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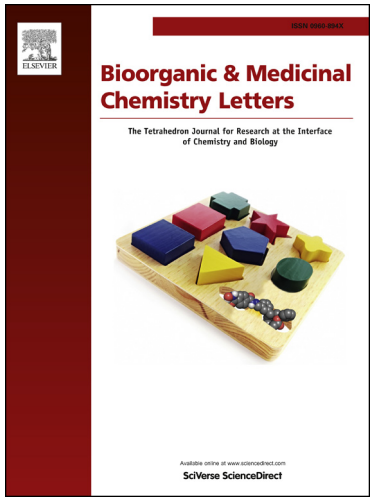

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Dinesh Addla, Anvesh Jallapally, Divya Gurram, Perumal Yogeeswari, Dharmarajan Sriram, Srinivas Kantevari

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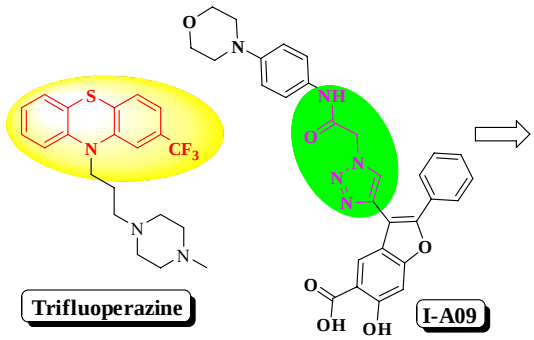

Compound	MIC (μg/mL)
5c	6.25
5l	6.25
5o	6.25

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Dinesh Addla[a], Anvesh Jallapally[a], Divya Gurram[a], Perumal Yogeeswari[b], Dharmarajan Sriram[b] and Srinivas Kantevari[a,c*]

[a] *Organic Chemistry Division-II (CPC Division), CSIR- Indian Institute of Chemical Technology, Hyderabad-500 007, INDIA.*
[b]*Medicinal Chemistry and Antimycobacterial Research Laboratory, Pharmacy Group, Birla Institute of Technology & Science-Pilani, Hyderabad Campus, Jawahar Nagar, Hyderabad-500078 INDIA.*
[c]*Academy of Scientific and innovative Research, CSIR- Indian Institute of Chemical Technology, Hyderabad-500 007, INDIA.*

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ABSTRACT

Molecular hybridization is an emerging structural modification tool to design molecules with better pharmacophoric properties. A series of novel 2-(trifluoromethyl)phenothiazine-1,2,3-triazoles **5a-v** designed by hybridizing two antitubercular drugs trifluoperazine and I-A09 in a single molecular architecture, were synthesized in very good yields using click chemistry. Among the all '22' compounds screened for *in vitro* antimycobacterial activity against *Mycobacterium tuberculosis* H37Rv (*Mtb*), three analogs **5c**, **5l** and **5o** were found to be most potent (MIC: 6.25µg/mL) antitubercular agents with good selectivity index.

Tuberculosis (TB) is an ancient chronic infectious disease caused mainly by pathogen *Mycobacterium tuberculosis* (*Mtb*).[1] According to the latest world health organization (WHO) report[2] there were 8.7 million TB cases, including 1.1 million cases among people with HIV. In 2011 alone 1.4 million people died because of TB, including half a million are women and 430,000 people co-infected with HIV.[3] Additionally, the evolution of its new virulent forms like multi drug resistant tuberculosis (MDR-TB) and extremely drug resistant tuberculosis (XDR-TB) has become a major threat to human kind.[4] All the above facts necessitated an urgent need to develop new, potent and fast acting antitubercular drugs to combat the spread of TB.[5] In this situation hybrid molecules[6] (designed by molecular hybridization[7] of different bioactive substances) were considered as one of the best and quicker way to access newer antitubercular agents preferably with novel mode of action.

