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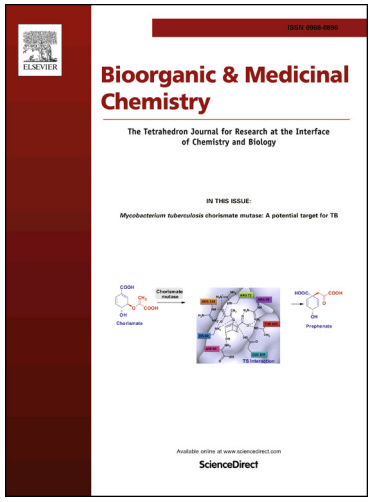

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Rudraraju Srilakshmi Reshma, Variam Ullas Jeankumar, Nidhi Kapoor, Shalini Saxena, Karyakulam Andrews Bobesh, Astakala Rishi Vachaspathy, Pappachan E Kolattukudy, Dharmarajan Sriram

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Mycobacterium tuberculosis lysine-ε-aminotransferase a potential target in dormancy: benzthiazole based inhibitors

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D. Sriram, Department of Pharmacy, Birla Institute of Technology & Science-Pilani, Hyderabad Campus, Jawaharnagar, Hyderabad - 500078. INDIA. Telephone: +91-40663030506; Fax: +91-4066303998; Email: dsriram@hyderabad.bits-pilani.ac.in

ABSTRACT:

MTB lysine-ε-aminotransferase (LAT) was found to play a crucial role in persistence and antibiotic tolerance. LAT serves as a potential target in the management of latent tuberculosis. In present work we attempted to derivatize the benzthiazole lead identified through high throughput virtual screening of Birla Institute of Technology and Science in house database. For Structure activity relationship purpose 22 derivatives were synthesized and characterized. Among synthesized compounds, eight compounds were found to be more efficacious in terms of LAT inhibition when compared to lead compound (IC_{50} 10.38 ± 1.21 µM). Compound **22** exhibits bactericidal action against nutrient starved *Mycobacterium tuberculosis* (MTB). It also exhibits significant activity in nutrient starvation model (2.9 log folds) and biofilm model (2.3 log folds).

Key words: Dormancy, *Mycobacterium tuberculosis*, Lysine-ε amino transferase, 3D granuloma model, Benzthiazole derivatives.

1. Introduction

Latent tuberculosis (TB) can be defined as a torpid noncontagious form of mycobacterium residing in host macrophages without symptoms of disease. One third of the global population has latent TB and chances upon reactivation to active TB are 5-10% [1]. The currently available option of treatment of latent TB is based on the monotherapy with Isoniazid (INH), for 6 to 9 months or 3- or 4-month INH + Rifampicin (Rif) combination therapy. Current treatment regimens are lengthy due to resistance, persistence of TB and also associated side effects in therapy is a major drawback.[2] Genetic expression profiling helped in identifying new targets associated with latency one such target is lysine-ε amino transferase (LAT) [3]. It has a key role in antibacterial resistance and persistence [4]. LAT is a PLP dependent type II aminotransferase enzyme which catalyzes reversible transamination from lysine to α-keto glutaric acid resulting in piperidine-6-carboxylic acid and glutamate.[4,24] In present work we focused our attention in expanding new class of benzthiazoles identified through virtual screening from our laboratory.[5] A set of twenty-two compounds were synthesized and evaluated for their potency against both replicative and non-replicating stages of bacteria. By molecular derivatization approach lead molecule (LAT IC_{50} 10.38 µM) was modified to develop new hit compound **21** (LAT IC_{50} 1.15 µM) with ten folds increases in inhibitory potency.

2. Results and discussion

2.1. Design and Synthesis

Recently we identified nine novel MTB LAT lead compounds by employing e-pharmacophore approach for crystal structure of LAT from MTB in internal aldimine form with a bound substrate of ⟨-ketoglutarate (PDB code: 2CJH) with 2.00 Å resolution.[5] Among the nine initial leads compound **1** (2-(benzo[*d*]thiazol-2-yl)-3-(4-hydroxyphenyl)acrylonitrile) was found to

have an IC_{50} of 10.38 µM. Modelling studies revealed that Compound **1** exhibited good binding affinity to active site of LAT enzyme. As the findings of the study are fascinating we attempted some modifications in compound **1** by introducing various changes in aromatic handle and the benzthiazole scaffold is unaltered. For structure activity relationship purpose various phenyl and heterocycles were introduced at R position (**Fig. 1**) and a library of twenty-two compounds were synthesized by two steps synthetic protocol (**Scheme 1**).

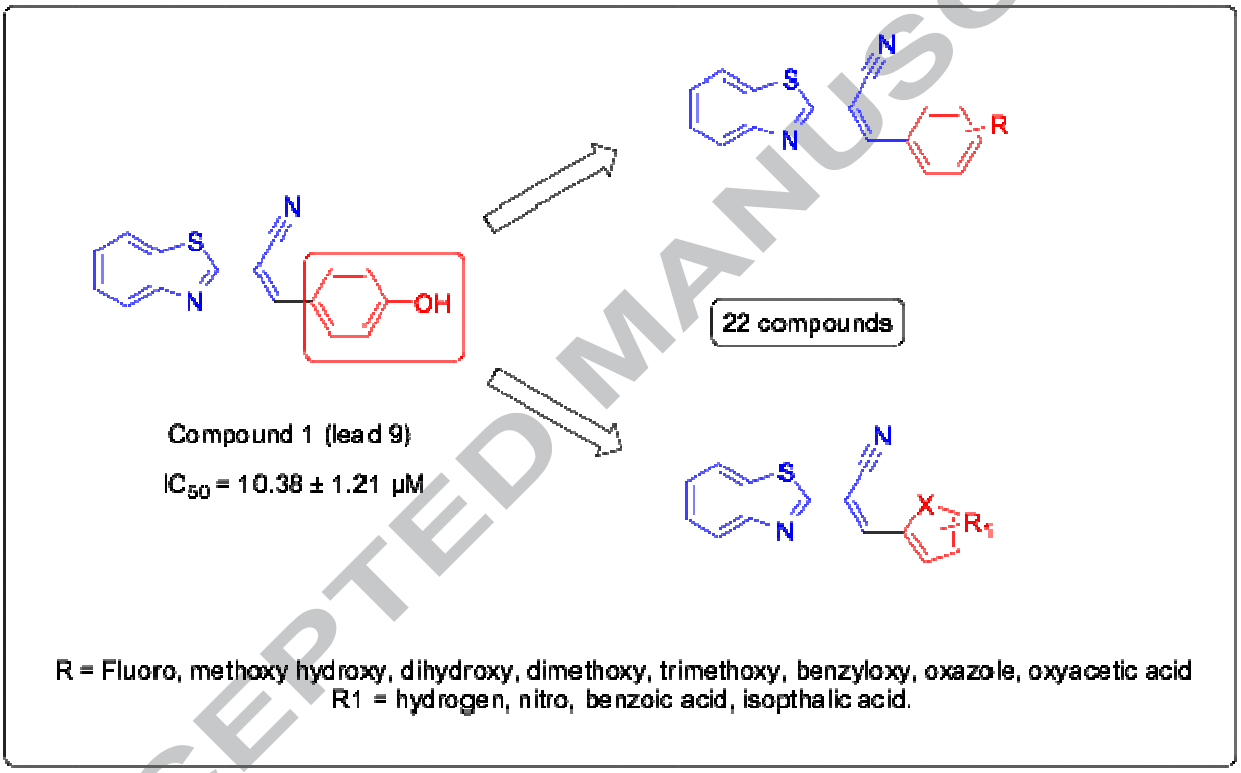
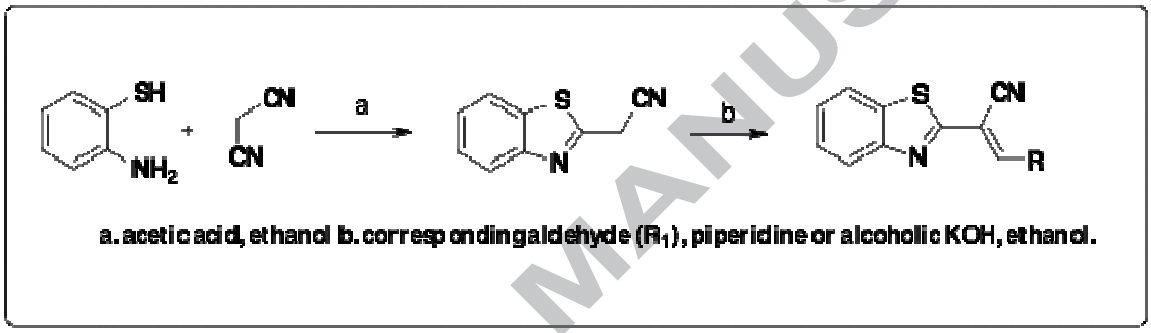


Figure 1: Design strategy employed for lead derivatization.

Synthesis started with the construction of benzothiazole ring; which was achieved by condensation of 2 aminothiophenol with malononitrile using ethanol as solvent in presence of catalytic amount of acetic acid. The second step involves Knoevenagel condensation of synthesized 2-(benzo[d]thiazol-2-yl) acetonitrile with substituted aryl/heteroaryl aldehydes. In this step nucleophilic addition of active hydrogen from acetonitrile part with carbonyl group of

substituted aldehydes followed by water elimination to form α-β unsaturated aldehydes (conjugated enone) as final compounds. In this step weak base piperidine used in catalytic amount. For SAR purpose Benzoxazole derivatives are prepared using similar protocol mentioned above. The compounds were characterized by mass, NMR spectroscopic methods and also by elemental analysis. NMR spectra and mass data were found to be unison with theoretical values confirming structure of final compounds. The elemental analysis results were within ±0.4% of the theoretical values.

