d. Details of the research work duly signed by the applicant, for which the Sun Pharma Research Award is claimed, including references and illustrations (not to exceed 6000 words)

I: Sulfur metabolism coordinating *Mycobacterium tuberculosis* respiration and redox balance

Metabolism of cysteine and methionine amino acids is critical for the survival of the most successful human pathogen, *Mycobacterium tuberculosis* (*Mtb)*. Cysteine functions as a biosynthetic precursor for essential cofactors such as Fe-S clusters and a primary antioxidant buffer, mycothiol. Fe-S cluster proteins and mycothiol coordinate *Mtb*'s ability to respire, resist oxidative stress, counteract anti-TB drugs, and establish infection. Therefore, it is essential to understand the pathways that *Mtb* utilizes to generate cysteine and its subsequent utilization to produce Fe-S clusters and mycothiol. We discovered that *Mtb* generates cysteine via the reverse transsulfuration pathway (RTS). We have biochemically, biophysically, and genetically characterized the first rate-limiting enzyme of the RTS pathway, cystathionine beta-synthase (Cbs). An important aspect of this work was characterization of Cbs by biophysical techniques, TEM and single-particle cryo-electron microscopy (cryo-EM) based atomic model determination of tetrameric assembly of native and S-adenosyl methionine (SAM)-bound *Mtb* Cbs, which provided unprecedented insight into the mechanism of allosteric activation of *Mtb* Cbs. We discovered that SAM depletion results in the degradation of Cbs, which triggers a non-canonical pathway for methionine biosynthesis in *Mtb.* Inhibition of RTS and the non-canonical methionine biosynthesis pathway killed *Mtb* by inducing oxidative stress. Lastly, Cbs is essential for *Mtb* to cause infection during HIV-TB coinfection.

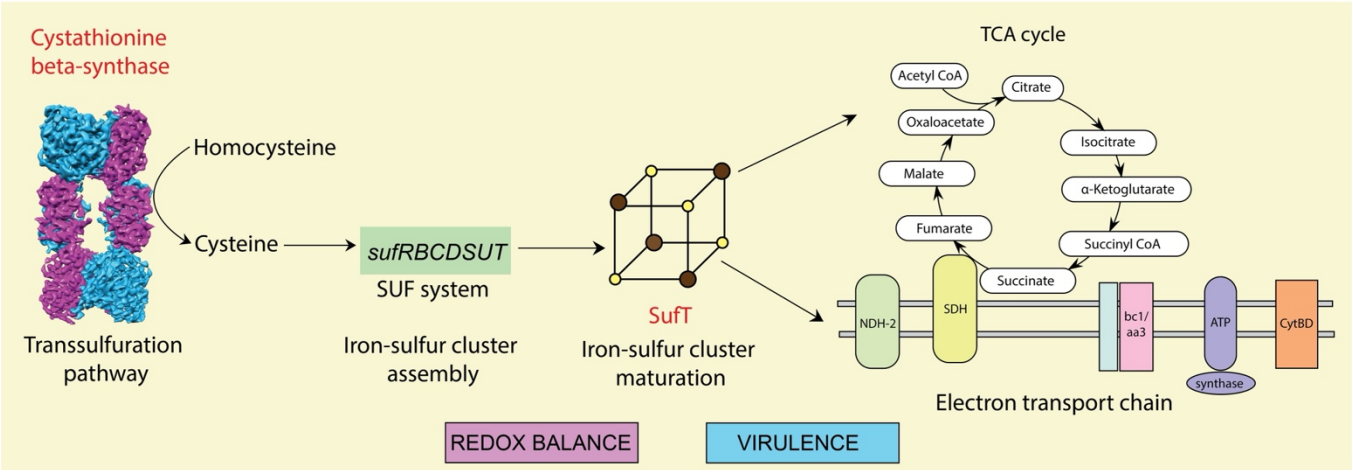

Fig. 1 : Reverse transsulfuration pathway rate limiting enzyme CBS is required for redox balance and virulence

II Fe- S cluster maturation is important for the survival of *M. tuberculosis*

Iron-sulfur (Fe-S) cluster proteins carry out essential cellular functions in diverse organisms, including the human pathogen *Mycobacterium tuberculosis* (*Mtb*). The mechanisms underlying Fe-S cluster biogenesis are poorly defined in *Mtb*. Here, we show that *Mtb* SufT (Rv1466), a DUF59 domain-containing essential protein, is required for the maturation of Fe-S clusters in *Mtb*. *Mtb* SufT interacts with SufS-SufU involved in Fe-S cluster biogenesis and the Fe-S cluster proteins, aconitase, and SufR. Further, a hyperactive cysteine present in the DUF59 domain of SufT is critical for its interaction with SufS/SufU/aconitase/SufR. Depleting SufT decreases aconitase's enzymatic activity under standard growth conditions and in response to oxidative stress and iron limitation. Depletion of SufT adversely