The activity of phenothiazines against *M. tuberculosis* has been known since 1913.[8] Some of the phenothiazine based successful drug candidates (Figure 1) for treating neurodegenerative disorders were also effective inhibiting *M. tuberculosis.*[9] Chlorpromazine, trifluoperazine (TPZ) and thioridazine are a few with phenathiazine architecture were found to act in synergy with *M. tuberculosis* susceptible to regular antibiotics rifampicin and streptomycin.[10] But these compounds are also known to exert toxic psychotropic effects by binding with a number of postsynaptic receptors. Among all, TPZ is comparatively less toxic and displays good antitubercular activity.[11] Besides this, triazole based antitubercular agents (Figure 2) may be regarded as a new class providing truly effective lead candidates[12] which are reported to inhibit bacteria. Among them I-A09 is presently in clinical trials.[13]

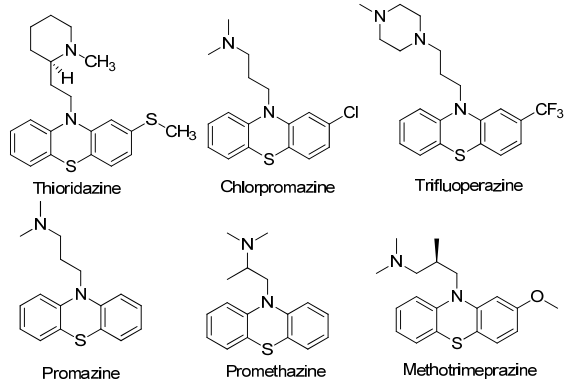


Figure 1: Representative phenothiazine based drug candidates

* Corresponding author. Tel.: +91-4027191437; fax: +91-4027198933; e-mail: kantevari@yahoo.com, kantevari@gmail.com.

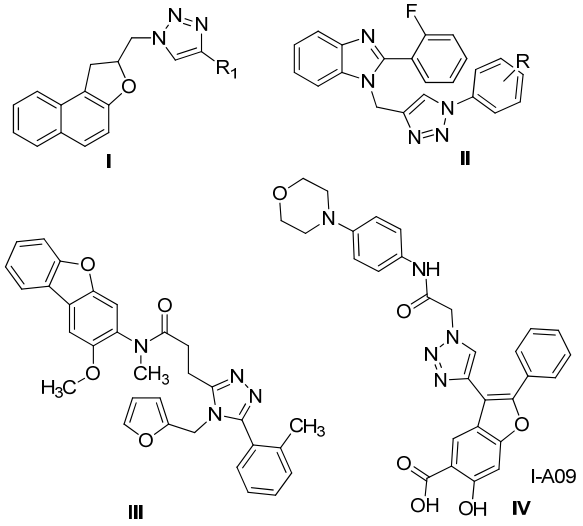


Figure 2: Triazole based antitubercular agents **I-IV**

It is therefore of our interest to integrate both 2-(trifluoromethyl)-10*H*-phenothiazine and triazole pharmacophoric units[14] in one molecular platform to generate a newer scaffold for biological evaluations. With the fact that 1,2,3-triazoles were efficiently made through Cu(I) catalyzed click chemistry,[15] we herein report an efficient synthesis of a series of novel 2-(trifluoromethyl) phenothiazine-1,2,3-triazole hybrids **5a-v** in very good yields. Screening all new compounds **5a-v** for *in vitro* activity against *M. tuberculosis* H37Rv resulted three compounds **5c**, **5l** and **5o** (MIC: 6.25µg/ mL) as most potent antitubercular agents with lower toxicity (selectivity index >10).

The designed scaffold (Figure 3) is in three parts: N-substituted 1,2,3-triazole as a central backbone, 2-(trifluoromethyl)-10*H*-phenothiazine for enhancing desired pharmacophoric behavior with drug like properties and aliphatic or aromatic groups appended to other side of 1,2,3-triazole moiety for liphophilicity control. Variations in the proposed scaffold could be accomplished with the choice of aliphatic or aromatic alkynes **4a-v.** The method adopted for synthesis of 1,2,3-triazole hybrids was based on a Huisgen 1,3-dipolar cycloaddition reaction (click reaction)[15] between azide **3** and alkynes **4a-v**.