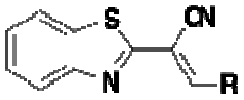


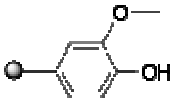
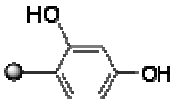
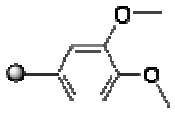
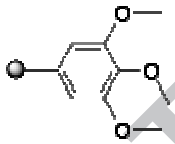
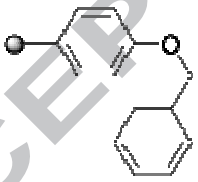
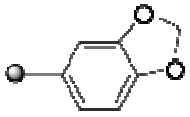
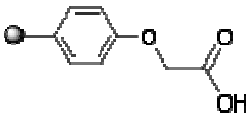
Scheme 1: Schematic representation of protocol employed for synthesis of benzthiazole derivatives.

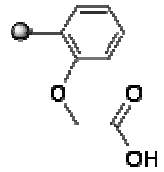
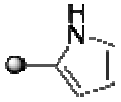
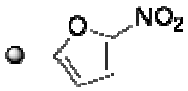
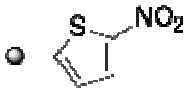
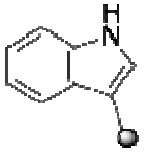
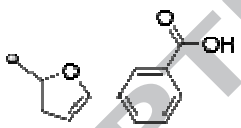
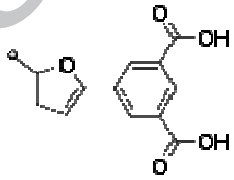
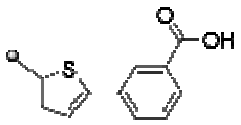
2.2. Biological Evaluation

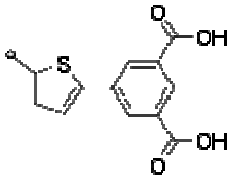
LAT catalyses reversible transamination from lysine to α-keto glutaric acid resulting in piperidine-6-carboxylic acid and glutamate. As the end products have absorbance maxima at 465 nm and 280 nm all compounds were evaluated for their MTB LAT inhibitory potency by spectroscopic method and the results are tabulated in **Tab 1**. Five compounds exhibited IC_{50} less than 5 µM and compound **19** was found to be most potent among all compounds with an IC_{50} value of 1.15 ± 0.27 µM. Compound **19** showed 10-fold reduction in IC_{50} when compared to lead compound **1** (10.38 ± 1.21 µM); eight compounds have more efficacy in terms of LAT inhibitory potency than lead compound **1**. Compounds **9, 17** and **22** also exhibited good inhibitory potency of 3.08 ± 0.37, 3.74 ± 0.27, 2.62 ± 0.37 µM respectively.

Table 1: Structure and biological results of studied compounds



CODE	R	LAT (IC_{50}) µM	MIC µM	MTT RAW cell lines 50 µg/ml
1		10.38 ± 1.21	>89.93	2.26 ± 0.11
2		4.11 ± 0.78	89.29	10.56 ± 0.63
3		7.83 ± 0.31	20.29	34.78 ± 0.82
4		23.19 ± 0.89	10.61	45.72 ± 0.21
5		61.41 ± 1.56	>77.64	32.45 ± 0.92
6		64.89 ± 2.31	>71.02	12.59 ± 0.38
7		47.93 ± 1.82	67.95	22.82 ± 0.21
8		65.98 ± 0.63	81.69	45.67 ± 0.91
9		3.08 ± 0.37	4.64	12.67 ± 0.35

10		53.78 ± 0.96	2.32	52.31 ± 1.92
11		16.23 ± 0.26	49.60	47.31 ± 1.81
12		92.57 ± 1.94	>93.28	17.34 ± 0.92
13		17.05 ± 1.21	>99.60	56.42 ± 0.97
14		19.59 ± 0.32	>84.18	19.64 ± 0.62
15		54.76 ± 0.21	>79.87	60.31 ± 1.23
16		37.91 ± 0.48	>83.06	45.69 ± 0.92
17		3.74 ± 0.27	>67.20	3.12 ± 0.67
18		14.06 ± 0.16	>67.20	15.24 ± 0.51
19		1.15 ± 0.27	60.09	14.38 ± 0.82
20		5.73 ± 0.79	64.43	18.94 ± 0.92

21		6.72 ± 0.27	2.01	5.64 ± 0.76
22		2.62 ± 0.37	>57.60	32.17 ± 1.56
INH		--	0.4	93.21 ± 1.25
RIF		--	0.5	23.48 ± 0.31

Nutrient starvation model was found to mimic microenvironment of granuloma as MTB exhibits significantly reduced intracellular ATP levels, a very low but continuous respiration and Loss of Ziehl-Neelsen staining characteristic [6,7]. This model is also characterized by resistance to commonly used antibiotics (INH, Rif, metronidazole, some fluoroquinolines) [8]. After 96hr of starvation 323 genes involved in amino acid biosynthesis, biosynthesis of cofactors, prosthetic groups and carriers, DNA replication, repair, recombination and restriction/modification, energy metabolism, lipid biosynthesis, translation and post-translational modification and virulence were down regulated. Genes which are found to be upregulated are involved in antibiotic production and resistance, insertion sequence elements, repeated sequences and phage, nucleotide biosynthesis and metabolism, putative enzymes and regulatory function [3]. LAT expressed by Rv3290c gene was found to be upregulated 41.86 folds as it involved in antibiotic resistance.[3] All the synthesized compounds were evaluated for their activities against dormant phase of mycobacterium at 10 µg/ml. Most of the compounds have well correlation between enzyme inhibitory concentration values and log reduction in this model. Compound **20** and **22** showed a bacterial log reduction of 2.9 fold and were more potent than first line TB drugs INH

(1.2 log fold) and Rif (1.3 log fold) and also Moxi (2.2 log fold). Compounds **9, 14, 19** also showed 2.8-log fold reduction and were more potent when compared to standard drugs (**Fig 2**).

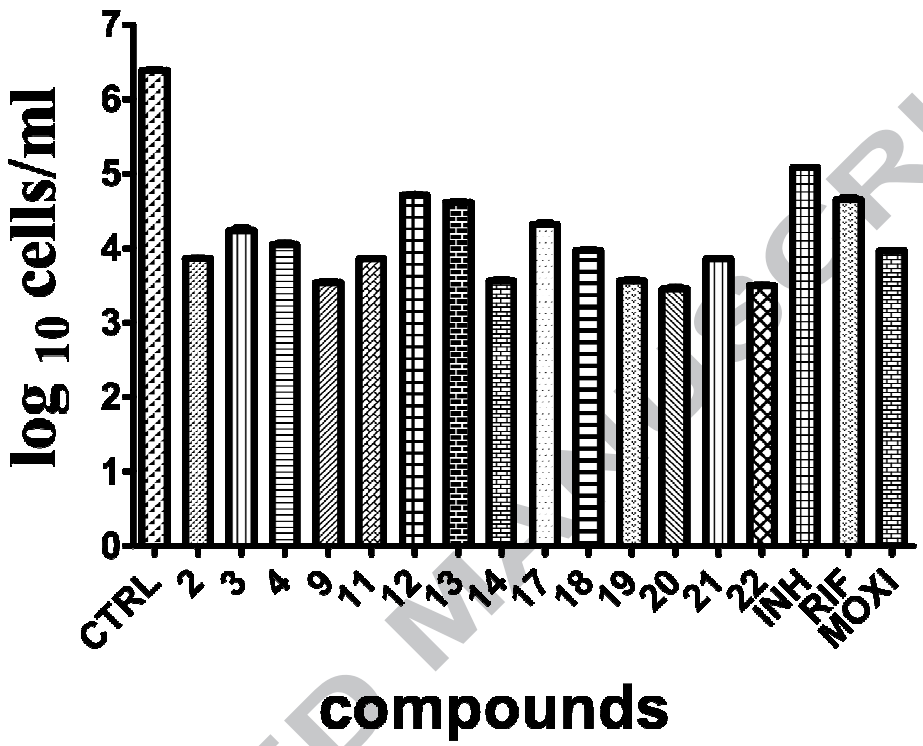


Figure 2: Biological activities of the synthesised compounds against *M. tuberculosis* in the nutrient starvation model. Bacterial count estimation (Mean ± S.D., n = 3) for control and treated groups conducted by using the MPN (most probable number) assay. most of the compounds gave significant inhibition of growth of *M. tuberculosis* in this model as compared to the control ($p < 0.0001$, two way ANOVA usingGraphPad Prism Software).

Biofilm formation explains the reasons for the long term persistence of MTB in human host and longer duration of treatment to completely recover from TB. Biofilm is a structured community of persistent cells (low nutrients and oxygen) enclosed in a self-produced polymeric matrix and adherent to an inert or living surface [9,10]. In case of MTB bacilli are embedded in a lipid-rich extracellular matrix containing free methoxy mycolic acids resulting in drug tolerance (50 times higher than MIC) and persistence [11]. Mycobacterial biofilms also called as pellicle or cords

forms a barrier for antibiotic penetration and environmental stress. Biofilm also plays a vital role in transmission, infection, persistence, chronic nature of disease, virulence, immunomodulation and relapse of TB. Within the primary granulomas in guinea pig lungs, an acellular rim has been observed adjacent to the edge of the mineralizing central necrosis containing drug-tolerant bacteria in micro colonies with features reminiscent of biofilms which are analogous to lesion environment in granuloma[12]. Identification of drugs that inhibit biofilm formation could enable the dramatic shortening of TB treatments using standard antibiotics, with substantial potential impact on global health and reduction of antibiotic resistance associated with non-compliance.[9-10] The effect of compounds 20 and 22 on viability of MTB biofilms was tested at a concentration of 10 µg/ml. Both compounds shown promising results and has 2.1 and 2.3 log reduction in bacterial count similar to standard drugs INH (1.9 log fold), Rif (2 log fold), Moxi (1.8 log fold) as shown in **Fig 3**.

