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Anti-tubercular activities of 5,6,7,8-tetrahydrobenzo[4,5]thieno[2,3-*d*]pyrimidin-4-amine analogues endowed with high activity toward non-replicative *Mycobacterium tuberculosis*

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ABSTRACT

Thirty three derivatives of 2-substituted 5,6,7,8-tetrahydrobenzo[4,5]thieno[2,3-*d*]pyrimidin-4-amine analogues were synthesized by molecular modification of a reported antimycobacterial molecule (GSK163574A). Compounds were evaluated in vitro against actively replicative and nutrient starved non-replicative *Mycobacterium tuberculosis* (MTB), enzymatic screening and cytotoxicity against RAW 264.7 cell line. Among the compounds, 2-ethyl-*N*-phenethyl-5,6,7,8-tetrahydrobenzo[4,5]thieno[2,3-*d*]pyrimidin-4-amine (**5c**) was found to be the most active compound against non-replicative MTB with 2.7 log reduction of bacteria at 10 μg/mL and was more potent than isoniazid (1.2 log reduction) and rifampicin (2.0 log reduction) at same dose level. Compound **5c** also showed activity against MTB alanine dehydrogenase enzyme with IC_{50} of 1.82 ± 0.42 μM and showed 25% cytotoxicity against RAW 264.7 cell line at 50 μg/mL.

1. Introduction

Mycobacterium tuberculosis (MTB), the causative agent of tuberculosis (TB), is one of the most lethal infectious agents affecting mankind.[1] In 2014, 9.6 million people fell ill with TB and 1.5 million died from the disease. About one-third of the world's population has latent TB, which means people have been infected by TB bacteria but without any signs and symptoms of the disease.[2] MTB infection is difficult to treat, requiring 6–9 months of chemotherapy with a cocktail of four antibiotics isoniazid, rifampicin, pyrazinamide and ethambutol. In addition to toxic side effects, the lengthy treatment regime results in poor patient compliance and thus drug resistant strains are beginning to emerge.[3] The World Health Organization estimates that globally in 2014, an estimated 480,000 people developed multidrug-resistant TB (MDR-TB).[2] This number continues to grow as 300,000 new MDR-TB cases are diagnosed each year with 79 percent of individuals showing resistance to three or more frontline drugs.[2] Taken together, the growing problem of MDR-TB and the lack of drugs that effectively target latent MTB, there seem to be an urgent need for identification of new antimicrobial targets and inhibitors. The challenges to antibacterial discovery have kept the output of novel antibacterial drug classes to extraordinarily low levels over the past 25 years, even though discovery programs have been in place at large and small pharmaceutical companies as well as academic laboratories over this period.[4] Target-based approaches are widely used in drug discovery; questions have been raised about the efficiency of this approach given the very high attrition rates that these projects have historically shown in the anti-infective field.[5] Compounds identified in whole-cell screens provide suitable chemical and biological starting points. The Tuberculosis Antimicrobial Acquisition and Coordinating Facility (TAACF) was established by the National Institute of Allergy and Infectious Diseases (NIAID) in 1994 to allow researchers access to high quality screening services in order to encourage anti-TB drug discovery research. TAACF reported anti-TB high-throughput results of large libraries of drug-like small molecules.[6–8] Similarly GSK reported 177 small molecules leads against TB,[9] one among them was the molecule GSK163574A (5-methyl-2-(6-methylpyridin-2-yl)-*N*-(pyrimidin-2-yl)thieno[2,3-*d*]pyrimidin-4-amine) that showed activity against MTBH37Rv with minimum inhibitory concentration (MIC) of 0.76 μM (Fig. 1).

We took GSK163574A as the starting point to designed novel series of 2-substituted 5,6,7,8-tetrahydrobenzo[4,5]thieno[2,3-*d*]pyrimidin-4-amine by modifying A, B, and C rings of lead followed by synthesis and biological evaluation (Fig. 1).

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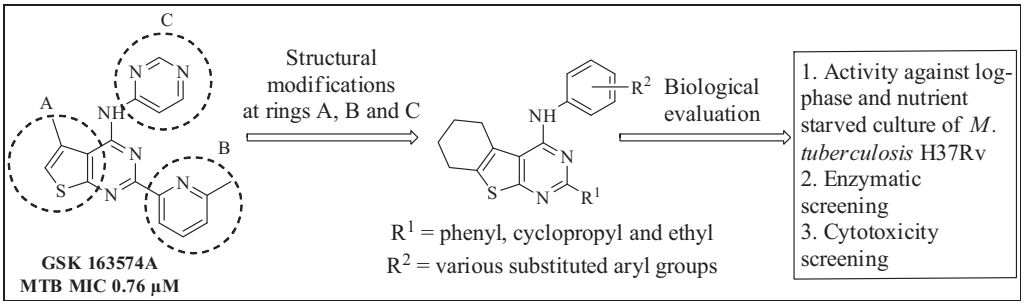


Figure 1. Design strategy and proposed plan of work.

2. Results and discussion

2.1. Chemistry

A library of thirty three molecules has been synthesised by following four step synthetic protocol as shown in Figure 2. In first step, commercially available cyclohexanone (**1**) was converted to 2-amino-4,5,6,7-tetrahydrobenzo[*b*]thiophene-3-carboxamide (**2**) using cyanoacetamide and elemental sulfur following Gewald reaction. In next step, N-acylation was carried out under basic conditions using benzoyl chloride, propionyl chloride and cyclopropylcarbonyl chloride to get three different *N*-acylated compounds (**3a–c**). In third step, cyclization was achieved under basic, reflux conditions by treating with aqueous NaOH, to yield tricyclic compound (**4a–c**). In final step, compounds (**4a–c**) were treated with eleven different substituted primary aryl amines under microwave conditions at high temperature to afford compounds (**5a–k**, **6a–k** and **7a–k**) (Table 1). All the compounds are stable to atmospheric air, stable on long standing in open atmosphere and they were stable to buffers whose pH range is ~1 to 12.

2.2. Biological evaluation

In the preliminary screening all the thirty three synthesized compounds were subjected to screening for their biological inhibition studies in actively replicating MTB using microplate alamar blue assay (MABA) assay method. Isoniazid, rifampicin, moxifloxacin, and ethambutol were used as positive controls and for comparison. 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 (Table 1). The tested compounds showed moderate activity with MIC ranging from 2.02 to 74.08 μM and only five compounds showed MIC of <10 μM. None of the compounds were more active than lead GSK163574A, isoniazid, rifampicin and moxifloxacin. Two compounds **7a** and **7e** showed better activity than standard first line anti-TB drug ethambutol. These results indicate that modification on A, B and C rings of GSK163574A reduces the actively replicative MTB. Compounds **4a–c** does not show any MTB activity.

In structure–MTB activity, we prepared three kinds of modification at 2nd position and among them in general phenyl ring showed better activity (**7a–k**; MIC 2.02–64.85 μM) followed by cyclopropyl ring (**6a–k**; MIC 8.78–71.53 μM) and then ethyl group (**5a–k**; MIC 18.18–77.29 μM). Within 2-phenyl substituted compounds (**7a–k**) we have prepared different 4th substituted compounds, among them simple phenyl (**7a**) showed MTB MIC of 4.36 μM. Extension of phenyl to benzyl (**7b**) and phenylethyl (**7c**) groups makes less active with MIC 33.65 and 64.85 μM respectively, whereas introduction of 2,5-dimethyl group (**7e**) makes twice potent (MIC 2.02 μM) but 2,4 and 2,6-dimethyl makes less potent than phenyl amino derivative.

Next stage we tested these compounds in nutrient starved MTB model, in this model MTB was grown in PBS for 6 weeks, using nutrient starvation, Betts et al.,[10] established a model in which MTB arrests growth, decreases its respiration rate and is resistant to isoniazid, rifampicin and metronidazole. After 6 weeks we treated nutrient starved non-replicative MTB with of synthesized compounds at 10 μg/mL for 1 week along with standard compounds isoniazid, rifampicin and moxifloxacin for comparison. The treated MTB cells 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

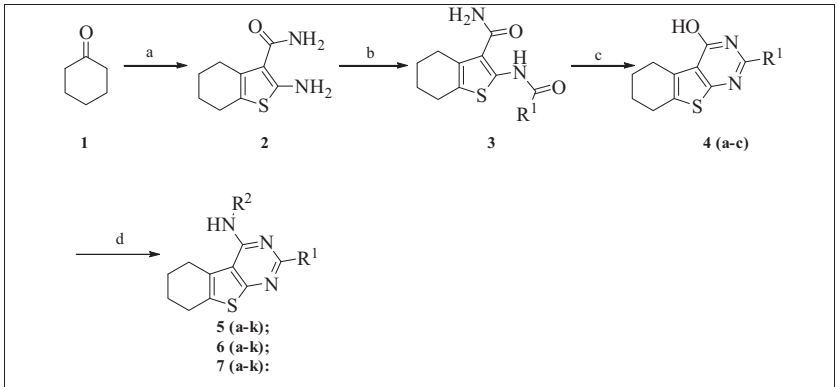
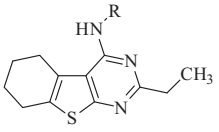
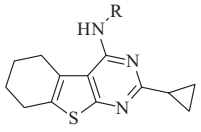


Figure 2. Synthetic protocol of the designed compounds.