affected *Mtb's* growth and altered the pool of tricarboxylic acid cycle intermediates, amino acids, and sulfur metabolites. Using Seahorse Extracellular Flux analyzer, we showed that the depletion of SufT diminishes glycolytic rate and oxidative phosphorylation in *Mtb*. The SufT-depleted strain of *Mtb* showed defective survival in response to oxidative stress and nitric oxide. Lastly, SufT depletion reduced the survival of *Mtb* in macrophages and attenuated the ability of *Mtb* to persist in mice. In summary, *Mtb* SufT assists in Fe-S cluster biogenesis and couples this process to core metabolism and respiration of *Mtb* for maintaining survival during conditions of low and high demand for Fe-S proteins.

III: Targeting heterogeneity in redox physiology of *Mycobacterium tuberculosis* to improve therapy outcome

A major obstacle in the clinical treatment of tuberculosis (TB) is the long therapy time (6-9 months) required to clear the infection. A plausible explanation for the protracted drug regimen is the development of heterogeneity- a process whereby a genetically identical population of *Mycobacterium tuberculosis* (*Mtb*: the pathogen that causes TB) diversifies to produce drug-tolerant subpopulations. This form of drug insensitivity is called as phenotypic drug tolerance, which represents the greatest hurdle to effective chemotherapy. Heterogeneity in bacterial population is likely to contribute to phenotypic drug tolerance in the sputum of active TB patients. Molecular basis of heterogeneity is largely derived from studies performed in liquid culture medium, which indicates the role of differences in cell sizes and growth rates (actively multiplying vs slow growth) in promoting drug tolerance [1, 2]. However, *in vitro* approaches have not yielded any effective strategies to target heterogeneity and drug tolerance in *Mtb*, indicating that the significance of bacterial heterogeneity and drug tolerance needs to be examined in the context of host environment during infection. Filling this knowledge gap will contribute to new avenues through which current anti-TB drugs could be used in combination with compounds that block the capacity of *Mtb* to promote heterogeneity *in vivo*.

We identified that host environment promotes variations in the redox physiology of *Mtb* population to tolerate anti-TB drugs during infection. We used a range of cutting-edge technologies such as redox biosensor, replication clock, flow sorting, intra-phagosomal RNA-sequencing, mass spectrometry, and animal models, to mechanistically dissect host and bacterial factors responsible for redox heterogeneity and multi-drug tolerance in *Mtb* population during infection. Our findings, for the first time, provide empirical evidences showing how host and bacterial mechanisms cross talk to induce bacterial heterogeneity and drug tolerance during infection. Based on our findings a new model of drug tolerance emerged. According to this model, limited phagosomal acidification inside naïve macrophages facilitates the emergence of a redox-altered drug-tolerant subpopulation of *Mtb*. This drug tolerant subpopulation is actively multiplying, metabolically active, and showed higher expression of genes responsible for rerouting of cysteine amino acid into pathways such as Fe-S cluster biogenesis and hydrogen sulfide gas generation. We provide genetic evidences that pathways coordinating cysteine flux are important contributors of acidic pH dependent redox heterogeneity and drug tolerance in *Mtb* (Fig. 1). More-importantly, redox heterogeneity and phagosomal pH also contributes to drug tolerance when *Mtb* infects macrophages co-infected with another human pathogen-HIV-1. Our findings have the potential to understand why high rates of TB therapy failure are clinically documented in humans co-infected with HIV-TB as compared to patient infected with TB alone [3].

