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Anti-tubercular agents. Part 3. Benzothiadiazine as a novel scaffold for anti-*Mycobacterium* activity

Ahmed Kamal,[a,*] K. Srinivasa Reddy,[a] S. Kaleem Ahmed,[a] M. Naseer A. Khan,[a] Rakesh K. Sinha,[b] J. S. Yadav[a] and Sudershan K. Arora[b]

[a]*Division of Organic Chemistry, Indian Institute of Chemical Technology, Hyderabad 500 007, India*
[b]*Lupin Laboratories Limited, Lupin Research Park, Pune 411 042, India*

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Abstract—In an effort to develop new and more effective therapies to treat tuberculosis, a series of benzothiadiazine 1,1-dioxide derivatives were synthesized and their in vitro activity against *Mycobacterium tuberculosis*, *Mycobacterium avium* and *Mycobacterium intracellulare* was evaluated. One of the compounds, **8c**, exhibited potent anti-tubercular activity, particularly for the resistant strains and thus prompted us to investigate its in vivo profile. However, the in vivo testing in a mouse model of tuberculosis infection did not show significant anti-tubercular activity, probably because of its poor bioavailability.

1. Introduction

Tuberculosis is one of the leading infectious causes of death in the world and has re-emanated as a growing global health problem. This is not only because of the lack of proper therapeutic agents for its treatment but also due to the development of drug-resistant strains.[1] Additionally, patients infected with HIV have a higher resistance of primary or reactivated tuberculosis, thus enhancing HIV replication and increasing the risk of death. The emergence of multidrug-resistant (MDR) tuberculosis[2,3] renders the present type of treatment much more difficult and may also become sometimes ineffective. Historically, no new drugs have been introduced in the clinic since the discovery of rifampin[4] in spite of major advances that have been made in the drug discovery process.

As a result, there is a dire need to develop novel, faster acting chemotherapeutics with lower toxicity that can be administered with other drugs for the treatment of HIV infections. In recent years, some new classes of compounds based on fluoroquinolones,[5] nitroimidazoles,[6] phenazines,[7] azoles[8] and oxazolidinones[9] have emerged for the treatment of tuberculosis. A new molecule R207910, a diarylquinoline[10] class of compounds, has been discovered recently that potently inhibits both drug-sensitive and drug-resistant *Mycobacterium tuberculosis*.

The discovery of sulfonamides as anti-bacterial agents in the early 1930s was the beginning of one of the most fascinating areas of chemotherapeutic agents. The sulfonamide group is considered as the pharmacophore which is present in a number of biologically active molecules, particularly in anti-microbial agents.[11] 4*H*-1,2,4-Benzothiadiazine 1,1-dioxides can be considered as a cyclic sulfonamide class of molecules and have been extensively studied as potassium channel openers, for example, diazoxide.[12] Moreover, this ring system has also been known for anti-microbial activity.[13,14] Some of the benzothiadiazine derivatives have attracted particular interest because of their action as partial allosteric modulators of α-amino-3-hydroxy-5-methyl isoxazolepropionic acid (AMPA) receptor desensitization to make them virtually devoid of neurotoxicity.[15,16] Similarly, this class of compounds has been shown to inhibit hepatitis C virus (HCV) replication effectively in cell based replication systems with no apparent cytotoxicity[17] (Fig. 1). Recently, a research programme has been initiated in this laboratory to develop new anti-tubercular agents based on different types of heterocyclic scaffolds[18,19] to explore their anti-tubercular potential. In view of the above facts and in continuation of our

* Corresponding author. Tel.: +91 40 27193157; fax: +91 40 27193189/27160512; e-mail addresses: ahmedkamal@iict.res.in; ahmedkamal@iictnet.org

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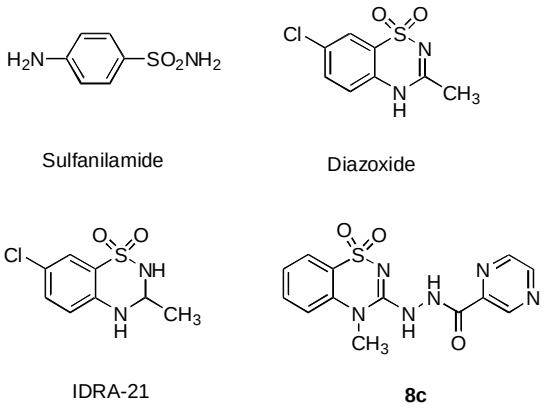


Figure 1. Chemical structures of sulfanilamide, diazoxide, IDRA-21 and considered 4*H*-1,2,4-benzothiadiazine 1, 1-dioxide.

search for anti-tubercular compounds, the encouraging anti-microbial properties of 4*H*-1,2,4-benzothiadiazine 1,1-dioxides have prompted us to synthesize new molecules based on the 4*H*-1,2,4-benzothiadiazine 1,1-dioxide ring system by incorporating pyridine and pyrazine moieties, which are known to exhibit anti-tubercular activity.[20,21] This paper describes the synthesis and anti-tuberculosis activity of this new class of compounds and also discusses efforts to develop the structure–activity relationship for this ring system.

2. Chemistry

The preparation of key intermediates 3-hydrazino-4-alkyl/aryl-4*H*-1,2,4-benzothiadiazine 1,1-dioxide and 3-chloro-4-alkyl/aryl-4*H*-1,2,4-benzothiadiazine 1,1-dioxide was accomplished by synthetic sequence illustrated in Scheme 1. The treatment of chlorosulfonyl isocyanate (**2**) with *N*-alkyl/aryl aniline (**1a**–**e**) in nitromethane, followed by cyclization with aluminium chloride, provided 4-alkyl/aryl-2*H*-1,2,4-benzothiadiazin-3(4*H*)-one 1,1-dioxide (**3a**–**e**).[22] This upon chlorination with PCl_5 afforded 3-chloro-4-alkyl/aryl-4*H*-1,2,4-benzothiadiazine 1,1-dioxide (**4a**–**e**) which on treatment with hydrazine hydrate in chloroform yielded 3-hydrazino-4-alkyl/aryl-4*H*-1,2,4-benzothiadiazine 1,1-dioxide (**5a**–**e**).[23] The synthesis of target compounds **8a**–**m** was carried out by refluxing 3-hydrazino-4-alkyl/aryl-4*H*-1,2,4-benzothiadiazine 1,1-dioxides (**5a**–**e**) with acid chlorides (**6a**–**d**) (nicotinoyl chloride hydrochloride, pyrazinoyl chloride, isonicotinoyl chloride hydrochloride and 6-chloro-nicotinoyl chloride) in THF and triethylamine as a base. Alternatively the synthesis of **8a**–**m** was also carried out by the reaction of 3-chloro-4-alkyl/aryl-4*H*-1,2,4-benzothiadiazine 1,1-dioxides (**4a**–**e**) with corresponding hydrazides (**7a**–**c**) (pyrazine-2-carbohydrazide, nicotinic hydrazide and isonicotinic hydrazide) in THF and triethylamine at room temperature (Scheme 2). However, the latter method was found to be more versatile in terms of yield and reaction conditions. The other target compounds, 3-amino substituted-4-alkyl/aryl-4*H*-1,2,4-benzothiadiazine 1,1-dioxides **10a**–**c**, were prepared by N-substitution of amide precursors (**9a**–**b**) (pyrazinamide, 6-chloro-nicotinamide) with 3-chloro-4-alkyl/aryl-4*H*-1,2,4-benzothiadiazine 1,1-dioxides (**4d**–**e**) in THF by employing sodium hydride (Scheme 3).

