Accepted Manuscript

Design, synthesis and biological evaluation of imidazo[2,1-*b*]thiazole and benzo[*d*]imidazo[2,1-*b*]thiazole derivatives as *Mycobacterium tuberculosis* pantothenate synthetase inhibitors

Ganesh Samala, Parthiban Brindha Devi, Shalini Saxena, Nikhila Meda, Perumal Yogeeswari, Dharmarajan Sriram

PII: S0968-0896(16)30072-4
DOI: http://dx.doi.org/10.1016/j.bmc.2016.01.059
Reference: BMC 12802

To appear in: *Bioorganic & Medicinal Chemistry*

Received Date: 10 December 2015
Revised Date: 29 January 2016
Accepted Date: 30 January 2016

Please cite this article as: Samala, G., Devi, P.B., Saxena, S., Meda, N., Yogeeswari, P., Sriram, D., Design, synthesis and biological evaluation of imidazo[2,1-*b*]thiazole and benzo[*d*]imidazo[2,1-*b*]thiazole derivatives as *Mycobacterium tuberculosis* pantothenate synthetase inhibitors, *Bioorganic & Medicinal Chemistry* (2016), doi: http://dx.doi.org/10.1016/j.bmc.2016.01.059

Design, synthesis and biological evaluation of imidazo[2,1-*b*]thiazole and benzo[*d*]imidazo[2,1-*b*]thiazole derivatives as *Mycobacterium tuberculosis* pantothenate synthetase inhibitors

Ganesh Samala, Parthiban Brindha Devi, Shalini Saxena, Nikhila Meda, Perumal Yogeeswari, Dharmarajan Sriram*

Department of Pharmacy, Birla Institute of Technology & Science-Pilani, Hyderabad Campus, Shameerpet, Hyderabad-500078, India.

ABSTRACT

In the present study, we have designed imidazo[2,1-*b*]thiazole and benzo[*d*]imidazo[2,1-*b*]thiazole derivatives from earlier reported imidazo[1,2-*a*]pyridine based *Mycobacterium tuberculosis* (MTB) pantothenate synthetase (PS) inhibitors. We synthesized thirty compounds and they were evaluated for MTB PS inhibition study, *in vitro* anti-TB activities against replicative and non-replicative MTB, *in vivo* activity using *M. marinum* infected Zebra fish and cytotoxicity against RAW 264.7 cell line. Among them compound 2-methyl-*N*'-(4-phenoxybenzoyl)benzo[*d*]imidazo[2,1-*b*]thiazole-3-carbohydrazide (**5bc**) emerged as potent compound active against MTB PS with IC_{50} of 0.53±0.13 µM, MIC of 3.53 µM, 2.1 log reduction against nutrient starved MTB, with 33% cytotoxicity at 50 µM. It also showed 1.5 log reduction of *M. marinum* load in Zebra fish at 10mg/kg.

Key words: Tuberculosis, Pantothenate synthetase, imidazo[2,1-*b*]thiazole, benzo[*d*]imidazo[2,1-*b*]thiazole.

*For Correspondence: Email- dsriram@hyderabad.bits-pilani.ac.in

Telephone: +91-4066303506; Fax: +91-4066303998

1. Introduction

The global threat to human health posed by the *Mycobacterium tuberculosis* (MTB) was recognized by the WHO, and it is estimated that one-third of the world's population is infected with the MTB (1). Synergy with the HIV, patient non-compliance, and the consequences of the development of drug and multidrug-resistant strains of MTB has made the situation ever more risk and it is widely acknowledged that novel intervention strategies are needed (2). Pantothenate synthetase (PS; EC 6.3.2.1) enzyme catalyses the last step of pantothenate biosynthesis, an ATP-dependent condensation of D-pantoate, and ß-alanine to form pantothenate (3). Pantothenic acid (vitamin B5) is an essential molecule required for the synthesis of coenzyme A and acyl carrier protein (ACP), that play important roles as acyl-group carriers in fatty-acid metabolism, cell signalling, the tricarboxylic-acid cycle, biosynthesis of polyketides, non-ribosomal peptides and several other reactions associated with intermediary metabolism (4). PS is not essential for *in vitro* growth but is essential for persistence of MTB in chronically infected mice. A strain of MTB with PS knock-out is severely attenuated in mice (5), thus making this enzyme an attractive target; moreover microorganisms need to synthesize pantothenate, while mammals obtain it from their diet. In the recent years many MTB PS inhibitors are reported which includes 2-methylimidazo[1,2-a]pyridine-3-carboxamides (6), thiazolidine derivatives (7), salicylanilide diethyl phosphates (8), tetrahydrothieno[2,3-c]pyridine-3-carboxamide (9), intermediate bi-substrate analogue (10), 3-biphenyl-4-cyanopyrrole-2-carboxylic acids (11), pyrazoles (12), 3-phenyl-4,5,6,7-tetrahydro-1H-pyrazolo[4,3-c]pyridine derivatives (13)and actinomycin D inhibitors (14). In the present study, we have designed, synthesized thirty imidazo[2,1-*b*]thiazole and benzo[*d*]imidazo[2,1-*b*]thiazole derivatives and evaluated for MTB PS enzyme inhibition study, *in vitro* anti-TB activities against replicative and nutrient starved non-replicative MTB, *in vivo* activity in Zebra fish using *M. marinum* and cytotoxicity against RAW 264.7 cell line.

2. Results and discussion

2.1. Designing of the compounds

In our previous work (6), we have used the crystal structure of the mycobacterial pantothenate synthetase (PS) in complex with 2-(2-(benzofuran-2-ylsulfonylcarbamoyl)-5-methoxy-1H-indol-1-yl)acetic acid inhibitor (PDB: 3IVX) having resolution of 1.73A° as a framework for virtual screening of known antitubercular compound database to identify lead compounds against this enzyme. Based on the hit identified, we undertook synthesis of analogues and evaluated for its biological activity. One of the lead had MTB PS activity of 1.90±0.12 µM and MIC of 4.53 µM. Based on these result and input from protein-ligand interactions observed in the structure of MTB PS with lead molecules, further modifications were explored as a ligand expansion step. Earlier we have reported imidazo[1,2-*a*]pyridine derivatives as MTB PS inhibitors with IC_{50} ranging from 1.90±0.12 to 9.20±0.96 µM and MTB with MIC ranging from 4.53 to 98.81 µM (6). In the present study, we have designed novel imidazo[2,1-*b*]thiazole and benzo[*d*]imidazo[2,1-*b*]thiazole derivatives by replacing pyridyl ring of imidazo[1,2-*a*]pyridine with thiazole and benzothiazole nucleus (**Figure 1**).

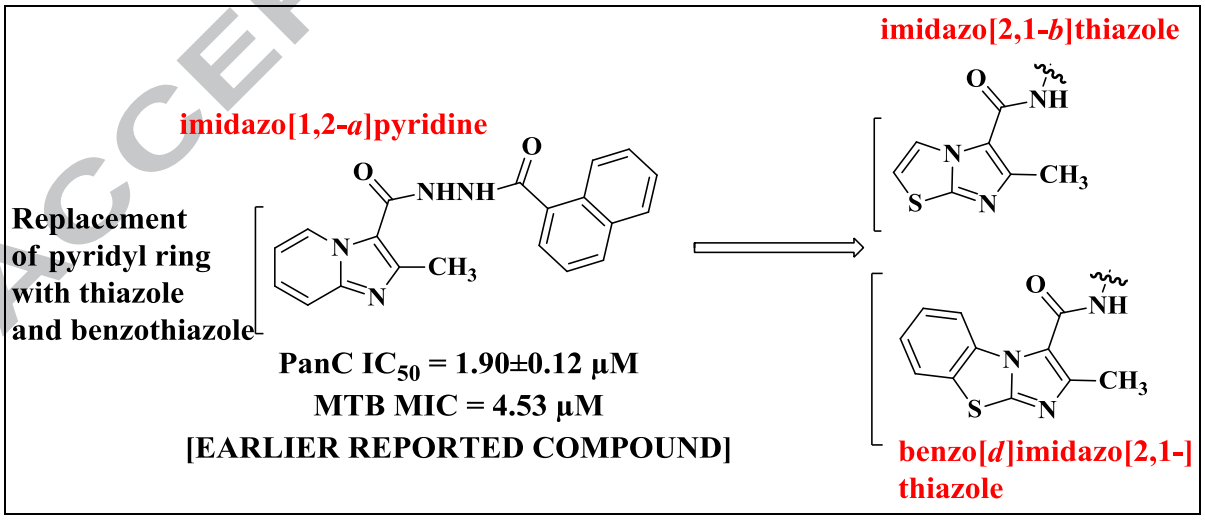
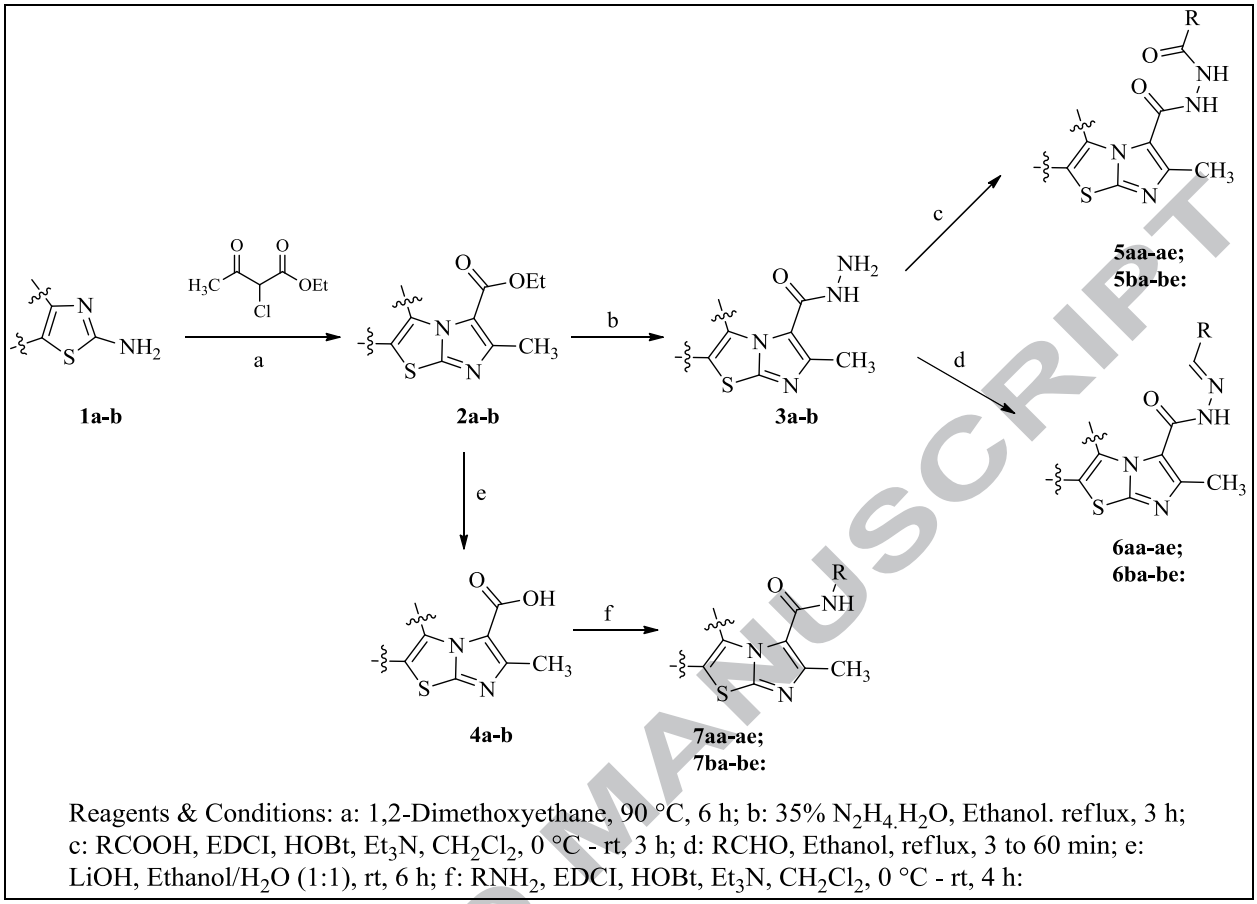