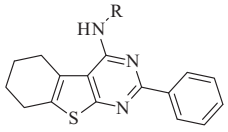
Table 1
Biological activities of the synthesized compounds



5a-k



6a-k



7a-k

Compd	R	Yield (%)	Mp (°C)	MTB MIC in μM	MTB ADH IC_{50} in μM	Cytotoxicity (RAW 264.7 cells) at 50 μg/mL % inhibition
5a	Phenyl	79	170–171	20.20	15.10 ± 1.80	7.82
5b	Benzyl	79	180–181	38.64	6.03 ± 0.97	7.05
5c	Phenethyl	91	191–192	37.04	1.82 ± 0.42	25.23
5d	2,4-Dimethylphenyl	69	141–142	74.08	7.41 ± 0.87	9.65
5e	2,5-Dimethylphenyl	76	196–197	74.08	>25	29.89
5f	2,6-Dimethylphenyl	84	201–202	18.52	5.02 ± 0.34	26.54
5g	4-Tolyl	87	182–183	77.29	3.26 ± 0.61	15.62
5h	4-Methoxyphenyl	74	243–244	73.65	4.68 ± 0.70	15.18
5i	4-Bromophenyl	78	290–291	32.19	6.54 ± 1.12	12.56
5j	4-Chlorophenyl	83	279–280	18.18	4.94 ± 0.48	15.62
5k	4-Flurophenyl	84	264–265	38.18	6.05 ± 0.68	13.98
6a	Phenyl	91	190–191	9.72	>25	8.80
6b	Benzyl	85	198–199	18.63	4.23 ± 0.96	23.05
6c	Phenethyl	78	204–205	35.77	17.03 ± 3.40	17.45
6d	2,4-Dimethylphenyl	92	213–214	71.53	9.00 ± 1.60	23.88
6e	2,5-Dimethylphenyl	87	222–223	35.77	2.07 ± 0.66	16.34
6f	2,6-Dimethylphenyl	80	116–117	17.88	7.55 ± 1.23	13.51
6g	4-Tolyl	93	218–219	74.52	13.43 ± 3.41	16.25
6h	4-Methoxyphenyl	91	209–210	17.78	>25	34.40
6i	4-Bromophenyl	71	234–235	15.61	8.93 ± 1.26	33.39
6j	4-Chlorophenyl	78	225–226	8.78	11.12 ± 3.46	15.49
6k	4-Flurophenyl	80	236–237	36.83	8.91 ± 1.66	4.82
7a	Phenyl	84	174–175	4.36	11.37 ± 4.51	7.15
7b	Benzyl	72	184–185	33.65	9.68 ± 0.96	26.52
7c	Phenethyl	70	190–191	64.85	5.60 ± 0.75	24.58
7d	2,4-Dimethylphenyl	79	196–197	16.21	9.96 ± 1.06	7.97
7e	2,5-Dimethylphenyl	81	156–157	2.02	5.82 ± 0.78	3.23
7f	2,6-Dimethylphenyl	64	150–151	8.11	8.82 ± 0.67	25.46
7g	4-Tolyl	72	190–191	33.65	7.97 ± 0.98	41.14
7h	4-Methoxyphenyl	68	180–181	16.13	8.72 ± 1.76	28.19
7i	4-Bromophenyl	88	210–211	28.64	8.70 ± 1.52	20.35
7j	4-Chlorophenyl	79	214–215	63.79	2.72 ± 0.42	20.80
7k	4-Flurophenyl	90	222–223	33.29	12.18 ± 0.87	33.41
Isoniazid				0.66	>25	12.16*
Rifampicin				0.23	>25	9.56*
Moxifloxacin				1.26	>25	ND
Ethambutol				7.64	>25	ND

* At 10 μg/mL; ND indicates not tested.

positive and MPN values were calculated using standard statistical methods.[11] At 10 μg/ml concentrations, standard first line anti-TB drugs isoniazid and rifampicin reduced ~1.2 and 2.0 log bacterial reductions respectively whereas fluoroquinolone antibacterial moxifloxacin showed high activity with ~2.7 log bacterial reduction. Minimum inhibitory concentration (MIC) of INH: 0.66 μM, RIF: 0.23 μM and moxifloxacin: 1.26 μM against actively replicative MTB. Among the thirty three tested compounds, twenty seven compounds showed activity with more than 1 log reduction of bacteria at 10 μg/ml (Fig. 3). Twenty compounds showed more bacterial log reduction than isoniazid, ten compounds showed activity better than rifampicin. One compound (**5c**) was equally active (2.7 log bacterial reduction) as moxifloxacin. Betts et al., also used microarray and proteome analysis to investigate the response of MTB to nutrient starvation. Proteome analysis of 6-week-starved cultures revealed the induction of several proteins which includes fumarate reductase flavoprotein subunit, pyruvate dehydrogenase E1 component subunit, L-alanine dehydrogenase (ADH), polyketide synthase, transcriptional regulator (ArsR family), sulfate transport system permease protein and lysine-ε-aminotransferase (LAT) enzymes over expressed >5 times.

As our synthesized compounds showed good activity against nutrient starved MTB, we decided to screen these molecules against few of the enzymes like ADH and LAT. In the case of LAT none of the molecules showed more than 50% inhibition at 25 μM. In MTB ADH screening,[12] thirty compounds showed 50% inhibition at 25 μM and IC_{50} of synthesized compounds reported in Table 1.

Twenty four compounds inhibited ADH with $IC_{50} < 10$ μM and compound **5c** (2-ethyl-*N*-phenethyl-5,6,7,8-tetrahydrobenzo[4,5]thieno[2,3-*d*]pyrimidin-4-amine) emerged as more potent with IC_{50} of 1.82 ± 0.42 μM which showed 2.7 log reduction in nutrient starved MTB also. We found very good correlation between ADH enzyme inhibition studies and bacterial log reduction nutrient starved MTB model. This enzyme ADH is 6.04 times over expressed in nutrient starved model. Increased levels of this enzyme has been linked to the generation of alanine for peptidoglycan biosynthesis[13] and the maintenance of the NAD^+ pool under conditions when the terminal electron acceptor oxygen become limiting.[14] NAD(H)-dependent L-AlaDH catalyze the oxidative deamination of L-alanine to pyruvate and ammonia (catabolic reaction) or, in the reverse

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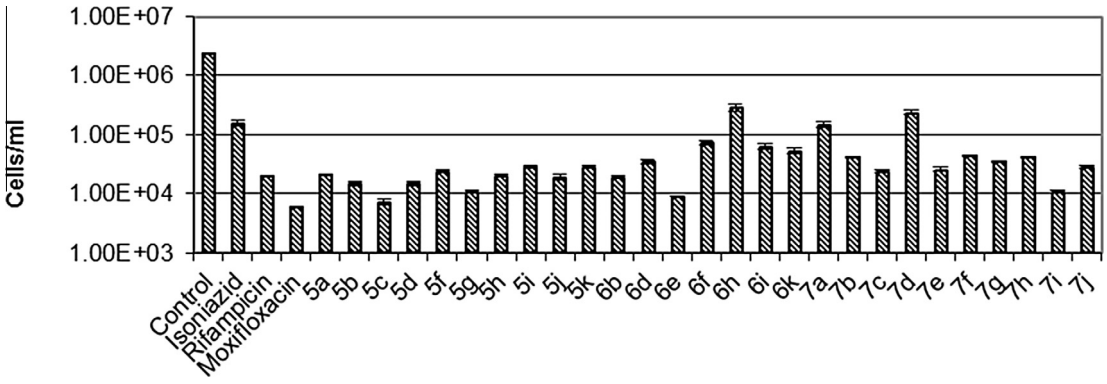

Figure 3. In vitro anti-TB activity against nutrient starved MTB at 10 μg/mL.

direction, the reductive amination of pyruvate to L-alanine (biosynthetic reaction).

