An Integration of Interdisciplinary Translational Research in Anti-TB Drug Discovery: Out of the University Research Laboratories to Combat *Mycobacterium tuberculosis*

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Tuberculosis (TB) is a dreadful infectious disease responsible for approximately two million human deaths and nine million new cases each year causing massive health and economic impact on global development [1]. One third of the world's population is latently infected by the pathogen, *Mycobacterium tuberculosis*. Emergence of total-drug-resistant (TDR)-TB on top of extensively-drug-resistant (XDR) and multidrug-resistant (MDR)-TB is undermining the efforts of many major national and international research consortia such as TB-Drug Discovery, UK (www.tbd-uk.org.uk), Antibiotic Action (www.antibiotic-action.com), New Medicines for Tuberculosis (www.nm4tb.org), Open Source Drug Discovery, India (OSDD; www.osdd.net), EuroLAC-TB consortium (www.eurolactb.org) and the Global TB alliance (www.tballiance.org) to resolve this global human health challenge.

Treatment against drug-resistant strains is too lengthy, complex, expensive and highly toxic which is often fatal to many MDR-TB patients and unfortunately effective treatment for XDR/TDR-TB does not exist [2]. New drugs with novel modes of action to tackle drug-resistance and dormant bacilli are urgently required. In resource-limited laboratory settings it takes significantly more time and effort to validate a single drug for a new TB regimen. Therefore a continual pipeline of novel inhibitors to treat drug-resistant and latent TB on a global scale is desperately needed.

Validation of a novel therapeutic target, target based discovery of novel leads and whole cell evaluation of novel chemical entities, are three key interdisciplinary research approaches in preclinical drug discovery. Because of the large investment for TB drug development which is nearly always considered a non-profit making programme for big Pharma-industries; many companies cannot afford to be involved in these efforts. To overcome these unmet medical needs, recently, we have noted that integration and co-operation among academic networks have shown significant outcomes from university based wet laboratories at the early stage of drug discovery. These endeavours are to de-risk and provide proof of concept studies which can pave the way for industry in new TB drug development.

Target Based Approach: Validation of Novel Therapeutic Targets in *Mycobacterium tuberculosis*.

The success of *M. tuberculosis* as a pathogen lies in its ability to survive within the hostile environment of human macrophages. For this reason, *M. tuberculosis* is equipped with an arsenal of endogenous functions that are vital for its survival [3].

Viable but non-replicating (VBNC) TB bacilli survive for an undefined period of time [3]. When the host is immunosuppressed, VBNC-bacilli are likely to transform to actively-dividing cells causing TB symptomatology. Resuscitation-promoting factor (Rpf) is the "bacterial cytokine" originally discovered in *Micrococcus luteus* that enables the bacteria to re-emerge from dormancy [4,5]. *M. tuberculosis* has five sequence homologues of Rpfs (Rpf A, B, C, D, E) [6]. Bioinformatic and structural studies have shown these proteins to be peptidoglycan degrading (hydrolase) enzymes [7,8] and their activity

has been verified *in vitro* [9] and *in vivo* within *M. tuberculosis* infected human tissues [10]. All Rpfs have some sequence variation at the *N*- and *C*-termini conferring the possibility of recognizing different substrates and interacting with other proteins involved in their regulation [11]. In mycobacteria, the serine/threonine protein kinases (STPKs) are responsible for the regulation of different cellular processes including cell development, survival inside macrophages, stress responses and host-pathogen interactions [12]. A serine/threonine protein kinase, PrkC in *Bacillus subtilis* [13] (a homologue of the *M. tuberculosis* PknB), has been reported to act as an effective signal leading to spore germination, which is considered to be a similar physiological process to mycobacterial resuscitation [14]. In a recent analysis of the phosphoproteome spectrum of *M. tuberculosis*, a putative hydrolase Rv3915 (a family that Rpfs are members of) has been identified as having a phosphorylation site at a threonine residue [15]. This indicates that regulation of resuscitation might be modulated through signaling by STPKs.

The Rpfs are the secretory products responsible for awaking bacilli from the persistent (dormant) state [4]*,* but their mechanism of regulation and endogenous substrates are yet to be determined. STPKs are involved in different metabolic pathways for regulation of cell division, survival inside macrophages, stress responses and host-pathogen interactions in mycobacteria but there is no direct evidence for their involvement in dormancy. To understand the physiology of *M. tuberculosis* during metabolic switching among active-dormant-reactive phases is an enthralling area of anti-TB drug discovery research.