Figure 1: Designing strategy of the compounds

2.2. Synthesis of designed compounds

The target molecules were synthesized by three-step synthetic protocol (**Scheme 1**), wherein the first step in the synthesis was the reaction between 2-aminothiazole (**1a**)/2-aminobenzothiazole (**1b**) and 2-chloroethylacetoacetate in 1,2-dimethoxyethane at 90 °C to yield the bi/tricyclic compounds **2a/2b**. In the next step, two types of reactions were carried out on ester group, one was the conversion of ester group into carboxylic acid (**4a/4b**) using LiOH in Ethanol/Water (1:1), and the other was the direct conversion of ester into acid hydrazide (**3a/3b**) using 35% aqueous solution of hydrazine hydrate in ethanol under reflux conditions. Reaction of compound **3a/3b** with various substituted aromatic carboxylic acids in presence of coupling agents EDCI, and HOBt produced the compounds (**5aa-5ae/5ba-5be**). In the third step, compound **3a/3b** on reaction with various substituted aldehydes in ethanol reflux conditions produced the acid hydrazones (**6aa-6ae/6ba-6be**) respectively in excellent yields. During the reaction we observed the formation of desired product as a solid then reaction mixture was filtered directly and washed with distilled water, cold ethanol and hexane to obtain pure products without further purification steps. In the case of simple amides, compound **4a/4b** was treated with substituted aromatic/aliphatic primary amines in presence of peptide coupling agent EDCI to produce final compounds (**7aa-7ae/7ba-7be)**. The purity of the synthesized compounds was checked by HPLC and elemental analyses and the structures were identified by spectral data. In the nuclear magnetic resonance spectra (^{1}H NMR and ^{13}C NMR), the signals of the respective protons of the prepared derivatives were verified on the basis of their chemical shifts, multiplicities, and coupling constants. The elemental analysis results were within ±0.4% of the theoretical values.



Scheme 1: Synthetic protocol of designed compounds

2.3. Pantothenate synthetase enzyme inhibition studies

All the synthesized compounds were assayed for MTB PS inhibition study, that couples the AMP produced in the condensation of β-alanine and pantoate with the oxidation of NADH to NAD+ through myokinase, pyruvate kinase and lactate dehydrogenase (3). The NAD+ produced can be monitored spectrophotometrically at 340 nm. In the initial screening at 25 µM, all compounds showed more than 50% inhibition against MTB PS and were further studied for IC_{50} measurements. All the compounds showed good IC_{50} in the range of 0.52±0.24 to 5.83±0.24 µM (**Table 1**); eight compounds (**5ad, 5bc, 6ab, 6ac, 7aa, 7ab, 7ba, 7bd**). inhibited MTB PS with nano molar concentration. Compounds *N*-(4-bromophenyl)-2-methylbenzo[*d*]imidazo[2,1-*b*]thiazole-3-carboxamide (**7ba**) and 2-Methyl-N'-(4-

phenoxybenzoyl)benzo[d]imidazo[2,1-b]thiazole-3-carbohydrazide **(5bc)** emerged as the most active compounds with an IC_{50} of 0.52±0.24 and 0.53±0.13 µM respectively.

Subsequently, all the molecule were docked into the crystal structure of MTB PS protein (PDB Id: 3IVX) to establish the structure activity relationship. All the compounds showed good binding energy in the range of -7.15 to -8.50 kcal/mol and showing good fitness with the MTB PS protein. Most of the compounds are showing important hydrogen bonding interaction with HIE44, Ser196, Ser197, Asp161, Gln72, HIE47 amino acid residues. Most of the compounds were further stabilized by *pi-pi* interaction with HIE44 analogous to that observed with the crystal ligand. One of the most active ligand *N*-(4-bromophenyl)-4-methyl-7-thia-2,5-diazatricyclo pentaene-3-carboxamide **(7ba)** having IC_{50} of 0.52±0.24 µM showing docking score of -7.66 kcal/mol. The ligand had two hydrogen bonding's with Asp161 and HIE44 also it showed a *pi-pi* interaction with HIE44 with a well fitted pose in the active site in the hydrophobic pocket within the vicinity of Leu50, Val187, Met195, Tyr82, Met40, and Pro38 and few polar amino acid residues HIE47, Ser196, Ser197, Gln72, Gln164, Thr39 and Thr186 respectively. The binding pattern within the active site pocket of the crystal ligand and reference ligand was quite similar and it is well fitted in the active site of the protein, additionally the van der Waal's forces were also observed which constituted for a stable binding profile of the molecule as shown in the **Figure 2**.

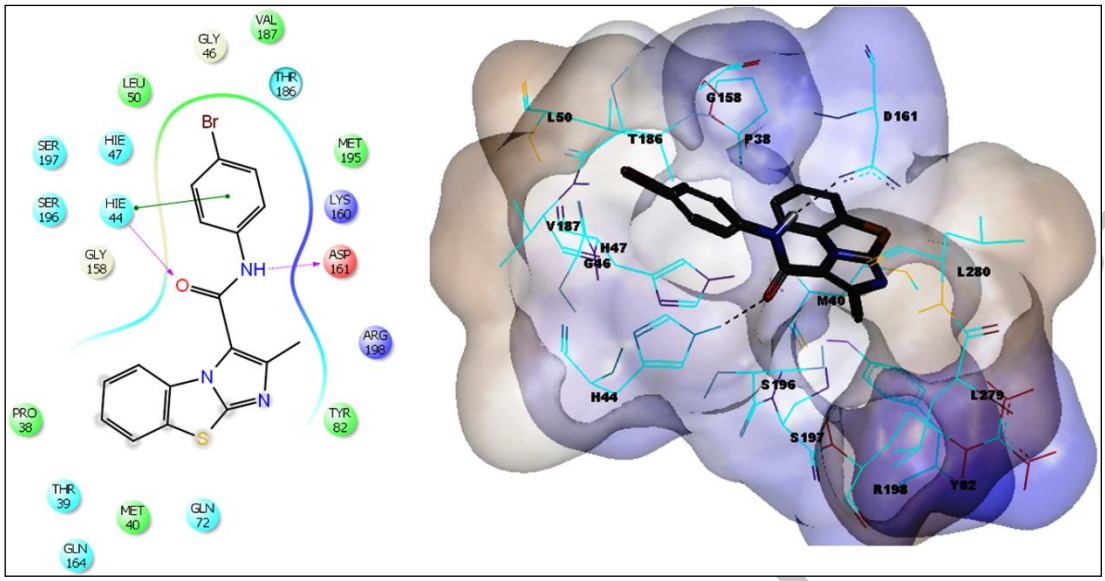
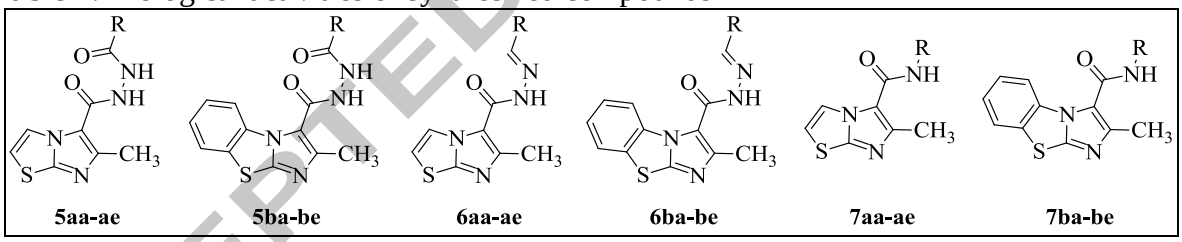


Figure 2: Binding pose and the interaction pattern of the compound **7aa** (In 2D ligplot, pink line represented hydrogen bond; green line represented pi-pi interaction, in surface representation the blue colour indicate the hydrophobic nature; gray colour indicate the hydrophilic nature of the active site).

