (pH 7.4) at 37°C for 45 min. The reaction mixture was then loaded on a silica-TLC plate. The chromatographic run was performed in the solvent A (iso-propanol:formic acid:water; 20:1:5). 100 μl reactions were made and 10 μl of it was loaded on TLC. The TLC plate was treated with 1% ninhydrin solution in methanol mixed with 5% citric acid solution. The plate is heat-dried, and the spots corresponding to Ornithine (identified by Ornithine standard) were densitometrically quantified by Multi Gauge V3.0 software, using Ornithine standard curve. All the experiments were done in triplicate, and the average was plotted using GraphPad Prism-7 software.

Fluorescence-based dye displacement assay

Fluorescence spectroscopic studies were performed on Jasco FP-6300 Spectrofluorometer. ANS (8-anilinonaphthalene-1-sulfonic acid; Sigma) was used as a dye to probe the hydrophobic region of MtArgJ. The excitation wavelength for ANS was 374 nM while the emission was recorded from 400 to 600 nM. The excitation and emission slit widths were 2.5 and 3 nM, respectively. Each spectrum was an average of six consecutive scans, and all the experiments were done in duplicates. Protein sample (1 μM) was diluted in PBS (pH 7.4) and was checked for the “inner filter effect” over the range of ANS as well as ligand concentrations. Blank containing equal concentration of fluorophore as that in samples was used as control, and the necessary corrections were done accordingly (Cardamone & Puri, 1992). ANS concentrations were determined spectroscopically using an extinction coefficient of 6.8 × 10^8 M⁻¹ cm⁻¹ at 370 nM. The protein was first titrated against varying concentrations of ANS to determine the kinetic parameters of ANS binding to MtArgJ. As the ANS forms a complex with protein, a blue shift was observed (from 520 to 470 nM). 5 mM of ANS was used with 1 μM of protein to attain maximum saturation at 4°C, and the total concentration of ANS and protein was kept constant throughout the experiment. Further, the ANS-MtArgJ complex was titrated against increasing concentrations of PRK/SRB in separate experiments. The decrease in fluorescence due to the displacement of ANS by PRK/SRB from hydrophobic pocket on the protein was monitored (Iyer et al., 2016). To determine the dissociation constant, net change in the relative fluorescence unit (RFU) was plotted against increasing PRK/SRB concentration (at constant protein and ANS concentrations). GraphPad Prism-7 was used for the analysis, and the non-linear regression (curve fit) model (least-squares ordinary fit) with the binding saturation “one site-specific binding” function was applied.

Fluorescence-based thermal shift assay (TSA)

A fluorescence microplate reader (iQ5, Bio-Rad iCycler Multicolor Real-Time PCR detection system) was used to monitor protein unfolding as a function of temperature. The detection involves increase in fluorescence of a fluorophore SYPRO Orange (Sigma, S5692) upon binding to the hydrophobic regions of the gradually unfolded protein. Protein samples were mixed with Hepes buffer (10 mM, pH 7.4) consisting of 1× SYPRO orange dye and appropriate concentrations of ligand over the range of several folds. Samples were incubated in 96-well PCR microplates (Bio-Rad) in the iCycler iQ5 Multicolor RT–PCR detection system. MtArgJ was kept constant at 10 μM, and the PRK/SRB concentrations were varied from 100 nM to 5 mM. DMSO was kept constant at 2% throughout the
screens. According to the experimental protocol, samples were heated at 0.5°C per minute, ranging from 10 to 95°C, and the fluorescence intensity was measured at the interval of 0.5°C (Niesen et al., 2007; Iyer et al., 2016). Cy5 filter with red-orange color intensity was selected for the SYPRO Orange detection. All the experiments were done in triplicate, and the average of three was plotted in the graphs. Appropriate buffer blanks were kept, and the intensities were subtracted for the same in each set. The fluorescence intensities were plotted as a function of temperature using GraphPad Prism-7 software, and the melting temperature of protein was determined using sigmoidal dose response function of GraphPad. The net change in $T_{m}$ was then plotted against increasing ligand concentration. The dissociation constants were determined by analyzing the data under nonlinear regression (curve fit) model (least-squares ordinary fit) using the binding saturation “one site-specific binding” function.