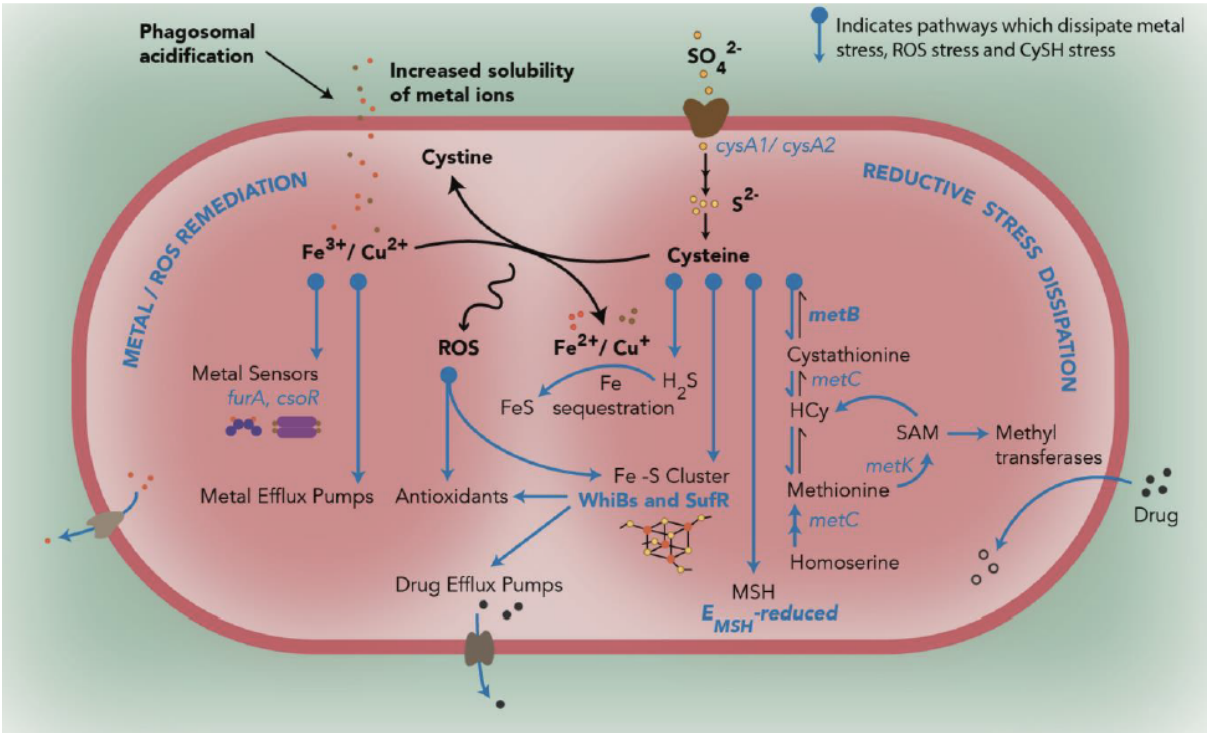

Fig. 2 Model depicting various mechanisms underlying redox-mediated drug tolerance in replicating *Mtb*. *Phagosomal acidification inside resting macrophages serves as a cue to induce redox heterogeneity and drug tolerance in Mtb. Gene expression data indicate that low pH induces accumulation of cysteine (CySH) causing reductive stress. Elevation of CySH is known to generate reactive oxygen species (ROS) through metal (Fe/Cu) catalysed oxidation to cystine (CyS2). Acidic pH also increases solubility of metals such as Fe and Cu, which drives generation of ROS via Fenton chemistry. Also, various anti-TB drugs induce oxidative stress inside Mtb during infection. To manage these stresses, Mtb efficiently channelizes the flux of CySH in reverse transsulfuration pathway (H2S generation), Fe-S cluster biogenesis (SufR/WhiBs), and MSH production, resulting a reductive shift in the EMSH of Mtb. All of these mechanisms are known to protect bacteria from drugs and oxidative stress by metal sequestration and activation of antioxidants. Increased expression of metal and drug efflux pumps further remediate metal/antibiotic triggered redox stress in Mtb. Induction of SAM-dependent methyl-transferase in EMSH-reduced bacteria can directly inactivate drugs by N-methylation. Impaired ability of metB, sufR, and whiB3 mutants in tolerating antibiotics suggests management of CySH flux as an important bacterial strategy to protect from drugs. Pharmacological inhibition of phagosomal acidity by CQ restores redox heterogeneity to subvert drug tolerance and post-therapeutic relapse.*

How can we relate our basic findings to develop new therapeutic strategies against human TB? Our study raises fascinating new possibilities for managing phenotypic antibiotic resistance. For example, inhibitors of host signal(s) that are sensed by *Mtb* to generate redox diversity could be exploited to restore phenotypic homogeneity and potentiate the killing activity of existing frontline antibiotics. On this basis, we reasoned that pharmacological inhibition of phagosomal acidification could preclude mobilization of redox-mediated drug-tolerant phenotype *in vivo*. We tested our hypothesis by using the antimalarial drug chloroquine (CQ), which is well known to increase vesicular pH [4]. We discovered that CQ in combination with front-line anti-TB drugs (isoniazid or rifampicin) eradicated drug-tolerant *Mtb*, ameliorated lung pathology, and reduced post-chemotherapeutic relapses in infected animals (mice and guinea pigs) (Fig. 2). The pharmacokinetic study shows no adverse interaction of CQ with the first line anti-TB drugs in mice. Since CQ is clinically used, has a longer half-life, is cost-effective, and highly

tolerable with fewer side effects [5], it can be conveniently repurposed to formulate new combinations with the current anti-TB regimen to reduce therapy duration.