Table 1: Biological activities of synthesized compounds



Compound	R	Yield (%)	MP (°C)	PanC IC_{50} in µM	MTB MIC in µM	Cytotoxicity at 50 µM (RAW 264.7 cells) % inhibition
5aa	Phenyl	81	166-167	5.31±0.12	40.12	10.34
5ab	4-Tolyl	79	171-172	4.99±0.21	5.96	39.25
5ac	4-Phenoxyphenyl	88	160-161	1.23±0.3	7.96	2.98
5ad	1-Naphthyl	69	162-163	0.64±0.10	17.83	27.47
5ae	Cyclohexyl	76	177-178	5.38±0.9	20.40	31.19
5ba	Phenyl	80	260-261	1.10±04	35.67	29.71
5bb	4-Tolyl	87	270-271	5.83±0.24	17.15	53.01
5bc	4-Phenoxyphenyl	74	214-215	0.53±0.13	3.53	10.40
5bd	1-Naphthyl	69	260-261	1.39±0.08	15.60	32.03
5be	Cyclohexyl	89	241-242	2.91±0.11	17.53	45.41
6aa	4-Bromophenyl	84	269-270	1.20±0.16	34.40	22.23
6ab	4-Trifluromethylphenyl	90	153-154	0.67±0.18	17.74	12.84
6ac	Phenyl	88	151-152	0.58±0.19	23.20	23.12

6ad	3,4,5-Trimethoxyphenyl	76	218-219	5.61±0.27	33.38	1.17
6ae	4-*N*,*N*-dimethylphenyl	92	119-120	2.50±0.3	38.18	6.75
6ba	4-Bromophenyl	87	252-253	1.02±0.13	15.12	10.06
6bb	4-Trifluromethylphenyl	93	271-272	5.31±0.11	16.53	16.11
6bc	Phenyl	90	246-247	2.15±0.8	9.35	34.57
6bd	3,4,5-Trimethoxyphenyl	91	249-250	2.07±0.20	29.45	16.18
6be	4-*N*,*N*-dimethylphenyl	82	256-257	1.46±0.12	4.13	12.06
7aa	4-Bromophenyl	78	213-214	0.69±0.04	2.32	32.32
7ab	Phenyl	82	109-110	0.74±0.21	12.14	14.07
7ac	4-Ethoxyphenyl	84	120-121	5.83±0.11	20.74	58.69
7ad	Benzyl	76	141-142	1.06±0.14	11.52	12.67
7ae	Cyclohexyl	69	146-147	2.00±0.16	11.85	32.10
7ba	4-Bromophenyl	63	255-256	0.52±0.04	16.18	20.87
7bb	Phenyl	81	200-201	1.03±0.11	40.67	33.21
7bc	4-Ethoxyphenyl	83	245-246	2.10±0.09	41.95	52.73
7bd	Benzyl	72	216-217	0.84±0.1	19.44	6.62
7be	Cyclohexyl	81	243-244	1.02±0.11	19.94	13.39
	Isoniazid			>25	0.72	NT
	Ethambutol			>25	7.64	NT

2.4. In vitro MTB screening

All the synthesized compounds were also screened for their *in vitro* anti-TB activity against log phase culture of MTB H37Rv (ATCC27294) using MABA assay method (15) with drug concentrations from 50 µg/mL to 0.78µg/mL (double dilution) in duplicates. The minimum inhibitory concentration (MIC) was determined for each compound which was measured as the minimum concentration of compound required to completely inhibit the bacterial growth. Isoniazid (INH) and ethambutol were used as reference compounds for comparison. The MIC values of the synthesized compounds along with the standard drugs for comparison are presented in **Table 1**. All the synthesized compounds showed activity against MTB with MIC ranging from 2.32 to 41.95 µM. Six compounds (**5ab, 5ac, 5bc, 6bc, 6be** and **7aa**) inhibited MTB with MIC of <10 µM; four compounds (**5ab, 5bc, 6be** and **7aa**) were found to be more potent than standard first line anti-TB drug ethambutol (MIC of 7.64 µM) and none were potent as INH. Compound **7aa** was found to be the most active compound *in vitro* with a MIC of 2.32 µM against log-phase culture of MTB. When compared to enzyme PS activity; MIC results were found to be many folds high; the reason might be due to MTB cell wall

penetration problem with these compounds or involvement of MTB efflux pumps. With respect to structure-MTB activity relationship; in the case of imidazo[2,1-*b*]thiazole derivatives, we have prepared five N,N'-diacylhydrazines (**5aa-5ae**), five N-acylhydrazones (**6aa-6ae**) and five amides (**7aa-7ae**). In general amides were more potent followed by N,N'-diacylhydrazines and then N-acylhydrazones. Within N,N'-diacylhydrazines substitution on phenyl ring increases MTB activity ~5-8 times (**5aa** vs **5ab** and **5ac**), similarly converting phenyl ring to cyclohexyl (**5ae**) and naphthyl (**5ad**) ring leads twice potent compounds. In the case of N-acylhydrazones substitution on phenyl ring (**6ac** vs others) in general is not favoured for MTB activity. In the case of amides, when compared to phenyl (**7ab**) introduction of electron withdrawing bromo group (**7aa**) makes six times more potent MTB activity and introduction of electron donating ethoxy group (**7ac**) makes less effective; whereas replacement of phenyl with cyclohexyl (**7ae**) or benzyl (**7ad**) does not alter any appreciable activity. In general imidazo[2,1-*b*]thiazoles were more potent than benzo[*d*]imidazo[2,1-*b*]thiazole derivatives.

Further we also tested two most active compounds (**5bc** and **7aa**) in nutrient starved MTB culture (16). In this model nutrient starvation caused MTB to arrest growth, minimized aerobic metabolism and became resistant to currently available anti-TB drugs while maintaining viability. Nutrient starvation may therefore mimic some of the features of MTB during the persistent state. Owing to its simplicity, reproducibility and ease of handling, we utilized this model for testing our few of the potent enzyme/MTB inhibitors aimed at non-replicative bacteria. In this model, cultures of MTB bacteria were subjected to nutrient depletion by growing the culture in PBS (Phosphate buffer saline) for 6 weeks. Nutrient starvation using this method triggered a dormancy response in the bacilli that was termed non-replicating persistence (NRP), a physiological state thought to mimic the one exhibited by MTB during various stages of persistent infection. After 6 weeks, the culture was treated

with the synthesized and standard drugs at a concentration of 10 µg/ml in a tube and incubated at 37 °C for 7 days exposure. The treated cell suspensions were diluted 10-fold up to 10^{-6} using Middlebrook 7H9 medium supplemented with OADC and were plated in 48 well plates in triplicates. The plates were incubated at 37 °C for 4 weeks and the wells with visible bacterial growth were counted as positive and maximum probable number (MPN) values were calculated using standard statistical methods (17). At 10 µg/ml concentration, standard first line anti-TB drugs INH and rifampicin (Rif) reduced ~1.1 and 2.1 log bacterial reduction respectively whereas moxifloxacin (Moxi) showed very good activity with ~2.5 log bacterial reduction (**Figure 3**). MIC of INH: 0.05 µg/ml, Rif: 0.1 µg/ml and moxifloxacin: 0.78 µg/ml against active MTB. Compounds **5ab** and **7 aa** reduced 2.1 and 2.0 log bacterial reduction respectively and were more potent than INH and almost equipotent as Rif. MTB PS is essential for biosynthesis of both CoA and ACP; which were in turn essential in fatty acid biosynthesis that plays a key role in persistent growth and pathogenicity of MTB (18); so these MTB PS inhibitors might be useful for treating both active and persistent TB infection.

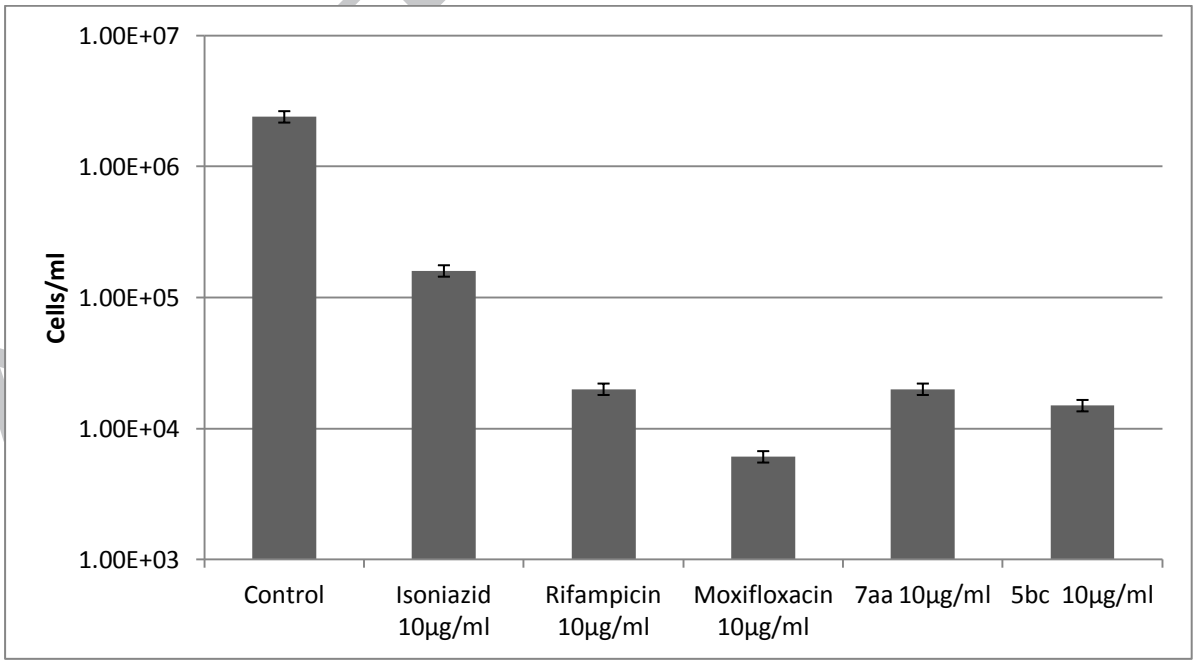


Figure 3: Inhibitors effect of tested compounds against nutrient starved MTB

2.5. Antimycobacterial screening using M. marinum induced adult zebra fish model (19)

In this model we used *M. marinum*, which is genetically close to MTB. It causes systemic tuberculosis-like granulomatous infection, superficial lesions which are examined similar to the MTB lesions clinically and pathologically (20) and also has less generation time (4-6 hr) when compared to MTB (20 hr). Adult Zebra fish model (*Danio rerio*) is infected with *M. marinum* which is known to produce normal pathogenesis similar to MTB; we observed granulomas-like lesions on the dorsal side and around the fins as sign of TB during the infection stage. Compound which showed good *in vitro* anti-TB activity (**5bc**) was tested in this model (after 7 days post infection) at 10mg/kg body weight along with INH (10mg/kg), Rif (5mg/kg), Moxi (5mg/kg), and negative control amoxicillin (10mg/kg). After 7 days of oral treatment fishes were sacrificed and bacterial count was done using MPN assay method. INH reduces ~1.8 log reduction of *M. marinum* load in Zebra fish at 10 mg/kg dose; whereas compound **5bc** showed ~1.5 log reduction of bacteria at same dose level. At the dose level of 5 mg/kg dose Rif and Moxi reduces 1.9 and 2.9 log reduction respectively (**Figure 4**). Negative control drug amoxicillin does not show any activity till 10 mg/kg dose.

