# Development of tuberculosis treatment

Tuberculosis, one among the socio-economic burdens in the developing countries including India, is primarily found to be associated with poor hygiene, malnutrition and impaired immune system. The multiple drug resistance in the causal organism i. e., *Mycobacterium spp.* , long term drug regimen induced hepatotoxicity/ renal toxicity and HIV pandemic are other compounding factors to aggravate the situation, hence need to be addressed on top priority. Therefore, in the present investigations we attempted to design and develop a more efficacious and biologically safe anti-tuberculosis drug, which could be utilized to target the pathogen specific cellular pathways, combating to drug resistance, shorten and/or simplify current treatment regimens, provide effective therapy for patients intolerant to current first-line drugs, and also to provide treatment to the patients with latent *Mycobacterium* infection. Based on the encouraging reports of SAR studies on symmetrical and asymmetrical cyclohexane-1, 2-diamine and cyclohexane-1, 3-diamine derivatives, we have also design and developed similar kind of anti-tuberculosis compounds using cyclohexane -1, 4-diamine as a synthone. We synthesized a library of symmetrical *trans* -cyclohexane-1, 4-diamine derivatives and evaluated them against *Mycobacterium tuberculosis* H $_{37}$ Rv strain to assess their applicability as potential anti-tuberculosis drug candidate molecules. Out of twenty seven symmetrically substituted *trans* -cyclohexane-1, 4-diamines screened, majority of the compounds showed a moderate to reasonably good anti-tubercular activity (MIC $_{99}$ = 50-25 µM). Only one compound -‘ 9u’ having *i* -propyl group substitution at *p* -position was found to show highly significant anti-tubercular activity against the *Mycobacterium tuberculosis* H $_{37}$ Rv strain with a MIC $_{99}$ value of 12. 5 µM. Four other synthesized compounds have also

shown moderate inhibitory activity against MRSA. In order to evaluate the therapeutic applicability of compound ‘ 9u’, showing highly significant activity against *Mycobacterium tuberculosis* H $_{37}$ Rv strain, toxicity evaluation in target host cells i. e., human lung epithelium cells-A549 was done at and above the anti-tuberculosis effective doses. We recorded the dose dependent toxic responses of test compound ‘ 9u’ at many fold higher than the effective doses against *Mycobacterium tuberculosis* H $_{37}$ Rv stain.

Genotoxicity assessment of the test compound- ‘ 9u’ has also been carried out employing two widely acceptable assays i. e., Micronucleus (MN) and Chromosomal aberration (CA) assays. Micronucleus (MN) formation is an indication of fragmentation in chromosomes, and that fragment is not incorporated into daughter nuclei during mitosis. On the other hand, chromosome aberration (CA) is denoted as a missing, extra, or an irregular portion of chromosomal DNA, which is not integrated into daughter nuclei. The xenobiotics exposure had been demonstrated to form an atypical number of chromosomes or a structural abnormality in one or more chromosomes due to MN and/ or CA. In MN assay, the cells were grown in DMEM/F-12 medium alone served as basal control and the other set of cells exposed to different concentrations of test compound ‘ 9u’ for different time periods were studied as treatment groups. The increase in the micronucleus (MN) frequency was observed as compared to the cells growing in normal medium. Our findings demonstrated that the test compound ‘ 9u’ has no mutagenic potential even at concentrations many fold higher to the therapeutically effective concentration against *Mycobacterium tuberculosis* H $_{37}$ Rv strain. No significant induction in the MN could be recorded in any

concentration of test compound except $10^{-3}$ and $10^{-4}$ M. The trends for CA were almost similar, as to that of MN assay. The most common aberrations were of chromatid gaps and break type, followed by higher concentrations of test compound. There were occasional incidences of aneuploidy in the cells exposed to higher concentrations, i. e., $10^{-3}$ and $10^{-4}$ M for longer duration i. e., 72 and 96 h. The induction of CA was dose dependent, i. e., 15±1. 73, 19±1. 15, 29±2. 31 and 37±2. 89 Aberrations/100 in cells exposed to $10^{-3}$ M for 24, 48, 72 and 96 h respectively.

So, the study results obtained indicate that the compound “ 9u” is safe from cytotoxicity and genotoxicity point of view in human lung epithelial cells-A549, even up to many folds higher concentrations than that are required to kill the *M. tuberculosis.*

However, anti-TB treatment requires a minimum of six months exposure to host systems and most of the available anti-TB drugs are known to induce apoptosis, oxidative stress and injury during such a long term exposure. Thus, our studies were extended to investigate the quantitative alterations, if any, in expression profile (mRNA and protein) of markers associated with apoptosis, injury and oxidative stress in the human lung epithelial cells-A549 receiving a chronic exposure of potential anti-TB compound ‘ 9u’. Healthy growing A549 cells were exposed to test drug i. e., *trans* -cyclohexane-1, 4-diamine derivative-9u at a therapeutic concentration ($10^{-5}$ M) for a period up to seven weeks. Our findings demonstrate that there was a statistically insignificant transit shift (till three weeks) in the markers of apoptosis (Bax, $Bcl_2$ , p53, caspase-3), cell injury (b-FGF, JNK, C-Jun C-Fos) and oxidative

stress (GSH, GPx, SOD, Catalase). Thereafter, expression changes came back to the basal, when compared with unexposed cells of respective time periods. The results also inferred that the transcriptional changes at mRNA level induced by compound ‘ 9u’ were translated into changes at the protein level and to further activity of the cells. There was linearity in the data obtained at both transcriptional and translational levels, only the changes were transient and statistically non-substantial and could be repaired by the cells themselves over a point of time during the chronic exposure of the test compound i. e., 7 weeks. The data confirm the therapeutic potential of compound ‘ 9u’ for even longer term treatment against *M. tuberculosis* without having any significant apoptosis, injury and oxidative stress.