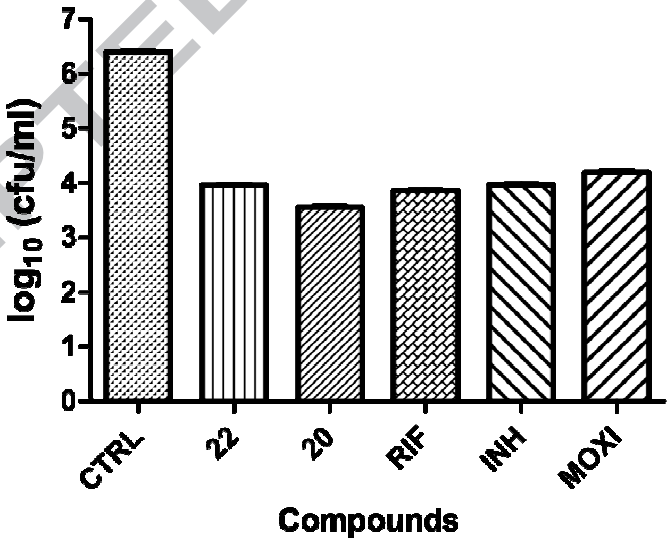


Figure 3: Biological activities of compounds **20** and **22** against biofilm forming *M. tuberculosis*. Bacterial count estimation (Mean ± S.D., n = 4) for control and treated groups conducted by using the MPN (most probable number) assay. Both compounds gave significant inhibition of growth of *M.*

tuberculosis in this model as compared to the control ($p < 0.0001$, two way ANOVA usingGraphPad Prism Software).

Kill curve experiments give an insight on nature of kill (bactericidal or static) and kinetics of kill under given set of conditions. MBC is defined as the lowest concentration of compound needed to kill 3 logs of MTB in 21 days under the given conditions. Compounds that are bactericidal may also exhibit time dependent killing or concentration-dependent killing. For compounds that have time-dependent kill, any concentration at or above the MBC will result in a constant rate of kill of the bacteria. For compounds that are concentration dependent, the rate of kill will increase as the concentration of compound increases. Knowledge of nature of kill and factors effecting kill can be related to pharmacokinetic parameters and is very much crucial in deciding appropriate antibiotic for therapy.[13] For further evaluation on its mechanism of action persistors obtained after 2 weeks' starvation were evaluated at 0, 7, 14, 21 days after drug treatment at varying drug concentrations (5, 10, 20 µg/ml). At all concentrations tested there was almost 3 log fold log reduction from 7th day to 21st day. Based on this observations kinetic profiling of compound **22** against dormant forms of mycobacteria was neither dependent on concentration nor time as observed in the **Fig 4**. It also has bactericidal effect against non-replicative stages of mycobacterium.

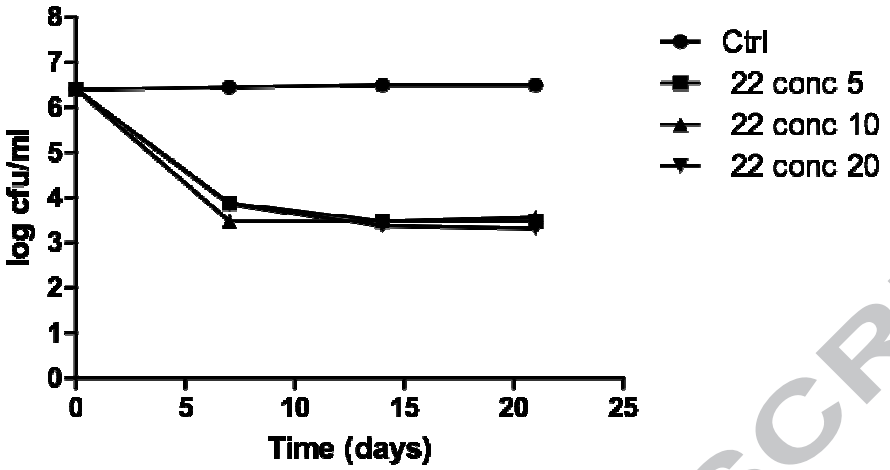


Figure 4: Kill kinetic curve of compound **22** at three different concentrations.

Zebra fish has genetic, physiological and immune (adaptive and innate) related similarities with mammalian host [14-16]. When infected with *M. marinum* it forms granulomas with central necrosis and hypoxia similar to human [17]. Oxidative stress stimulates the expression of bacterial efflux pumps in active stage of mycobacterium in zebra fish model [14,18]. Crucial virulence factors, host genes and immune cell types implicated in human MTB pathogenesis are retained in zebrafish–*M. marinum* model [19]. The natural heterogeneity of the zebrafish population proved to be beneficial as it helps in understanding of genetic differences of different individuals. It also serves as a link between *in vitro* and *in vivo* models as it provides insight into pharmacodynamic and kinetic parameters [19,20]. *M. marinum* is relatively safe for humans as it is restricted to topical lesions, has genetic similarity with MTB and also has higher replication rate than *M. tuberculosis*. Activity and dosage of antimycobacterial compounds in zebrafish closely resemble characteristics in humans. Owing to all the mentioned advantages and ease of handling, cost effectiveness zebra fish model can be used for high throughput screening of antimycobacterial agents.[17,19] Most potent molecule in the series **22** was tested for *in vivo*

antimycobacterial potency by well validated *M. marinum* induced zebra fish model. It showed 1.2 log bacterial reduction whereas INH and Moxi showed 2.8 and 2.7 log reduction (**Fig 5**).

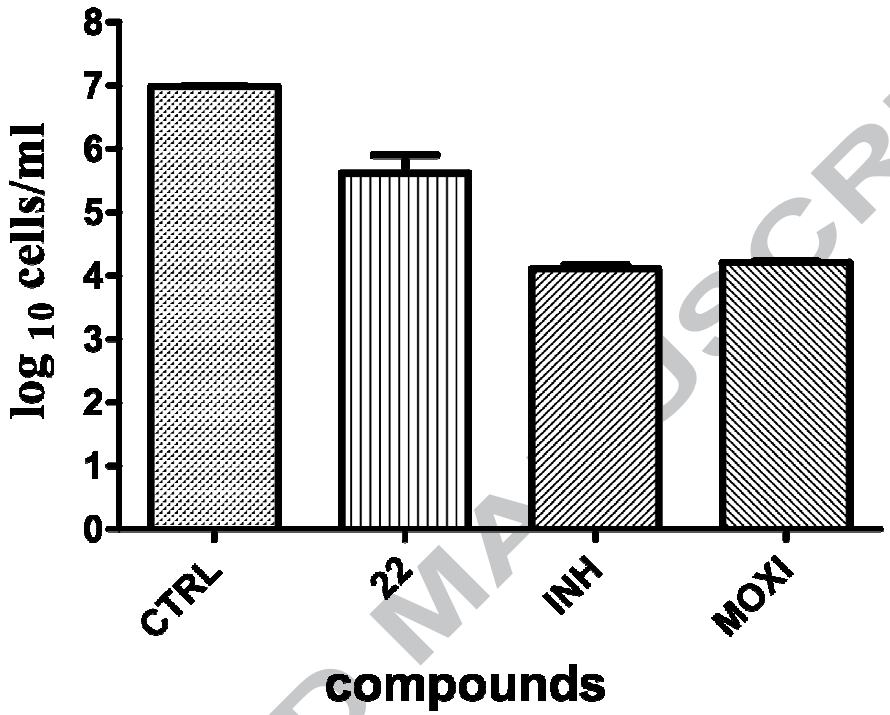


Figure 5: Bacterial count estimation (Mean ± S.E.M., n = 6) for control and treated groups by zebra fish model conducted by using MPN (most probable number) assay. The statistical significance ($p < 0.001$) with respect to infected control group has been analyzed by Two-way ANOVA using GraphPad Prism Software.

3D granuloma model is a biomimetic model of human granuloma dormancy as well as reactivation in favorable conditions such as immunosuppression. The mycobacterium in this model exhibits characteristics of dormancy such as loss of acid fast staining, accumulation of fats, Rifampicin tolerance and gene expression changes. The other features of model which makes it a replica of human granuloma are as follows- granuloma formation, multinucleated giant cell formation, decrease in CD4 T cell counts, unchanged CD8 T cell values, increase in

$CD4^+$ $CD25^+$ T cells, decrease in activated macrophage cells, increase in cytokine and chemokine secretion by host immune cells in response to MTB infection, and resuscitation upon immunosuppression by treatment with anti-TNFα mAbs. [21] After formation of 3D granuloma it was treated with compound 22 for 4 days followed by Rifampicin for 3 days. Then the granuloma was hydrolyzed followed by enumeration of % rifampicin tolerant persisters and MTB cfu. When the granuloma was treated with compound **22** alone (30.85 $X10^5$) cfu were much higher but when treated in combination with Rif, the cfu counts were less (2.11X 10^5), as compared to the cfu counts for MTB from granulomas treated with Rif alone (3.3X10^5) as shown in **Fig 6** and **7**. This indicates that 22, is a more effective killer in the presence of Rif.

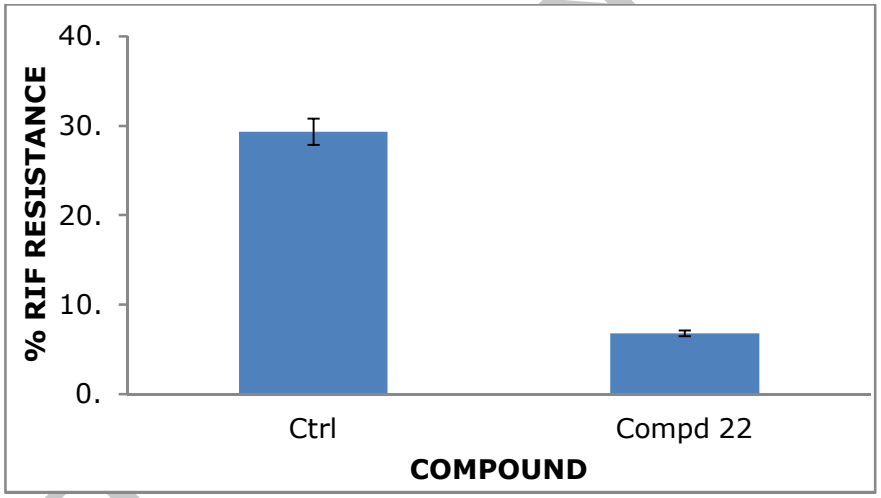


Figure 6: After the formation of granuloma, the granulomas were either left untreated or were treated with the compound **22** for 4 days and then with Rif for further 3 days. The graph represents the % rif resistance of *Mtb* recovered from these granulomas.