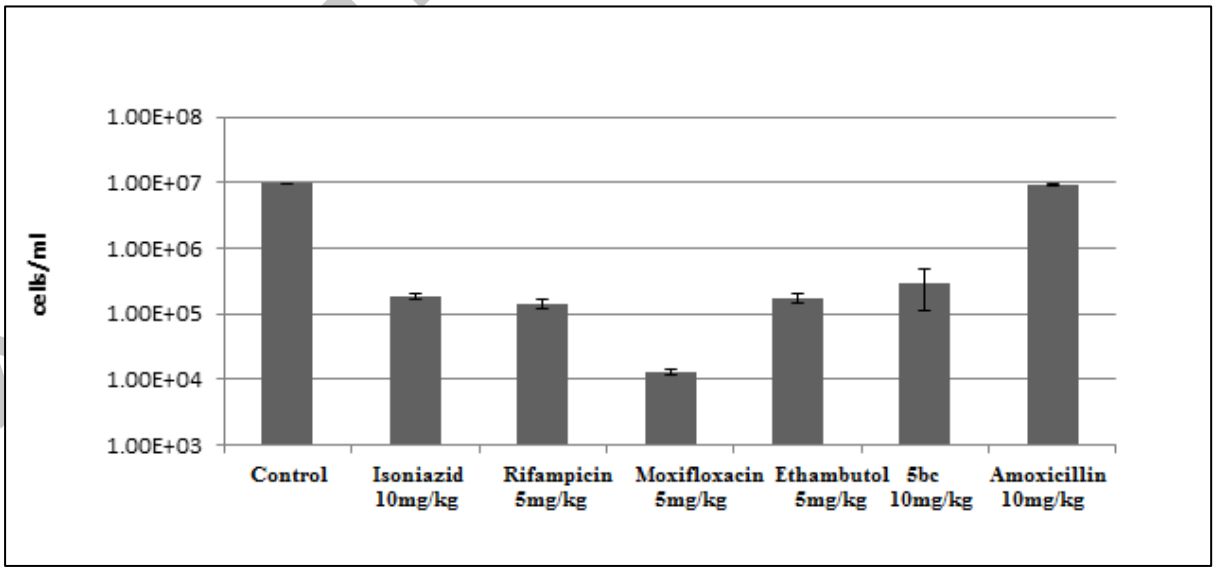


Figure 4: Antimycobacterial screening using *M. marinum* induced adult zebra fish model

2.6. In vitro cytotoxicity studies

All the compounds were also tested for *in vitro* cytotoxicity against RAW 264.7 cells (mouse macrophage cell line) at 50 μM concentration using (4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. As MTB survives inside macrophages any new molecules should not show any toxicity to macrophages; so we carried out this study *in vitro*. Percentage inhibitions of cells were reported in **Table 1**. The most promising anti-TB compound **5bc** showed only 10.4% cytotoxicity at 50μM.

3. Conclusion

We designed and synthesized novel compounds from our earlier reported MTB PS lead inhibitors and evaluated for their biological evaluation. Among them seventeen compounds (**5ac, 5ad, 5ba, 5bc, 5bd, 6aa, 6ab, 6ac, 6ba, 6be, 7aa, 7ab, 7ad, 7ba, 7bb, 7bd** and **7be**) showed more activity than lead against MTB PS and three compounds (**5bc, 6be** and **7aa**) were found to be more potent than lead against MTB MIC. Most active compounds (**5bc** and **7aa**) also showed appreciable activity in nutrient starved MTB and *M. marinum* infected Zebra fish model. As we mentioned earlier PS is not essential for *in vitro* survival of MTB; further these molecules to be tested *in vivo* to prove their specificity towards MTB PS.

4. Experimental Section

4.1. Chemistry

Reagents and solvents obtained from commercial sources were used without further purification. All the reactions were monitored by thin layer chromatography (TLC) on silica gel 40 F254 (Merck, Darmstadt, Germany) coated on aluminium plates. All ^{1}H and ^{13}C NMR spectra were recorded on a Bruker AM-400 and 100 MHz spectrometer, Bruker BioSpin Corp., Germany. Chemical shifts are reported in parts per million (ppm) using tetramethyl silane (TMS) as an internal standard. Temperatures are reported in degrees Celsius and are uncorrected. Compounds were analysed for C, H, N using Elementar vario EL III analyser and analytical results obtained were within ±0.4% of the calculated values for the formula

shown. Purity of the final compounds were >95%; checked by HPLC (Agilent technologies – Reverse phase C-18 column; run time 25 min; 0.1 TFA in CH_3CN, 0.1 TFA in H_2O as eluent). Molecular weights of the synthesised compounds were checked by (Shimadzu, LCMS-2020) ESI-MS method.

4.1.1. Preparation of ethyl 6-methylimidazo[2,1-b]thiazole-5-carboxylate (2a)

2-aminothiazole (3.00 g, 29.99 mmol) and 2-chloroethylacetoacetate (4.96 mL, 35.99 mmol) were taken in 1,2-dimethoxyethane (30 mL) and heated at 90 °C for 6 h. The reaction mixture was concentrated under reduced pressure, diluted with EtOAc (80 mL), washed the organic layer with H_2O (3 × 30 mL). The separated organic layer was dried over anhy Na_2SO_4 and concentrated under *vacuo* to get crude compound. The crude compound was purified by column chromatography using 20% EtOAc in Hexanes as eluent to get ethyl 6-methylimidazo[2,1-*b*]thiazole-5-carboxylate (**2a**) (5.20 g, 82%) as an Off-white solid. ESI-MS showed 211 $[M+H]^+$ and carried to next step.

4.1.2. Preparation of ethyl 2-methylbenzo[d]imidazo[2,1-b]thiazole-3-carboxylate (2b)

2-aminobenzothiazole (3.00 g, 19.97 mmol) and 2-chloroethylacetoacetate (3.30 mL, 23.96 mmol) were taken in 1,2-dimethoxyethane (30 mL) and heated at 90 °C for 6 h. The reaction mixture was concentrated under reduced pressure, diluted with EtOAc (80 mL), washed the organic layer with H_2O (3 × 30 mL). The separated organic layer was dried over anhy Na_2SO_4 and concentrated under vacuo to get crude compound. The crude compound was purified by column chromatography using 25% EtOAc in Hexanes as eluent to get ethyl 2-methylbenzo[*d*]imidazo[2,1-*b*]thiazole-3-carboxylate (**2b**) (4.32 g, 83%) as an Off-white solid. ESI-MS showed 261 $[M+H]^+$ and carried to next step.

4.1.3. Preparation of 6-methylimidazo[2,1-b]thiazole-5-carbohydrazide (3a)

To the stirred solution of ethyl 6-methylimidazo[2,1-*b*]thiazole-5-carboxylate (**2a)** (5.20 g) in Ethanol (40 mL) was added 35% aqueous solution of $N_2H_4.H_2O$ (40 mL) and refluxed for 3

h. The reaction mixture was concentrated to half volume and cooled on ice bath, the solids formed were filtered and dried in vacuum oven to get 6-methylimidazo[2,1-*b*]thiazole-5-carbohydrazide (**3a**) (4.30 g, 89%) as an Off-white solid. ESI-MS showed 197 $[M+H]^+$.

4.1.4. Preparation of 2-methylbenzo[d]imidazo[2,1-b]thiazole-3-carbohydrazide (3b)

To the stirred solution of ethyl 2-methylbenzo[*d*]imidazo[2,1-*b*]thiazole-3-carboxylate (**2b)** (4.32 g) in Ethanol (40 mL) was added 35% aqueous solution of $N_2H_4.H_2O$ (35 mL) and refluxed for 3 h. The reaction mixture was concentrated to half volume and cooled on ice bath, the solids formed were filtered and dried in vacuum oven to get 2-methylbenzo[*d*]imidazo[2,1-*b*]thiazole-3-carbohydrazide (**3b**) (3.69 g, 90%) as an Off-white solid. ESI-MS showed 247 $[M+H]^+$.

4.1.5. Preparation of 6-methylimidazo[2,1-b]thiazole-5-carboxylic acid (4a)

To the stirred solution of ethyl 6-methylimidazo[2,1-*b*]thiazole-5-carboxylate (**2a)** (3.00 g) in ethanol/water (1:1) (30 mL) was added LiOH (4.00 g) and stirred at room temperature for 4 h. The reaction mixture was concentrated to half volume, and added 6N HCl at 0 °C till the reaction mixture turned to $_P$H ~ 6, the solids formed were filtered and dried in vacuum oven to get 6-methylimidazo[2,1-*b*]thiazole-5-carboxylic acid (**4a**) (2.10 g, 80%) as an Off-white solid. ESI-MS showed 183 $[M+H]^+$.

4.1.6. Preparation of 2-methylbenzo[d]imidazo[2,1-b]thiazole-3-carboxylic acid (4b)

To the stirred solution of ethyl 2-methylbenzo[*d*]imidazo[2,1-*b*]thiazole-3-carboxylate (**2b)** (3.00 g) in ethanol/water (1:1) (30 mL) was added LiOH (4.00 g) and stirred at room temperature for 4 h. The reaction mixture was concentrated to half volume, and added 6N HCl at 0 °C till the reaction mixture turned to $_P$H ~ 6, the solids formed were filtered and dried in vacuum oven to get 2-methylbenzo[*d*]imidazo[2,1-*b*]thiazole-3-carboxylic acid **(4b)** (2.05 g, 76%) as an Off-white solid. ESI-MS showed 233 $[M+H]^+$.

General procedure for the synthesis of final molecules (5aa-ae** and **5ba-be)

To the stirred solution of R-COOH (1.0 equiv), in CH_2Cl_2 at 0 °C was added EDCI (1.2 equiv), HOBt (1.2 equiv) and Et_3N (2.0 equiv) stirred for few minutes then was added 6-methylimidazo[2,1-*b*]thiazole-5-carbohydrazide (for **5aa-ae**)/ 2-methylbenzo[*d*]imidazo[2,1-*b*]thiazole-3-carbohydrazide (for **5ba-be**) (1.2 equiv), and allowed stir at rt for 3 h, The reaction mixture was diluted with CH_2Cl_2 and washed with H_2O and the separated organic layer was concentrated under reduced pressure, purified by column chromatography.