3. Results and discussion

The anti-mycobacterial activity of the compounds was determined with the objective of identifying the compounds having inhibitory activity against susceptible (sensitive strains; inhibited by the front line anti-tubercular drug viz. isoniazid) and resistant strains (not inhibited by isoniazid) of *M. tuberculosis* (causative agent of human tuberculosis). In addition to *M. tuberculosis*, the anti-mycobacterial activity was also evaluated against *Mycobacterium avium* and *Mycobacterium intracellulare*, which are primary causative agents for avian tuberculosis but are also associated with the disease in humans in the developed countries in AIDS patients and immunocompromised individuals for the selection

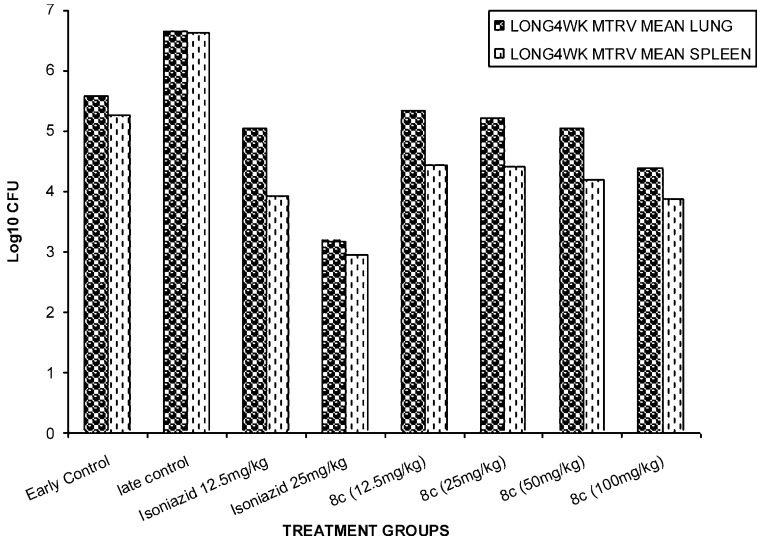
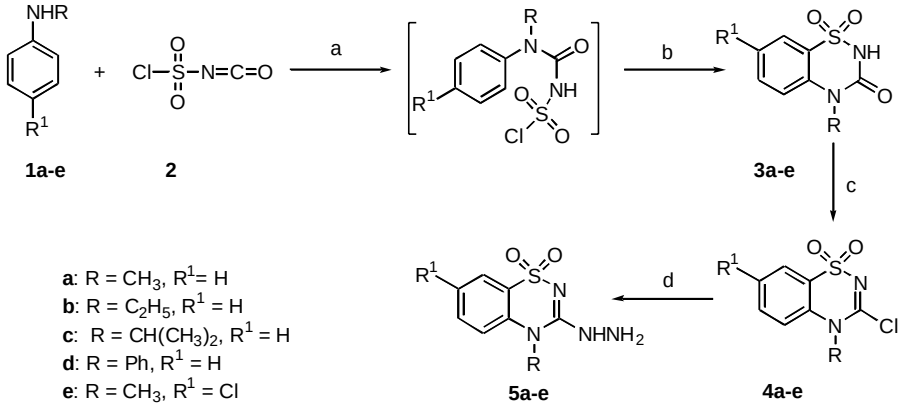
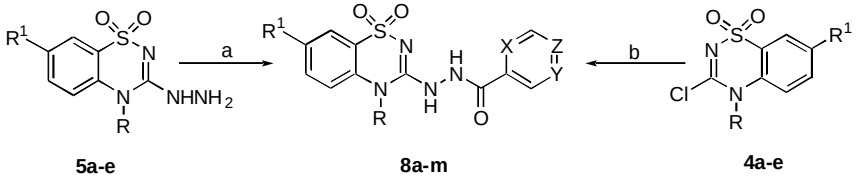


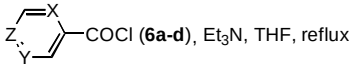
Figure 2. In vivo efficacy of compound **8c** in intravenous mouse model against *M. tuberculosis* (sensitive strain) (treatment period: 4 weeks).



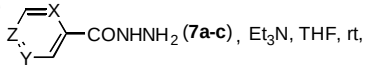
Scheme 1. Reagents and conditions: (a) CH_3NO_2, 0 °C—rt, 30 min; (b) $AlCl_3$, 120 °C, 30 min; (c) PCl_5, 190 °C, 30 min; (d) $N_2H_4{\cdot}H_2O$, $CHCl_3$, 10 °C.



Scheme 2. Reagents and conditions:

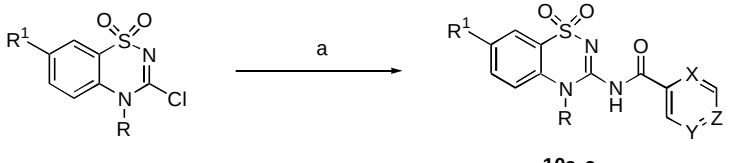
a  COCl (**6a-d**), Et_3N, THF, reflux

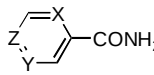
6a: X = Z = CH, Y = N
6b: X = Y = CH, Z = N
6c: X = Y = N, Z = CH
6d: X = CH, Y = N, Z = CCl

b  $CONHNH_2$ (**7a-c**), Et_3N, THF, rt, 24 h.