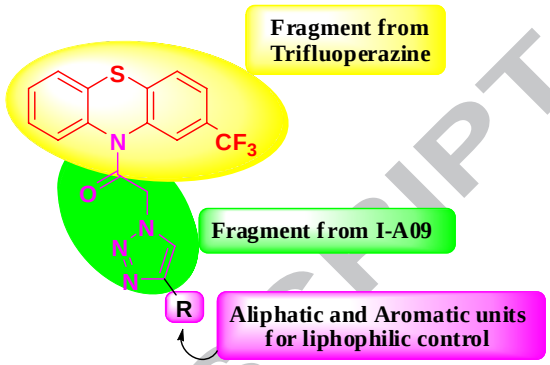
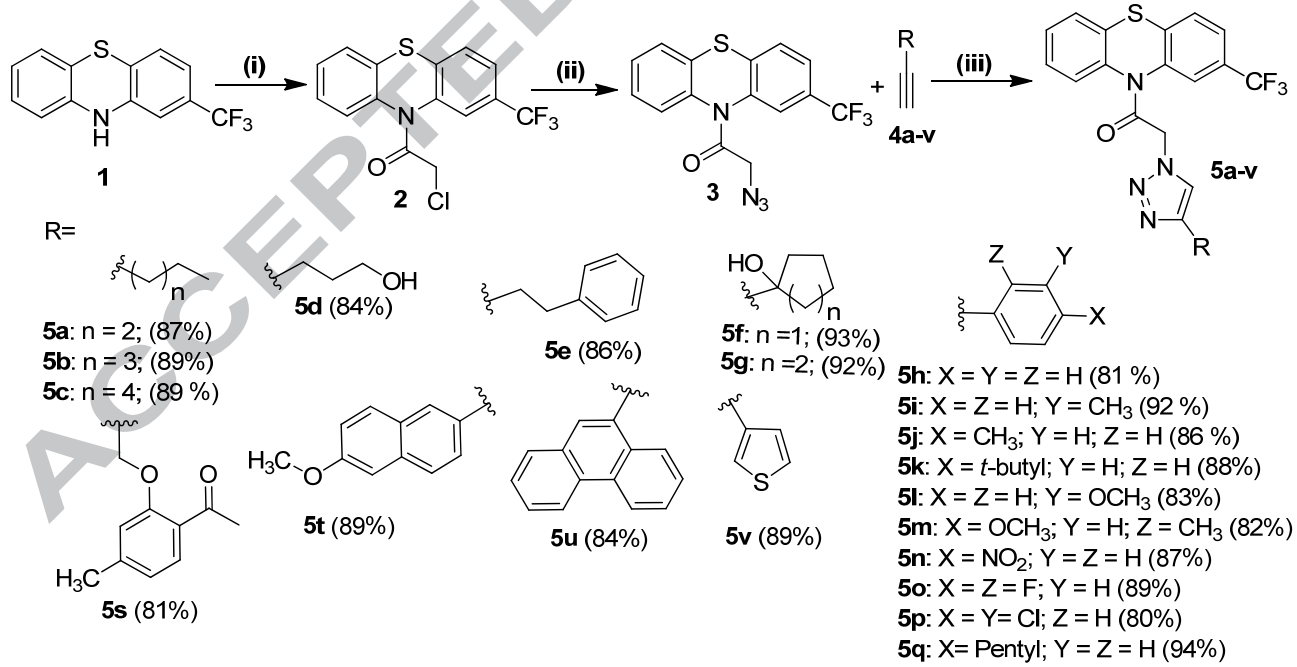