4.1.8. N'-Benzoyl-6-methylimidazo[2,1-b]thiazole-5-carbohydrazide (5aa): To the stirred solution of Benzoic acid (0.4 g, 3.27 mmol), in CH_2Cl_2 at 0 °C was added EDCI (0.76 g, 3.92 mmol), HOBt (0.53 g, 3.92 mmol), and Et_3N (1.02 mL, 7.19 mmol) stirred for few minutes then was added 6-methylimidazo[2,1-*b*]thiazole-5-carbohydrazide (0.71 g, 3.60 mmol), and allowed stir at rt for 3 h, The reaction mixture was diluted with CH_2Cl_2 (20 mL) and washed with H_2O (3 × 20 mL) and the separated organic layer was concentrated under reduced pressure, purified by column chromatography using 25% EtOAc/Hexanes as eluent. MS(ESI) *m/z* 301 $[M+H]^+$. ^{1}H NMR (400 MHz, DMSO-d_6): δ 9.48 (s, 1H), 8.49 (d, *J* = 8.0 Hz, 1H), 7.92–7.78 (m, 3H), 7.54–7.46 (m, 3H), 7.30 (d, *J* = 8.0 Hz, 1H), 2.60 (s, 3H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 168.3, 161.8, 153.4, 145.6, 142.2, 139.7, 137.9, 133.1, 127.6 (2C), 126.3(2C), 119.2, 17.8. Anal. calcd for $C_{14}H_{12}N_4O_2S$: C, 55.99; H, 4.03; N, 18.65 % Found C, 56.03; H, 4.12; N, 18.71%.

4.1.9. 6-Methyl-N'-(4-methylbenzoyl)imidazo[2,1-b]thiazole-5-carbohydrazide (5ab) MS(ESI) *m/z* 315 $[M+H]^+$. ^{1}H NMR (400 MHz, DMSO-d_6): δ 9.36 (s, 1H), 8.50 (d, *J* = 8.0 Hz, 1H), 7.99–7.81 (m, 3H), 7.56 (d, *J* = 7.6 Hz, 2H), 7.29 (d, *J* = 8.0 Hz, 1H), 2.61 (s, 3H), 2.39 (s, 3H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 169.9, 162.3, 154.6, 146.2, 141.3, 140.4, 138.5, 133.6, 128.4 (2C), 126.9(2C), 120.6, 20.9, 18.2. Anal. calcd for $C_{15}H_{14}N_4O_2S$: C, 57.31; H, 4.49; N, 17.82 % Found C, 57.43; H, 4.52; N, 17.91%.

4.1.10. 6-Methyl-N'-(4-phenoxybenzoyl)imidazo[2,1-b]thiazole-5-carbohydrazide (5ac)

MS(ESI) *m/z* 393 $[M+H]^+$. ^1H NMR (400 MHz, DMSO-d_6): δ 9.63 (s, 1H), 8.51 (d, *J* = 8.0 Hz, 1H), 7.91 (d, *J* = 7.6 Hz, 2H), 7.76–7.63 (m, 3H), 7.56–7.45 (m, 3H), 7.42–7.30 (m, 3H), 2.58 (s, 3H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 166.9, 164.4, 160.3, 154.6, 151.7, 144.0, 139.6, 136.3, 135.1, 129.4 (2C), 127.1(2C), 124.9, 124.2 (2C), 122.4(2C), 119.1, 18.0. Anal. calcd for $C_{20}H_{16}N_4O_3S$: C, 61.21; H, 4.11; N, 14.28 % Found C, 61.33; H, 4.20; N, 14.31%.

4.1.11. N'-(1-Naphthoyl)-6-methylimidazo[2,1-b]thiazole-5-carbohydrazide (5ad)

MS(ESI) *m/z* 351 $[M+H]^+$. ^1H NMR (400 MHz, DMSO-d_6): δ 10.63 (s, 1H), 9.31 (d, *J* = 8.0 Hz, 1H), 8.47 (d, *J* = 8.0 Hz, 1H), 7.81–7.72 (m, 2H), 7.68–7.54 (m, 3H), 7.47–7.32 (m, 3H), 2.61 (s, 3H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 169.0, 166.2, 162.0, 152.4, 149.7, 139.0, 137.3, 134.2, 133.9, 133.0, 129.4, 128.3, 126.4, 125.7, 125.1, 124.4, 119.3, 17.2. Anal. calcd for $C_{18}H_{14}N_4O_2S$: C, 61.70; H, 4.03; N, 15.99 % Found C, 61.73; H, 4.09; N, 16.09%.

4.1.12. N'-(Cyclohexanecarbonyl)-6-methylimidazo[2,1-b]thiazole-5-carbohydrazide (5ae)

MS(ESI) *m/z* 307 $[M+H]^+$. ^1H NMR (400 MHz, DMSO-d_6): δ 9.54 (s, 1H), 8.61 (d, *J* = 8.0 Hz, 1H), 7.92 (s, 1H), 7.32 (d, *J* = 8.0 Hz, 1H), 2.56 (s, 3H), 2.18–2.14 (m, 1H), 1.78–1.50 (m, 10H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 174.2, 164.8, 160.2, 148.3, 144.8, 138.4, 118.2, 47.9, 31.3(2C), 26.6(3C), 16.9. Anal. calcd for $C_{14}H_{18}N_4O_2S$: C, 54.88; H, 5.92; N, 18.29 % Found C, 54.93; H, 5.98; N, 18.39%.

4.1.13. N'-Benzoyl-2-methylbenzo[d]imidazo[2,1-b]thiazole-3-carbohydrazide (5ba):

MS(ESI) *m/z* 351 $[M+H]^+$. ^1H NMR (400 MHz, DMSO-d_6): δ 9.44 (s, 1H), 8.09–7.81 (m, 3H), 7.74–7.63 (m, 4H), 7.56–7.44 (m, 3H), 2.63 (s, 3H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 167.2, 163.5, 158.6, 149.3, 143.6, 142.7, 138.6, 136.7, 134.2, 129.6, 128.0 127.8 (2C), 126.4(2C), 124.6, 120.4, 17.8. Anal. calcd for $C_{18}H_{14}N_4O_2S$: C, 61.70; H, 4.03; N, 15.99 % Found C, 61.73; H, 4.12; N, 16.11%.

4.1.14. 2-Methyl-N'-(4-methylbenzoyl)benzo[d]imidazo[2,1-b]thiazole-3-carbohydrazide (5bb)

MS(ESI) *m/z* 365 $[M+H]^+$. ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.08–7.92 (m, 3H), 7.86 (d, *J* = 8.0 Hz, 2H), 7.72–7.64 (m, 2H), 7.54–7.39 (m, 3H), 2.60 (s, 3H), 2.42 (s, 3H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 168.6, 163.2, 155.8, 146.9, 140.4, 138.4, 137.3, 133.8, 129.6 (2C), 128.3, 127.4(2C), 126.5, 124.7, 124.0, 119.7, 22.3, 17.8. Anal. calcd for $C_{19}H_{16}N_4O_2S$: C, 62.62; H, 4.43; N, 15.37 % Found C, 62.63; H, 4.52; N, 15.41%.

4.1.15. 2-Methyl-N'-(4-phenoxybenzoyl)benzo[d]imidazo[2,1-b]thiazole-3-carbohydrazide (5bc)

MS(ESI) *m/z* 443 $[M+H]^+$. ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.10–7.96 (m, 4H), 7.90–7.74 (m, 3H), 7.63 (d, *J* = 8.0 Hz, 2H), 7.60 (s, 1H), 7.56–7.45 (m, 5H), 2.62 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 167.4, 165.6, 162.5, 157.4, 153.0, 145.5, 140.3, 137.4, 136.2, 133.2, 128.9 (2C), 127.4(2C), 126.6, 125.7, 124.9(2C), 124.2, 123.9(2C), 121.4, 117.9, 17.1. Anal. calcd for $C_{24}H_{18}N_4O_3S$: C, 65.14; H, 4.10; N, 12.66 % Found C, 65.23; H, 4.20; N, 12.71%.

4.1.16. N'-(1-Naphthoyl)-2-methylbenzo[d]imidazo[2,1-b]thiazole-3-carbohydrazide (5bd)

MS(ESI) *m/z* 401 $[M+H]^+$. ^{1}H NMR (400 MHz, DMSO-d_6): δ 9.28 (d, *J* = 8.0 Hz, 1H), 8.04–7.87 (m, 4H), 7.81–7.74 (m, 4H), 7.66–7.53 (m, 4H), 2.59 (s, 3H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 168.3, 167.1, 163.5, 155.7, 150.4, 139.5, 138.2, 136.4, 135.6, 133.6, 129.4, 129.2, 128.6, 127.4, 126.4, 126.2, 125.8, 125.0, 124.8, 124.3, 117.8, 17.2. Anal. calcd for $C_{22}H_{16}N_4O_2S$: C, 65.98; H, 4.03; N, 13.99 % Found C, 66.03; H, 4.09; N, 14.09%.

4.1.17. N'-(Cyclohexanecarbonyl)-2-methylbenzo[d]imidazo[2,1-b]thiazole-3-carbohydrazide (5be)

MS(ESI) *m/z* 357 $[M+H]^+$. ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.07–7.96 (m, 3H), 7.69–7.58 (m, 3H), 2.58 (s, 3H), 2.16–2.11 (m, 1H), 1.76–1.51 (m, 10H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 172.9, 163.3, 162.7, 152.2, 139.4, 137.6, 126.8, 125.4, 124.7, 124.1, 119.4, 47.2, 31.6(2C), 25.9(3C), 16.7. Anal. calcd for $C_{18}H_{20}N_4O_2S$: C, 60.65; H, 5.66; N, 15.72 % Found C, 60.73; H, 5.68; N, 15.89%.

General procedure for the synthesis of final molecules (6aa-ae** and **6ba-be)

To the stirred solution of 6-methylimidazo[2,1-*b*]thiazole-5-carbohydrazide (**3a**) (for **6aa-ae**)/ 2-methylbenzo[*d*]imidazo[2,1-*b*]thiazole-3-carbohydrazide (3**b)** (for **6ba-be**) (1.0 equiv), aldehyde (1.1 equiv), conc. H_2SO_4 (cat) were taken in Ethanol and refluxed for 1 h. The formed solids were filtered, dried and triturated with CH_2Cl_2/hexanes to get pure products.