A key unique feature of TB causing bacilli is their complex cell walls, which consist of an unusual mycolyl-arabinogalactan-peptidoglycan (MAGP) complex. Peptidoglycan provides shape and structural integrity to the bacterial cell. This innermost layer of the bacterial cell wall is present in virtually all bacteria (with the exception of Archea). The glycan backbone of peptidoglycan (PG) is comprised of alternating *N*-acetyl glucosamine (NAG) and *N*-acetyl muramic acid (NAM) (*N*-glycolyl muramic acid is also present in *M. tuberculosis*) residues, which are linked to each other by β-(1,4)-glycosidic bonds; the muramic acid residues containing a polypeptide side chain.

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Neighbouring peptide-substituted glycan strands are cross linked by peptide bridges which extend from the carboxyl group of the terminal D-alanine of one peptide unit to the side chain amino group of the di-amino acid residue of another unit. The nature of the di-amino acid residue of the peptide units, the composition and length of the peptide bridges and the extent of peptidoglycan cross-linking vary according to the bacterial species [16,17]. In addition, peptidoglycan is covalently linked to lipoarabinomannan (LAM) and arabinogalactan (AG) via phosphodiester bonds and in turn attached to mycolates (M) and other complex lipids in *M. tuberculosis*.

Primarily based on the locale of biochemical reactions, PG synthesis, degradation and remodeling occur at different rates in different physiological stages of *M. tuberculosis*. The biosynthesis of PG starts in the cytoplasm through production of activated lipid-linked precursors. The transport of these hydrophilic precursors across the inner membrane is facilitated through coupling to a hydrophobic carrier, namely undecaprenyl phosphate (also known as bactoprenol), which is catalysed by the MraY and MurG transferases respectively. The third and final stage, involves the insertion of the disaccharide-pentapeptide precursor into the growing peptidoglycan polymer, and consists of *trans*-glycosylation (which lengthens the saccharide chains) and *trans*-peptidation (which forms the peptide cross bridges), which occur in the extra-cellular space and is catalysed by penicillin-binding-proteins (PBPs) [18].

The Mur enzymes (MurA-F) act during the initial cytoplasmic stages of PG biosynthesis. Among them MurC, D, E and F are members of the ATP-dependent ligase family. These enzymes contribute to the synthesis of UDP *N*-acetyl muramoyl pentapeptide through a sequential peptide-ligase reaction by using UDP-MurNAc, ATP and different amino acid substrates. Crystal structures of the recombinant Mur ligases in *E. coli* have been determined [19-22]. All these Mur ligases have been found to be essential in a number of pathogenic bacteria. Sequence alignments at the gene and amino acid level, however, clearly indicate significant dissimilarities between these Mur ligases from *M. tuberculosis* and from other micro-organisms including *E. coli* [23]. In order to characterize the key biochemical pathway involved in the initial cytoplasmic stages of mycobacterial PG biosynthesis, to date, the *mur C, D, E* and *F* genes have been cloned from *M. tuberculosis* H$_{37}$Rv and the recombinant Mtb-Mur proteins purified from the soluble fraction were shown to be functional. Mtb-MurC was found not only to be able to add L-Ala, its natural substrate, but also glycine and L-serine. Mtb-MurD, E and F were strictly specific towards their substrates and only showed activity with their respective target amino acids: D-glutamic acid, *meso*-diaminopimelic acid and D-alanine-D-alanine respectively. Furthermore, presence of divalent cations was found to be an absolute requirement for their activities. Also higher concentrations of ATP and UDP-sugar substrates were inhibitory for the activities of all ATP dependent Mur ligases suggesting a stringent control of the cytoplasmic steps of peptidoglycan biosynthetic pathway in *M. tuberculosis* [23-25].

All the four Mur ligases in *M. tuberculosis* are a part of the division/cell wall (*dcw*) operon where the *dcw* gene cluster spans from *murE* until *ftsQ*. This analysis also identified a new promoter driving the co-transcription of *mur* ligases along with key cell division genes such as *ftsQ* and *ftsW*. As cell division and PG biosynthesis genes share the *dcw* cluster, and that the PG biosynthetic protein PBP3 has been reported earlier to interact with both FtsW and FtsZ, therefore forming a trimeric complex in mycobacteria [26], it was hypothesized that an interaction between the PG synthesis and cell division proteins could exist. In addition to a confirmation to that, it was further reported that the regulation of mycobacterial Mur ligases were through protein-protein interactions with Ser/Thr Protein kinases and via phosphorylation [25,27].

