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Preliminary SAR and biological evaluation of antitubercular triazolothiadiazine derivatives against drug-susceptible and drug-resistant *Mtb* strains

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ABSTRACT

Following up the SAR study of triazolothiadiazoles for their antitubercular activities targeting *Mt* SD in our previous study, on the principle of scaffold hopping, the C3 and C6 positions of triazolothiadiazine were examined systematically to define a preliminary structure−activity relationship (SAR) with respect to biological activity. This study herein highlights the potential of two highly potent advanced leads **6c-3**, **6g-3** and several other compounds with comparable potencies as promising new candidates for the treatment of TB (**6c-3**, MIC-H37Rv = 0.25 μg/mL; MIC-MDRTB = 2.0 μg/mL; MIC-RDRTB = 0.25 μg/mL; *Mt* SD-IC_{50} = 86.39 μg/mL; and **6g-3**, MIC-H37Rv = 1.0 μg/mL; MIC-MDRTB = 4.0 μg/mL; MIC-RDRTB = 2.0 μg/mL; *Mt* SD-IC_{50} = 73.57 μg/mL). Compounds **6c-3** and **6g-3** possessed a para-nitro phenyl at the 6 position showed low Vero and HepG2 cells toxicity, turning out to be two excellent lead candidates for preclinical trials. In addition, *In vitro Mt* SD inhibitory assay indicates that *Mt* SD is at least one of the targets for their antitubercular activity. Thus, they may turn out to be promising multidrug-resistance-reversing agents.

Keywords:

Shikimate dehydrogenase

Antitubercular agent

3,6-Disubstituted triazolothiadiazine

SAR

1. Introduction

Tuberculosis (TB) caused by *Mycobacterium tuberculosis* (*Mtb*) remains a leading cause of morbidity and mortality due to contagious and insidious disease worldwide. Despite its known etiology, TB still remains one of the most threatening health problems globally, claiming approximately 9.6 million cases in 2014, of which 480,000 were multi-drug-resistant (MDR). [1] The only FDA-approved TB drug, Bedaquiline, has been recently announced for the treatment of MDR-TB since the 1960s. [2] Unfortunately, like other known second-line antitubercular drugs, it has been reported to possess unwanted side effects, in particular its ability to cause QT (the time interval measured from the beginning of the QRS complex to the end of the T wave in an electrocardiogram) prolongation could lead to an abnormal and potentially fatal heart rhythm. [3] The rise of MDR and extremely drug-resistant (XDR) strains of *M. tuberculosis* [4] has prompted a search for new tuberculosis drug candidates and new druggable targets. Consequently, in the development of novel antitubercular chemotherapeutics, the lack of cross-resistance with existing drugs remains pivotal, along with good candidate potency and little or no toxicity. [5]

In order to discover novel nontoxic antitubercular drugs with least chances of pre-existing resistance, it is imperative to choose such a novel target which is not only essential for the survival but is also exclusive to *Mtb*. The shikimate pathway is one such target which is found in many human pathogens, including *Mycobacterium tuberculosis*, for the production of chorismate, a biosynthetic precursor for aromatic amino acids and other aromatic compounds. Shikimate dehydrogenase (SD), the fourth enzyme of this pathway is involved in the conversion of 3-dehydroshikimate to shikimate using NADPH as the cosubstrate. Since, it is proven to be necessary for the survival of *Mtb* and is absent in mammals. [6] *M. tuberculosis* shikimate dehydrogenase (*Mt* SD) is therefore a potential target for the treatment of human disease, including attractive target for new TB drug and vaccine development [7]. The novel mode of action