Figure 3: Design strategy for new phenathiazine-1,2,3-triazole hybrids

As a starting point for the study, 2-azido-1-(2-(trifluoromethyl)-10*H*-phenothiazin-10-yl)ethanone **3** required for the preparation of 1,2,3-triazole hybrids was synthesized from 2-trifluoromethyl phenathiazine **1** (Scheme 1) by modifying the literature procedures.[16] Reaction of 2-chloro-1-(2-(trifluoromethyl)-10*H*-phenothiazin-10-yl)ethanone (**2**)[17] (obtained by reacting **1** with chloroacetyl chloride in toluene), with sodium azide in the presence of *tetra*-n-butylammonium bromide produced 2-azido-1-(2-(trifluoromethyl)-10*H*-phenothiazin-10-yl)ethanone **3**[18] in 98% yield. The azide **3** was fully characterized by ^{1}H, ^{13}C NMR and mass (ESI and HR-MS) spectral data. Alkynes **4a-v** required were procured from commercial sources and were used as such in the click reaction with azide **3**.



***Reaction conditions*:** (i) Chloroacetyl chloride, toulene, reflux, 6h, 97%; (ii) NaN_3, tetra-n-butylammonium bromide, dichloromethane: H_2O (1:1), 98%; (iii) $CuSO_4.5H_2O$, sodium ascorbate, *t*-BuOH, H_2O (1:1), 1-2 h, RT, 80-94%.

Scheme 1: Synthesis of 2-(trifluoromethyl) phenothiazine-1, 2, 3-triazole hybrids **5a-v**

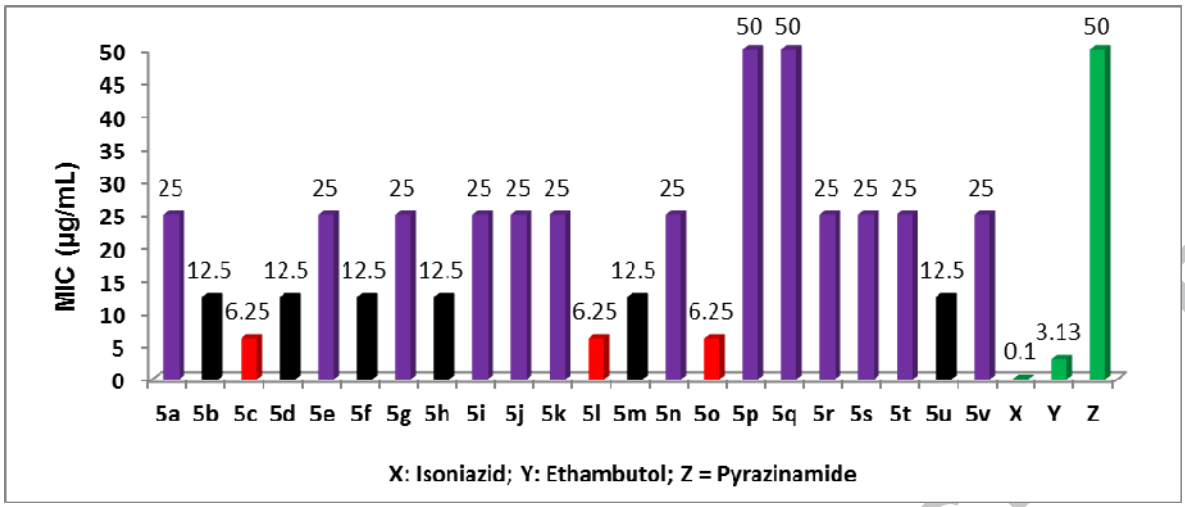


Figure 4: Antitubercular activity of phenothiazine analogues **5a-v**

Having both alkynes **4a-v** and azide **3** in hand, we employed Huisgen's (3+2) cycloaddition reaction in the presence of $CuSO_4$ catalyst, sodium ascorbate in *t*-butanol and water (1:1, v/v). All alkynes **4a-v** were reacted well with 2-azido-1-(2-(trifluoro methyl)-10*H*-phenothiazin-10-yl)ethanone **3** to give 1,2,3-triazole hybrids **5a-v** in excellent yields (Scheme 1).[19] Triazoles **5a-v** obtained was fully characterized by ^{1}H, ^{13}C NMR and mass (ESI and HR-MS) spectral data.[19] Purity of all the new compounds **5a-v** (>95%) was determined by HPLC analysis.