We also tried to dock these molecules with crystal structure of the mycobacterial MTB L-ADH in complex with *N*6-methyl adenosine (PDB ID: 4LMP)[15] having resolution of 1.95 Å and crystal structure of the protein ADH bound with co-factor NAD^+ and substrate pyruvate (PDB ID: 2VHW)[15] using Glide XP docking (Glide, version 5.7, Schrçdinger, LLC, New York, NY, 2011). The inhibitors are not docked properly on both crystal structures indicates that they may bind in allosteric site. To prove this concept, here we represent the active molecules from each representative core (**5c**, **6e** and **7j**). Compounds were showing the docking score in the range of −3.49 to −4.80 in the ADH protein complex with *N*6-methyl adenosine (PDB ID: 4LMP). Binding analysis of these compounds revealed that all the three active compounds were mainly involved in hydrophobic interactions with the ADH protein (Fig. 4). All the compounds were fitted in the hydrophobic pocket within the vicinity of Ile199, Pro247, Leu255, Leu249, Leu197, Ile174, Ala126, Leu127, Ala179, Leu130, Ala176 and few polar amino acid residues Gln121, Asp198, Thr178, Ser220 respectively. Though these compounds were able to fit in to active site but failed to interact with active site residues through hydrogen bonding. This is the reason these molecules were showing low docking score.

To confirm the interaction between ADH and inhibitors we used differential scanning fluorimetry (DSF) which is a widely used method for screening libraries of small molecules for interactions with proteins.[16] DSF is being increasingly used as a robust method for initial screening of proteins for interacting small molecules, either for identifying physiological partners or for hit discovery. This technique has the advantage that it requires only a PCR machine suitable for quantitative PCR. In this method, ADH was incubated with a fluorescent dye (Sypro Orange dye), which alters its fluorescence upon binding to the hydrophobic regions of the proteins. The protein–dye sample is then heated, and the fluorescence monitored as the heat rises. The unfolding of the protein, and exposure of hydrophobic parts of the protein, gives rise to a characteristic pattern in the fluorescence as a function of temperature (Fig. 5). A higher or positive shift of melting temperature (T_m) of protein–ligand complex compared to the native protein T_m signify a better stabilization of the protein–ligand complex, which in turn would reflect on the inhibitor binding. The most potent compound **5c** display positive shifts towards the MTB ADH protein (1.4 °C). The native protein melting temperature is 46.20 °C; whereas the protein complexed with inhibitor showed melting temperature of 47.60 °C proves the stabilization of the ligand towards the desired protein MTB L-AlaDH.

The safety profile of all the synthesized compounds was evaluated by testing their in vitro cytotoxicity in mouse macrophage cell line (RAW 264.7) cells at 50 μg/mL concentration using (4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.[17] Since MTB reside inside the macrophage, the monocyte macrophage cell lines (RAW 264.7) were mostly used

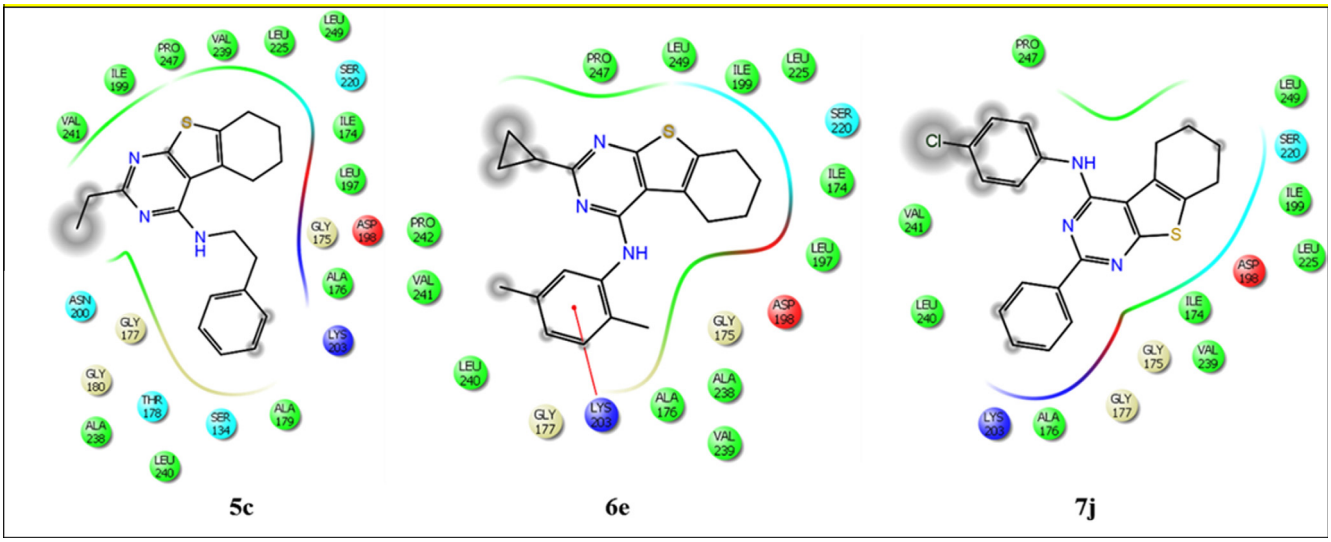

Figure 4. Binding analysis of active compounds from the series.

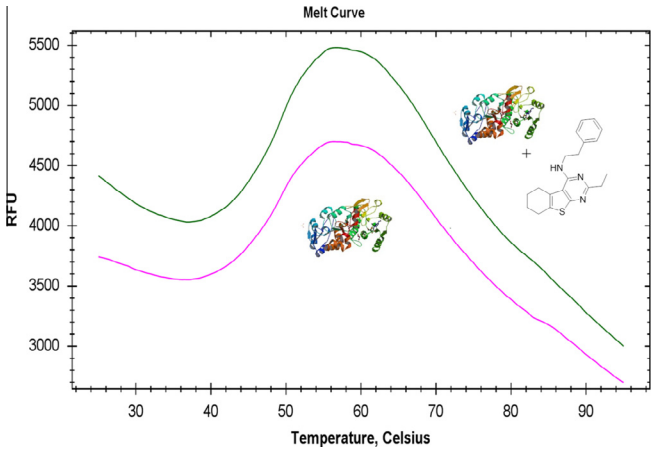

Figure 5. DSF experiment for compound **5c** (protein–ligand complex, green) showing an increase in the thermal shift of 1.4 °C when compared to the native ADH protein (red).

for tuberculosis research; in order to check whether the screened compounds were not toxic towards macrophages but toxic to the bacteria. All the compounds showed less than 50% inhibition with good safety profile with low enzyme/MTB inhibitory potential.

3. Conclusion

In the present study we tried to modify reported MTB inhibitor GSK163574A, but none of the molecules showed promising in actively replicative MTB. Most of the molecules showed good potency in nutrient starved non-replicative MTB. Most of the molecules also showed MTB ADH enzyme which was 6.04 times over expressed in the nutrient starved MTB. We also supported the enzyme target with DSF studies. This kind of molecules might be starting point for developing new drugs for dormant TB for which currently available anti-Tb drugs were in effective.

4. Experimental section

4.1. Chemistry

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 ^1H and ^{13}C NMR spectra were recorded on a Bruker AM (400 MHz, 100 MHz) NMR spectrometer, Bruker BioSpin Corp, Germany. Molecular weights of the synthesized compounds were checked by LCMS 6100B series Agilent Technology with electrospray ionization (ESI+). 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 using CHN Analyzer (Vario MICRO cube in CHN mode).

4.1.1. Preparation of 2-amino-4,5,6,7-tetrahydrobenzo[*b*]thiophene-3-carboxamide (2)

To the stirred solution of Compound **1** (3.0 g, 30.56 mmol), 2-cyanoacetamide (2.56 g, 30.56 mmol), sulfur powder (0.97 g, 30.56 mmol) in ethanol (40 mL) was added morpholine (5.31 mL, 61.11 mmol) and stirred the reaction mixture at room temperature for 6 h. The reaction mixture was concentrated, diluted with EtOAc and washed the organic layer with H_2O (2 × 30 mL). The separated organic layer was dried over anhydrous Na_2SO_4, evaporated and purified by column chromatography to get Compound **2** (5.40 g, 90%) as an light yellow solid. ESI-MS found 197 $[M+H]^+$.

4.1.2. General procedure for the preparation of 3a–c

To the stirred solution of compound **2** (1.0 equiv) in CH_2Cl_2 at 0 °C was added Et_3N (2.0 equiv) followed by R^1COCl (1.2 equiv) and allowed to stir at room temperature for 6 h. The reaction mixture was diluted with CH_2Cl_2 and washed with satd $NaHCO_3$, H_2O and dried over anhyd Na_2SO_4 and evaporated under vacuo to get compound **3** as an off-white solid.