Two crystal structures of Mtb-MurE have been solved with different substrates bound to it, and essential residues have been determined for this enzyme by mutation studies [23,24]. A number of initial hits against the Mtb-MurE enzyme have been reported by us recently [28-30] and some of these agents are also antibacterial against whole-cell *M. tuberculosis*, are now part of medicinal chemistry projects to optimize these hits. Among these compounds, natural product aporphine alkaloids and their novel synthetic analogues have raised further hope and offer further confidence in concept of enzymatic and whole cellular combined screening strategies in TB research (Bhakta S and Hailes HC; unpublished results).

Next to MurE on the *dcw* operon, the ATP-dependent Mur Ligase, MurF, acts at the final stage of UDP sugar-linked polypeptide synthesis. The product of this reaction, UDP *N*-acetyl muramoylpentapeptide, is the key substrate for the membrane bound transition stage of PG biosynthesis. The central biochemical reactions in PG biosynthesis appear to be essential and unique in *M. tuberculosis*. Consequently our knowledge on the structure of the enzymes involved in these reaction steps and their function and regulation, on the cell function and physiology will lead us towards validating them as novel therapeutic targets and target-based discovery of new drugs.

Whole Cell Phenotypic Approach: An Efficient Predictive Model for High-Throughput Whole-Cell Phenotypic Intracellular Screening of Anti-Tuberculars

Whilst a large number of novel chemical libraries are waiting for comprehensive phenotypic evaluation at the preclinical stage of TB drug development, the search for novel chemotherapeutics against drug-resistant TB is severely impeded by the slow growth of this organism and the need to work in highly stringent and expensive biosafety level-3 laboratories with the additional need of further training for the health and safety for the researchers. This poses considerable obstacles, such as complex handling, expensive set up and special training requirements that are major bottlenecks towards extensive drug screening research. In order to alleviate these critical issues, surrogates have been introduced in the drug discovery process, of which non-pathogenic, fast-growing *Mycobacterium aurum* is one of the most useful and closely related surrogate strains. This is because of its similar cell wall constitution, drug susceptibility profile and similar drug-resistance mechanisms to *M. tuberculosis* [31,32]. The whole-cell high-throughput screening (HTS) platform, a rapid but gold-standard assay for characterising anti-tubercular compounds is always invaluable for effectively selecting potential hits at a very early stage of TB drug discovery program. Phagocytosis and survival of the TB pathogen through interaction of the host and bacterial cell receptors inside the macrophage environment are two key consequences. In the spot culture growth inhibition (SPOTi) assay [33], a different physiology of the mycobacteria is represented as a biofilm on the surface of solid media mimicking the *in vivo* growth of the pathogen which is typically seen in histopathogical *M. tuberculosis* lesions. By exploiting the characteristics of *M. aurum* and by the use of the HT-SPOTi assay, Gupta and Bhakta recently established an integrated surrogate drug screening model for intracellular screening of inhibitors [32]. Inhibitors work with greater impact in a lower oxygen level intracellular natural redox environment which is created by the immune cells representing asymptomatic latent TB infection with viable but non-replicating TB bacteria [3]. In addition, the sensitivity to an acidic pH environment

and the ability to multiply inside RAW 264.7 macrophages provides additional advantages for implementing the surrogate *M. aurum* in intracellular drug-screening model methods. Using this model one can screen approximately twenty compounds in one set of experiments and the model is, as yet, only against actively dividing bacterial cells.

As a step forward to explore new anti-TB molecules in a rapid and more efficient assay method, one must develop and characterise a high throughput intracellular screening model for both the actively dividing and dormant *Mycobacterium sp.* as a gold standard to substitute for the highly virulent, extremely slow-growing *M. tuberculosis* in the early stage of an anti-TB screening program.

Articulated translational and co-operative research interventions from interdisciplinary research laboratory settings are rays of hope to combat the *M. tuberculosis* enemy which has been a threat to human health and well being for millennia.

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