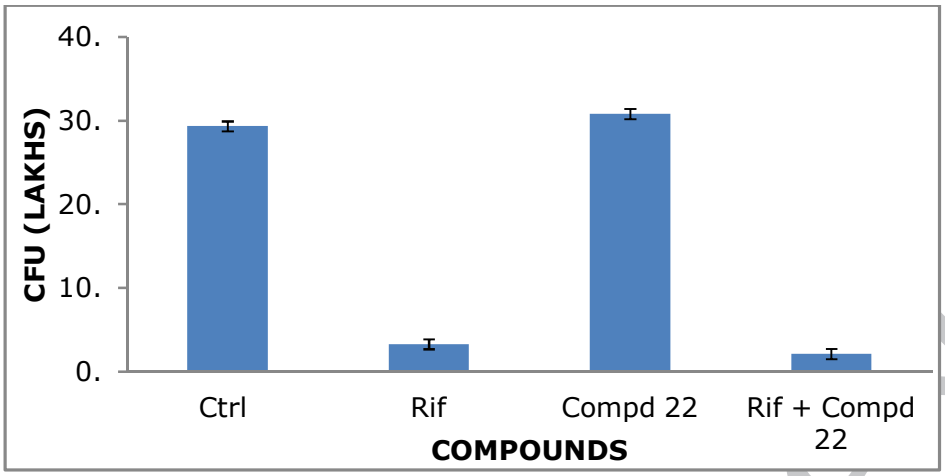


Figure 7: Data showing cfu counts for *Mtb* recovered from granulomas treated either with no drugs or treated with Rif, compd **22** alone or treated with compd **22**+ Rif.

When tested against active forms of mycobacterium the MIC were in the range of 2.01 to 99.60 µM. surprisingly compounds **21** and **9** has good potency against both replicating and non-replicating stages of bacteria as their MIC values are 2.01 and 4.64 µM respectively (**Tab 1**). Compound **10** was found to be inactive against LAT enzyme but has low MIC of 2.32 µM indicating that its mechanism of action might be different than LAT.

Compound **22** ((E)-4-(5-(2-(benzo[d]thiazol-2-yl)-2-cyanovinyl)thiophen-2-yl)isophthalic acid) on docking gave a glide score of -6.12 kcal/mol. The ligand had four hydrogen bonding with Arg422, Lys300 and GLn274 with a well fitted pose in the active site. The ligand found in the hydrophobic pocket within the vicinity of Val30, Leu24, Met28, Leu28, Leu414, Pro415, Phe167 and Val63. The binding pattern within the active site pocket of the crystal ligand and reference ligand was quite similar and additionally the solvent exposure area around the ligand constituted for a stable binding profile of the molecule as shown in the **Fig 8**. SAR analysis revealed that when compared to substituted phenyl ring, heterocycles are favoring activity. Heterocycles attached with isophthalic acid were more promising hits than

heterocycles attached to benzoic acid. When attempts were made to modify the benzthiazole core with benzoxazole ring it resulted in complete loss of activity.

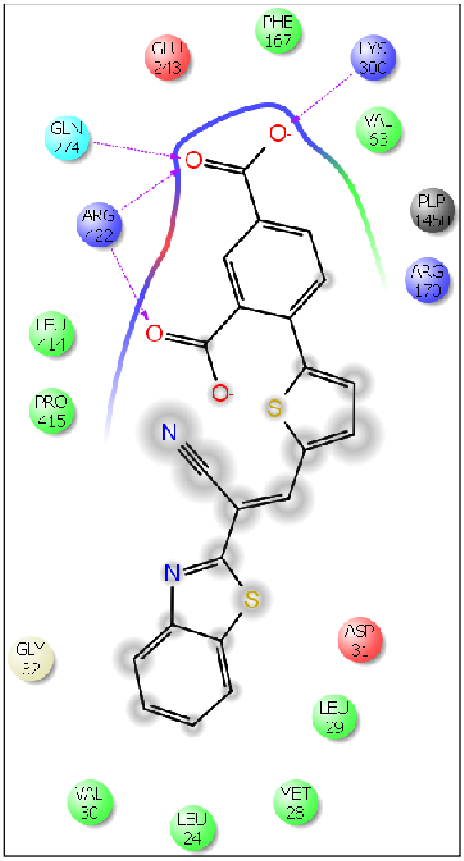


Figure 8: Interactions of most active inhibitor **22** with the active site residues of MTB LAT.

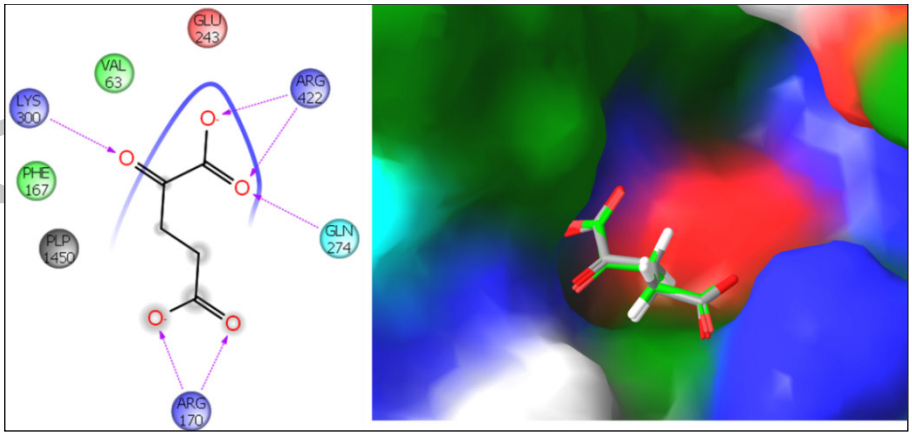


Figure 9: Interactions of reference inhibitor with the active site residues of MTB LAT and superimposition of docked pose of the reference ligand to the original pose of the ligand.

To know the effect of compound on macrophages where the parasite resides RAW cell lines were used for MTT studies. All the compounds were tested at a concentration of 50 µg/ml and the percentage inhibition were found to be in range of 2.26 to 60.31% (**Tab 1**). Most of the compounds are devoid of effects on metabolism.

3. Conclusions

In present work an existing lead was modified to develop more active compound against MTB LAT a crucial enzyme for regulation of amino acid pool which in turn key for antibiotic tolerance and persistence. In our attempts we also unexpectedly arrived at an interesting compound **21** ((*E*)-4-(5-(2-(benzo[*d*]thiazol-2-yl)-2-cyanovinyl)thiophen-2-yl)benzoic acid) which even though has moderate activity against persistent phase of mycobacterium it has significant potency against active phase. In the entire series compound **22** emerged as potent molecule with LAT IC_{50} of 2.62 µM. It has a significant log reduction of 2.9 and 2.3 log fold against nutrient starved and biofilm forming mycobacteria. It was found to be inactive against active MTB and also in *M. marinum* induced zebra fish model indicating that it acts through dormant targets. Compound **22** was also found to possess bactericidal effect which is independent of concentration and time. It was found to be effective in combination with Rif in 3D granuloma model. All these parameters make it a better candidate for further development.

4. Materials and methods

4.1. Synthesis

Benzthiazole scaffold was prepared by condensation of *ortho*-aminothiophenol with malanonitrile. Knoevenagel condensation of active methylene group with corresponding aldehydes resulted in final compounds as shown in **Scheme 1**. [22,23]

4.1.1. General:

All commercially available chemicals and solvents were used without further purification. TLC experiments were performed on alumina-backed silica gel 40 F254 plates (Merck, Darmstadt, Germany). The homogeneity of the compounds was monitored by thin layer chromatography (TLC) on silica gel 40 F254 coated on aluminum plates, visualized by UV light and $KMnO_4$ treatment. All ^{1}H and ^{13}C NMR spectra were recorded on a Bruker AM-300 (300.12 MHz, 75.12 MHz) NMR spectrometer, BrukerBioSpin Corp, Germany. Molecular weights of the synthesized compounds were checked by LCMS 6100B series Agilent Technology. Chemical shifts are reported in ppm (δ) with reference to the internal standard TMS. The signals are designated as follows: s, singlet; d, doublet; dd, doublet of doublets; t, triplet; m, multiplet. Elemental analyses were carried out on an automatic Flash EA 1112 Series, CHN Analyzer (Thermo).

4.1.2. **2-(Benzo[d]thiazol-2-yl)acetonitrile**: To solution of 2-aminobenzenethiol (2.5g, 19.97 mmol) in absolute ethanol (25ml), malanonitrile (1.32g, 19.97 mmol) was added, stirred and refluxed at 50 ^{0}C for 2hrs along with catalytic amount of glacial acetic acid. the solvent was evaporated under reduced pressure and the residue was extracted with chloroform and purified by column chromatography using hexane–ethylacetate as eluent and decolourised with activated carbon to afford **2-(benzo[d]thiazol-2-yl)acetonitrile** (2.3g, 66.11%) as yellow solid.