7a: X = Z = CH, Y = N
7b: X = Y = CH, Z = N
7c: X = Y = N, Z = CH

8a: R = CH_3, R^1 = H, X = Z = CH, Y = N
8b: R = CH_3, R^1 = H, X = Y = CH, Z = N
8c: R = CH_3, R^1 = H, X = Y = N, Z = CH
8d: R = CH_3, R^1 = H, X = CH, Y = N, Z = CCl
8e: R = CH_3, R^1 = Cl, X = Z = CH, Y = N
8f: R = CH_3, R^1 = Cl, X = Y = N, Z = CH
8g: R = C_2H_5, R^1 = H, X = Z = CH, Y = N
8h: R = C_2H_5, R^1 = H, X = Y = CH, Z = N
8i: R = C_2H_5, R^1 = H, X = Y = N, Z = CH
8j: R = $CH(CH_3)_2$, R^1 = H, X = Y = N, Z = CH
8k: R = Ph, R^1 = H, X = Z = CH, Y = N
8l: R = Ph, R^1 = H, X = Y = CH, Z = N
8m: R = Ph, R^1 = H, X = Y = N, Z = CH



Scheme 3. Reagents and conditions: (a)  $CONH_2$ (**9a-b**) NaH, THF, rt, 2 h.

9a: X = CH, Y = N, Z = CCl
9b: X = Y = N, Z = CH

10a: R = CH_3, R^1 = Cl, X = CH, Y = N, Z = CCl
10b: R = CH_3, R^1 = Cl, X = Y = N, Z = CH
10c: R = Ph, R^1 = H, X = CH, Y = N, Z = CCl

of the compounds possessing broad-spectrum activity. Since the resistant strains of *M. tuberculosis*, *M. avium* and *M. intracellulare* are not inhibited by the isoniazid (control drug), it was our objective to develop molecules having activity primarily against *M. tuberculosis* and additionally against the opportunistic pathogens *M. avium* and *M. intracellulare*.

The results of anti-mycobacterial activity (MIC) of evaluated compounds are summarized along with

Table 1. Anti-mycobacterial activity of 4*H*-1,2,4-benzothiadiazine 1,1-dioxide analogues against *Mycobacterium* sp.

Compound	MIC (μg/mL)				
	M. tb. H_{37}Rv ATCC 27294	*M. tb.* clinical isolates		*M. a.* ATCC 49601	*M. i.* ATCC 13950
		Sensitive	Resistant		
8a	>16.0	>16.0	>16.0	>16.0	>16.0
8b	>16.0	>16.0	>16.0	>16.0	>16.0
8c	**0.5**	**0.5–1.0**	**0.5–2.0**	**2.0**	**2.0**
8d	>16.0	>16.0	>16.0	>16.0	>16.0
8e	4.0	4.0–8.0	4.0–8.0	>16.0	>16.0
8f	>16.0	>16.0	>16.0	>16.0	>16.0
8g	>16.0	>16.0	>16.0	>16.0	>16.0
8h	>16.0	>16.0	>16.0	>16.0	>16.0
8i	8.0	8.0–16.0	8.0–16.0	16.0	8.0
8j	>16.0	>16.0	>16.0	>16.0	>16.0
8k	>16.0	>16.0	>16.0	>16.0	>16.0
8l	>16.0	>16.0	>16.0	>16.0	>16.0
8m	8.0	8.0–16.0	8.0–16.0	>16.0	>16.0
10a	>16.0	>16.0	>16.0	>16.0	>16.0
10b	>16.0	>16.0	>16.0	>16.0	>16.0
10c	>16.0	>16.0	>16.0	>16.0	>16.0
Isoniazid	0.25	0.125–0.25	8.0->16.0	8.0->16.0	8.0

M. tb., *Mycobacterium tuberculosis*; *M. a.*, *Mycobacterium avium*; *M. i.*, *Mycobacterium intracellulare*.

standard drug isoniazid in Table 1. Among the screened compounds, four compounds were found to be active in the preliminary screen, that is, they showed inhibition of *M. tuberculosis* at either of the three concentrations (12.5, 25 and 50 μg/mL). These four molecules were then tested by agar dilution assay[24] to determine the minimum inhibitory concentration against a panel of sensitive and resistant clinical isolates. Three of four compounds **8e**, **8i** and **8m** exhibited activity against *Mycobacterium* isolates having 4.0–16.0 μg/mL, while compound **8c** containing pyrazine-2-carbohydrazide as a substituent at the three position of 4*H*-1,2,4-benzothiadiazine 1,1-dioxide demonstrated excellent anti-mycobacterial activity with MIC of 0.5–2.0 μg/mL against both drug-resistant and drug-sensitive clinical isolates of *M. tuberculosis* (H_{37}Rv ATCC 27294), and a MIC of 2.0 μg/mL against *M. avium* (ATCC 49601) and *M. intracellulare* (ATCC 13950). Of the four active compounds **8c**, **8i** and **8m** are substituted with pyrazine-2-carbohydrazides and their activity is in the order of R = CH_3 (**8c**) > R = C_2H_5 (**8i**) ~ R = Ph (**8m**) > R = $CH(CH_3)_2$ (**8j** data not shown). It is known that hydrazide of pyrazine-2-carboxylic acid has not exhibited any anti-mycobacterial activity;[25] however, N′-substitution of pyrazine-2-carbohydrazide moiety with 4*H*-1,2,4-benzothiadiazine 1,1-dioxide ring system shows anti-mycobacterial activity. Therefore, combination of 4*H*-1,2,4-benzothiadiazine 1,1-dioxide ring system with pyrazine-2-carbohydrazide in present investigation has produced a new class of structures possessing in vitro anti-tubercular activity. Another observation is that the presence of electron-withdrawing chloro substitution at the seventh position on the benzene moiety of **8c** makes this compound devoid of any anti-mycobacterial activity (**8f**). Further, the other related compounds, that is, amide derivatives of 4*H*-1,2,4-benzothiadiazine ring system (**10a–c**) were not found to be active.