4.1.3. General procedure for the preparation of 4a–c

To a solution of compound **3** in MeOH (2.0 vol) was added a solution of 1 N NaOH (10.0 vol) and the mixture was refluxed for 3 h. then the mixture was poured into water and neutralised with a concentrated solution of HCl to give a precipitate which was filtered and washed with water and dried to obtain desired compound.

4.1.4. General procedure for the synthesis of 5a–k, 6a–k and 7a–k

The mixture of compound **4a** (for **5a**–**k**)/**4b** (for **6a**–**k**)/**4c** (for **7a**–**k**) (1.0 equiv), substituted primary amine (R^2NH_2) (1.1 equiv) and *p*-toluenesulfonic acid (catalytic) were taken in methanol (3 volumes) and subjected to microwave irradiation (temperature 135 °C, pressure and power automatic) for 45 min. Ice water was added to the reaction mixture and the obtained solids were filtered, washed with water, cold ethanol and hexanes to get compounds (**5a**–**k**, **6a**–**k** and **7a**–**k**) respectively.

4.1.5. 2-Ethyl-*N*-phenyl-5,6,7,8-tetrahydrobenzo[4,5]thieno[2,3-*d*]pyrimidin-4-amine (5a)

To the mixture of 2-ethyl-5,6,7,8-tetrahydrobenzo[4,5]thieno[2,3-*d*]pyrimidin-4-ol (**4a**) (0.3 g, 1.28 mmol), aniline (0.13 mL, 1.41 mmol) and PTSA (catalytic) was added methanol (0.9 mL) and subjected to microwave irradiation (temperature 135 °C, pressure and power automatic) for 45 min. Ice water was added to the reaction mixture and the obtained solids were filtered, washed with water, cold ethanol and hexanes to get title compound (0.31 g, 79%) as an Off-white solid. MS(ESI) *m*/*z* 310 $[M+H]^+$. ^1H NMR (400 MHz, DMSO-d_6): δ 9.24 (s, 1H), 7.92 (d, *J* = 7.6 Hz, 2H), 7.81–7.74 (m, 3H), 3.11 (q, *J* = 7.2 Hz, 2H), 3.06 (t, *J* = 7.2 Hz, 2H), 2.83 (t, *J* = 7.2 Hz, 2H), 1.86 (t, *J* = 6.8 Hz, 4H), 1.29 (t, *J* = 7.2 Hz, 3H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 174.8, 158.4, 146.1, 140.3, 139.8, 133.3(2C), 129.0, 128.4, 124.3(2C), 118.4, 33.4, 25.2, 24.7, 24.1(2C), 12.5. Anal. Calcd for $C_{18}H_{19}N_3S$: C, 69.87; H, 6.19; N, 13.58. Found C, 69.98; H, 6.29; N, 13.66.

4.1.6. *N*-Benzyl-2-ethyl-5,6,7,8-tetrahydrobenzo[4,5]thieno[2,3-*d*]pyrimidin-4-amine (5b)

MS(ESI) *m*/*z* 324 $[M+H]^+$. ^1H NMR (400 MHz, DMSO-d_6): δ 8.66 (s, 1H), 7.56–7.48 (m, 5H), 4.41 (s, 2H), 3.13 (q, *J* = 7.2 Hz, 2H), 3.03 (t, *J* = 6.8 Hz, 2H), 2.84 (t, *J* = 7.2 Hz, 2H), 1.88 (t, *J* = 6.8 Hz, 4H), 1.25 (t, *J* = 7.2 Hz, 3H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 172.9, 160.4, 146.5, 141.2, 138.6, 133.0, 130.3(2C), 128.4, 126.6 (2C), 119.6, 49.3, 33.1, 24.8, 24.3, 23.4(2C), 12.3. Anal. Calcd for $C_{19}H_{21}N_3S$: C, 70.55; H, 6.54; N, 12.99. Found C, 70.68; H, 6.69; N, 13.06.

4.1.7. 2-Ethyl-*N*-phenethyl-5,6,7,8-tetrahydrobenzo[4,5]thieno[2,3-*d*]pyrimidin-4-amine (5c)

MS(ESI) *m*/*z* 338 $[M+H]^+$. ^1H NMR (400 MHz, DMSO-d_6): δ 8.31 (s, 1H), 7.60–7.49 (m, 5H), 3.49 (s, 2H), 3.11–2.96 (m, 4H), 2.89–2.74 (m, 4H), 1.83 (t, *J* = 6.8 Hz, 4H), 1.23 (t, *J* = 6.8 Hz, 3H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 170.8, 160.1, 144.9, 139.3, 136.4,

128.5(2C), 127.3, 125.3(2C), 123.4, 118.2, 48.1, 36.3, 34.6, 25.2, 24.3, 23.8(2C), 13.2. Anal. Calcd for $C_{20}H_{23}N_3S$: C, 71.18; H, 6.87; N, 12.45. Found C, 71.28; H, 6.99; N, 12.58.

4.1.8. *N*-(2,4-Dimethylphenyl)-2-ethyl-5,6,7,8-tetrahydrobenzo [4,5]thieno[2,3-*d*]pyrimidin-4-amine (5d)

MS(ESI) *m*/*z* 338 $[M+H]^+$. ^1H NMR (400 MHz, DMSO-d_6): δ 8.34 (s, 1H), 7.63 (s, 1H), 7.38 (d, J = 7.2 Hz, 1H), 7.18 (d, J = 7.2 Hz, 1H), 3.09–2.94 (m, 4H), 2.81 (t, J = 6.8 Hz, 2H), 2.43 (s, 3H), 2.36 (s, 3H), 1.88 (t, J = 6.8 Hz, 4H), 1.24 (t, J = 7.2 Hz, 3H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 173.6, 157.3, 146.8, 138.3, 137.5, 136.4, 133.3, 129.3, 127.4, 124.3, 119.5, 117.3, 34.2, 24.9, 24.3, 23.8(2C), 22.2, 20.9, 12.6. Anal. Calcd for $C_{20}H_{23}N_3S$: C, 71.18; H, 6.87; N, 12.45. Found C, 71.28; H, 6.99; N, 12.56.

4.1.9. *N*-(2,5-Dimethylphenyl)-2-ethyl-5,6,7,8-tetrahydrobenzo [4,5]thieno[2,3-*d*]pyrimidin-4-amine (5e)

MS(ESI) *m*/*z* 338 $[M+H]^+$. ^1H NMR (400 MHz, DMSO-d_6): δ 7.63–7.54 (m, 2H), 7.24 (s, 1H), 7.18 (d, J = 6.8 Hz, 1H), 3.14 (t, J = 6.8 Hz, 2H), 2.87–2.80 (m, 4H), 2.41 (s, 3H), 2.32 (s, 3H), 1.89 (t, J = 6.8 Hz, 4H), 1.26 (t, J = 6.8 Hz, 3H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 172.4, 156.1, 145.9, 137.8, 136.5, 136.2, 133.1, 127.1, 125.3, 123.9, 120.4, 118.6, 34.4, 25.1, 24.6, 24.2(2C), 23.4, 20.7, 13.0. Anal. Calcd for $C_{20}H_{23}N_3S$: C, 71.18; H, 6.87; N, 12.45. Found C, 71.26; H, 6.94; N, 12.61.

4.1.10. *N*-(2,6-Dimethylphenyl)-2-ethyl-5,6,7,8-tetrahydrobenzo [4,5]thieno[2,3-*d*]pyrimidin-4-amine (5f)

MS(ESI) *m*/*z* 338 $[M+H]^+$. ^1H NMR (400 MHz, DMSO-d_6): δ 9.21 (s, 1H), 7.31 (d, J = 6.8 Hz, 2H), 7.09 (t, J = 6.4 Hz, 1H), 3.08 (t, J = 6.8 Hz, 2H), 2.90–2.82 (m, 4H), 2.31 (s, 6H), 1.86 (t, J = 6.8 Hz, 4H), 1.28 (t, J = 6.8 Hz, 3H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 173.0, 152.4, 144.9, 136.9, 137.5(2C), 133.8(2C), 131.1, 129.4, 124.4, 119.3, 34.6, 25.3, 24.9(2C), 24.5(2C), 21.2, 12.7. Anal. Calcd for $C_{20}H_{23}N_3S$: C, 71.18; H, 6.87; N, 12.45. Found C, 71.24; H, 6.90; N, 12.64.