4.1.3. General procedure for the synthesis of benzothiazole acrylonitrile derivatives:

Procedure A: To a warm solution of the corresponding 2-(benzo[d]thiazol-2-yl) acetonitrile (1 mmol) in absolute ethanol (8 mL) was added the corresponding aldehyde (1 mmol) and catalytic amount of piperidine (0.2 mmol). The reaction mixture was then stirred and heated to 80^{o}C for 0.5-1 h, (as monitored by TLC and LCMS for completion), the precipitate formed was collected

by suction and recrystallised from ethanol to give the desired product in good yield as mentioned below

Procedure B: To a warm solution of the corresponding 2-(benzo[d]thiazol-2-yl) acetonitrile (1 mmol) in absolute ethanol (8 mL) was added the corresponding aldehyde (1 mmol) and catalytic amount of 10% methanolic KOH (0.2 mmol). The reaction mixture was then stirred and heated to 80^{o}C for 0.5-1 h, (as monitored by TLC and LCMS for completion), the precipitate formed was collected by suction and recrystallised from ethanol to give the desired product in good yield as mentioned below

4.1.3.1. (*E*)-2-(Benzo[*d*]thiazol-2-yl)-3-(4-hydroxyphenyl)acrylonitrile (1): The compound was synthesized according to the above general procedure **A** using 2-(benzo[d]thiazol-2-yl) acetonitrile (0.25g, 1.44mmol), 4-hydroxy benzaldehyde(0.18g, 1.44mmol), piperidine (0.025g, 0.29mmol) to afford **1** (0.32g, 80.2%) as yellow coloured solid. M.p: 218-220^{o}C. . ^{1}H NMR (DMSO-d_6): $\delta_{H.}$ 8.34 (s, 1H), 8.16 (t, *J* = 7.4Hz, 1H), 8.05 (t, *J* = 7.8Hz, 1H), 7.62 – 7.49 (m, 4H), 6.82 (m, 2H), 5.46 (s, 1H). ^{13}C NMR (DMSO-d6): δc. 163.4, 158.2, 152.5(2C), 134.6, 130.3(2C), 128.1, 126.6, 123.4(2C), 122.3, 120.4, 116.3, 114.8, 105.7.ESI-MS *m/z* 279.3 $(M+H)^{+}$. Anal Calcd.for $C_{16}H_{10}N_2OS$; C, 69.04; H, 3.62; N, 10.06; Found: C, 69.13; H, 3.61; N, 10.07.

4.1.3.2. (*E*)-2-(Benzo[*d*]thiazol-2-yl)-3-(4-fluorophenyl)acrylonitrile (2): The compound was synthesized according to the above general procedure **A** using 2-(benzo[*d*]thiazol-2-yl)acetonitrile (0.25g, 1.44mmol), 4-fluoro benzaldehyde(0.18g, 1.44mmol), piperidine (0.025g, 0.29mmol) to afford **2** (0.32g, 79.6 %) as red coloured solid. M.p: 174-176^{o}C. ^{1}H NMR (DMSO-d_6): $\delta_{H.}$ 8.37 (s, 1H), 8.18 (t, *J* = 8.0Hz, 1H), 8.04 (t, *J* = 8.2Hz, 1H), 7.84 - 7.53 (m, 4H), 7.25 (m, 2H). ^{13}C NMR (DMSO-d6): δc. 164.1, 162.4, 155.7(2C), 138.7, 131.1(2C), 125.6, 123.6,

122.8(2C), 120.4, 118.7, 115.3(2C), 106.5.ESI-MS *m/z* 281.4 $(M+H)^+$. Anal Calcd.for $C_{16}H_9FN_2S$; C, 68.55; H, 3.24; N, 9.99; Found: C, 68.38; H, 3.23; N, 10.01.

4.1.3.3. (*E*)-2-(Benzo[*d*]thiazol-2-yl)-3-(4-hydroxy-3-methoxyphenyl)acrylonitrile (3): The compound was synthesized according to the above general procedure **A** using 2-(benzo[*d*]thiazol-2-yl)acetonitrile (0.25g, 1.44mmol), 4-hydroxy-3-methoxy benzaldehyde (0.218g, 1.44mmol), piperidine (0.025g, 0.29mmol) to afford **3** (0.39g, 88.04%) as green coloured solid. M.p: 175-177°C. ^{1}H NMR (DMSO-d6): $\delta_{H.}$ 8.31 (s, 1H), 8.16 (t, *J* = 7.8Hz, 1H), 8.05 (t, *J* = 7.4Hz, 1H), 7.55 – 7.18 (m, 4H), 7.02 (d, *J* = 8.2Hz, 1H), 5.41 (s, 1H), 3.84 (s, 3H). ^{13}C NMR (DMSO-d6): δc. 161.2, 153.8(2C), 148.8, 148.3, 137.6, 129.4, 124.2, 123.6(2C), 122.6, 119.6, 118.3, 115.9, 112.1, 107.5, 57.9.ESI-MS *m/z* 309.4$(M+H)^+$. Anal Calcd.for $C_{17}H_{12}N_2O_2S$; 66.22; H, 3.92; N, 9.08; Found: C, 66.17; H, 3.91 N, 9.10.

4.1.3.4. (*E*)-2-(Benzo[*d*]thiazol-2-yl)-3-(2,4-dihydroxyphenyl)acrylonitrile (4): The compound was synthesized according to the above general procedure **A** using 2-(benzo[*d*]thiazol-2-yl)acetonitrile (0.25g, 1.44mmol), 2,4-dihydroxy benzaldehyde (0.198g, 1.44mmol), piperidine (0.025g, 0.29mmol) to afford **4** (0.35g, 82.9%) as orange coloured solid. M.p: 151-153°C. ^{1}H NMR (DMSO-d6): $\delta_{H.}$ 8.43 (s, 1H), 8.15 (t, *J* = 8.0Hz, 1H), 8.03 (t, *J* = 7.4Hz, 1H), 7.95 -6.56 (m, 4H), 6.42 (s, 1H), 5.38 (s, 2H).^{13}C NMR (DMSO-d6): δc. 161.1, 160.2, 157.4, 153.9(2C), 137.9, 131.4, 126.9, 124.2(2C), 120.4, 118.7, 110.2, 108.6, 107.8, 103.2.ESI-MS *m/z* 295.4 $(M+H)^+$. Anal Calcd.for $C_{16}H_{10}N_2O_2S$; C, 65.29; H, 3.42; N, 9.52; Found: C, 65.17; H, 3.43; N, 9.51.

4.1.3.5. (*E*)-2-(Benzo[*d*]thiazol-2-yl)-3-(3,4-dimethoxyphenyl)acrylonitrile (5): The compound was synthesized according to the above general procedure **A** using 2-

(benzo[*d*]thiazol-2-yl)acetonitrile (0.25g, 1.44mmol), 3,4-dimethoxy benzaldehyde (0.239g, 1.44mmol), piperidine (0.025g, 0.29mmol) to afford **5** (0.27g, 58.32%) as orange coloured solid. M.p: 147-149^{0}C. ^{1}H NMR (DMSO-d6): $\delta_{H.}$ 8.36 (s, 1H), 8.16 (t, *J* = 8.8Hz, 1H), 8.06 (t, *J* = 7.2Hz, 1H), 7.81 – 7.47 (m, 4H), 7.19 (d, *J* = 8.4Hz, 1H), 3.87 (s, 3H), 3.84 (s, 3H). ^{13}C NMR (DMSO-d6): δc. 163.3, 153.5, 152.8, 149.3, 146.8, 134.7, 126.8, 126.6, 125.7, 125.4, 123.3, 121.6, 117.1, 111.3, 111.1, 102.3, 56.1(2C).ESI-MS *m/z* 323.4(M+H)$^+$. Anal Calcd.for $C_{18}H_{14}N_2O_2S$; C, 67.06; H, 4.38; N, 8.69; Found: C, 66.92; H, 4.39 N, 8.71.

4.1.3.6. ***(E)*-2-(Benzo[*d*]thiazol-2-yl)-3-(3,4,5-trimethoxyphenyl)acrylonitrile (6):** The compound was synthesized according to the above general procedure **A** using 2-(benzo[*d*]thiazol-2-yl)acetonitrile (0.25g, 1.44mmol), 3,4,5-trimethoxy benzaldehyde (0.283g, 1.44mmol), piperidine (0.025g, 0.29mmol) to afford **6** (0.31g, 61.27%) as yellow coloured solid. M.p 262-264^{0}C. ^{1}H NMR (DMSO-d6): $\delta_{H..}$ 8.16 (s, 1H), 8.07 (t, *J* = 7.2Hz, 1H), 8.05 (t, *J* = 7.6Hz, 1H), 7.57 – 7.32 (m, 4H), 3.95 (s, 3H), 3.94 (s, 6H). ^{13}C NMR (DMSO-d6): δc. 163, 153.7(2C), 153.5(2C), 135.0, 127.6, 127.1, 126.1, 123.6(2C), 121.8, 117.0, 107.9, 104.1(2C), 61.2, 56.4(2C).ESI-MS *m/z* 353.42 (M+H)$^+$. Anal Calcd.for $C_{19}H_{16}N_2O_3S$; C, 64.76; H, 4.58; N, 7.95; Found: C, 64.63; H, 4.57; N, 7.97.

4.1.3.7. ***(E)*-2-(Benzo[*d*]thiazol-2-yl)-3-(4-benzyloxyphenyl)acrylonitrile (7):** The compound was synthesized according to the above general procedure **A** using 2-(benzo[*d*]thiazol-2-yl)acetonitrile (0.25g, 1.44mmol), benzyloxybenzaldehyde (0.31g, 1.44mmol), piperidine (0.025g, 0.29mmol) to afford **7** (0.37g, 69.94%) as brown coloured solid. M.p 217-219 ^{0}C. ^{1}H NMR (DMSO-d6): $\delta_{H..}$ 8.28 (s, 1H), 8.16 (t, *J* = 8.2Hz, 1H), 8.06 (t, *J* = 7.8Hz, 1H), 7.74 (d, *J* = 7.8Hz, 2H), 7.51 – 7.37 (m, 7H), 7.04 (d, *J* = 7.8Hz, 2H), 5.28 (s, 2H). ^{13}C NMR (DMSO-d6): δc. 162.3, 159.8, 157.4, 153.8(2C), 136.8, 135.9, 129.8(2C), 128.6(2C), 127.5(2C), 127.4, 126.3,

124.2(2C), 120.1, 118.8, 114.3(2C), 107.8, 69.7.ESI-MS *m/z* 369.45$(M+H)^+$. Anal Calcd.for $C_{23}H_{16}N_2OS$; C, 74.98; H, 4.38; N, 7.60; Found: C, 75.08; H, 4.39; N, 7.62.