4.1.18. N'-(4-Bromobenzylidene)-6-methylimidazo[2,1-b]thiazole-5-carbohydrazide (6aa): 6-methylimidazo[2,1-*b*]thiazole-5-carbohydrazide (0.4 g, 2.03 mmol), 4-bromobenzaldehyde (0.22 mL, 2.23 mmol), conc. H_2SO_4 (3 drops) were taken in ethanol (7 mL) and refluxed for 30 min. The solids in the reaction mixture were filtered, washed with Water, cold Ethanol, Hexanes and dried in vacuum oven to get (0.54 g, 72%) title compound.

MS(ESI) *m/z* 364 $[M+H]^+$. ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.61 (s, 1H), 8.32 (d, *J* = 7.2 Hz, 1H), 7.81 (d, *J* = 7.6 Hz, 2H), 7.68–7.49 (m, 3H), 7.29 (d, *J* = 7.6 Hz, 1H), 2.62 (s, 3H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 166.3, 162.1, 149.8, 146.1, 141.4, 130.6, 128.7, 127.4(2C), 125.4(2C), 124.1, 118.6, 17.3. Anal. calcd for $C_{14}H_{11}BrN_4OS$: C, 46.29; H, 3.05; N, 15.42 % Found C, 46.33; H, 3.09; N, 15.51%.

4.1.19. 6-Methyl-N'-(4-(trifluoromethyl)benzylidene)imidazo[2,1-b]thiazole-5-carbohydrazide (6ab)

MS(ESI) *m/z* 353 $[M+H]^+$. ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.63 (s, 1H), 8.49 (d, *J* =7.6 Hz, 1H), 7.74 (d, *J* =7.6 Hz, 2H), 7.63 (d, *J* =7.6 Hz, 2H), 7.48 (s, 1H), 7.30 (d, *J* =7.6 Hz, 1H), 2.60 (s, 3H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 164.4, 162.1, 148.9, 146.5, 137.6, 133.3, 130.6, 129.1, 127.3(2C), 126.2(2C), 124.4, 118.6, 17.4. Anal. calcd for $C_{15}H_{11}F_3N_4OS$: C, 51.13; H, 3.15; N, 15.90 % Found C, 51.23; H, 3.19; N, 15.96%.

4.1.20. N'-Benzylidene-6-methylimidazo[2,1-b]thiazole-5-carbohydrazide (6ac)

MS(ESI) *m/z* 285 $[M+H]^+$. ^{1}H NMR (400 MHz, DMSO-d_6): δ 9.34 (s, 1H), 8.52 (d, *J* = 8.1 Hz, 2H), 8.12 (s, 1H), 7.69–7.36 (m, 5H), 2.64 (s, 3H); ^{13}C NMR (100 MHz, DMSO-d_6) δ

168.4, 164.9, 152.9, 148.3, 139.4, 129.8, 128.3, 124.9(2C), 124.0(2C), 123.7, 119.1, 18.9. Anal. calcd for $C_{14}H_{12}N_4OS$: C, 59.14; H, 4.25; N, 19.70 % Found C, 59.33; H, 4.29; N, 19.91%.

4.1.21. 6-Methyl-N'-(3,4,5-trimethoxybenzylidene)imidazo[2,1-b]thiazole-5-carbohydrazide (6ad)

MS(ESI) *m/z* 375 $[M+H]^+$. ^{1}H NMR (400 MHz, DMSO-d_6): δ 9.45 (s, 1H), 8.53 (s, 1H), 8.39 (d, *J* = 8.0 Hz, 1H), 7.33 (d, *J* = 8.0 Hz, 1H), 7.27 (s, 2H), 3.94 (s, 9H), 2.61 (s, 3H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 162.5, 161.9, 156.3(2C), 144.8, 143.1, 142.3, 140.6, 139.5, 137.4, 121.2(2C), 120.1, 63.4, 60.9(2C), 17.1. Anal. calcd for $C_{17}H_{18}N_4O_4S$: C, 54.53; H, 4.85; N, 14.96 % Found C, 54.63; H, 4.92; N, 14.98%.

$C_{15}H_{11}F_3N_4OS$: C, 51.13; H, 3.15; N, 15.90 % Found C, 51.23; H, 3.19; N, 15.96%.

4.1.22.N'-(4-(Dimethylamino)benzylidene)-6-methylimidazo[2,1-b]thiazole-5-carbohydrazide (6ae)

MS(ESI) *m/z* 328 $[M+H]^+$. ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.55 (s, 1H), 8.46 (d, *J* = 8.0 Hz, 1H), 7.56–7.47 (m, 3H), 7.29 (d, *J* = 8.0 Hz, 1H), 7.11 (d, *J* = 8.0 Hz, 2H), 3.15 (s, 6H), 2.62 (s, 3H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 163.4, 162.5, 156.3, 146.2, 143.8, 141.4, 136.3, 129.3(2C), 125.1, 120.4, 115.2(2C), 44.1(2C), 17.3. Anal. calcd for $C_{16}H_{17}N_5OS$: C, 58.70; H, 5.23; N, 21.39 % Found C, 58.73; H, 5.29; N, 21.51%.

4.1.23. N'-(4-Bromobenzylidene)-2-methylbenzo[d]imidazo[2,1-b]thiazole-3-carbohydrazide (6ba)

MS(ESI) *m/z* 414 $[M+H]^+$. ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.58 (s, 1H), 8.04–7.81 (m, 3H), 7.71 (d, *J* = 7.6 Hz, 2H), 7.64–7.51 (m, 4H), 2.60 (s, 3H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 164.7, 163.3, 160.8, 144.0, 139.5, 137.3, 136.8, 135.4, 133.0, 130.7(2C), 129.0, 128.6(2C), 126.7, 123.4, 120.4, 17.4. Anal. calcd for $C_{18}H_{13}BrN_4OS$: C, 52.31; H, 3.17; N, 13.56 % Found C, 52.33; H, 3.29; N, 13.71%.

4.1.24. 2-Methyl-N'-(4-(trifluoromethyl)benzylidene)benzo[d]imidazo[2,1-b]thiazole-3-carbohydrazide (6bb)

MS(ESI) *m/z* 403 $[M+H]^+$. ^1H NMR (400 MHz, DMSO-d_6): δ 8.58 (s, 1H), 8.09–7.94 (m, 3H), 7.76 (d, *J* =8.0 Hz, 2H), 7.65–7.48 (m, 4H), 2.62 (s, 3H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 163.6, 163.0, 156.6, 140.1, 138.4, 137.1, 134.3, 131.8, 129.5, 128.2, 128.9(2C), 128.3, 127.5(2C), 126.6, 123.9, 119.1, 16.9. Anal. calcd for $C_{19}H_{13}F_3N_4OS$: C, 56.71; H, 3.26; N, 13.92 % Found C, 56.83; H, 3.29; N, 14.06%.

4.1.25. N'-Benzylidene-2-methylbenzo[d]imidazo[2,1-b]thiazole-3-carbohydrazide (6bc)

MS(ESI) *m/z* 335 $[M+H]^+$. ^1H NMR (400 MHz, $CDCl_3$): δ 8.55 (d, *J* = 8.0 Hz, 1H), 8.02–7.91 (m, 3H), 7.78 (d, *J* = 8.0 Hz, 2H), 7.65–7.51 (m, 5H), 2.62 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 163.3, 162.6, 158.9, 146.2, 139.0, 137.5, 136.8, 134.5, 133.0, 128.4(2C), 126.2, 125.7(2C), 124.2, 123.4, 119.6, 17.4. Anal. calcd for $C_{18}H_{14}N_4OS$: C, 64.65; H, 4.22; N, 16.75 % Found C, 64.73; H, 4.29; N, 16.91%.

4.1.26. 2-Methyl-N'-(3,4,5-trimethoxybenzylidene)benzo[d]imidazo[2,1-b]thiazole-3-carbohydrazide (6bd)

MS(ESI) *m/z* 425 $[M+H]^+$. ^1H NMR (400 MHz, DMSO-d_6): δ 8.56 (s, 1H), 7.99–7.90 (m, 2H), 7.54–7.42 (m, 3H), 7.21 (s, 2H), 3.96 (s, 9H), 2.60 (s, 3H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 163.6, 162.9, 160.2, 150.3(2C), 146.4, 142.6, 139.3, 136.2, 135.1, 134.6, 129.8, 126.3, 124.6, 121.4, 114.6(2C), 62.1, 60.6(2C), 17.3. Anal. calcd for $C_{21}H_{20}N_4O_4S$: C, 59.42; H, 4.75; N, 13.20 % Found C, 59.63; H, 4.82; N, 13.38%.

$C_{15}H_{11}F_3N_4OS$: C, 51.13; H, 3.15; N, 15.90 % Found C, 51.23; H, 3.19; N, 15.96%.

4.1.27. N'-(4-(Dimethylamino)benzylidene)-2-methylbenzo[d]imidazo[2,1-b]thiazole-3-carbohydrazide (6be)

MS(ESI) *m/z* 328 $[M+H]^+$. ^1H NMR (400 MHz, DMSO-d_6): δ 9.79 (s, 1H), 8.61 (s, 1H), 8.01–7.90 (m, 2H), 7.69–7.54 (m, 4H), 7.15 (d, *J* = 8.0 Hz, 2H), 3.13 (s, 6H), 2.61 (s, 3H);

^{13}C NMR (100 MHz, DMSO-d_6) δ 164.3, 161.1, 158.4, 153.6, 146.5, 137.8, 135.1, 133.8, 130.4, 128.2(2C), 126.0, 125.3, 124.6, 122.4(2C), 118.1, 43.2(2C), 16.9. Anal. calcd for $C_{20}H_{19}N_5OS$: C, 63.64; H, 5.07; N, 18.55 % Found C, 63.73; H, 5.19; N, 18.71%.

General procedure for the synthesis of final molecules (7aa-ae** and **7ba-be)

To the stirred solution of 6-methylimidazo[2,1-*b*]thiazole-5-carboxylic acid (for **7aa-ae**)/ 2-methylbenzo[*d*]imidazo[2,1-*b*]thiazole-3-carboxylic acid (for **7ba-be**) (1.0 equiv), in CH_2Cl_2 at 0 °C was added EDCI (1.2 equiv), HOBt (1.2 equiv) and Et_3N (2.0 equiv) stirred for few minutes then was added R-NH_2 (1.2 equiv), and allowed stir at rt for 3 h, the reaction mixture was diluted with CH_2Cl_2 and washed with H_2O and the separated organic layer was concentrated under reduced pressure, purified by column chromatography.