4.1.11. 2-Ethyl-*N*-(*p*-tolyl)-5,6,7,8-tetrahydrobenzo[4,5]thieno [2,3-*d*]pyrimidin-4-amine (5g)

MS(ESI) *m*/*z* 325 $[M+H]^+$. ^1H NMR (400 MHz, $CDCl_3$): δ 8.91 (s, 1H), 7.46 (d, J = 7.6 Hz, 2H), 7.36 (d, J = 7.6 Hz, 2H), 3.12 (t, J = 7.2 Hz, 2H), 2.96–2.87 (m, 4H), 2.46 (s, 3H), 1.87 (t, J = 6.8 Hz, 4H), 1.29 (t, J = 6.8 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 174.2, 156.1, 147.3, 137.6, 136.8, 133.9, 129.5(2C), 126.8, 122.6(2C), 120.3, 34.5, 26.0, 24.5(2C), 23.2, 20.5, 13.2. Anal. Calcd for $C_{19}H_{21}N_3S$: C, 70.55; H, 6.54; N, 12.99. Found C, 70.64; H, 6.60; N, 13.04.

4.1.12. 2-Ethyl-*N*-(4-methoxyphenyl)-5,6,7,8-tetrahydrobenzo [4,5]thieno[2,3-*d*]pyrimidin-4-amine (5h)

MS(ESI) *m*/*z* 340 $[M+H]^+$. ^1H NMR (400 MHz, $CDCl_3$): δ 8.46 (s, 1H), 7.54 (d, J = 7.6 Hz, 2H), 7.42 (d, J = 7.6 Hz, 2H), 3.96 (s, 3H), 3.08 (t, J = 6.8 Hz, 2H), 2.99–2.88 (m, 4H), 1.91 (t, J = 6.8 Hz, 4H), 1.30 (t, J = 6.8 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 173.9, 158.0, 142.4, 139.1, 134.6, 132.6, 131.4, 127.3(2C), 124.9(2C), 119.9, 61.2, 33.3, 25.6, 24.8(2C), 23.8, 12.7. Anal. Calcd for $C_{19}H_{21}N_3OS$: C, 67.23; H, 6.24; N, 12.38. Found C, 67.34; H, 6.33; N, 12.44.

4.1.13. *N*-(4-Bromophenyl)-2-ethyl-5,6,7,8-tetrahydrobenzo [4,5]thieno[2,3-*d*]pyrimidin-4-amine (5i)

MS(ESI) *m*/*z* 388 $[M+H]^+$, 390 $[MH+2]^+$. ^1H NMR (400 MHz, DMSO-d_6): δ 8.36 (s, 1H), 7.56 (d, J = 8.0 Hz, 2H), 7.48 (d, J = 8.0 Hz, 2H), 3.13 (t, J = 6.8 Hz, 2H), 2.89 (t, J = 6.8 Hz, 2H), 2.74 (t, J = 6.8 Hz, 2H), 1.86 (t, J = 7.2 Hz, 4H), 1.28 (t, J = 6.8 Hz, 3H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 174.4, 156.7, 146.3, 140.1, 138.3, 136.5, 134.3, 130.4(2C), 126.3(2C), 120.1, 34.2, 26.1, 25.3(2C), 24.6, 13.2. Anal. Calcd for $C_{18}H_{18}BrN_3S$: C, 55.67; H, 4.67; N, 10.82. Found C, 55.74; H, 4.73; N, 10.94.

4.1.14. *N*-(4-Chlorophenyl)-2-ethyl-5,6,7,8-tetrahydrobenzo [4,5]thieno[2,3-*d*]pyrimidin-4-amine (5j)

MS(ESI) *m*/*z* 344 $[M+H]^+$, 346 $[MH+2]^+$. ^1H NMR (400 MHz, DMSO-d_6): δ 8.32 (s, 1H), 7.60 (d, J = 8.0 Hz, 2H), 7.49 (d, J = 8.0 Hz, 2H), 3.12 (t, J = 6.8 Hz, 2H), 2.91 (t, J = 6.8 Hz, 2H), 2.77 (t, J = 6.8 Hz, 2H), 1.89 (t, J = 7.2 Hz, 4H), 1.32 (t, J = 6.8 Hz, 3H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 176.4, 155.4, 147.6, 141.3, 139.0, 136.2, 131.2(2C), 127.1(2C), 124.3, 120.9, 34.6, 27.3, 26.0(2C), 24.9, 13.9. Anal. Calcd for $C_{18}H_{18}ClN_3S$: C, 62.87; H, 5.28; N, 12.22. Found C, 62.94; H, 5.33; N, 12.34.

4.1.15. 2-Ethyl-*N*-(4-fluorophenyl)-5,6,7,8-tetrahydrobenzo[4,5] thieno[2,3-*d*]pyrimidin-4-amine (5k)

MS(ESI) *m*/*z* 328 $[M+H]^+$. ^1H NMR (400 MHz, DMSO-d_6): δ 9.63 (s, 1H), 7.65 (d, J = 8.0 Hz, 2H), 7.54 (d, J = 8.0 Hz, 2H), 3.16 (t, J = 7.2 Hz, 2H), 2.94 (t, J = 7.2 Hz, 2H), 2.81 (t, J = 6.8 Hz, 2H), 1.93 (t, J = 7.2 Hz, 4H), 1.33 (t, J = 6.8 Hz, 3H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 177.3, 156.7, 146.3, 142.0, 138.4, 137.1, 132.6(2C), 128.3(2C), 125.7, 121.4, 35.3, 28.1, 26.6(2C), 24.3, 13.3. Anal. Calcd for $C_{18}H_{18}FN_3S$: C, 66.03; H, 5.54; N, 12.83. Found C, 66.14; H, 5.63; N, 12.94.

4.1.16. 2-Cyclopropyl-*N*-phenyl-5,6,7,8-tetrahydrobenzo[4,5] thieno[2,3-*d*]pyrimidin-4-amine (6a)

MS(ESI) *m*/*z* 322 $[M+H]^+$. ^1H NMR (400 MHz, DMSO-d_6): δ 8.64 (s, 1H), 7.82 (d, J = 7.6 Hz, 2H), 7.61–7.54 (m, 3H), 3.06 (t, J = 7.2 Hz, 2H), 2.80 (t, J = 7.2 Hz, 2H), 1.84 (t, J = 6.8 Hz, 4H), 1.56–1.51 (m, 1H), 1.29–1.11 (m, 4H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 170.8, 156.2, 144.0, 139.3, 136.4, 128.9(2C), 128.1, 126.5, 123.6(2C), 119.4, 25.4, 24.9, 22.4(2C), 13.3, 10.2(2C). Anal. Calcd for $C_{19}H_{19}N_3S$: C, 70.99; H, 5.96; N, 13.07. Found C, 71.08; H, 6.04; N, 13.16.

4.1.17. *N*-Benzyl-2-cyclopropyl-5,6,7,8-tetrahydrobenzo[4,5] thieno[2,3-*d*]pyrimidin-4-amine (6b)

MS(ESI) *m*/*z* 336 $[M+H]^+$. ^1H NMR (400 MHz, DMSO-d_6): δ 8.53 (s, 1H), 7.58–7.49 (m, 5H), 4.43 (s, 2H), 3.04 (t, J = 7.2 Hz, 2H), 2.86 (t, J = 7.2 Hz, 2H), 1.85 (t, J = 6.8 Hz, 4H), 1.54–1.49 (s, 1H), 1.27–1.14 (m, 4H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 170.9, 159.4, 146.3, 139.4, 137.9, 136.3, 128.3(2C), 126.1, 125.8(2C), 117.9, 47.8, 24.6, 24.0, 23.6(2C), 12.9. 9.99 (2C). Anal. Calcd for $C_{20}H_{21}N_3S$: C, 71.61; H, 6.31; N, 12.53. Found C, 71.68; H, 6.39; N, 12.66.

4.1.18. 2-Cyclopropyl-*N*-phenethyl-5,6,7,8-tetrahydrobenzo[4,5] thieno[2,3-*d*]pyrimidin-4-amine (6c)

MS(ESI) *m*/*z* 350 $[M+H]^+$. ^1H NMR (400 MHz, DMSO-d_6): δ 9.31 (s, 1H), 7.74 (t, J = 7.2 Hz, 2H), 7.62–7.54 (m, 3H), 3.47 (s, 2H), 3.13–2.97 (m, 4H), 2.83 (t, J = 6.8 Hz, 2H), 1.86 (t, J = 6.8 Hz, 4H), 1.56–1.50 (m, 1H), 1.26–1.16 (m, 4H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 170.4, 159.6, 144.7, 138.6, 137.2, 129.2(2C), 128.1, 126.3(2C), 125.3, 117.4, 47.3, 36.9, 25.4, 24.7, 24.2(2C), 13.0, 10.3 (2C). Anal. Calcd for $C_{21}H_{23}N_3S$: C, 72.17; H, 6.63; N, 12.02. Found C, 72.28; H, 6.69; N, 12.08.