Table 2. Mean viable counts of *M. tuberculosis* H_{37} Rv recovered from the target organs (lung and spleen) of mice treated with different concentrations of the compound **8c**

Treatment groups	Log_{10} CFU of *M. tuberculosis* recovered from target organs	
	Mean lung	Mean spleen
Early control	5.6 ± 0.27	5.26 ± 0.30
Late control	6.65 ± 0.65	6.62 ± 0.57
Isoniazid 12.5 mg/kg	5.06 ± 0.87	3.93 ± 0.88
Isoniazid 25 mg/kg	3.18 ± 0.76	2.97 ± 0.80
8c		
(12.5 mg/kg)	5.36 ± 0.48	4.45 ± 0.48
(25 mg/kg)	5.22 ± 0.15	4.43 ± 0.16
(50 mg/kg)	5.06 ± 0.78	4.21 ± 0.77
(100 mg/kg)	4.41 ± 0.39	3.88 ± 0.39

Since compound **8c** demonstrated good in vitro activity against *M. tuberculosis* isolates and also has moderate activity against *M. avium* and *M. intracellulare*, it was selected for in vivo activity studies. The in vivo efficacy of the compound **8c** was determined in murine model of pulmonary tuberculosis. Treatment of *M. tuberculosis* infected animals showed no reduction in the load (viable count) of tubercle bacilli in the target organs (lungs and spleen) of all animals treated with four different concentrations of compound **8c** (Table 2, Fig. 2). However, animals treated with 25 mg/kg dose of isoniazid >2 log reduction in the viable count of tubercle bacilli were observed compared to control. The inactivity of compound **8c** (in vivo) may be due to its poor bioavailability.

4. Conclusion

The synthesis and screening of anti-mycobacterial activity for a novel series of 4*H*-1,2,4-benzothiadiazine 1,1-dioxides has been investigated. These heterocyclic compounds with pyrazine-2-carbohydrazide as a substituent at three position have emerged as potential compounds

endowed with moderate to good activity. The possible improvement of anti-tubercular activity of this basic benzothiadiazine structure through suitable modulation of the ring substituents as well as additional functionalization suggests further exploration of this class of compounds. In summary, it has been shown that the good activity of these molecules makes them possible leads for synthesizing new compounds that possess better activity and required bioavailability.

5. Experimental section

5.1. General

Melting points were determined with an electrothermal melting point apparatus and are reported uncorrected. ^{1}H NMRs were recorded on a Bruker UXNMR/XWIN-NMR (200 MHz) or Varian VXR-Unity (400 MHz) with TMS (0 ppm) as an internal standard. Coupling constants are reported in Hertz (Hz). IR (as KBr discs) spectra were taken on a Thermo Nicolet Nexus 670 spectrometer. EI mass spectra were recorded on a VG-7070H Micromass mass spectrometer at 200 °C, 70 eV, with a trap current of 200 μA and 4 kV of acceleration voltage. FAB mass spectra were recorded on a LSIMS-VG-AUTOSPEC-Micromass spectrometer. Analytical TLC of all reactions was performed on Merck prepared plates (silica gel 60 F-254 on glass). Column chromatography was performed using Acme silica gel (100–200 mesh, unless otherwise mentioned). Yields were not optimized. THF was distilled under argon from sodium benzophenone ketyl prior to use. Pyrazine-2-carbohydrazide,[26] pyrazinoyl chloride[27] and pyrazine-2-carboxamide[28] were prepared according to the literature procedures reported elsewhere. All remaining compounds carbohydrazides (isonicotinic hydrazide, nicotinic hydrazide), 6-chloro-nicotinamide, acid chlorides (nicotinoyl chloride hydrochloride, isonicotinoyl chloride hydrochloride and 6-chloro-nicotinoyl chloride) and commercially available N-substituted anilines were purchased from Lancaster. All solvents and reagents were used without further purification unless otherwise specified. Elemental analysis was within ±0.4% of the theoretical values.

6. General procedures

6.1. Synthesis of 3-hydrazino substituted-4-alkyl/aryl-4*H*-1,2,4-benzothiadiazine 1,1-dioxide (*8a–m*)

6.1.1. Method A. To a magnetically stirred solution of 3-hydrazino-4-alkyl/aryl-4*H*-1,2,4-benzothiadiazine 1,1-dioxide (**5a–c**)[23] (1 mmol) and acid chloride hydrochloride (1 mmol) in dry THF (10 mL) was added triethylamine (0.35 mL, 2.51 mmol) at 0 °C under nitrogen atmosphere. The resulting mixture was allowed to stir at reflux temperature for 3 h. The reaction mixture was then cooled to room temperature and concentrated under reduced pressure. The residue thus obtained was dissolved in water (50 mL). The aqueous layer was extracted with ethyl acetate (4× 25 mL). The combined organic layer was washed with 10% aqueous $NaHCO_3$ solution, brine and dried over anhydrous Na_2SO_4. The resulting product was purified on column chromatography employing $CHCl_3/CH_3OH$ (19:1) as an eluent.

6.1.2. Method B. To a stirred solution of carbohydrazide (**7a–c**) (1 mmol) and triethyl amine (0.17 mL, 1.22 mmol) in THF (10 mL) at room temperature was added a solution of 3-chloro-4-alkyl/aryl-4*H*-1,2,4-benzothiadiazine 1,1-dioxide (**4a–e**)[23] (1 mmol) in THF (10 mL). The resulting mixture was stirred overnight at room temperature and then concentrated under reduced pressure. The residue thus obtained was dissolved in water (50 mL). The aqueous layer was extracted with ethyl acetate (4× 25 mL). The combined organic layer was washed with brine solution and dried over anhydrous Na_2SO_4. The resulting product was purified on column chromatography employing $CHCl_3/CH_3OH$ (19:1) as an eluent. However, the yields of the procedure are higher in method B when compared to method A.

6.2. *N*′-(4-Methyl-1,1-dioxido-4*H*-1,2,4-benzothiadiazin-3-yl)nicotinohydrazide (8a)

The title compound was obtained from 3-hydrazino-4-methyl-4*H*-1,2,4-benzothiadiazine 1,1-dioxide (**5a**, 226 mg, 1 mmol), nicotinoyl chloride hydrochloride (**6a**, 178 mg, 1 mmol) and triethyl amine (0.35 mL, 2.51 mmol) as described in method A (yield 68%) or from 3-chloro-4-methyl-4*H*-1,2,4-benzothiadiazine 1,1-dioxide (**4a**, 231 mg, 1 mmol) and nicotinic hydrazide (**7a**, 137 mg, 1 mmol) as described in method B (yield 75%).

Mp 308–310 °C; ^{1}H NMR (400 MHz, $CDCl_3$ + DMSO-d_6): δ 10.9 (s, 1H), 9.9 (br s, 1H), 9.2 (br s, 1H), 8.7 (d, 1H, J = 3.0 Hz), 8.3 (d, 1H, J = 8.0 Hz), 7.8 (m, 1H), 7.64 (t, 1H, J = 8.05 Hz), 7.45 (m, 1H), 7.35 (m, 2H), 3.68 (s, 3H); FABMS *m*/*z* 332 (M^++1, 50); IR (KBr) (ν_{max}/cm^{-1}) 3267, 3005, 2914, 1706, 1594, 1540, 1478, 1386, 1267, 1169, 1103. Anal. Calcd for $C_{14}H_{13}N_5O_3S$: C, 50.75; H, 3.95; N, 21.14%. Found: C, 50.72; H, 3.85; N, 21.26%.