Conclusion:

Upon successful culmination of the subject area, as per the targets envisaged in the outline, the major findings can be concluded as under:

- A library of twenty seven symmetrical *trans* -cyclohexane-1, 4-diamine derivatives have been synthesized with high purity and effectively characterized.
- The majority of compounds have shown moderate to fairly good antitubercular activity with a $MIC_{99}$ range between 50-25 µM.
- Out of twenty seven compounds screened, four compounds have shown moderate inhibitory activity against MRSA.
- The test compound -‘ 9u’ having *i* -propyl group substitution at *p* -position was found to possess highly significant anti-tubercular activity

against the *Mycobacterium tuberculosis* H $_{37}$ Rv strain with a MIC $_{99}$ value of 12. 5 µM.

- The *in vitro* cytotoxicity/ genotoxicity studies carried out in human lung epithelium cells-A549 with the test compound ‘ 9u’ identify the test compound ‘ 9u’ biologically safe at the concentrations many fold higher than its effective concentration against *Mycobacterium tuberculosis* H $_{37}$ Rv strain.
- Our findings also demonstrate that there was a statistically insignificant transit shift in the markers of apoptosis, injury and oxidative stress at initial exposure of A549 cells to test compound ‘ 9u’, which could be restored by the cells themselves over a period of time during the chronic exposure of the test compound.

At the same time, no toxic responses of compound ‘ 9u’ at and above the therapeutic doses in the cells of target organs may be a step towards the development of potential alternative therapeutic entities that can be used both to reduce the duration of therapy as well as to combat the growing problem of clinical drug resistance. However, the study recommends *in vivo* investigations using experimental models to further investigate the possible anti-tuberculosis potential of test compound ‘ 9u’ at biological safe doses.

When this work was initiated, Rv3014c had been tentatively annotated in the Tuberculist genomic database as a putative NAD+ -dependent DNA ligase. At the outset we wanted to verify that the gene really coded for a functional LigA. Additionally, we wanted to solve the crystal structures of the full length or specific domains of the protein to probe interesting aspects of the structure – function relationship and also to exploit the structure(s) in the

rational identification of novel inhibitors. This objective was in line with the promise of NAD+ -dependent DNA ligase as a novel drug target. Chapter 1 places the current work in the light of already known and studied nucleotidyltransferase family of proteins of which DNA ligases are a member. Chapter 2 covers the various techniques and experimental approaches used to clone, purify and characterize the proteins/peptides in the present work. It also deals with the methods used to solve the structure of the adenylation domain of M tuberculosis LigaseA, analysis of the kinetic parameters of different inhibitors as also in silica docking I screening approaches. Chapter 3 The results obtained while cloning, purifying and characterization of the enzyme are detailed here. The preparation of the domain deleted mutants and their functional characterization are also reported here Chapter 4 deals with the crystallization of functional domains as also the fulllength ligaseA. The structure solution and analysis of the 36 kDa adenylation domain of M. tuberculosis LigaseA along with bound co-factor is also reported here. Chapter 5 deals with the identification of novel inhibitors of M tuberculosis LigaseA through docking/virtual screening techniques. In vitro and antibacterial assays reported here have led to the identification of a novel class of inhibitory compounds, which bind to the co-factor binding region. These compounds inhibit M. tuberculosis NAD+ ligase with higher specificity compared to bacteriophage T4 ATP ligase as well as human DNA ligase I. 111 A part of the results of this thesis have already been reported in the following publications. PUBLICATIONS & PATENTS NAD+ -dependent ligase (Rv3014c) from M. tuberculosis H37Rv: Crystal structure of the adenylation domain and identification of novel inhibitors. Sandeep Kumar Srivastava, Rama Pati Tripathi and Ravishankar Ramachandran. Journal of Biological

Chemistry May 17, PMID 15901723, (2005). Substituted glycosyl ureides and amines as potent inhibitors of Mycobacterium tuberculosis NAD+ -dependent DNA ligase. Sandeep Kumar Srivastava, Divya Dube, Neetu Tewari, Namrata Dwivedi, Rama Pati Tripathi and Ravishankar Ramachandran. (Manuscript Communicated) Novel Glycosyl Ureides Useful as Inhibitors of NAD+ DNA Ligase from M. tuberculosis H37Rv. Sandeep Kumar Srivastava, Neetu Tiwari, Rama Pati Tripathi and Ravishankar Ramachandran. (Patent has been filed, 2005)