4.1.19. 2-Cyclopropyl-*N*-(2,4-dimethylphenyl)-5,6,7,8-tetrahydrobenzo[4,5]thieno[2,3-*d*]pyrimidin-4-amine (6d)

MS(ESI) *m*/*z* 350 $[M+H]^+$. ^1H NMR (400 MHz, DMSO-d_6): δ 8.36 (s, 1H), 7.49 (s, 1H), 7.36 (d, J = 7.2 Hz, 1H), 7.12 (d, J = 7.2 Hz, 1H), 3.09 (d, J = 7.2 Hz, 1H), 2.94 (d, J = 7.2 Hz, 1H), 2.41 (s, 3H), 2.37 (s, 3H), 1.85 (t, J = 6.8 Hz, 4H), 1.52–1.48 (m, 1H), 1.25–1.11 (m, 4H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 172.6, 154.3, 144.3, 139.4, 138.1, 136.3, 132.8, 129.3, 128.2, 126.6, 125.4, 119.6, 25.8,

25.0, 24.6(2C), 24.3, 23.8, 12.6, 9.3(2C). Anal. Calcd for $C_{21}H_{23}N_3S$: C, 72.17; H, 6.63; N, 12.02. Found C, 72.28; H, 6.79; N, 12.06.

4.1.20. 2-Cyclopropyl-*N*-(2,5-dimethylphenyl)-5,6,7,8-tetrahydrobenzo[4,5]thieno[2,3-*d*]pyrimidin-4-amine (6e)

MS(ESI) *m*/*z* 350 $[M+H]^+$. ^1H NMR (400 MHz, DMSO-d_6): δ 8.46 (s, 1H), 7.45 (d, J = 7.6 Hz, 1H), 7.39–7.31 (m, 2H), 3.11 (d, J = 7.2 Hz, 1H), 2.97 (d, J = 7.2 Hz, 1H), 2.44 (s, 3H), 2.39 (s, 3H), 1.88 (t, J = 7.2 Hz, 4H), 1.53–1.47 (m, 1H), 1.26–1.14 (m, 4H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 172.9, 155.2, 145.1, 138.8, 138.1, 135.4, 133.2, 129.2, 127.6, 126.2, 126.0, 120.0, 25.6, 25.3, 24.9 (2C), 24.6, 22.2, 11.8, 9.1(2C). Anal. Calcd for $C_{21}H_{23}N_3S$: C, 72.17; H, 6.63; N, 12.02. Found C, 72.26; H, 6.72; N, 12.09.

4.1.21. 2-Cyclopropyl-*N*-(2,6-dimethylphenyl)-5,6,7,8-tetrahydrobenzo[4,5]thieno[2,3-*d*]pyrimidin-4-amine (6f)

MS(ESI) *m*/*z* 350 $[M+H]^+$. ^1H NMR (400 MHz, DMSO-d_6): δ 7.42 (d, J = 7.6 Hz, 2H), 7.27–7.19 (m, 2H), 3.04 (d, J = 7.2 Hz, 1H), 2.94 (d, J = 7.2 Hz, 1H), 2.33 (s, 6H), 1.91 (t, J = 7.2 Hz, 4H), 1.51–1.46 (m, 1H), 1.24–1.12 (m, 4H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 171.9, 153.6, 146.3, 138.4, 136.3, 133.1, 130.2(2C), 129.5(2C), 126.2, 120.4, 25.2, 24.7, 23.8(2C), 21.4(2C), 11.6, 9.0(2C). Anal. Calcd for $C_{21}H_{23}N_3S$: C, 72.17; H, 6.63; N, 12.02. Found C, 72.26; H, 6.72; N, 12.09.

4.1.22. 2-Cyclopropyl-*N*-(*p*-tolyl)-5,6,7,8-tetrahydrobenzo[4,5]thieno[2,3-*d*]pyrimidin-4-amine (6g)

MS(ESI) *m*/*z* 325 $[M+H]^+$. ^1H NMR (400 MHz, DMSO-d_6): δ 8.36 (s, 1H), 7.42 (d, J = 8.0 Hz, 2H), 7.33 (d, J = 8.0 Hz, 2H), 3.08 (t, J = 7.6 Hz, 2H), 2.98 (t, J = 7.6 Hz, 2H), 2.41 (s, 3H), 1.84 (t, J = 7.2 Hz, 4H), 1.52–1.47 (m, 1H), 1.23–1.08 (m, 4H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 173.5, 154.3, 146.3, 138.4, 137.3, 131.8, 128.7(2C), 127.3, 123.6(2C), 116.1, 26.1, 25.3(2C), 24.6, 22.3, 12.3, 9.3(2C). Anal. Calcd for $C_{20}H_{21}N_3S$: C, 71.61; H, 6.31; N, 12.53. Found C, 71.66; H, 6.40; N, 12.64.

4.1.23. 2-Cyclopropyl-*N*-(4-methoxyphenyl)-5,6,7,8-tetrahydrobenzo[4,5]thieno[2,3-*d*]pyrimidin-4-amine (6h)

MS(ESI) *m*/*z* 352 $[M+H]^+$. ^1H NMR (400 MHz, $CDCl_3$): δ 8.39 (s, 1H), 7.63 (d, J = 7.6 Hz, 2H), 7.48 (d, J = 7.6 Hz, 2H), 4.06 (s, 3H), 3.06 (t, J = 7.2 Hz, 2H), 2.91 (t, J = 7.2 Hz, 2H), 1.87 (t, J = 6.8 Hz, 4H), 1.48–1.43 (m, 1H), 1.26–1.05 (m, 4H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.8, 156.3, 155.4, 144.3, 138.3, 135.2, 128.2, 126.4 (2C), 124.2(2C), 123.2, 61.9, 25.2, 24.4(2C), 23.4, 12.4, 9.7(2C). Anal. Calcd for $C_{20}H_{21}N_3OS$: C, 68.35; H, 6.02; N, 11.96. Found C, 68.39; H, 6.13; N, 12.04.

4.1.24. *N*-(4-Bromophenyl)-2-cyclopropyl-5,6,7,8-tetrahydrobenzo[4,5]thieno[2,3-*d*]pyrimidin-4-amine (6i)

MS(ESI) *m*/*z* 400 $[M+H]^+$, 402 $[MH+2]^+$. ^1H NMR (400 MHz, DMSO-d_6): δ 9.42 (s, 1H), 7.81 (d, J = 7.6 Hz, 2H), 7.54 (d, J = 7.6 Hz, 2H), 3.01 (t, J = 7.2 Hz, 2H), 2.81 (t, J = 7.2 Hz, 2H), 1.89 (t, J = 7.2 Hz, 4H), 1.53–1.44 (m, 1H), 1.27–1.08 (m, 4H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 172.6, 154.6, 145.9, 140.3, 136.9, 134.6 (2C), 132.0, 124.3(2C), 120.4, 118.6, 25.5, 24.7(2C), 24.1, 11.6, 10.1(2C). Anal. Calcd for $C_{19}H_{18}BrN_3S$: C, 57.00; H, 4.53; N, 10.50. Found C, 57.04; H, 4.63; N, 10.64.

4.1.25. *N*-(4-Chlorophenyl)-2-cyclopropyl-5,6,7,8-tetrahydrobenzo[4,5]thieno[2,3-*d*]pyrimidin-4-amine (6j)

MS(ESI) *m*/*z* 356 $[M+H]^+$, 358 $[MH+2]^+$. ^1H NMR (400 MHz, DMSO-d_6): δ 9.63 (s, 1H), 7.77 (d, J = 7.6 Hz, 2H), 7.56 (d, J = 7.6 Hz, 2H), 3.05 (t, J = 7.2 Hz, 2H), 2.88 (t, J = 7.2 Hz, 2H), 1.87 (t, J = 7.2 Hz, 4H), 1.52–1.46 (m, 1H), 1.25–1.06 (m, 4H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 171.7, 155.5, 146.4, 139.4, 135.3, 132.3 (2C), 130.1, 128.5, 126.7(2C), 119.3, 24.9, 24.3(2C), 23.5, 11.7, 9.4 (2C). Anal. Calcd for $C_{19}H_{18}ClN_3S$: C, 64.12; H, 5.10; N, 11.81. Found C, 64.24; H, 5.23; N, 11.94.