6.3. *N*′-(4-Methyl-1,1-dioxido-4*H*-1,2,4-benzothiadiazin-3-yl)isonicotinohydrazide (8b)

The title compound was obtained from 3-hydrazino-4-methyl-4*H*-1,2,4-benzothiadiazine 1,1-dioxide (**5a**, 226 mg, 1 mmol), isonicotinoyl chloride hydrochloride (**6b**, 178 mg, 1 mmol) and triethyl amine (0.35 mL, 2.51 mmol) as described in method A (yield 65%) or from 3-chloro-4-methyl-4*H*-1,2,4-benzothiadiazine 1,1-dioxide (**4a**, 231 mg, 1 mmol) and isonicotinic hydrazide (**7b**, 137 mg, 1 mmol) as described in method B (yield 75%).

Mp 175–178 °C; ^{1}H NMR (200 MHz, $CDCl_3$ + DMSO-d_6): δ 11.2 (br s, 1H), 10.1 (br s, 1H), 8.85 (d, 2H, J = 5.2 Hz), 8.0 (d, 2H, J = 5.2 Hz), 7.7 (m, 2H), 7.45 (m, 2H), 3.7 (s, 3H); FABMS *m*/*z* 332 (M^++1, 60); IR (KBr) (ν_{max}/ cm^{-1}) 2955, 1680, 1604, 1585, 1559, 1466, 1355, 1312, 1171, 1099. Anal. Calcd for $C_{14}H_{13}N_5O_3S$: C, 50.75; H, 3.95; N, 21.14%. Found: C, 50.84; H, 3.85; N, 21.24%.

6.4. *N*′-(4-Methyl-1,1-dioxido-4*H*-1,2,4-benzothiadiazin-3-yl)pyrazine-2-carbohydrazide (8c)

The title compound was obtained from 3-hydrazino-4-methyl-4*H*-1,2,4-benzothiadiazine 1,1-dioxide (**5a**, 226 mg, 1 mmol), pyrazinoyl chloride (**6c**, 143 mg, 1 mmol) and triethyl amine (0.17 mL, 1.22 mmol) as described in method A (yield 65%) or from 3-chloro-4-methyl-4*H*-1,2,4-benzothiadiazine 1,1-dioxide (**4a**, 231 mg, 1 mmol) and pyrazine-2-carbohydrazide (**7c**, 137 mg, 1 mmol) as described in method B (yield 70%).

Mp 266–268 °C; ^{1}H NMR (400 MHz, $CDCl_3$ + DMSO-d_6): δ 10.8 (s, 1H), 10.0 (br s, 1H), 9.2 (s, 1H), 8.8 (d, 1H, J = 2.3 Hz), 8.7 (d, 1H, J = 2.3 Hz), 7.7 (d, 1H, J = 7.6 Hz), 7.6 (t, 1H, J = 7.6 Hz), 7.35 (m, 2H), 3.65 (s, 3H); FABMS *m*/*z* 333 (M^++1, 60); IR (KBr) (ν_{max}/cm^{-1}) 3357, 3085, 3009, 1711, 1592, 1546, 1505, 1475, 1374, 1291, 1176, 1097; Anal. Calcd for $C_{13}H_{12}N_6O_3S$: C, 46.98: H, 3.64: N, 25.29%. Found: C, 46.61; H, 3.65; N, 25.34%.

6.5. 6-Chloro-*N*′-(4-methyl-1,1-dioxido-4*H*-1,2,4-benzothiadiazin-3-yl)nicotinohydrazide (8d)

The title compound was obtained from 3-hydrazino-4-methyl-4*H*-1,2,4-benzothiadiazine 1,1-dioxide (**5a**, 226 mg, 1 mmol), 6-chloro-nicotinoyl chloride (**6d**, 176 mg, 1 mmol) and triethyl amine (0.17 mL, 1.22 mmol) as described in method A. Yield 68%; mp 226–228 °C; ^{1}H NMR (400 MHz, $CDCl_3$ + DMSO-d_6): δ 10.95 (s, 1H), 9.9 (br s, 1H), 9.0 (s, 1H), 8.3 (d, 1H, J = 8.2 Hz), 7.7 (d, 1H, J = 8.2 Hz), 7.56 (t, 1H, J = 8.2 Hz), 7.42 (m, 1H), 7.3 (m, 2H), 3.62 (s, 3H); FABMS *m*/*z* 366 (M^++1, 60); IR (KBr) (ν_{max}/cm^{-1}) 3218, 3063, 2963, 1693, 1593, 1543, 1465, 1375, 1267, 1160, 1099; Anal. Calcd for $C_{14}H_{12}ClN_5O_3S$: C, 45.97; H, 3.31; N, 19.15%. Found: C, 45.97; H, 3.31; N, 19.15%.

6.6. *N*′-(7-Chloro-4-methyl-1,1-dioxido-4*H*-1,2,4-benzothiadiazin-3-yl)nicotinohydrazide (8e)

The title compound was obtained from 3,7-dichloro-4-methyl-4*H*-1,2,4-benzothiadiazine 1,1-dioxide (**4e**, 265 mg, 1 mmol) and nicotinic hydrazide (**7a**, 137 mg, 1 mmol) as described in method B.

Yield 75%; mp 248 °C (charred) 284–286 °C (melted); ^{1}H NMR (400 MHz, $CDCl_3$ + DMSO-d_6): δ 11.0 (br s, 1H), 10.0 (br s, 1H), 9.2 (s, 1H), 8.8 (d, 1H, J = 4.4 Hz), 8.3 (d, 1H, J = 8.1 Hz), 7.7 (s, 1H), 7.5 (m, 1H), 7.4 (m, 1H), 7.3 (dd, 1H, J = 8.8 Hz, 0.74 Hz), 3.6 (s, 3H); FABMS *m*/*z* 365 (M^++1, 20). Anal. Calcd for $C_{14}H_{12}ClN_5O_3S$: C, 45.97; H, 3.31; N, 19.15%. Found: C, 45.92; H, 3.28; N, 19.27%.

6.7. *N*′-(7-Chloro-4-methyl-1,1-dioxido-4*H*-1,2,4-benzothiadiazin-3-yl)pyrazine-2-carbohydrazide (8f)

The title compound was obtained from 3,7-dichloro-4-methyl-4*H*-1,2,4-benzothiadiazine 1,1-dioxide (**4e**, 265 mg, 1 mmol) and pyrazine-2-carbohydrazide (**7c**, 138 mg, 1 mmol) as described in method B.