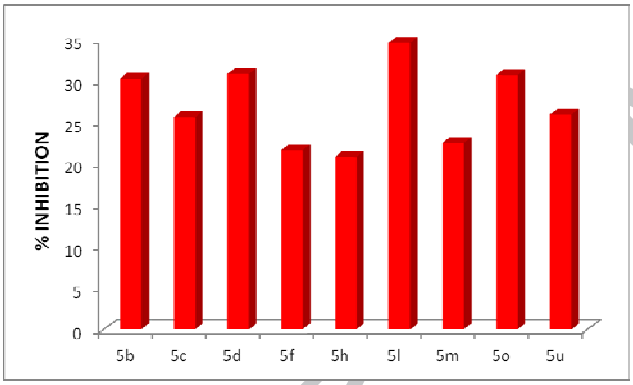


Figure 5: Percentage inhibition of HEK-293Tcells at a concentration of 50µg/ mL phenothiazine analogues

The antimycobacterial activity of the synthesized phenathiazine-1,2,3-triazole hybrids **5a-v** has been screened against *M. tuberculosis* H37Rv (ATCC27294) by agar dilution method[20] for the determination of MIC in triplicates. The minimum inhibitory concentration (MIC) is defined as the minimum concentration of compound required to completely inhibit the bacterial growth. The MIC values (µg/mL) of **5a-v** along with the standard drugs for comparison are furnished in Figure 4. Twenty two new compounds screened have showed *in vitro* activity against *Mtb* with MIC ranging from 6.25 - 50.0 µg/mL. When compared to first line anti-TB drugs Isoniazid (0.1 µg/mL), Ethambutol (MIC 3.13 µg/mL), all the 22 compounds were found to be less potent than Ethambutol and Isoniazid. But, all these triazole hybrids except **5p-q,** are more potent (≤ 25µg/mL) when compared to another anti-TB drug Pyrazinamide (50.0 µg/mL). Among all these phenothiazine hybrids, eleven derivatives **5a, 5e, 5g, 5i-k, 5n, 5r-t** and **5v** exhibited MIC 25µg/mL and six derivatives **5b, 5d, 5f, 5h, 5m** and **5u** exhibited MIC 12.5µg/mL. Three phenathiazine-triazole hybrids **5c**, **5l**, and **5o** displayed MIC 6.25µg/mL, a value postulated by the global program for the discovery of new antitubercular drugs as threshold for the evaluation of new *M. tuberculosis* therapies. Structure-activity correlations of new compounds **5a-c** with respect to their antitubercular activity revealed that the increase in inhibition of *Mtb* activity is attributed to the increase in alkyl chain length appended to 1,2,3-triazole nucleus. Also to note that alkyl chain with hydroxyl group (in **5d**) and phenyl group (in **5e**) displayed reduced *Mtb* inhibition activity. Among phenothiazine-triazole hybrids **5h-v** with substituted aryls appended to 1, 2, 3-triazole nucleus revealed that two compounds **5l** bearing electron donating methoxy group on phenyl ring and **5o** bearing two fluoro substituents on phenyl ring are most active inhibiting *Mtb* activity.