4.1.28. N-(4-Bromophenyl)-6-methylimidazo[2,1-b]thiazole-5-carboxamide (7aa): To the stirred solution of 6-methylimidazo[2,1-*b*]thiazole-5-carboxylic acid (0.4 g, 2.19 mmol), in CH_2Cl_2 at 0 °C was added EDCI (0.50 g, 2.64 mmol), HOBt (0.36 g, 2.64 mmol), and Et_3N (0.62 mL, 4.38 mmol) stirred for few minutes then was added 4-bromoaniline (0.45 g, 2.64 mmol), and allowed stir at rt for 3 h, The reaction mixture was diluted with CH_2Cl_2 (25 mL) and washed with H_2O (3 × 20 mL) and the separated organic layer was concentrated under reduced pressure, purified by column chromatography using 40% EtOAc/hexanes as eluent. MS(ESI) *m/z* 335 $[M+H]^+$. ^{1}H NMR (400 MHz, DMSO-d_6): δ 9.70 (s, 1H), 8.57 (d, *J* = 8.0 Hz, 1H), 7.81 (d, *J* = 8.4 Hz, 2H), 7.67 (d, *J* = 8.0 Hz, 2H), 7.29 (d, *J* = 7.6 Hz, 1H), 2.61 (s, 3H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 164.5, 162.3, 143.2, 141.7, 133.9, 132.8, 130.2, 128.3, 126.5(2C), 121.4, 118.4, 16.9. Anal. calcd for $C_{13}H_{10}BrN_3OS$: C, 46.44; H, 3.00; N, 12.50 % Found C, 46.53; H, 3.12; N, 12.71%.

4.1.29. 6-Methyl-N-phenylimidazo[2,1-b]thiazole-5-carboxamide (7ab)

MS(ESI) *m/z* 258 $[M+H]^+$. ^{1}H NMR (400 MHz, DMSO-d_6): δ 10.21 (s, 1H), 8.52 (d, *J* = 8.0 Hz, 1H), 7.74–7.56 (m, 5H), 7.24 (d, *J* = 8.0 Hz, 1H), 2.64 (s, 3H); ^{13}C NMR (100 MHz,

DMSO-d_6) δ 166.9, 156.3, 146.5, 138.0, 132.2, 129.4, 127.7 (2C), 126.2, 124.4(2C), 119.4, 16.9. Anal. calcd for $C_{13}H_{11}N_3OS$: C, 60.68; H, 4.31; N, 16.33 % Found C, 60.73; H, 4.42; N, 16.51%.

4.1.30. N-(4-Methoxyphenyl)-6-methylimidazo[2,1-b]thiazole-5-carboxamide (7ac)

MS(ESI) *m/z* 302 $[M+H]^+$. ^{1}H NMR (400 MHz, $CDCl_3$): δ 10.34 (s, 1H), 8.49 (d, *J* = 8.0 Hz, 1H), 7.62 (d, *J* = 7.6 Hz, 2H), 7.32 (d, *J* = 7.6 Hz, 1H), 7.02 (d, *J* = 8.0 Hz, 2H), 4.12 (q, *J* = 7.2 Hz, 2H), 2.63 (s, 3H), 1.38 (t, *J* = 7.2 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 165.2, 160.2, 158.4, 145.3, 137.4, 134.6, 126.3(2C), 123.2, 119.4(2C), 118.1, 71.3, 17.2, 15.8. Anal. calcd for $C_{15}H_{15}N_3O_2S$: C, 59.78; H, 5.02; N, 13.94 % Found C, 59.93; H, 5.12; N, 14.11%.

4.1.31. N-Benzyl-6-methylimidazo[2,1-b]thiazole-5-carboxamide (7ad)

MS(ESI) *m/z* 272 $[M+H]^+$. ^{1}H NMR (400 MHz, $CDCl_3$): δ 9.45 (s, 1H), 8.52 (d, *J* = 8.0 Hz, 1H), 7.51–7.38 (m, 5H), 7.36 (d, *J* = 8.0 Hz, 1H), 4.23 (s, 2H), 2.62 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 163.4, 160.6, 144.4, 142.3, 137.3, 136.2, 127.4(2C), 126.5(2C), 125.9, 119.4, 51.3, 16.8. Anal. calcd for $C_{14}H_{13}N_3OS$: C, 61.97; H, 4.83; N, 15.49 % Found C, 62.03; H, 4.92; N, 15.61%.

4.1.32. N-Cyclohexyl-6-methylimidazo[2,1-b]thiazole-5-carboxamide (7ae)

MS(ESI) *m/z* 264 $[M+H]^+$. ^{1}H NMR (400 MHz, $CDCl_3$): δ 9.27 (s, 1H), 8.49 (d, *J* = 7.6 Hz, 1H), 7.31 (d, *J* = 7.6 Hz, 1H), 3.34–3.28 (m, 1H), 2.61 (s, 3H), 1.71–1.63 (m, 4H), 1.48–1.24 (m, 6H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 162.7, 158.3, 146.2, 142.3, 136.2, 119.4, 61.3, 34.2(2C), 27.3, 25.2(2C), 16.2. Anal. calcd for $C_{13}H_{17}N_3OS$: C, 59.29; H, 6.51; N, 15.96 % Found C, 59.43; H, 6.64; N, 16.11%.

4.1.33. N-(4-Bromophenyl)-2-methylbenzo[d]imidazo[2,1-b]thiazole-3-carboxamide (7ba):

MS(ESI) *m/z* 386 $[M+H]^+$. ^{1}H NMR (400 MHz, DMSO-d_6): δ 10.71 (s, 1H), 8.03–7.90 (m, 2H), 7.83 (d, *J* = 7.2 Hz, 2H), 7.69–7.54 (m, 4H), 2.59 (s, 3H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 162.7, 160.2, 156.4, 138.4, 136.9, 133.6, 132.2, 131.5, 130.3(2C), 128.2, 126.5, 126.1,

123.3(2C), 119.5, 15.6. Anal. calcd for $C_{17}H_{12}BrN_3OS$: C, 52.86; H, 3.13; N, 10.88 % Found C, 52.93; H, 3.22; N, 10.99%.

4.1.34. 2-Methyl-N-phenylbenzo[d]imidazo[2,1-b]thiazole-3-carboxamide (7bb)

MS(ESI) *m/z* 308 $[M+H]^+$. ^{1}H NMR (400 MHz, DMSO-d_6): δ 10.35 (s, 1H), 8.02 (d, *J* = 7.2 Hz, 1H), 7.72–7.63 (m, 3H), 7.58 (d, *J* = 7.6 Hz, 2H), 7.47–7.35 (m, 3H), 2.62 (s, 3H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 163.4, 159.5, 156.2, 139.4, 137.6, 136.1, 135.6, 134.2, 128.8(2C), 127.4, 127.0, 126.5, 125.3(2C), 120.4, 16.4. Anal. calcd for $C_{17}H_{13}N_3OS$: C, 66.43; H, 4.26; N, 13.67 % Found C, 66.70; H, 4.42; N, 13.81%.

4.1.35. N-(4-Ethoxyphenyl)-2-methylbenzo[d]imidazo[2,1-b]thiazole-3-carboxamide (7bc)

MS(ESI) *m/z* 352 $[M+H]^+$. ^{1}H NMR (400 MHz, $CDCl_3$): δ 9.34 (s, 1H), 8.09 (d, *J* = 6.8 Hz, 1H), 8.04 (d, *J* = 6.8 Hz, 1H), 7.77–7.63 (m, 4H), 7.22 (d, *J* = 7.6 Hz, 2H), 4.14 (q, *J* = 7.2 Hz, 2H), 2.61 (s, 3H), 1.36 (t, *J* = 7.2 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 164.6, 161.3, 156.5, 152.4, 139.3, 136.1, 134.2, 133.6, 130.3, 127.2, 124.4, 122.4(2C), 119.9, 119.4(2C), 69.4, 16.6, 15.4. Anal. calcd for $C_{19}H_{17}N_3O_2S$: C, 64.94; H, 4.88; N, 11.96 % Found C, 64.99; H, 4.92; N, 12.09%.

4.1.36. N-Benzyl-2-methylbenzo[d]imidazo[2,1-b]thiazole-3-carboxamide (7bd)

MS(ESI) *m/z* 322 $[M+H]^+$. ^{1}H NMR (400 MHz, $CDCl_3$): δ 10.33 (s, 1H), 8.10–8.03 (m, 2H), 7.69–7.42 (m, 7H), 4.19 (s, 2H), 2.63 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 162.5, 160.5, 158.9, 140.1, 138.6, 136.8, 135.2, 129.1(2C), 127.4, 126.5(2C), 125.1, 123.5, 123.0, 119.4, 52.2, 16.9. Anal. calcd for $C_{18}H_{15}N_3OS$: C, 67.27; H, 4.70; N, 13.07 % Found C, 67.33; H, 4.82; N, 13.21%.

4.1.37. N-Cyclohexyl-2-methylbenzo[d]imidazo[2,1-b]thiazole-3-carboxamide (7be)

MS(ESI) *m/z* 314 $[M+H]^+$. ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.07 (d, *J* = 6.8 Hz, 1H), 8.01 (d, *J* = 6.8 Hz, 1H), 7.67–7.55 (m, 3H), 3.33–3.28 (m, 1H), 2.63 (s, 3H), 1.74–1.66 (m, 4H), 1.51–1.27 (m, 6H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 164.5, 162.8, 158.4, 137.2, 135.9, 135.2, 127.5,

126.3, 126.0, 122.2, 60.3, 34.6(2C), 27.8, 26.0(2C), 16.9. Anal. calcd for $C_{17}H_{19}N_3OS$: C, 65.15; H, 6.11; N, 13.41 % Found C, 65.23; H, 6.14; N, 13.61%

4.2. Biological activity

4.2.1. MTB PS screening

The MTB panC gene (Rv3602c) encoding the pantothenate synthetase was cloned and transformed into BL21 (DE3) cells and the expression of the protein was performed. For the assay, to each well of a 96-well plate, 60µl of PS reaction mixture consisting of 0.4 mM NADH, 5 mM pantoic acid, 10 mM $MgCl_2$, 5mM β-alanine, 10 mM ATP, 1 mM potassium phosphoenolpyruvate, and 20 µl of enzyme mixture consists of 18 units/ml each of chicken muscle myokinase, rabbit muscle pyruvate kinase, and rabbit muscle lactate dehydrogenase diluted in 100 mM HEPES buffer were added. The reaction mixture and enzyme mixture were added to the plate to a final volume of 100 µl with 100 mM HEPES buffer (pH 7.8). Concentration of enzyme was determined based on the range finding experiments by varying the concentration of enzymes. Compounds were then added to the plates (from 25 µM to lower concentration) and the reaction was initiated with the addition of 10 µL of 4.32 pM of MTB PS, diluted in buffer. The test plate was immediately transferred to a microplate reader and the depletion of NADH was measured at 340 nm. The reaction components except MTB PS were mixed in the well and the background reaction was measured; MTB PS was then added and the reaction kinetics was monitored. Reactions were carried out at 37°C in a heat-controlled PerkinElmer Victor X3 Spectrophotometer. % Inhibitions were calculated using following formula: 100 x [(1 - compound rate - background rate) / (full reaction rate - background rate)]