against *Mtb* would ensure that the compounds targeting SD will be least prone to developing resistance. Consequently, we set out to devise SD inhibitors that could be used as selective antitubercular drugs. We previously developed a high-throughput screening (HTS) model targeting *Mt* SD to identify competitive *Mt* SD inhibitors. [8] One 3,6-disubstituted triazolothiadiazole (**1**, MIC-H37Rv = 8.0 μg/mL; MIC-MDRTB (Isoniazid and Rifampin resistant strains) = 8.0 μg/mL; MIC-RDRTB (Rifampin resistant strains) = 8.0 μg/mL; *Mt* SD-IC_{50} = 23.00 μg/mL) (Fig. 1) has been identified as a promising lead for antitubercular drug development. We have previously reported the structure-activity relationships study of 3,6-disubstituted triazolothiadiazoles, which gave a good number of hit compounds through screening against *Mtb* H37Rv strain at 8.0 μg/mL. Moreover, selected lead compounds from these hits were found to exhibit excellent MIC values in the range of 0.25−8.0 μg/mL against drug sensitive strains (H37Rv) as well as MDRTB and RDRTB. These lead compounds exhibit potential inhibitory activity on *Mt* SD. Subsequently, triazolothiadiazoles **2** and **3** (Fig. 1) were found to be bactericidal and also active against *Mt* SD.[9]

The triazolothiadiazine core is a well-known privileged structure in medicinal chemistry as it is a versatile heterocycle, possessing a wide spectrum of biological activities including antimicrobial, [10-15] antitumor, [16-19] antioxidant, [10] anti-HIV, [18] antiviral, [20] analgesic [21], acetyl- and butyryl-cholinesterases inhibitory [19,22] , alkaline phosphatase inhibitory [19], monoamine oxidase inhibitory [22], 15-lipo-oxygenase inhibitory [23], and SIRT1 inhibitory [24] activities. We were interested in the triazolothiadiazine class of compounds because triazolothiadiazines were previously identified as having activity against *M. tuberculosis* [14, 25]. Suresh Kumar et al. reported clubbed isopropylthiazole triazolothiadiazines with activity against *M. tuberculosis* and pursued antitubercular screening so as to obtain a potent and low-toxicity lead **4** [14] (Fig. 1). Kincaid et al. identified triazolothiadiazines with activity against *M. tuberculosis* by means of virtual screening

for UDP-galactopyranose mutase ligands. [25] The triazolothiadiazines reported in these studies represent a variety of pharmacophores with having extensive variation at the C-3 and C-6 positions for modulation of activity. Therefore, it is an important pharmacophore for the discovery of new drugs. However, against *Mt* SD, no compound has emerged as a promising lead in the past years besides triazolothiadiazoles previously reported by us [9].

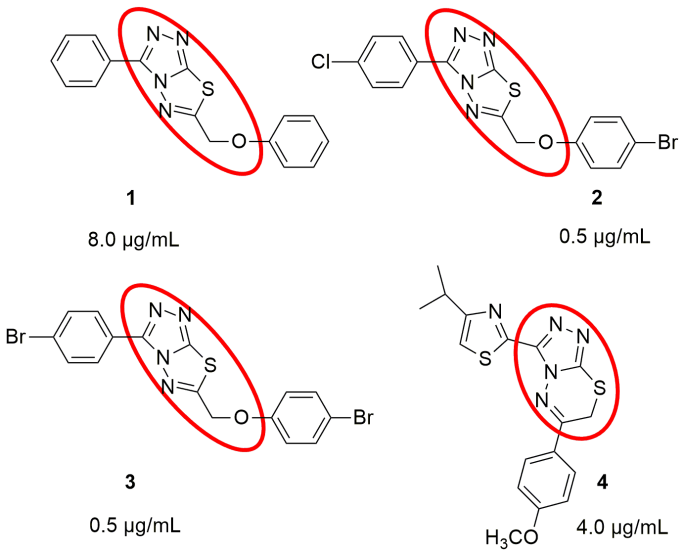


Fig. 1. Lead compounds from triazolothiadiazines.

The scaffold hopping approach is one of the strategies included within the rational design protocol for identification of new biologically relevant small molecules. Since we have previously studied the SAR study of 3,6-disubstituted triazolothiadiazoles [9], in our continuous efforts to discover novel antitubercular scaffolds, scaffold hopping was used to find other antitubercular inhibitors against *Mt* SD. With this in mind, a series of 3,6-disubstituted triazolothiadiazines that maintain structural elements of the parent compound **1** have been synthesized whereby the triazolothiadiazole is replaced with the triazolothiadiazine. Considering above facts and the principles of group replacement and bioisosterism, we set out for optimization of the triazolothiadiazine through systematic structural modifications at the 3 and 6