**Drug sensitivity assay**

Minimum inhibitory concentration (MIC$_{90}$) for the inhibitors was determined using a microplate Alamar blue assay (MABA; Franzblau, 2000). The Alamar blue assay was performed in a sterile 96-well flat-bottom transparent plates. $Mtb$ strains were cultured in 7H9 medium supplemented with 10% OADC and grown till exponential phase ($OD_{600} = 0.4$). Approximately $10^{8}$ bacteria were taken per well in a total volume of 200 μl of media. Auto-fluorescence control was set for the control wells with no $Mtb$. Additional controls were taken for the wells with $Mtb$ cells without inhibitor and the wells with only inhibitor and media. After 5-day incubation at 37°C, 20 μl of 10× Alamar blue (Kinetic blue—Krishgen Biosystems—cat. no. CC1100) was added to each well, and the plates were re-incubated for 24 h. The fluorescence intensity was then recorded in a SpectraMax M3 plate reader (Molecular Device) in top-reading mode with excitation at 530 nm and emission at 590 nm. Percentage inhibition was calculated based on the change in relative fluorescence units with increasing inhibitor concentration. The minimum inhibitory concentration that resulted in at least 90% inhibition was identified as MIC$_{90}$.

**$Mtb$ internalization and cell death assay by FACS**

For FACS experiments, a GFP-expressing strain of $Mtb$ was used to infect macrophages in similar manner as described above except that $10^{5}$ cells were seeded per well in a 24-well plate. The adherent macrophages were treated with trypsin, washed twice with PBS, and analyzed with flow cytometry using BD FACSaria FUSION (BD). The samples were excited at 405 and 488 nm lasers at a constant emission (510 nm). The program BD FACS suite software was used to analyze flow cytometry data. For propidium iodide (PI; Sigma cat. no. P4170) staining, macrophages harvested after trypsinization were washed with PBS and incubated with PI (1 μg/ml) dye for 30 s and subjected to flow cytometer.

**Apoptosis detection assay**

**Caspase 3 assay**

The caspase 3/7 levels in the supernatant were determined by using Caspase-Glow® 3/7 Assay reagent (Promega G8091). The macrophages (THP1 and Raw264.7) were seeded in 96-well plate and infected with $Mtb$ H37Rv as described earlier at the MOI of 2. The infected cells were treated with two different concentrations of PRK and incubated for varying time points till 48 h. Post-incubation, the supernatant was collected and incubated with Caspase-Glow 3/7 assay reagent (1:1) for 1 h at 37°C. Post-incubation, the wells were subjected to chemiluminescence and the intensity was recorded using a multi-channel ELISA plate reader (Thermo Scientific—Verioscan reader). All the experiments were done in triplicate and plotted using GraphPad Prism-7 software.

**Arginine supplementation assay**

$Mtb$ H37Rv cells were incubated in 7H9 media with only 1% of OADC. The secondary culture was divided into four sets and each one treated with either PRK (1 μg/ml), PRK + arginine (1 mM), Rif (0.4 μg/ml), or Rif + arginine (1 mM) for 24 h. The cells were then washed thrice with PBS and plated on 7H10 agar supplemented with 1% ADC at three different dilutions. The CFU counts were calculated based on the varying dilution of the same sample.

**Mass spectrometric analysis**

$Mtb$ H37Rv cells were grown as mentioned, and a secondary culture of 100 ml was incubated in a roller incubator chamber in roller bottles. Equal number of cells were taken and divided into five sets, each treated with either DMSO or PRK at four different concentrations (0.1, 0.5, 1, and 5 μg/ml) for 4 h. An internal standard of heavy isotope-labeled ($^{13}$C$_6$) L-arginine (m/z 180; from Cambridge isotope laboratories) was used as an internal control for extraction efficiency and comparability in the metabolite levels from different conditions. After 4-h treatment, cells were harvested, washed twice with PBS, and resuspended in the extraction solution ($H_2O$/acetoni-trile/methanol in the ratio of 40:40:20) and lysed by bead beating. The extracted metabolite samples were passed through a Sep-PAC column prior to ESI/MS analysis for identification of the arginine levels (m/z 174). Bruker HTC Ultra (ETDII) ESI ion trap instrument was used for mass spectrometry, and samples were passed through C-18 column through a mobile phase of acetonitrile and water with 0.1% formic acid. Flow rate was maintained at 0.2 ml/min, and sample volume injected was 10 μl. The peak was confirmed for arginine by fragmentation pattern analysis (MS/MS of the selected peak).