4.2.2. In vitro MTB screening

The compounds were further screened for their *in vitro* antimycobacterial activity against *M. tuberculosis* H37Rv by microplate Alamar blue assay method (15). Briefly, the inoculum was

prepared from fresh LJ medium re-suspended in 7H9-S medium (7H9 broth, 0.1% casitone, 0.5% glycerol, supplemented oleic acid, albumin, dextrose, and catalase [OADC]), adjusted to a McFarland tube No. 1, and diluted 1:20; 100 µl was used as inoculum. Each drug stock solution was thawed and diluted in 7H9-S at four-fold the final highest concentration tested. Serial two-fold dilutions of each drug were prepared directly in a sterile 96-well microtiter plate using 100 µl 7H9-S. A growth control containing no antibiotic and a sterile control were also prepared on each plate. Sterile water was added to all perimetre wells to avoid evaporation during the incubation. The plate was covered, sealed in plastic bags and incubated at 37^{o}C in normal atmosphere. After 7 days incubation, 30 ml of alamar blue solution was added to each well, and the plate was re-incubated overnight. A change in colour from blue (oxidised state) to pink (reduced) indicated the growth of bacteria, and the MIC was defined as the lowest concentration of drug that prevented this change in colour.

4.2.3. In vitro cytotoxicity screening

Some compounds were further examined for toxicity in a RAW 264.7 cell line at the concentration of 50 µM. After 72 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.

4.2.4. In vitro nutrient starved MTB screening

Culture of *Mycobacterium tuberculosis* H37RV were grown in Middlebrook 7H9 medium supplemented with OADC (nutrient rich medium) was pelleted and washed twice with PBS (Phosphate buffer saline, HiMedia Laboratories). The pellet was re-suspended in PBS in sealed bottles and is incubated at 37 °C for 6 weeks. Six week starved cultures were treated with standard drugs like Isoniazid, Rifampicin and Moxifloxacin along with synthesized drugs for 7 days at a concentration of 10ug/ml. The treated cell suspensions were diluted 10-fold up to 10^{-6} using Middlebrook 7H9 medium supplemented with OADC and 100 µl of

each dilutions were plated in 48 well plates in triplicates along with 900 µl of Midllebrook 7H9 medium (HiMedia Laboratories) supplemented with OADC (HiMedia Laboratories). The microplates were incubated at 37 °C for 28 days without agitation. Wells with visible bacterial growth were counted as positive, and MPN values were calculated using standard statistical methods.

4.2.5. 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. Fish were initially weighed and monitored for its locomotor activities and were grouped into control and treatment groups (n=6). All the fish were infected by intraperitoneal injection with 20 µl of thawed bacterial stocks (around 0.75 million bacteria). They were observed for lesions, reduction in swimming activities and squamous eruptions in the initial 7 day infection stage which was followed by treatment stage. The drug solutions were prepared based on the fish's body weight and oral dosing amount of 5 µL. Fish were then administered drug orally using micropipette for 7 days. After treatment they were allowed to swim in 1.5mg/mL solution of kanamycin sulphate before proceeding for sacrifice at the end of study i.e., 14^{th} day. Finally, all of them were sacrificed using homogenization technique and the tissue sample was prepared in Middlebrook 7H9 broth. The collected homogenate was serially diluted to 10^{-6} times and plated into 48-well plates, incubated at 30 °C for 24 hours. The plates were checked for the bacterial counts using MPN assay method.

Acknowledgements

Authors acknowledge DST, India for its financial support to carry out this work. Ganesh Samala (SRF) is thankful to CSIR, India for fellowship; DS acknowledges University Grants

Commission, New Delhi, India for UGC Research Award. We also acknowledge Dr David Eisenberg and Dr Shuishu Wang, Department of Biochemistry & Molecular biology, USUHS, Maryland for providing MTB PS clone.

References

1. WHO Global tuberculosis report 2015, http://www.who.int/tb/country/en/.
2. R. Zheng, J.S. Blanchard, Biochemistry. 40 (2001) 12904-12.
3. C. Lienhardt, A. Vernon, M.C. Raviglione, Curr Opin Pulm Med. 16 (2010) 186-193.
4. R. Chapman, E. Shephard, H. Stutz, N. Douglass, V.K. Sambandamurthy, I. Garcia, B. Ryffel, W. Jacobs, A.L. Williamson, PLoS One. 7 (2012) e32769.
5. V. K. Sambandamurthy, S. C. Derrick, K. V. Jalapathy, B. Chen, R. G. Russell, S. L. Morris, W. R. Jacobs, Jr. Infect Immun. 73 (2005) 1196–1203.
6. G. Samala, R. Nallangi, P.B. Devi, S. Saxena, R. Yadav, J.P. Sridevi, P. Yogeeswari, D. Sriram, Bioorg Med Chem. 22 (2014) 4223-32.
7. P.B. Devi, G. Samala, J.P. Sridevi, S. Saxena, M. Alvala, E.G. Salina, D. Sriram, P. Yogeeswari, ChemMedChem. 9 (2014) 2538-47.
8. M. Krátký, E. Novotná, S. Saxena, P. Yogeeswari, D. Sriram, M. Švarcová, J. Vinšová, ScientificWorldJournal. (2014) 703053.
9. G. Samala, P.B. Devi, R. Nallangi, J.P. Sridevi, S. Saxena, P. Yogeeswari, D. Sriram, Bioorg Med Chem. 22 (2014) 1938-47.
10. Z. Xu, W. Yin, L.K. Martinelli, J. Evans, J. Chen, Y. Yu, D.J. Wilson, V. Mizrahi, C. Qiao, C.C. Aldrich, Bioorg Med Chem. 22 (2014) 1726-35.
11. A. Kumar, A. Casey, J. Odingo, E.A. Kesicki, G. Abrahams, M. Vieth, T. Masquelin, V. Mizrahi, P.A. Hipskind, D.R. Sherman, T. Parish, PLoS One. 8 (2013) e72786.
12. F. Ntie-Kang, S. Kannan, K. Wichapong, L.C. Owono, W. Sippl, E. Megnassan. Mol Biosyst. 10 (2014) 223-39.

13. G. Samala, P.B. Devi, R. Nallangi, P. Yogeeswari, D. Sriram, Eur J Med Chem. 69 (2013) 356-64.

14. Y. Yang, P. Gao, Y. Liu, X. Ji, M. Gan, Y. Guan, X. Hao, Z. Li, C. Xiao, Bioorg Med Chem Lett. 21 (2011) 3943-6.

15. L. Collins, S.G. Franzblau, Antimicrob Agents Chemother. 41 (1997) 1004-1009.

16. J.C. Betts, P.T. Lukey, L.C. Robb, R.A. McAdam, K. Duncan, Mol Microbiol 43 (2002) 717–731.

17. J.C. De Man, J Appl Microbiol 1 (1975) 67–78.

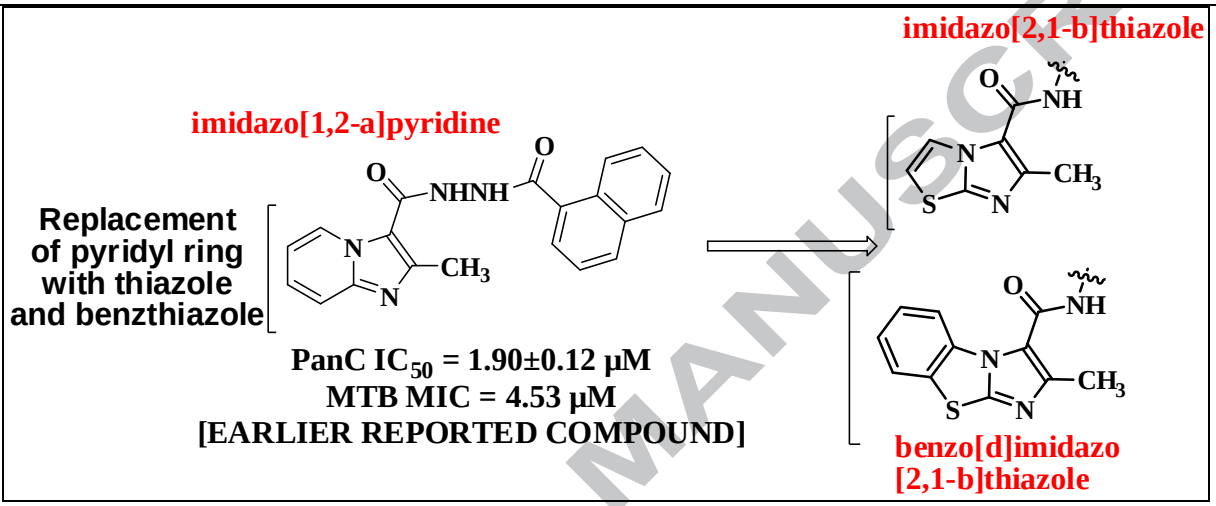
18. S. Wang, D. Eisenberg, Protein Sci. 12 (2003) 1097–1108.

19. J.P. Sridevi, H.S. Anantaraju, P. Kulkarni, P. Yogeeswari, D. Sriram, Int J Mycobact 3 (2014) 259-267.

20. D.M. Tobin, L. Ramakrishnan, Cell Microbiol 10 (2008) 1027–1039.

Design, synthesis and biological evaluation of imidazo[2,1-*b*]thiazole and benzo[d]imidazo[2,1-b]thiazole derivatives as *Mycobacterium tuberculosis* pantothenate synthetase inhibitors

Ganesh Samala, Parthiban Brindha Devi, Shalini Saxena, Nikhila Meda Perumal Yogeeswari, Dharmarajan Sriram*



Compound **5bc** emerged as potent compound active against MTB PS with IC_{50} of 0.53±0.13 µM, MIC of 3.53 µM, 2.1 log reduction against nutrient starved MTB. It also showed 1.5 log reduction of *M. marinum* load in Zebra fish at 10mg/kg.