Yield 72%; mp 284–286 °C; 1H NMR (400 MHz, $CDCl_3$ + DMSO-d_6): δ 10.6 (s, 1H), 9.3 (s, 1H), 8.8 (d, 1H, J = 2.2 Hz), 8.6 (s, 1H), 7.75 (d, 1H, J = 2.2 Hz), 7.55 (dd, 1H, J = 8.92 Hz, 2.2 Hz), 7.3 (d, 1H, J = 8.9 Hz), 3.6 (s, 3H); FABMS *m*/*z* 367 (M^++1, 60); IR (KBr) (ν_{max}/cm^{-1}) 3274, 3031, 2940, 1690, 1592, 1551, 1477, 1409, 1367, 1285, 1154, 1114. Anal. Calcd for $C_{13}H_{11}ClN_6O_3S$: C, 42.57; H, 3.02; N, 22.91%. Found: C, 42.54; H, 3.12; N, 22.58%.

6.8. *N*′-(4-Ethyl-1,1-dioxido-4*H*-1,2,4-benzothiadiazin-3-yl)nicotinohydrazide (8g)

The title compound was obtained from 3-chloro-4-ethyl-4*H*-1,2,4-benzothiadiazine 1,1-dioxide (**4b**, 245 mg, 1 mmol) and nicotinic hydrazide (**7a**, 137 mg, 1 mmol) as described in method B.

Yield 78%; mp 184–186 °C; ^{1}H NMR (400 MHz, $CDCl_3$ + DMSO-d_6): δ 10.95 (s, 1H), 9.8 (br s, 1H), 9.2 (s, 1H), 8.7 (br s, 1H), 8.3 (d, 1H, J = 7.0 Hz), 7.8 (d, 1H, J = 7.0 Hz), 7.6 (m, 1H), 7.4 (m, 2H), 7.3 (t, 1H, J = 7.0 Hz) 4.2 (q, 2H, J = 7.0 Hz), 1.4 (t, 3H, J = 7.0 Hz); EIMS *m*/*z* 346 (M^++1); IR (KBr) (ν_{max}/cm^{-1}) 3205, 2990, 1664, 1594, 1532, 1391, 1282, 1170, 1100. Anal. Calcd for $C_{15}H_{15}N_5O_3S$: C, 52.16; H, 4.38; N, 20.28%. Found: C, 52.24; H, 4.44; N, 20.32%.

6.9. *N*′-(4-Ethyl-1,1-dioxido-4*H*-1,2,4-benzothiadiazin-3-yl)isonicotinohydrazide (8h)

The title compound was obtained from 3-chloro-4-ethyl-4*H*-1,2,4-benzothiadiazine 1,1-dioxide (**4b**, 245 mg, 1 mmol) and isonicotinic hydrazide (**7b**, 137 mg, 1 mmol) as described in method B.

Yield 78%; mp 102–104 °C; ^{1}H NMR (200 MHz, $CDCl_3$ + DMSO-d_6): δ 11.0 (br s, 1H), 9.8 (br s, 1H), 8.8 (d, 2H, J = 5.07 Hz), 7.9 (d, 2H, J = 5.07 Hz) 7.6–7.8 (m, 2H), 7.3–7.5 (m, 2H), 4.2 (q, 2H, J = 6.3 Hz), 1.45 (t, 3H, J = 6.3 Hz); FABMS *m*/*z* 344 (M^+−1, 50). Anal. Calcd for $C_{15}H_{15}N_5O_3S$: C, 52.16; H, 4.38; N, 20.28%. Found: C, 52.18; H, 4.32; N, 20.21%.

6.10. *N*′-(4-Ethyl-1,1-dioxido-4*H*-1,2,4-benzothiadiazin-3-yl)pyrazine-2-carbohydrazide (8i)

The title compound was obtained from 3-chloro-4-ethyl-4*H*-1,2,4-benzothiadiazine 1,1-dioxide (**4b**, 245.0 mg, 1 mmol) and pyrazine-2-carbohydrazide (**7c**, 138 mg, 1 mmol) as described in method B (yield 70%) or from 3-hydrazino-4-ethyl-4*H*-1,2,4-benzothiadizine 1,1-dioxide (**5b**, 240 mg, 1 mmol) and pyrazinoyl chloride (**6c**, 143 mg, 1 mmol) and triethyl amine (0.167 mL, 1.2 mmol) as described in method A (yield 66%).

Mp 234–236 °C; ^{1}H NMR (400 MHz, $CDCl_3$ + DMSO-d_6): δ 10.6 (br s, 1H), 9.3 (s, 1H), 8.8 (d, 1H, J = 2.3 Hz), 8.7 (d, 1H, J = 2.3 Hz), 7.8 (d, 1H, J = 8.4 Hz), 7.6 (t, 1H, J = 8.4 Hz), 7.3 (m, 2H), 4.2 (q, 2H, J = 6.9 Hz), 1.4 (t, 3H, J = 6.9 Hz); EIMS *m*/*z* 346 (M^+, 20); IR (KBr) (ν_{max}/ cm^{-1}) 3392, 3265, 3065, 2984, 1709, 1594, 1547, 1501, 1393, 1276, 1167. Anal. Calcd for

$C_{14}H_{14}N_6O_3S$: C, 48.55; H, 4.07; N, 24.26%. Found: C, 48.19; H, 4.05; N, 24.32%.

6.11. *N*′-(4-Isopropyl-1,1-dioxido-4*H*-1,2,4-benzothiadiazin-3-yl)pyrazine-2-carbohydrazide (8j)

The title compound was obtained from 3-chloro-4-isopropyl-4*H*-1,2,4-benzothiadiazine 1,1-dioxide (**4c**, 259 mg, 1 mmol) and pyrazine-2-carbohydrazide (**7c**, 138 mg, 1 mmol) as described in method B.

Yield 71%; mp 269–271 °C; ^{1}H NMR (400 MHz, $CDCl_3$ + DMSO-d_6): δ 10.6 (br s, 1H), 9.3 (s, 1H), 8.8 (d, 1H, J = 2.2 Hz), 8.6 (d, 1H, J = 2.2 Hz), 7.8 (d, 1H, J = 7.4 Hz), 7.5 (m, 2H), 7.3 (t, 1H, J = 7.4 Hz), 4.7 (q, 1H, J = 7.4 Hz), 1.6 (d, 6H, J = 7.4 Hz); FABMS *m*/*z* 361 (M^++1, 20); IR (KBr) (ν_{max}/ cm^{-1}) 3340, 3241, 1696, 1589, 1538, 1480, 1353, 1293, 1169, 1113. Anal. Calcd for $C_{15}H_{16}N_6O_3S$: C, 49.99; H, 4.47; N, 23.32%. Found: C, 49.83; H, 4.51; N, 23.14%.