positions as shown in Fig. 2 to evaluate their steric and electronic effects on the antimycobacterial activity and to deduce SAR. As a result, we have discussed the effect of unsubstitution and different substitution of aromatic and hetero-aromatic groups at the 3 and 6 positions on the potency against *Mtb*. Moreover, to explore the chemical space around the C3 aryl moiety of the triazolothiadiazines, a methylene spacer is inserted between carbon atom at the 3 position and the sterically encumbered β-naphthalene ring. In detail, we conducted an exploratory study to understand the preliminary SAR of a triazolothiadiazine series regarding antitubercular activity, which has led to the identification of two highly potent lead compounds **6c-3** (MIC-H37Rv = 0.25 μg/mL), **6g-3** (MIC-H37Rv = 1.0 μg/mL) and others with comparable potencies. Additionally, the several potent compounds were also screened for their *in vitro Mt* SD inhibition and were tested to assess their apparent cytotoxicity toward Vero and HepG2 cells. The results of this study are summarized in this paper.

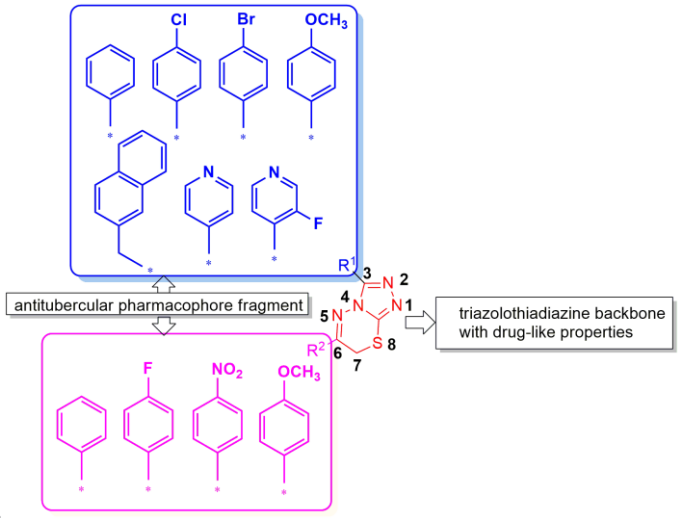
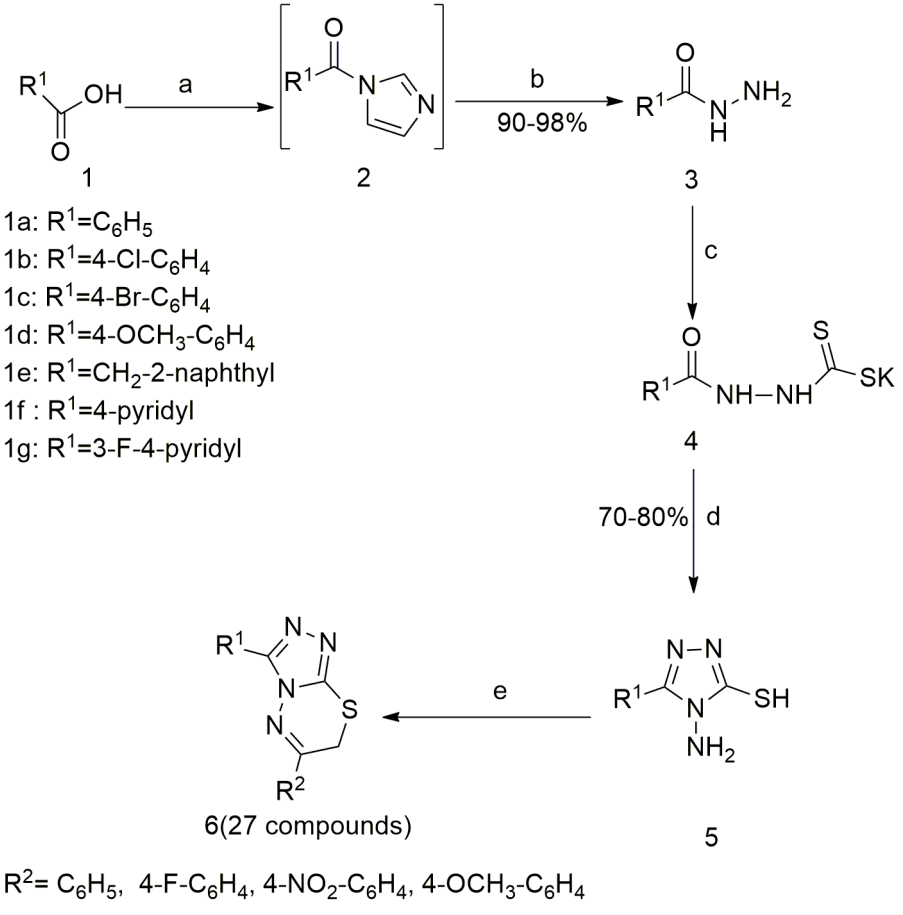