4.1.26. 2-Cyclopropyl-*N*-(4-fluorophenyl)-5,6,7,8-tetrahydrobenzo[4,5]thieno[2,3-*d*]pyrimidin-4-amine (6k)

MS(ESI) *m*/*z* 340 $[M+H]^+$. ^1H NMR (400 MHz, DMSO-d_6): δ 8.35 (s, 1H), 7.56 (d, J = 7.6 Hz, 2H), 7.44 (d, J = 7.6 Hz, 2H), 3.10 (t, J = 7.2 Hz, 2H), 2.92 (t, J = 7.2 Hz, 2H), 1.88 (t, J = 7.2 Hz, 4H), 1.49–1.45 (m, 1H), 1.23–1.10 (m, 4H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 173.4, 158.4, 149.6, 144.2, 137.2, 136.7, 128.3, 126.1 (2C), 124.7(2C), 121.3, 25.4, 24.3, 24.0(2C), 11.6, 10.3(2C). Anal. Calcd for $C_{19}H_{18}FN_3S$: C, 67.23; H, 5.35; N, 12.38. Found C, 67.34; H, 5.63; N, 12.44.

4.1.27. *N*,2-Diphenyl-5,6,7,8-tetrahydrobenzo[4,5]thieno[2,3-*d*]pyrimidin-4-amine (7a)

MS(ESI) *m*/*z* 358 $[M+H]^+$. ^1H NMR (400 MHz, DMSO-d_6): δ 8.37 (d, J = 7.6 Hz, 2H), 7.82–7.69 (m, 6H), 7.54–7.42 (m, 3H), 3.03 (t, J = 7.6 Hz, 2H), 2.85 (t, J = 7.2 Hz, 2H), 1.87 (t, J = 7.2 Hz, 4H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 166.4, 155.2, 147.5, 142.7, 139.2, 137.4, 133.2, 128.1(2C), 127.3(2C), 126.0, 125.2, 124.6(2C), 120.8 (2C), 117.2, 25.6, 24.3, 23.6(2C). Anal. Calcd for $C_{22}H_{19}N_3S$: C, 73.92; H, 5.36; N, 11.75. Found C, 73.99; H, 5.63; N, 12.04.

4.1.28. *N*-Benzyl-2-phenyl-5,6,7,8-tetrahydrobenzo[4,5]thieno[2,3-*d*]pyrimidin-4-amine (7b)

MS(ESI) *m*/*z* 358 $[M+H]^+$. ^1H NMR (400 MHz, DMSO-d_6): δ 8.36 (s, 1H), 7.81 (d, J = 7.2 Hz, 2H), 7.67–7.56 (m, 3H), 7.48–7.32 (m, 5H), 4.42 (s, 2H), 3.08 (t, J = 7.2 Hz, 2H), 2.89 (t, J = 7.2 Hz, 2H), 1.84 (t, J = 7.2 Hz, 4H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 164.9, 156.6, 146.3, 141.3, 138.8, 137.2, 133.6, 127.7(2C), 127.1(2C), 125.8, 125.3, 124.3(2C), 121.2(2C), 118.4, 45.2, 24.9, 24.1, 23.2 (2C). Anal. Calcd for $C_{23}H_{21}N_3S$: C, 74.36; H, 5.70; N, 11.31. Found C, 74.49; H, 5.83; N, 11.44.

4.1.29. *N*-Phenethyl-2-phenyl-5,6,7,8-tetrahydrobenzo[4,5]thieno[2,3-*d*]pyrimidin-4-amine (7c)

MS(ESI) *m*/*z* 386 $[M+H]^+$. ^1H NMR (400 MHz, DMSO-d_6): δ 9.43 (s, 1H), 8.11 (d, J = 7.2 Hz, 2H), 7.77–7.68 (m, 4H), 7.62–7.48 (m, 4H), 3.46 (t, J = 6.8 Hz, 2H), 3.04 (t, J = 6.8 Hz, 2H), 2.89–2.65 (m, 4H), 1.87 (t, J = 6.8 Hz, 4H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 166.2, 158.3, 144.6, 140.3, 139.2, 137.6, 134.2, 127.9(2C), 127.2 (2C), 126.2, 125.5, 124.1(2C), 122.1(2C), 119.3, 46.1, 36.9, 24.7, 23.8, 23.4(2C). Anal. Calcd for $C_{24}H_{23}N_3S$: C, 74.77; H, 6.01; N, 10.90. Found C, 74.89; H, 6.13; N, 11.04.

4.1.30. *N*-(2,4-Dimethylphenyl)-2-phenyl-5,6,7,8-tetrahydrobenzo[4,5]thieno[2,3-*d*]pyrimidin-4-amine (7d)

MS(ESI) *m*/*z* 386 $[M+H]^+$. ^1H NMR (400 MHz, DMSO-d_6): δ 9.16 (s, 1H), 7.84 (d, J = 7.2 Hz, 2H), 7.72–7.66 (m, 3H), 7.56 (s, 1H), 7.49–7.41 (m, 2H), 3.03 (d, J = 6.8 Hz, 2H), 2.91 (d, J = 6.8 Hz, 2H), 2.43 (s, 3H), 2.34 (s, 3H), 1.82 (t, J = 6.8 Hz, 4H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 174.9, 155.3, 145.7, 138.6, 137.4, 136.7, 133.9, 130.3, 129.6, 128.6, 127.3, 126.6(2C), 125.9, 125.1, 123.5 (2C), 118.4, 25.3, 24.7, 24.2(2C), 23.1, 22.2. Anal. Calcd for $C_{24}H_{23}N_3S$: C, 74.77; H, 6.01; N, 10.90. Found C, 74.88; H, 6.09; N, 11.06.

4.1.31. 2-Cyclopropyl-*N*-(2,5-dimethylphenyl)-5,6,7,8-tetrahydrobenzo[4,5]thieno[2,3-*d*]pyrimidin-4-amine (7e)

MS(ESI) *m*/*z* 386 $[M+H]^+$. ^1H NMR (400 MHz, DMSO-d_6): δ 8.37 (s, 1H), 7.85 (d, J = 7.2 Hz, 2H), 7.68–7.58 (m, 3H), 7.53–7.47 (m, 3H), 3.04 (d, J = 6.8 Hz, 2H), 2.92 (d, J = 6.8 Hz, 2H), 2.41 (s, 3H), 2.33 (s, 3H), 1.82 (t, J = 6.8 Hz, 4H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 168.7, 156.6, 146.3, 139.5, 138.7, 136.8, 134.8, 133.9, 133.0, 130.6, 128.6(2C), 127.9(2C), 125.7, 123.1, 120.4, 119.2, 25.9, 24.8,

24.2(2C), 23.1, 21.4. Anal. Calcd for $C_{24}H_{23}N_3S$: C, 74.77; H, 6.01; N, 10.90. Found C, 74.86; H, 6.12; N, 10.99.

4.1.32. *N*-(2,6-Dimethylphenyl)-2-phenyl-5,6,7,8-tetrahydrobenzo[4,5]thieno[2,3-*d*]pyrimidin-4-amine (7f)

MS(ESI) *m*/*z* 386 $[M+H]^+$. ^1H NMR (400 MHz, DMSO-d_6): δ 8.37 (d, J = 7.2 Hz, 2H), 7.87–7.69 (m, 3H), 7.63 (d, J = 7.2 Hz, 2H), 7.58–7.53 (m, 2H), 3.06 (d, J = 6.8 Hz, 2H), 2.91 (d, J = 6.8 Hz, 2H), 2.31 (s, 6H), 1.86 (t, J = 6.8 Hz, 4H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 172.4, 154.3, 145.8, 139.0, 137.1, 135.8, 133.6(2C), 132.6, 130.6(2C), 129.3(2C), 128.7(2C), 128.2, 126.7, 121.3, 24.7, 24.3, 23.4(2C), 19.9(2C). Anal. Calcd for $C_{24}H_{23}N_3S$: C, 74.77; H, 6.01; N, 10.90. Found C, 74.86; H, 6.12; N, 11.01.