6.12. *N*′-(1,1-Dioxido-4-phenyl-4*H*-1,2,4-benzothiadiazin-3-yl)nicotinohydrazide (8k)

The title compound was obtained from 3-chloro-4-phenyl-4*H*-1,2,4-benzothiadiazine 1,1-dioxide (**4d**, 293 mg, 1 mmol) and nicotinic hydrazide (**7a**, 137 mg, 1 mmol) as described in method B.

Yield 75%; mp 143–145 °C; ^{1}H NMR (200 MHz, $CDCl_3$ + DMSO-d_6): δ 11.1 (br s, 1H), 9.15 (s, 1H), 8.7 (d, 1H, J = 6.3 Hz), 8.3 (d, 1H, J = 7.8 Hz), 7.95 (m, 1H), 7.6–7.8 (m, 4H), 7.2–7.4 (m, 4H), 6.4 (d, 1H, J = 7.8 Hz); FABMS *m*/*z* 392 (M^+−1, 100). Anal. Calcd for $C_{19}H_{15}N_5O_3S$: C, 58.00; H, 3.84; N, 17.80%. Found: C, 58.31; H, 3.78; N, 17.51%.

6.13. *N*′-(1,1-Dioxido-4-phenyl-4*H*-1,2,4-benzothiadiazin-3-yl)isonicotinohydrazide (8l)

The title compound was obtained from 3-chloro-4-phenyl-4*H*-1,2,4-benzothiadiazine 1,1-dioxide (**4d**, 293 mg, 1 mmol) and isonicotinic hydrazide (**7b**, 137 mg, 1 mmol) as described in method B.

Yield 76%; mp 273–275 °C; ^{1}H NMR (200 MHz, $CDCl_3$ + DMSO-d_6): δ 11.0 (br s, 1H), 8.7 (d, 2H, J = 5.0 Hz), 7.7–7.9 (m, 6H), 7.6 (m, 2H), 7.3 (m, 2H), 6.4 (d, 1H, J = 6.3 Hz); EIMS *m*/*z* 393 (M^+, 80). Anal. Calcd for $C_{19}H_{15}N_5O_3S$: C, 58.00; H, 3.84; N, 17.80%. Found: C, 58.24; H, 3.87; N, 17.94%.

6.14. *N*′-(1,1-Dioxido-4-phenyl-4*H*-1,2,4-benzothiadiazin-3-yl)pyrazine-2-carbohydrazide (8m)

The title compound was obtained from 3-chloro-4-phenyl-4*H*-1,2,4-benzothiadiazine 1,1-dioxide (**4d**, 293 mg, 1 mmol) and pyrazine-2-carbohydrazide (**7c**, 138 mg, 1 mmol) as described in method B.

Yield 70%; mp 264–266 °C; ^{1}H NMR (400 MHz, $CDCl_3$ + DMSO-d_6): δ 10.6 (s, 1H), 9.2 (s, 1H), 8.8 (d, 1H, J = 2.2 Hz), 8.7 (d, 1H, J = 2.2 Hz), 7.82 (dd, 1H, J = 7.4 Hz, 1.5 Hz), 7.64–7.74 (m, 3H), 7.5–7.55 (m, 2H), 7.38 (dt, 1H, J = 7.4 Hz, 1.5 Hz), 7.32 (t, 1H, J = 7.43 Hz), 6.4 (d, 1H, J = 8.2 Hz); FABMS *m*/*z* 395 (M^++1, 15); IR (KBr) (ν_{max}/cm^{-1}) 3352, 3086, 1694, 1593, 1553, 1498, 1347, 1292, 1166, 1132. Anal. Calcd for $C_{18}H_{14}N_6O_3S$: C, 54.81; H, 3.58; N, 21.31%. Found: C, 54.54; H, 3.68; N, 21.62%.

6.15. Synthesis of 3-amino substituted-4-alkyl/aryl-4*H*-1,2,4-benzothiadiazine 1,1-dioxide (10a–c)

To a stirred solution of carboxamide (**9a**–**b**) (1 mmol) and a suspension of sodium hydride (60% oil suspension, 48 mg, 1.2 mmol) in dry THF (10 mL) was added a solution of 3-chloro-4-alkyl/aryl-4*H*-1,2,4-benzothiadiazine 1,1-dioxide (**4d**–**e**) (1 mmol) in dry THF (10 mL) at 0 °C under nitrogen atmosphere. The resulting mixture was allowed to warm to room temperature slowly and then stirred at room temperature for 2 h. The final reaction mixture was slowly poured into water (50 mL). Two layers separated and water layer was extracted with ethyl acetate (3× 25 mL). The combined organic layer was washed with brine solution, dried over Na_2SO_4, concentrated under reduced pressure and purified on column chromatography employing $CH_3OH/CHCl_3$ (19:1) as eluent.

6.16. 6-Chloro-*N*-(7-chloro-4-methyl-1,1-dioxido-4*H*-1,2,4-benzothiadiazin-3-yl)nicotinamide (10a)

The title compound was obtained from 3,7-dichloro-4-methyl-4*H*-1,2,4-benzothiadiazine 1,1-dioxide (**4e**, 265 mg, 1 mmol) and 6-chloro-nicotinamide (**9a**, 157 mg, 1 mmol) by following the above procedure.

Yield 75%; mp 264–265 °C; ^{1}H NMR (400 MHz, $CDCl_3$ + DMSO-d_6): δ 11.8 (br s, 1H), 9.0 (s, 1H), 8.4 (dd, 1H, J = 8.3 Hz, 2.5 Hz), 7.9 (s, 1H), 7.7 (m, 1H), 7.6 (d, 1H, J = 8.3 Hz), 7.5 (d, 1H, J = 8.3 Hz), 3.6 (s, 3H); FABMS *m*/*z* 385 (M^++1, 45). Anal. Calcd for $C_{14}H_{10}Cl_2N_4O_3S$: C, 43.65; H, 2.62; N, 14.54%. Found: C, 43.64; H, 2.83; N, 14.57%.