Fig. 2. Optimization of 3,6-disubstituted triazolothiadiazines.

2. Chemical synthesis

Compounds for the optimization library of 3,6-disubstituted triazolothiadiazines (27 compounds in total) were synthesized according to Scheme 1. The commercially available appropriate aromatic acid was firstly activated by 1,1'-carbonyldiimidazole and then hydrazinolysized to give corresponding aroyl hydrazides **3a−h** in 79−95% yields in one-pot, which then reacted with carbon disulfide, in presence of potassium hydroxide in ethanol to afford the respective potassium dithiocarbazinate **4a−h**, which later cyclized to desired 4-amino-3-mercapto-1,2,4-triazole **5a−h** by reacting with hydrazine hydrate (80%) in 60−70% yields. The resulting triazoles **5a−h** were further converted to corresponding triazolothiadiazines **6** in one pot-reaction by condensation with the appropriate phenacyl bromides in the presence of absolute ethanol through microwave irradiation (MWI) in 90−99% yields.



Scheme 1. Synthesis of 3,6-disubstituted triazolothiadiazines. Reagents and conditions: (a)1,1'-carbonyldiimidazole, THF, 2 h, room temperature (rt); (b) $N_2H_4H_2O$, rt, overnight; (c) CS_2, KOH, EtOH, rt, 8 h; (d) $N_2H_4H_2O$, rt, H_2O, 2 h, rt/5 h, reflux; (e) $ArCOCH_2Br$, EtOH, MWI, 30 min, 95 °C

3. Results and discussions

3.1. Evaluation of antitubercular activity and SAR study

The library of 3,6-disubstituted triazolothiadiazines **6a-g** (27 compounds) was subjected to evaluated for their activity against *Mtb* H37Rv, MDRTB and RDRTB by the BacT/ALERT 3D liquid culture technology[26] using Rifampin (RFP) and Isoniazid (INH) as the standard drugs to determine the MIC. Results are summarized in Table 1 and Table 2. Compounds were measured at a concentration of 8.0 μg/mL (MIC) for the preliminary assessment of the activity against *Mtb* H37Rv, wherein 17 compounds are inactive at 8.0 μg/mL concentration (Table 1), 4 compounds is comparable in activity to the lead compound **1** (Table 1), whereas 6 compounds are found to inhibit the growth of *Mtb* H37Rv, MDRTB and RDRTB at variable concentrations (Table 2). Some of the most representative compounds are also tested toward Vero and HepG2 cells to ascertain the cytotoxicity profile. To further analyze the biological profile of these promising compounds, *in vitro Mt* SD inhibitory assay has been carried out as well. These modifications lead to a variable range of activities and allow us to construct a plausible SAR as will be described herein.

The activity of selected 6 derivatives was assessed for their ability to inhibit the growth of the acquired clinical MDRTB and RDRTB strains from Jiangsu province hospital, China (Table 2). To our delight, these triazolothiadiazines showed potent activity MIC = 0.25 ~ 4 μg/mL against two tested drug-resistant strains (Table2). Control data for INH, RFP and the lead compound **1** is also reported (Table 2) for comparison.