**Mycobacterium tuberculosis infection of mice through aerosol and drug treatment**

For chronic model of infection, BALB/c mice were infected via aerosol through Madison chamber aerosol generation instrument calibrated to deliver 100 CFU. Mice were infected with $Mtb$ H37Rv strain at 100 CFU of bacilli per mouse. Mice were caged for 4 weeks for establishment of infection. At specific time points (0, 12, and 24 days) post-treatment, mice were sacrificed (cervical dislocation) and their lungs were removed and processed for investigation of bacillary load. Once the infection was established, as determined by bacterial CFU count, mice were divided into five sets, untreated, PBS-treated, Rif-treated (10 mg/kg body weight), PRK-treated (40 mg/kg body weight), and PRK + Rif-treated. The dosage for PRK was determined by the MIC$_{90}$ obtained in the cell-based studies. The pharmacokinetics of PRK is already reported in various studies and is safe for IP administration (Asano et al., 2009; Ye et al., 2017). The clearance rate from the body is also reported to be very
efficient and safe. Each group contained 18 mice, 6 sacrificed at 0 day of treatment, 6 at 12\textsuperscript{th} day of treatment and 6 at 24\textsuperscript{th} day of treatment. CFUs were determined by homogenizing the lungs and plating appropriate serial dilutions (10-, 100-, and 1,000-fold) on 7H11 supplemented with OADC and PANTA (BD-245114) plates. Colonies were observed and counted after 21 days of incubation at 37°C. Lungs were analyzed for the formation of tubercular granulomas in treated versus untreated conditions. Histopathology analysis was performed as described previously (Singh et al., 2003). Briefly, sections of lungs, spleen, and liver were fixed in 10% neutral buffered formalin for embedding in paraffin, sectioning, and staining with hematoxylin and eosin (H&E). A blinded examination of at least three serial sections from each mouse was carried out to evaluate the number of granulomas (lungs) and tissue-associated histopathology (spleen and liver).

All the animal experiments were performed strictly according to the guidelines of the animal ethics committee (AEC). A license to perform the mentioned animal experiments was taken from the institute’s AEC well in advance before the commencement of the animal experiments. All the mice used were BALB/c female mice, aged 3–4 weeks at the beginning of the experiment. Animals were caged throughout the experiment in well-ventilated cages in clean rooms of BSL3 facility, in complete isolation. Once humanely sacrificed for the study by cervical dislocation, the carcasses were double-auto-claved and incinerated separately in an incineration chamber.

**qPCR primer sequence**

5-LO_Fwd: 5’ CTACGATGTCAACGGTGATG 3’
5-LO_Rev: 5’ GTGTCGCTGGAGGATATGA 3’
COX2_Fwd: 5’ GCTCTCAATGATGTTGATC 3’
COX2_Rev: 5’ GCTGGCCCTCCTGTTATGA 3’
FLAP_Fwd: 5’ TCTACACTGCCAACAGAC 3’
FLAP_Rev: 5’ ACGGACATGAGGACACCG 3’
CYSLTR1_Fwd: 5’ GGT GCT GAG GTA CCA GAT AG 3’
CYSLTR1_Rev: 5’ CAT GTT CTC CAG GAA TGG CT 3’
MCPI_Fwd: 5’ GGA CCA TTC ACG TGT TGG C 3’
MCPI_Rev: 5’ ACA GCT TCT TTG GGA CAC C 3’

**Statistical analysis**

All the data were derived from at least three independent experiments. Statistical analyses were conducted using GraphPad Prism software, and values were presented as mean ± SD. The statistical significance of the differences between experimental groups was determined by two-tailed, unpaired Student’s t-test unless specified. Differences with a P-value of < 0.01 were considered significant. An account of the P-values and “n” for all the experiments are given in Appendix Table S7.

Rest of the methods involved in this study are given in detail in the Appendix information provided.

**Expanded View** for this article is available online.

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**Author contributions**

AM and AS conceived and designed the study. AS provided the entire infrastructure and supported the research. AM performed experiments and analyzed the data. AM and AS wrote the manuscript. AS corrected the manuscript. ASM performed and analyzed computational studies. RSR and AM performed animal experiments. AR and RR helped with the biochemical experiments and microbiology. All authors reviewed the results and approved the final version of the manuscript.

**Conflict of interest**

The authors declare that they have no conflict of interest.

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