4.1.3.8. (*E*)-3-(Benzo[d][1,3]dioxol-5-yl)-2-(benzo[d]thiazol-2-yl)acrylonitrile (**8**): The compound was synthesized according to the above general procedure **B** using 2-(benzo[*d*]thiazol-2-yl)acetonitrile (0.25g, 1.44mmol), piperonal (0.22g, 1.44mmol), alc. KOH (0.02g, 0.29mmol) to afford **8** (0.22 g, 50.11%) as red coloured solid. M.p219-221 ^{0}C.^{1}H NMR (DMSO-d6): $\delta_{H.}$ 8.30 (s, 1H), 8.15 (t, *J* = 7.8Hz, 1H), 8.07 (t, *J* = 7.2Hz, 1H), 7.83 (d, *J* = 7.4Hz, 1H), 7.60 (m, 2H), 7.37 (s, 1H), 7.02 (d, *J* = 7.6Hz, 1H), 6.12 (s, 2H). ^{13}C NMR (DMSO-d6): δc. 158.4, 154.4(2C), 149.1, 147.8, 137.6, 129.1, 126.7, 123.3(2C), 122.3, 119.2, 118.4, 112.1, 108.4, 107.3, 100.8.ESI-MS *m/z* 307.32 $(M+H)^+$. Anal.Calcd.for $C_{17}H_{10}N_2O_2S$; C, 66.65; H, 3.29; N, 9.14; Found: C, 66.59; H, 3.28; N, 9.12.

4.1.3.9. *(E)*-2-(-4-(2-(Benzo[d]thiazol-2-yl)-2-cyanovinyl)phenoxy)acetic acid (9): The compound was synthesized according to the above general procedure **A** using 2-(benzo[*d*]thiazol-2-yl)acetonitrile (0.25g, 1.44mmol), 4-formyl-phenoxy acetic acid(0.26 g, 1.44mmol), piperidine (0.025g, 0.29mmol) to afford **9** (0.27g, 55.9%) as green coloured solid. M.p 231-233^{0}C. ^{1}H NMR (DMSO-d6): $\delta_{H.}$ 12.68 (s, 1H), 8.38 (s, 1H), 8.15 (t, *J* = 7.6Hz, 1H), 8.07 (d, *J* = 7.8Hz, 1H), 7.65 – 7.56 (m, 4H), 7.06 (d, *J* = 7.4Hz, 2H), 4.44 (s, 2H). ^{13}C NMR (DMSO-d6): δc. 169.7, 162.8, 157.6, 153.8(2C), 137.6, 130.4(2C), 127.4, 126.9, 123.1(2C), 122.8, 118.7, 114.5(2C), 107.9, 63.9.ESI-MS *m/z* 335.36 $(M-H)^+$. Anal Calcd.for $C_{18}H_{12}N_2O_3S$; C, 64.27; H, 3.60; N, 8.33; Found: C, 64.32; H, 3.61; N, 8.31.

4.1.3.10. *(E)*-2-(-2-(2-(Benzo[*d*]thiazol-2-yl)-2-cyanovinyl)phenoxy)acetic acid (10): The compound was synthesized according to the above general procedure **A** using 2-(benzo[*d*]thiazol-2-yl)acetonitrile (0.25g, 1.44mmol), 2-formyl-phenoxy acetic acid(0.26g,

1.44mmol), piperidine (0.025g, 0.29mmol) to afford **10** (0.21g, 43.48%) as green coloured solid. M.p 257-259 ^{0}C. ^{1}H NMR (DMSO-d6): $\delta_{H.}$ 12.63 (s, 1H), 8.72 (s, 1H), 8.17 (t, *J* = 7.2Hz, 2H), 8.09 (d, *J* = 8.0Hz, 1H), 7.59 (t, *J* = 7.8Hz, 1H), 7.52 (t, *J* = 8.2Hz, 2H), 7.09 (t, *J* = 8.2Hz, 1H), 7.01 (d, *J* = 8.2Hz, 1H), 4.42 (s, 2H).^{13}C NMR (DMSO-d6): *δ*c.170.7, 163.4, 158.8, 152.9(2C), 143.3, 134.0, 127.7, 127.0, 126.1, 123.0, 122.4, 120.8, 120.0, 116.3, 113.6, 104.1, 68.5. ESI-MS *m/z* 335.36(M-H)$^{+}$. Anal Calcd.for $C_{18}H_{12}N_2O_3S$; C, 64.27; H, 3.60; N, 8.33; Found: C, 64.22; H, 3.59; N, 8.34.

4.1.3.11. ***(E)*-2-(Benzo[*d*]thiazol-2-yl)-3-(furan-2-yl)acrylonitrile (11):** The compound was synthesized according to the above general procedure **B** using 2-(benzo[*d*]thiazol-2-yl)acetonitrile (0.25g, 1.44mmol), furaldehyde (0.14g, 1.44mmol), alc. KOH (0.02g, 0.29mmol) to afford **11** (0.28 g, 77.78%) as yellow coloured solid. M.p: 137-139^{o}C. ^{1}H NMR (DMSO-d6): $\delta_{H.}$ 8.22 (s, 1H), 8.14 (t, *J* = 7.8Hz, 1H), 8.07 (t, *J* = 8.0Hz, 1H), 7.82 (d, *J* = 7.2Hz, 1H), 7.52 (m, 2H), 7.03 (d, *J* = 8.0Hz, 1H), 6.67 (t, *J* = 8.2Hz, 1H). ^{13}C NMR (DMSO-d6): *δ*c. 162.4, 152.2, 150.6, 144.8, 143.6, 135.3, 124.5, 123.4(2C), 120.1, 118.9, 113.8, 112.9, 109.4. ESI-MS *m/z* 253.3(M+H)$^{+}$. Anal Calcd.for $C_{14}H_8N_2OS$; C, 66.65; H, 3.20; N, 11.10; Found: C, 66.58; H, 3.21; N, 11.12.

4.1.3.12. **(*E*)-2-(Benzo[d]thiazol-2-yl)-3-(thiophen-2-yl)acrylonitrile** (**12**): The compound was synthesized according to the above general procedure **B** using 2-(benzo[*d*]thiazol-2-yl)acetonitrile (0.25g, 1.44mmol), 2-thiophenecarboxaldehyde (0.16g, 1.44mmol), alc. KOH (0.02g, 0.29mmol) to afford **12** (0.13 g, 33.77%) as yellow coloured solid. M.p 149-151 ^{0}C. ^{1}H NMR (DMSO-d6): $\delta_{H.}$ 8.26 (s, 1H), 8.13 (t, *J* = 8.2Hz, 1H), 8.05 (t, *J* = 7.8Hz, 1H), 7.73 (d, *J* = 8.2Hz, 1H), 7.59 (m, 2H), 7.26 (t, *J* = 8.8Hz, 1H), 7.11 (d, *J* = 7.6Hz, 1H). ^{13}C NMR (DMSO-d6): *δ*c. 162.1, 154.7, 142.3, 137.6, 135.3, 133.2, 129.8, 128.6, 124.7, 123.2(2C), 120.2, 118.3,

113.8. ESI-MS *m/z* 269.41 $(M+H)^+$. Anal.Calcd.for $C_{14}H_8N_2S_2$; C, 62.66; H, 3.00; N, 10.44; Found: C, 62.75; H, 2.99; N, 10.42.

4.1.3.13. *(E)*-2-(Benzo[*d*]thiazol-2-yl)-3-(1*H*-pyrrol -2-yl)acrylonitrile (13): The compound was synthesized according to the above general procedure **B** using 2-(benzo[*d*]thiazol-2-yl)acetonitrile (0.25g, 1.44mmol), pyrrole-2-carboxaldehyde(0.14g, 1.44mmol), alc. KOH (0.02g, 0.29mmol) to afford **13** (0.19g, 52.63%) as brown coloured solid. M.p: 176-178^{o}C. ^{1}H NMR (DMSO-d6): $\delta_{H.}$ 9.12 (s, 1H), 8.21 (s, 1H), 8.12 (t, *J* = 7.2Hz, 1H), 8.05 (t, *J* = 7.8Hz, 1H), 7.57 (m, 2H), 7.03 (d, *J* = 8.0Hz, 1H), 6.67 (d, *J* = 8.2Hz, 1H), 6.26 (t, *J* = 7.4Hz, 1H). ^{13}C NMR (DMSO-d6): δc. 162.6, 154.1, 145.4, 137.0, 126.6, 125.4(2C), 122.8, 120.5, 118.7, 118.0, 113.9, 112.0, 107.9. ESI-MS *m/z* 252.4 $(M+H)^+$. Anal Calcd.for $C_{14}H_9N_3S$; C,66.91; H, 3.61; N, 16.72; Found: C, 66.87; H, 3.62; N, 16.74.

4.1.3.14. (E)-2-(Benzo[d]thiazol-2-yl)-3-(5-nitrofuran-2-yl)acrylonitrile (**14**): The compound was synthesized according to the above general procedure **B** using 2-(benzo[*d*]thiazol-2-yl)acetonitrile (0.25g, 1.44mmol), 5-nitrofurfural (0.20g, 1.44mmol), alc. KOH (0.02g, 0.29mmol) to afford **14** (0.28g, 65.57%) as orange coloured solid. M.p 245-247 ^{0}C.^{1}H NMR (DMSO-d6): $\delta_{H.}$ 8.26 (s, 1H), 8.15 (t, *J* = 7.2Hz, 1H), 8.04 (t, *J* = 7.8Hz, 1H), 7.64 – 7.58 (m, 3H), 6.97 (d, *J* = 8.0Hz, 1H). ^{13}C NMR (DMSO-d6): δc. 161.3, 155.9, 154.2, 153.9, 144.8, 137.6, 124.6, 123.7(2C), 122.8, 118.1, 116.5, 114.8, 113.6. ESI-MS *m/z* 298.3 $(M+H)^+$. Anal.Calcd.for $C_{14}H_7N_3O_3S$; C, 56.56; H, 2.37; N, 14.13; Found: C, 56.48; H, 2.36; N, 14.12.

4.1.3.15. (*E*)-2-(Benzo[d]thiazol-2-yl)-3-(5-nitrothiophen-2-yl)acrylonitrile (**15**): The compound was synthesized according to the above general procedure **B** using 2-(benzo[*d*]thiazol-2-yl)acetonitrile (0.25g, 1.44mmol), 5-nitrothiophene-2-carboxaldehyde (0.23g, 1.44mmol), alc. KOH (0.02g, 0.29mmol) to afford **15** (0.32g, 71.27%) as green coloured solid.