6.17. *N*-(7-Chloro-4-methyl-1,1-dioxido-4*H*-1,2,4-benzothiadiazin-3-yl)pyrazine-2-carboxamide (10b)

The title compound was obtained from 3,7-dichloro-4-methyl-4*H*-1,2,4-benzothiadiazine 1,1-dioxide (**4e**, 265 mg, 1 mmol) and pyrazine-2-carboxamide (**9b**, 123 mg, 1 mmol) by following the same procedure as used for **10a**.

Yield 70%; mp 286–288 °C; ^{1}H NMR (400 MHz, $CDCl_3$ + DMSO-d_6): δ 11.1 (br s, 1H), 9.3 (s, 1H), 8.85 (d, 1H, J = 2.3 Hz), 8.7 (s, 1H), 7.9 (d, 1H, J = 2.3 Hz), 7.7 (m, 1H), 7.5 (m, 1H), 3.7 (s, 3H); FABMS *m*/*z* 352 (M^++1, 30); IR (KBr) (ν_{max}/cm^{-1}) 3284, 3066, 2924, 1732, 1546, 1486, 1372, 1313, 1247, 1158, 1102. Anal. Calcd for $C_{13}H_{10}ClN_5O_3S$:

C, 44.39; H, 2.87; N, 19.91%. Found: C, 44.21; H, 2.84; N, 20.12%.

6.18. 6-Chloro-*N*-(1,1-dioxido-4-phenyl-4*H*-1,2,4-benzothiadiazin-3-yl)nicotinamide (10c)

The title compound was obtained from 3-chloro-4-phenyl-4*H*-1,2,4-benzothiadiazine 1,1-dioxide (**4d**, 293 mg, 1 mmol) and 6-chloro-nicotinamide (**9a**, 157 mg, 1 mmol) by following the procedure as used for **10a**.

Yield 72%; mp 152–154 °C; ^{1}H NMR (400 MHz, $CDCl_3$ + DMSO-d_6): δ 11.5 (br s, 1H), 8.9 (s, 1H), 8.25 (dd, 1H, J = 8.2 Hz, 1.5 Hz), 7.95 (s, 1H), 7.9 (d, 1H, J = 8.2 Hz), 7.1–7.4 (m, 6H), 7.0 (t, 1H, J = 7.4 Hz), 6.84 (t, 1H, J = 7.4 Hz); FABMS *m*/*z* 413 (M^++1); IR (KBr) (ν_{max}/cm^{-1}) 3377, 3165, 1702, 1587, 1504, 1451, 1353, 1256, 1145. Anal. Calcd for $C_{19}H_{13}ClN_4O_3S$: C, 55.28; H, 3.17; N, 13.57%. Found: C, 55.13; H, 2.93; N, 13.81%.

7. Microbiology

7.1. Growth and maintenance of mycobacterial strains

Cultures of *M. tuberculosis* (H_{37}Rv ATCC 27294), *M. avium* (ATCC 49601), *M. intracellulare* (ATCC 13950) and clinical isolates were obtained from various medical institutions. These cultures were grown on LJ medium and maintained at −70 °C. The cultures revived from −70 °C were subcultured on Middlebrook 7H9 broth for 10 days and stored at 4 °C until used.

7.2. Drug and compound preparation

Stock solutions were made in dimethylsulfoxide (DMSO). It has been verified that DMSO did not suppress or delay *M. avium* or *M. tuberculosis* strains growth when added undiluted producing 5% concentration in the medium. Isoniazid was employed as reference drug.

8. In vitro studies

8.1. Agar diffusion assay

The ability of the compounds to inhibit the growth of *Mycobacterium* species was determined by agar diffusion assay. Briefly, reference strains *M. tuberculosis* (H_{37}Rv ATCC 27294), *M. avium* (ATCC 49601) and *M. intracellulare* (ATCC 13950) were grown in Middlebrook 7H9 broth containing 10% ADC supplement at 37 °C on a rotary shaker at 150 rpm for 10 days. The turbidity of the culture was adjusted to 0.5 McFarland, 0.50 mL of the individual cultures was then added to the molten Middlebrook 7H10 in 150 mm petri plates. Uniform holes were then made in the media in which the different concentrations (50, 25 and 12.5 μg/mL) of individual compounds were added. The plates were then incubated at 37 °C for 21–28 days. Compounds showing zone of inhibition greater than or equal to the standard were considered active.

8.2. Agar dilution assay

Minimum inhibitory concentrations (MIC in μg/mL) of compounds against strains of *Mycobacterium* were determined by a reference agar dilution method as per the NCCLS-M24-T2 recommendations.[24] The compounds and reference drug were dissolved in DMSO and diluted twofold to obtain 10 serial dilutions of each compound. Appropriate volumes of compounds were incorporated into duplicate plates of Middlebrook 7H10 agar medium supplemented with 10% Middlebrook supplement oleic acid–albumin–dextrose–catalase (OADC) enrichment at concentration of 0.03–16 μg/mL. Test organisms (*Mycobacterium* strains) were grown in Middlebrook 7H9 broth containing 0.05% Tween 80 and 10% ADC supplement. After 10 days of incubation at 37 °C, the broths were adjusted to the turbidity of 0.5 McFarland standard. The organisms were further diluted 10-fold in sterile water containing 0.10% Tween 80. The resulting mycobacterial suspensions were spotted (3–5 μL/spot) onto drug supplemented 7H10 media plates. The plates were sealed and incubated at 37 °C for 3–4 weeks in upright position. The MIC was recorded as the highest dilution/lowest concentration of the drug that completely inhibited the growth of mycobacterial cultures.

9. In vivo studies

The in vivo efficacy of **8c** was determined in a murine model of pulmonary tuberculosis. Four-week-old female out bred Swiss albino mice housed in a pathogen free; biosafety level 3 environments within micro isolator cages were infected by intravenous injection of 10^6 CFU of *M. tuberculosis* suspension. Following infection mice were randomly distributed in different groups of six each. All animals were treated with once daily dose after 14 days of infection. Therapy was given seven days a week for 4 weeks. All agents were administered by oral gavage and were dosed with 3–4 different concentrations 100, 50, 25 and 12.5 mg/kg of body weight. Control group of infected but untreated mice was killed at the initiation of therapy (baseline control) or at the end of the treatment period (late control) along with treated animals. Mice were sacrificed by cervical dislocation 5 days after the administration of the last dose of drug. The spleens and lungs were removed aseptically and homogenized in tissue homogenizer. At least 6 serial 10-fold dilutions of the homogenate were plated onto selective Middlebrook 7H11 agar plates in duplicate. The colony counts were recorded after incubation at 37 °C for 4 weeks. The viable cell counts were converted to Log_{10} values. A compound showing complete absence of growth >2 Log reduction in viable counts compared to the base line control was considered significant.

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