In conclusion, our study is a clear example of how fundamental research can generate translational opportunities to target one of the global human pathogen, which kills ~ 1.7 million people annually. Finally, redox-mediated multi-drug tolerance may be relevant to other chronic pathogens. For example, heightened antioxidant capacity is linked to the acquisition of phenotypic antibiotic resistance in the human pathogens *Pseudomonas aeruginosa* [6]. Thus our findings may have broad relevance to several human pathogens where a sterilizing cure is therapeutically challenging.

IV: Tackling HIV using Hydrogen sulphide gas

We found that a clinically used antioxidant N-acetyl cysteine (NAC) suppresses HIV reactivation from a latently infected cells. Later it was reported that NAC partly acts by releasing hydrogen sulfide (H_2S) gas, which also a potent antioxidant and in low amounts help improve the function of mitochondria and reduces inflammation. We discovered that reactivation of HIV-1 from latency is associated with depletion in the endogenous levels of H_2S. We further found that as long as cells maintains higher levels of H_2S, virus remains locked in the latent state. Interfering with a main cellular enzyme responsible for H_2S production results in breakdown of latency and reactivation of HIV. We observed a direct effect of H_2S on suppressing HIV reactivation and replication along with all its other beneficial effects, such as maintenance of mitochondrial health and dissipation of oxidative stress in our

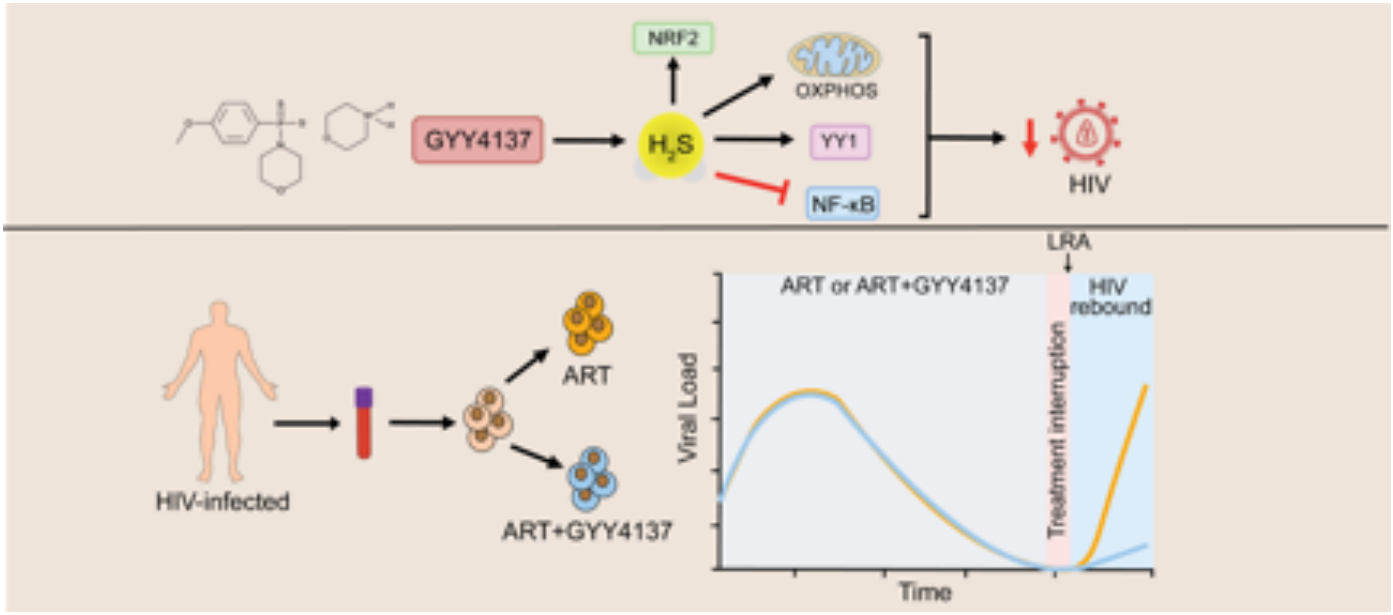

Fig. 3: H_2S donors (GYY4137) locks HIV in latency. GYY4137 regulates HIV latency and reactivation by activating NRF2-Keap1 system and epigenetic modulator, YY1, and suppressing NF-KB pathway.

Lastly, exposing latent cells derived from the HIV infected individuals with a combination of a pharmacological donor of H_2S (GYY4137) and ART locked HIV in a latent state and prevented virus rebound when ART was stopped. Our study open the door to supplementing clinically used ART with chemical donors of H_2S to lock HIV in a state of deep latency, potentially improving the lives of millions infected with the virus. Since H_2S donors are already undergoing clinical trials for other diseases, they can quickly be repurposed for HIV treatment.

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(Amit Singh- Signature)