The *in vitro* cytotoxicity of hybrid analogues evaluated for anti-TB activity with MIC ≤12.5µg/mL were also assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (MTT) assay[21] against Human Embryonic Kidney (HEK-293T) cells at 50µg/mL concentration. Percentage inhibition of cells was reported in Figure 5. The most promising anti-TB compounds **5c, 5l** and **5o** exhibited 25.6%, 34.6% and 30.6% inhibition respectively at 50µg/mL with selectivity index of approximately >10. Compounds that exhibited selectivity Index (SI) values greater than 10 in HEK-293Tcells were considered nontoxic. The results demonstrated that the compounds **5c, 5l** and **5o** with high inhibitory activity against *M. tuberculosis* (6.25 µg/mL) also exhibited lowest toxicity, i.e., high SI (>10) against HEK-293Tcells.

In conclusion we have designed a series of novel 2-(trifluoro methyl) phenothiazine-1,2,3-triazoles **5a-v** by hybridizing two antitubercular drugs trifluoperazine and I-A09. The required azide building block **3** was prepared from 2-(trifluoromethyl) phenathiazine in two steps. New analogues **5a-v** were synthesized using Huisgen's (3+2) cycloaddition reaction between azide **3** and alkynes **4a-v** in presence of copper sulphate and sodium ascorbate. Evaluation of all the new hybrids **5a-v** against *M. tuberculosis* H37Rv (*Mtb*) and cytotoxicity revealed that three compounds **5c**, **5l** and **5o** are best active antitubercular agents with MIC 6.25 µg/mL and with selectivity index >10. The results described here demonstrate the potential utility of molecular hybridization in designing new hybrid analogues of 2-(trifluoromethyl) phenathiazine with appended triazole fragment as potent antitubercular agents for further optimization.