4.1.33. 2-Phenyl-*N*-(*p*-tolyl)-5,6,7,8-tetrahydrobenzo[4,5]thieno[2,3-*d*]pyrimidin-4-amine (7g)

MS(ESI) *m*/*z* 372 $[M+H]^+$. ^1H NMR (400 MHz, DMSO-d_6): δ 9.43 (s, 1H), 8.26 (d, J = 7.2 Hz, 2H), 7.82–7.74 (m, 3H), 7.67–7.56 (m, 4H), 3.03 (t, J = 7.2 Hz, 2H), 2.89 (t, J = 7.2 Hz, 2H), 2.40 (s, 3H), 1.83 (t, J = 6.8 Hz, 4H), 1.52–1.47 (m, 1H), 1.23–1.08 (m, 4H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 170.7, 155.4, 144.8, 139.2, 138.0, 136.3, 134.2, 130.4(2C), 129.3(2C), 127.8(2C), 127.1, 126.3(2C), 120.4, 117.9, 25.4, 24.7(2C), 23.8, 22.5. Anal. Calcd for $C_{23}H_{21}N_3S$: C, 74.36; H, 5.70; N, 11.31. Found C, 74.46; H, 5.80; N, 11.44.

4.1.34. *N*-(4-Methoxyphenyl)-2-phenyl-5,6,7,8-tetrahydrobenzo[4,5]thieno[2,3-*d*]pyrimidin-4-amine (7h)

MS(ESI) *m*/*z* 388 $[M+H]^+$. ^1H NMR (400 MHz, $CDCl_3$): δ 8.19 (d, J = 7.6 Hz, 2H), 7.83 (d, J = 7.6 Hz, 2H), 7.68–7.60 (m, 6H), 3.96 (s, 3H), 3.12 (t, J = 7.2 Hz, 2H), 2.96 (t, J = 7.2 Hz, 2H), 1.89 (t, J = 6.8 Hz, 4H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 169.9, 158.5, 156.5, 146.1, 139.6, 138.3, 136.2, 134.7, 130.6(2C), 129.1(2C), 127.8, 127.2(2C), 124.7(2C), 121.1, 60.3, 25.1, 24.3(2C), 22.8. Anal. Calcd for $C_{23}H_{21}N_3OS$: C, 71.29; H, 5.46; N, 10.84. Found C, 71.38; H, 5.62; N, 10.94.

4.1.35. *N*-(4-Bromophenyl)-2-phenyl-5,6,7,8-tetrahydrobenzo[4,5]thieno[2,3-*d*]pyrimidin-4-amine (7i)

MS(ESI) *m*/*z* 436 $[M+H]^+$, 438 $[MH+2]^+$. ^1H NMR (400 MHz, DMSO-d_6): δ 8.91 (s, 1H), 8.31 (d, J = 6.8 Hz, 2H), 7.87 (d, J = 6.8 Hz, 2H), 7.62–7.56 (m, 5H), 3.14 (t, J = 6.4 Hz, 2H), 2.87 (t, J = 6.4 Hz, 2H), 1.83 (t, J = 6.4 Hz, 4H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 165.3, 157.2, 152.8, 144.6, 139.7, 136.7, 135.1, 129.2(2C), 127.8(2C), 127.0, 126.1(2C), 125.3, 124.2(2C), 118.0, 25.1, 24.4, 23.8(2C). Anal. Calcd for $C_{22}H_{18}BrN_3S$: C, 60.55; H, 4.16; N, 9.63. Found C, 60.69; H, 4.29; N, 9.74.

4.1.36. *N*-(4-Chlorophenyl)-2-phenyl-5,6,7,8-tetrahydrobenzo[4,5]thieno[2,3-*d*]pyrimidin-4-amine (7j)

MS(ESI) *m*/*z* 392 $[M+H]^+$, 394 $[MH+2]^+$. ^1H NMR (400 MHz, DMSO-d_6): δ 8.33–8.28 (m, 3H), 7.81 (d, J = 7.2 Hz, 2H), 7.53–7.48 (m, 5H), 3.13 (t, J = 6.8 Hz, 2H), 2.81 (t, J = 6.8 Hz, 2H), 1.87 (t, J = 6.8 Hz, 4H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 165.3, 156.1, 146.4, 141.3, 138.9, 137.8, 134.0, 129.2(2C), 128.3(2C), 127.3, 126.6(2C), 125.3, 124.2(2C), 118.7, 25.3, 24.8, 24.1(2C). Anal. Calcd for $C_{22}H_{18}ClN_3S$: C, 67.42; H, 4.63; N, 10.72. Found C, 67.49; H, 4.69; N, 10.84.

4.1.37. *N*-(4-Fluorophenyl)-2-phenyl-5,6,7,8-tetrahydrobenzo[4,5]thieno[2,3-*d*]pyrimidin-4-amine (7k)

MS(ESI) *m*/*z* 376 $[M+H]^+$. ^1H NMR (400 MHz, DMSO-d_6): δ 9.21 (s, 1H), 8.30 (d, J = 6.8 Hz, 2H), 7.85 (d, J = 6.8 Hz, 2H), 7.60–7.54 (m, 5H), 3.10 (t, J = 6.4 Hz, 2H), 2.85 (t, J = 6.4 Hz, 2H), 1.84 (t, J = 6.4 Hz, 4H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 164.7, 156.6, 151.4, 144.2, 138.4, 137.2, 134.4, 129.4(2C), 127.1(2C), 126.5, 125.2(2C), 124.1, 123.4(2C), 118.4, 25.6, 24.7, 24.3(2C). Anal. Calcd for $C_{22}H_{18}FN_3S$: C, 70.38; H, 4.83; N, 11.19. Found C, 70.49; H, 4.99; N, 11.24.

4.2. Biological screening

4.2.1. MTB MABA assay

The compounds were screened for their in vitro antimycobacterial activity against *Mycobacterium tuberculosis* H37Rv by microplate Alamar blue assay method. 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 °C in normal atmosphere. After 7 days of incubation, 30 ml of alamar blue solution was added to each well, and the plate was re-incubated overnight. A change in color from blue (oxidized 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 color.

4.2.2. In vitro dormant MTB model

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 Middlebrook 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.

4.2.3. In vitro MTB ADH enzyme inhibition assay

The enzyme inhibition studies were performed in microtitre plate containing desired substrate and enzyme concentration as explained above. The spectrophotometric determination of the reaction product NADH that accompanies the conversion of L-alanine into pyruvate in the oxidative deamination was measured at 340 nm in heat controlled microplate reader PerkinElmer Victor X3 instrument. The synthesized compounds were added to the plate with different concentrations from 25 µM to 0.5 µM in order to determine IC_{50} values for the all the synthesized compounds. The reaction mixture except MTB ADH was added and background reactions were measured. Reactions were carried out at 37 °C in a heat-controlled PerkinElmer Victor X3 Spectrophotometer. Further the IC_{50} values were calculated using GraphPad Prism analysis software.

4.2.4. Differential scanning fluorimetry

A thermal shift assay, also called differential scanning fluorimetry (DSF) is a thermal-denaturation assay that measures the thermal stability of a target protein and a subsequent increase in protein melting temperature upon binding of a ligand to the pro-

tein. We used Sypro Orange dye (Sigma–aldrich) in a Real time PCR instrument (Bio-Rad iCycle5), which binds to exposed core residues of a denatured protein and results in an increased fluorescence signal. The instrument was programmed to equilibrate the samples at 25 °C for 3 min and further rise in temperature till 95 °C, with every 0.1 °C rice using a LED/Photodiode set matched with dye excitation and emission wavelengths. Required concentration of MTB L-AlaDH and dye was determined by varying different concentrations of protein and dye in analysis buffer containing 100 mM HEPES–NaOH, pH-7.8. The selected top active compounds were diluted in <10% DMSO. Later, in a 96 well PCR plate, 20 µl of reaction volume containing 10 µl of MTB L-AlaDH (100 µg/ml) in analysis buffer, 6 µl of 15× dye (Diluted from 5000× stock with sub-stock of 50× in DMSO). Based on their dilutions, the compounds were diluted and added to the subsequent wells. The melting temperature of the protein and protein complexed with ligand was determined as the lowest point of first derivative plot and calculated by the software within the instrument.

4.2.5. In vitro cytotoxicity screening

The synthesized compounds were further examined for its cytotoxicity in mouse macrophage cell line (RAW 264.7) at 50 µg/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. Mouse macrophages were grown in RPMI medium supplemented with 10% fetal bovine serum (FBS), 10,000 units' penicillin and 10 mg streptomycin per ml in T25 flasks to attain 80–90% confluency. Cells were scraped and seeded into wells approx 5000 cells per well in poly-L-lysine coated plates. The microtiter plates were incubated at 37 °C, 5% CO_2, 95% air and 100% relative humidity for 24 h prior to addition of experimental drugs. The test compounds at 50 µM concentration were then added to cells and incubated at 37 °C for 48 h; later 10 µL of 0.5 mg/ml concentration of MTT was added and incubated for 3 h at 37 °C and the final product Formazon crystals were measured at 595 nm.

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