M.p 260-262 ^{0}C.^{1}H NMR (DMSO-d6): $\delta_{H.}$ 8.20 (s, 1H), 8.18 (d, *J* = 8.0Hz, 1H), 8.14 (t, *J* = 7.2Hz, 1H), 8.06 (t, *J* = 7.8Hz, 1H), 7.50 (m, 2H), 7.41 (d, *J* = 8.0Hz, 1H). ^{13}C NMR (DMSO-d6): *δ*c.162.2, 156.3, 154.7, 147.2, 141.8, 138.2, 135.3, 129.8, 126.8, 123.6(2C), 119.7, 116.5, 111.2. ESI-MS *m/z* 314.37 $(M+H)^{+}$. Anal.Calcd.for $C_{14}H_7N_3O_2S_2$; C, 53.66; H, 2.25; N, 13.41; Found: C, 53.62; H, 2.24; N, 13.39..

4.1.3.16. *(E)*-2-(Benzo[*d*]thiazol-2-yl)-3-(1*H*-indol-3-yl)acrylonitrile (16): The compound was synthesized according to the above general procedure **B** using 2-(benzo[*d*]thiazol-2-yl)acetonitrile (0.25g, 1.44mmol), indole-3-carboxaldehyde(0.21g, 1.44mmol), alc. KOH (0.02g, 0.29mmol) to afford **16** (0.22g, 50.81%) as green coloured solid. M.p: 289-291^{o}C . ^{1}H NMR (DMSO-d6): $\delta_{H.}$ 11.52 (s, 1H), 8.31 (s, 1H), 8.10 (t, *J* = 7.8Hz, 1H), 8.02 (t, *J* = 7.2Hz, 1H), 7.82 (s, 1H), 7.73 – 7.62 (m, 3H), 7.12 – 7.05 (m, 3H). ^{13}C NMR (DMSO-d6): *δ*c. 161.2, 154.1(2C), 137.3, 136.1, 129.4, 126.9, 125.0, 124.7(2C), 123.2, 121.8, 119.6, 118.8, 117.9, 113.8, 111.3, 110.7. ESI-MS *m/z* 302.37 $(M+H)^{+}$. Anal Calcd.for $C_{18}H_{11}N_3S$; C,71.74; H, 3.68; N, 13.94; Found: C, 71.65; H, 3.67; N, 13.96.

4.1.3.17. (*E*)-3-(5-(2-(Benzo[*d*]thiazol-2-yl)-2-cyanovinyl)furan-2-yl)benzoic acid (17): The compound was synthesized according to the above general procedure **A** using 2-(benzo[*d*]thiazol-2-yl)acetonitrile (0.25g, 1.44mmol), 3-(5-formyl furan-2-yl) benzoic acid (0.31g, 1.44mmol), piperidine (0.025g, 0.29mmol) to afford **17** (0.31g, 58.05%) as orange coloured solid. M.p:279-281^{o}C. ^{1}H NMR (DMSO-d6): $\delta_{H.}$ 13.12 (s, 1H), 8.65 (s, 1H), 8.53 (d, *J* = 7.2Hz, 1H), 8.21 (s, 1H), 8.23 – 8.13 (m, 2H), 8.06 (t, *J* = 7.2Hz, 1H), 7.84 (t, *J* = 8.2Hz,1H), 7.51 (m, 2H), 7.14 (d, *J* = 8.2Hz, 1H), 6.94 (d, *J* = 7.8Hz, 1H). ^{13}C NMR (DMSO-d6): *δ*c. 171.3, 162.3, 156.3, 152.7, 150.8, 144.8, 137.3, 131.1, 130.9, 129.9, 129.8(2C), 126.9, 124.2,

123.1(2C), 122.1, 118.6, 114.7, 110.2, 106.1. ESI-MS *m/z* 371.05(M-H)$^+$. Anal Calcd.for $C_{21}H_{12}N_2O_3S$; C,67.73; H, 3.25; N, 7.52; Found: C, 67.69; H, 3.24; N, 7.50.

4.1.3.18. (*E*)-4-(5-(2-(Benzo[*d*]thiazol-2-yl)-2-cyanovinyl)furan-2-yl)benzoic acid (18): The compound was synthesized according to the above general procedure **A** 2-(benzo[*d*]thiazol-2-yl)acetonitrile (0.25g, 1.44mmol), 4-(5-formyl furan-2-yl) benzoic acid(0.31g, 1.44mmol), piperidine (0.025g, 0.29mmol) to afford **18** (0.35g, 65.54%) as yellow coloured solid. M.p:109-111^{o}C . ^{1}H NMR (DMSO-d6): $\delta_{H.}$ 13.07 (s, 1H), 8.27 (s, 1H), 8.19 – 8.07 (m, 6H), 7.51 (m, 2H), 7.16 (d, *J* = 8.0Hz, 1H), 6.96 (d, *J* = 8.2Hz, 1H). ^{13}C NMR (DMSO-d6): δc. 170.6, 161.0, 155.7, 153.3, 151.2, 143.2, 136.8, 134.3, 130.8, 126.9(2C), 125.1, 124.4(2C), 123.1(2C), 120.5, 117.7, 114.7, 110.8, 107.1. ESI-MS *m/z* 371.05 (M-H)$^+$. Anal Calcd.for $C_{21}H_{12}N_2O_3S$; C,67.73; H, 3.25; N, 7.52; Found: C, 67.81; H, 3.24; N, 7.50.

4.1.3.19. (*E*)-5-(5-(2-(Benzo[d]thiazol-2-yl)-2-cyanovinyl)furan-2-yl)isophthalic acid (19): The compound was synthesized according to the above general procedure **A** using 2-(benzo[*d*]thiazol-2-yl)acetonitrile (0.25g, 1.44mmol), 5-(5-formylfuran-2-yl)isophthalic acid (0.38 g, 1.44 mmol),piperidine (0.025 g, 0.29 mmol) to afford **19** (0.38 g, 61.87%) as yellow coloured solid. M.p: 269-271 ^{0}C. ^{1}H NMR (DMSO-d6): $\delta_{H.}$ 12.96 (s, 1H), 12.87 (s, 1H), 9.27 (s, 2H), 8.67 (s, 1H), 8.31 (s, 1H), 8.15 (t, *J* = 7.2Hz, 1H), 8.03 (t, *J* = 7.0Hz,1H), 7.48 (m, 2H), 7.12 (d, *J* = 8.2Hz, 1H), 6.96 (d, *J* = 8.4Hz, 1H). ^{13}C NMR (DMSO-d6): δc. 170.9(2C), 161.8, 155.8, 154.6, 150.3, 144.8, 136.9, 135.9(2C), 130.8(3C), 130.6, 126.2, 125.1(2C), 120.4, 117.7, 114.9, 110.3, 106.6. ESI-MS *m/z* 415.41 (M-H)$^+$. Anal.Calcd.for $C_{22}H_{12}N_2O_5S$; C, 63.46; H, 2.90; N, 6.73; Found: C, 63.39; H, 2.89; N, 6.75.

4.1.3.20. (*E*)-3-(5-(2-(Benzo[*d*]thiazol-2-yl)-2-cyanovinyl)thiophen-2-yl)benzoic acid (20): The compound was synthesized according to the above general procedure **A** using 2-

(benzo[*d*]thiazol-2-yl)acetonitrile (0.25g, 1.44mmol), 3-(5-formyl thiophen-2-yl) benzoic acid (0.33g, 1.44mmol), piperidine (0.025g, 0.29mmol) to afford **20** (0.36g, 64.6%) as brown coloured solid. M.p:283-285^{o}C . ^{1}H NMR (DMSO-d6): $\delta_{H.}$ 13.32 (s, 1H), 8.63 (s, 1H), 8.25 (s, 1H), 8.16 (t, *J* = 8.0Hz, 1H), 8.12 - 7.98 (m, 4H), 7.86 (d, *J* = 4.0Hz, 1H), 7.64 – 7.50 (m, 3H). ^{13}C NMR (DMSO-d6): δc. 171.2, 162.4, 155.3, 144.5, 142.7, 139.7, 135.4, 133.5, 132.3, 130.2(2C), 130.1, 129.9, 127.6, 125.6, 124.3, 122.8(2C), 120.1, 118.2, 113.5. ESI-MS *m/z* 387.1(M-H)$^{+}$. Anal Calcd.for $C_{21}H_{12}N_2O_2S_2$; C, 64.93; H, 3.11; N, 7.21; Found: C, 64.98; H, 3.12; N, 7.23.

4.1.3.21. (*E*)-4-(5-(2-(Benzo[*d*]thiazol-2-yl)-2-cyanovinyl)thiophen-2-yl)benzoic acid (21): The compound was synthesized according to the above general procedure **A** using 2-(benzo[*d*]thiazol-2-yl)acetonitrile (0.25g, 1.44mmol), 4-(5-formyl thiophen-2-yl) benzoic acid (0.33g, 1.44mmol), piperidine (0.025g, 0.29mmol) to afford **21** (0.29g, 52.07%) as brown coloured solid. M.p:289-291^{o}C. ^{1}H NMR (DMSO-d6): $\delta_{H.}$ 13.01 (s, 1H), 8.34 (s, 1H), 8.20 (t, *J* = 7.2Hz, 1H), 8.16 (t, *J* = 8.0Hz, 1H), 8.11 – 7.87 (m, 4H), 7.67 (m, 2H), 7.28 (d, *J* = 8.0 Hz, 2H). ^{13}C NMR (DMSO-d6): δc. 170.1, 161.7, 152.4, 143.9, 141.5, 139.8, 138.4, 135.1, 131.0, 130.9, 128.4, 127.9, 126.8(2C), 125.1, 124.9(2C), 123.7, 120.4, 117.4, 113.2.ESI-MS *m/z* 387.51 (M-H)$^{+}$. Anal Calcd for $C_{21}H_{12}N_2O_2S_2$; C, 64.93; H, 3.11; N, 7.21; Found: C, 64.86; H, 3.12; N, 7.23.