Acknowledgments

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17. **2-Chloro-1-(2-(trifluoromethyl)-10H-phenothiazin-10-yl)ethanone** (**2**): To a solution of 2-trifluoromethyl phenathiazine **1** (2.0g, 7.49 mmol) in toluene (30 mL) was added chloroacetyl chloride (0.88mL, 11.23 mmol) at 0°C and then heated at 80°C for 12h. The reaction mixture was cooled to RT, concentrated under reduced pressure and the crude residue was dissolved in dichloromethane (50 mL), washed with water (2x50mL) dried over anhydrous sodium sulphate and evaporated to give 2-Chloro-1-(2-(trifluoromethyl)-10H-phenothiazin-10-yl)ethanone (**2**) as white solid (2.5g, 97%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.91 (s, 1H), 7.47-7.57(m, 4H), 7.28-7.42 (m, 1H), 7.17 (d, J=7.55Hz 1H), 4.25 (d, J= 12.8Hz 1H), 4.12 (d, J=12.8Hz, 1H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 165.4, 138.0, 137.1, 128.9, 128.3, 128.2, 127.9, 126.1, 125.2, 123.99, 123.96, 41.5. IR (KBr) 3003, 2950, 1692, 1608, 1467, 1329, 1244, 1168, 1132, 1086, 824, 747, 641 cm^{-1}. MS (ESI) m/z 344 $[M+H]^+$.
18. **2-Azido-1-(2-(trifluoromethyl)-10*H*-phenothiazin-10-yl)ethanone** (**3**): Compound **2** (2.0g, 5.83 mmol) in dichloromethane (15 mL) was added sodium azide (0.75g,11.66 mmol) in water (15 mL) and *tetra*-n-butyl ammonium bromide (0.04 g, 0.12 mmol) and stirred at RT for 12h. The organic layer was separated, washed with water (3x30mL), dried over sodium sulfate and concentrated under reduced pressure to give product **3** (2.01g, 98 %) as colorless solid. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.86(bs, 1H), 7.55-7.57(m, 1H),7.44-7.52(m, 3H), 7.38 (t, J=7.32Hz, 1H), 7.32(t, J= 7.47Hz, 1H), 4.07(d, J= 15.1Hz, 1H), 3.87 (d, J= 15.8Hz, 1H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 166.6, 137.8, 136.6, 132.4, 129.8, 129.4, 128.4, 128.3, 128.0, 127.8, 126.3, 123.9, 50.8. IR (KBr) 2936, 2102, 1619, 1467, 1330, 1248, 1164, 1123, 1087, 887, 767, 629 cm^{-1}. MS (ESI) m/z 351 $[M+H]^+$.
19. **Synthesis of 2-(trifluoromethyl)phenothiazine-1,2,3-triazole hybrids 5a-v:** Azide **3** (1.0 mmol), alkynes **4a-4v** (1.0 mmol), copper sulphate.$5H_2O$ (20 mol %) and sodium ascorbate (20 mol%) in *t*-butanol & water (1:1, v/v, 4mL), was stirred at RT for 1-2h. After completion (TLC), the reaction mixture was diluted with ethyl acetate (20 mL) and water (5 mL), the organic layer was separated, washed with brine solution (2 x 10 mL), dried over anhydrous sodium sulphate and concentrated under reduced pressure. The crude residue thus obtained was purified over silica gel column chromatography eluted with ethyl acetate/ hexane (1:2) to give pure 1,2,3-triazole hybrids **5a-v.**
Representative spectral data for products **5a-v: 2-(4-butyl-1*H*-1,2,3-triazol-1-yl)-1-(2-(trifluoromethyl)-10*H*-phenothiazin-10-yl)ethanone (5a)**: M.P:126 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.89 (s, 1H), 7.57-7.60 (m, 2H), 7.52 (d, J=7.93Hz 2H), 7.42-7.45 (m, 2H), 7.36 (t, J=7.62Hz, 1H), 5.55 (bd, 2H), 2.71 (t, J=7.62Hz, 2H), 1.61-1.68(m, 2H), 1.33-1.42 (m, 2H), 0.91 (t, J=7.32Hz, 3H), ^{13}C NMR (75 MHz, $CDCl_3$) δ 164.6, 148.7, 137.6, 136.3, 128.6, 128.3, 128.1, 124.1, 122.2, 51.3, 31.3, 25.3, 22.2, 13.7. IR (KBr) 3156, 2923, 2855, 1704, 1608, 1467, 1328, 1251, 1127, 1086, 829, 755, 641 cm^{-1}. MS (ESI) m/z 433$[M+H]^+$; HR-MS (ESI) Calcd for $C_{21}H_{20}N_4OF_3S$ $[M+H]^+$:433.13044, found:433.12986.
20. **Antitubercular evaluation assay**: Two-fold serial dilutions (50.0, 25.0, 12.5, 6.25, 3.13, 1.56, 0.78 and 0.4 µg/mL) of each test compounds **5a-v** and drugs were prepared and incorporated into Middlebrook 7H11 agar medium with OADC Growth Supplement. Inoculum of *M. tuberculosis* H37Rv ATCC 27294 was prepared from fresh Middlebrook 7H11 agar slants with OADC (oleic acid, albumin, dextrose and catalase; Difco) Growth Supplement adjusted to 1 mg/mL (wet weight) in Tween 80 (0.05%) saline diluted to 10^{-2} to give a concentration of ~ 10^7 cfu/mL. A 5 µL amount of bacterial suspension was spotted into 7H11 agar tubes containing 10-fold serial dilutions of drugs per mL. The tubes were incubated at 37 °C, and final readings were recorded after 28 days. This method is similar to that recommended by the National Committee for Clinical Laboratory Standards for the determination of MIC in triplicate.
21. **Evaluation of Cytotoxicity:** Antitubercular active compounds with MIC ≤ 12.5µg/mL were further examined for toxicity in a HEK-293T cell line at the concentration of 50 µg/mL. 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.

Supplementary Material

General experimental details, ^{1}H, ^{13}C NMR and mass (ESI & HRMS) spectral data and copies of ^{1}H, ^{13}C NMR and HRMS spectra of all the new compounds **2, 3 & 5a-v** can be obtained free of charge from the internet.