4.1.3.22. (E)-4-(5-(2-(Benzo[d]thiazol-2-yl)-2-cyanovinyl)thiophen-2-yl)isophthalicacid (22): The compound was synthesized according to the above general procedure **A** using 2-(benzo[*d*]thiazol-2-yl)acetonitrile (0.25g, 1.44mmol), 4-(5-formyl thiophen-2-yl) isophthalic acid (0.39g, 1.44mmol), piperidine (0.025g, 0.29mmol) to afford **22** (0.38g, 61.19%) as yellow solid. M.p:281-283^{o}C . ^{1}H NMR (DMSO-d6): $\delta_{H.}$ 13.01 (s, 1H), 12.96 (s, 1H), 8.52 – 8.48 (m, 2H),

8.27 (s, 1H), 8.18 (m, 1H), 8.14 (t, *J* = 7.4Hz, 1H), 8.03 (d, *J* = 7.2Hz, 1H), 7.92 (d, *J* = 7.6Hz, 1H), 7.58 (m, 2H), 7.43 (d, *J* = 8.0Hz, 1H). ^{13}C NMR (DMSO-d6): δc. 170.4, 169.1, 158.3, 152.4, 144.2, 141.5, 139.2, 138.5, 135.4, 132.8, 131.3, 129.7(2C), 128.4, 128.1, 126.8, 125.1, 124.2(2C), 120.4, 117.4, 113.0. ESI-MS *m/z* 431.44(M-H)$^+$. Anal Calcd for $C_{22}H_{12}N_2O_4S_2$; C, 61.10; H, 2.80; N, 6.48; Found: C, 61.14; H, 2.79; N, 6.50.

4.2. Enzyme inhibitory assay

LAT enzyme has been cloned and purified as per procedure reported in literature. MTB LAT enzymatic assay was performed in 100 µl volume containing 200 mM phosphate buffer (pH 7.2), 1.5 mM L-lysine, 1.5 mM ⟨-ketoglutarate, 15 ⌈M pyridoxal 5-phosphate with MTB LAT for 1hr at 37°C. The compounds were added to the plates with different concentrations from 50 ⌈M to 1 ⌈M. Reactions were terminated by adding 10% trichloroacetic acid in ethanol. The MTB LAT activity was monitored and the end product piperidene 6-carboxylate and glutamate was measured due to its colour intensity of its adduct with o-aminobenzaldehyde at 465 nm and 280 nm [5,24]. Reactions were carried out in a heat-controlled Perkin Elmer Victor X3 spectrophotometer. [5]

4.3. MIC determination

All compounds were screened for their activity against replicative stage of MTB by MABA method in duplicates. The bacterial inoculums of H37Rv (active TB) was prepared from fresh LJ medium and re-suspended in Middlebrook 7H9 broth supplemented with OADC growth supplement. This culture was grown at 37°C until the turbidity matched with McFarland no.1 turbidity standard. The culture was diluted to 1:25 with media and 100 µL was used as an inoculum. Each drug stock solution was diluted four-fold highest concentration tested. Sterile medium with and without bacteria as control maintained on each plate. After 7 days of

incubation, 50 µL of 1:1 mixture of alamar blue solution and sterile tween 80 were added to each well and the plate was re-incubated overnight. [25]

4.4. MTT assay

The synthesized compounds were further examined for its effects on metabolism in mouse macrophage cell line (RAW 264.7) at 50µg/ml concentration. After 48 h of exposure, viability was assessed on the basis of cellular conversion of MTT into a formazan product using the Promega Cell Titer 96 non-radioactive cell proliferation assay. The compounds are tested at 50 µg/ml concentration for 48 hrs. [26]

4.5. Nutrient starvation model

A culture of *M. tuberculosis* H37Rv (O.D. of 0.8 - 1.0) grown in Middlebrook 7H9 medium supplemented with OADC was pelleted and washed twice with PBS. The pellet was resuspended in PBS in sealed bottles and incubated at 37 °C for 6 weeks. Aliquots of these cultures were then treated with standard drugs like INH, Rif and Moxifloxacin (Mox) and the lead compounds for 7 days at a concentration of 10 µg/ml. The frequency of persistors was enumerated by MPN assay.[3]

4.6. Biofilm formation and determination of frequency of persistors

4.5 ml of Sautons media inoculated with MTB culture with an O.D. of 0.7 - 1.0 (1:100 dilution) was added in each well and sealed with parafilm then incubated at 37 ^{0}C for 5 weeks. To the matured biofilm, compound to be evaluated was injected at desired concentration into media and swirled (n=4). The plates were incubated in incubator for 7 days after sealing with parafilm. At the end of the incubation, Tween-80 (0.1% volume/volume) was added, swirled and incubated at room temperature for 15 minutes. The content of each well was mixed with pipette several times

and centrifuged in 15mL conical tube. The pellet was re-suspended in 5mL of fresh wash buffer (PBS with 10% glycerol and 0.05% Tween-80). Washing was repeated three times and the pellet was re-suspended in 5mL of wash buffer. The tubes were kept on rocker for overnight at room temperature. Pass the whole contents of tube through syringe fitted with sterile microtip (2-200µl) for 5-6 times to attain fairly homogenous suspension. The frequency of persisters in the biofilm population was determined by comparing antibiotic treated plates to solvent treated plates by MPN assay.[11]

4.7. Kill kinetics

MTB culture (5ml) (O.D.0.6-1.0) was centrifuged at 2,500 rpm for 15 min. The supernatant was discarded and suspended in 1ml of 10% PBS-Tyloxapol. The culture was diluted with PBS-Tyloxapol till it attains O.D. of 0.1 and it was starved for 2 weeks prior to the study. For each compound to be tested 4 tubes were labeled -3 as concentrations (5, 10, 20 µg/ml) to be tested and one served as DMSO control. To each tube 5ml of PBS-Tyloxapol and 50µl of starved culture were added. To compound tubes 100 µl of stock solution (50x) of compound was added to attain desired concentration. To tube labeled as control 100 µl DMSO was added. The contents of tubes were mixed and incubated at 37 ^{0}C. The treated cell suspensions were tested at 0, 7, 14, 21 days intervals. The bacterial count was noted down by using standard statistical methods using MPN assay.[13]

4.8. Antimycobacterial screening using Mycobacterium marinum induced adult zebra fish screening

One of the most active compounds was further evaluated for its *in vivo* activity using adult zebra fish model. We used *Mycobacterium marinum strain* (ATCC BAA-535) grown at 30 °C in

Middlebrook 7H9 broth. Fishes were initially weighed and were divided into control and treatment groups (n=6). All the fishes were infected by intraperitoneal injection with 20 µl of thawed bacterial stocks (around 0.75 million bacteria). After 7 days fishes were administered drug (5 µl) orally using micropipette for 7 days at a dose of 10 mg/kg (for INH 5 mg/kg). Finally, all of them were using homogenization technique and the tissue sample was prepared in Middlebrook 7H9 broth. The plates were checked for bacterial counts using MPN assay method.[27]

4.9. Human 3D granuloma model

An extracellular matrix (ECM) was prepared by mixing 0.95ml Purecol collagen solution, 50⌈l 10x DPBS (Lonza, USA), 4⌈l fibronectin (BD Biosciences, USA) and 10⌈l 1N NaOH (Sigma, USA) per ml of matrix solution and kept on ice (pH 7.0). PBMC cells were mixed at room temperature (RT) with ECM at $5x10^5$cells/50⌈l/well of 96-well plate. Assuming 5% macrophages in PBMCs, H37Rv strain of *Mtb* was added to the ECM at multiplicities of infection (MOI) of 0.1. ECM was allowed to set by incubating at 37^0C, CO_2 incubator for 45min. RPMI media containing 20% human Serum was added to the set ECM containing PBMCs and *Mtb* and incubated in 37^0C, CO_2 incubator. Media was changed after 2 days.

The granulomas were either left untreated or were treated with the compound 22 for 4 days and then with rif for further 3 days. To add compounds media was removed, and media containing the compound 22 was added to the experimental wells for 4 days. Then to some wells Rif was added. After 3 additional days, media was removed and wells treated with 50⌈l/well collagenase (Sigma, USA) for 40 min at 37^0C to isolate host PBMC cells. Samples from five wells were pooled in 1.8ml micro centrifuge tubes and host cells were lysed with 200⌈l of 0.1% triton X-100

solution. MTB pellet was obtained by centrifuging at 3500 Xg for 12 min. the MTB pellet was suspended in 1ml 7H9 media and 10-fold serial dilutions were made in Middlebrook 7H9 media containing 0.05% tween-80 and 100⌈l samples plated on Middlebrook 7H10 agar plates. Plates were incubated at 37^0C. Colony forming units (cfu) were determined after four weeks. % Rifampicin-tolerance is calculated by formula - %Rif-tolerance = cfu (Rif)/cfu(untreated) x 100.[21]

4.10. Docking studies (validation of active site)

Subsequently, the active molecules were docked into the crystal structure of LAT from MTB in internal aldimine form with bound substrate of 2-ketoglutarate (PDB code: 2CJH) with 2.00 Å resolution. The docking (grid 15 Å) was done by Glide XP (extra precision module of Schrodinger 9.3, Glide, version 5.7, Schrodinger, LLC, New York, NY, 81 2011) protocol where the output gives a docking pose with XP glide score with rmsd (root mean square deviation) to crystal ligand at active site to establish the structure activity relationship. [19]

Closer analysis of the crystal structure bound with ligand was found in the vicinity of the Arg422, Gln274, Lys300, Arg170, Phe167, Glu243 amino acid residues. While the hydrophobic pocket of MTB LAT is defined by the side chains of Phe415, Leu414, Val63, and Phe167. Further, re-docking results with ligand preparation showed that this ligand exhibited similar interactions as that of the original crystal structure with an RMSD of 0.24 Å with highest glide score of -6.04 kcal/mol as illustrated in **Fig. 9**.

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Mycobacterium tuberculosis **lysine-ε-aminotransferase a potential target in dormancy: benzthiazole based inhibitors**	

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