As Table 1 and Table 2 shows, the substituents at both 3- and 6-position are significantly influential on antibacterial activity. Disappointingly, it is very clear that neither the weak electron-withdrawing chloro group nor the strong electron-donating methoxy group at the para-position of the phenyl ring attached at 3-position of conjugated skeleton is favor of the potency

of compounds (**6b-1−4**, **6d-1−4**). On the contrary, bromo substituent (electron-withdrawing group) attached at para position of the phenyl ring at the C-3 position results in a sharp increase in potency, leading to the discovery of two highly active lead compounds **6c-1** and **6c-3** (MIC-H37Rv = 0.25 μg/mL), which are 32-fold more potent than the lead compound **1**. It could be seen that **6c-1** and **6c-3** were significantly more potent than other triazolothiadiazines. This increase in activity is not surprising in light of triazolothiadiazoles previously reported. [9] On the other hand, the nature of the phenyl ring substituent at the C-6 position exhibits notable effects on the potency. It is evident that most of the hybrid compounds bearing 4-nitrophenyl at the C-6 position are significantly active against H37Rv, MDRTB and RDRTB (**6c-3**, **6e-3**, **6f-3**, **6g-3**). The introduction of electron-withdrawing fluoro group at para-position of phenyl ring attached to C-6 of heteroaromatic core leads to reduced activity as compared to the lead compound **1** (MIC > 8.0 μg/mL).

In the 3-phenyl series of compounds (**6a-1−4**), the activity requirement for the substitution pattern of the 6-phenyl moiety is less well defined and merits further investigation. The compounds with electron-withdrawing fluoro, nitro and electron-donating methoxy at the para-position of the phenyl ring attached at 6-position do not show appreciable antitubercular activity (MIC > 8.0 μg/mL) whereas compound **6a-1** incorporating unsubstituent phenyl ring attached at 6-position shows better potency than the parent compound **1** (MIC-H37Rv = 2.0 μg/mL; MIC-MDRTB = 4.0 μg/mL; MIC-RDRTB = 2.0 μg/mL). Thus, the fact that any activity at all was observed is somewhat intriguing to us and suggests compounds with further improvements in potency may be obtainable.

In the 3-(4-bromophenyl) series of compounds (**6c-1−4**), quite surprisingly, almost all compounds (barring **6c-2**) examined possess good to excellent antitubercular activities. It is obvious that unsubstituent and 4-bromo of the benzene ring at the 6 position are beneficial to

increase the potency, highlighting **6c-1** and **6c-3** as the most potent compounds in the series with MIC value of 0.25 μg/mL which is 32-fold more active as compared to the parent compound **1**. Meanwhile, **6c-4** is comparable in activity to the lead compound **1**. The results clearly suggest that 4-bromophenyl group to the 3 position is crucial for biological activity. The most potent compound in this series, at present, is **6c-3** (MIC-MDRTB = 2.0 μg/mL; MIC-RDRTB = 0.25 μg/mL). This result indicates that 4-bromo attached to the benzene ring at the 3 position is important for growth inhibition activity as even compound **6c-2** with an 4-fluorophenyl group at the 6 position showed an MIC-H37Rv > 8.0 μg/mL.

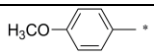
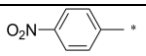
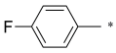
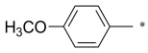
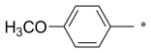
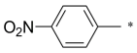
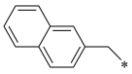
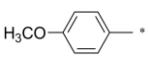
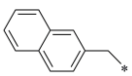
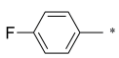
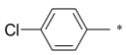
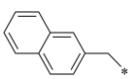
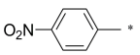
On the basis of above data, we next turn our attention to inserting a methylene spacer between carbon atom at the 3 position and the sterically encumbered β-naphthalene ring in order to increase molecule flexibility. Unfortunately, unsubstituent, small electron-donating methoxy and weak electron-withdrawing fluoro at the para-position of the phenyl ring attached at 6-position have a negative impact on activity (MIC-H37Rv > 8.0 μg/mL). However, compound **6e-3** with 4-nitrophenyl at the C-6 position, is the most active compound of the series (MIC-MDRTB = 4.0 μg/mL; MIC-RDRTB = 4.0 μg/mL). It suggests that a sterically hindered group at the para position of phenyl ring at the C-3 position of triazolothiadiazine moiety has a slight impact on activity.

On the basis of the principle of bioisosterism, aiming to improve the water solubility by introduction of ionizable nitrogen groups, **6f-1−3** have been synthesized whereby the benzene ring at the 3 position is replaced with a pyridine ring. Fortunately, **6f-3** incorporating 4-methoxyphenyl attached at 6-position is emerged as an efficient candidate (MIC-MDRTB = 4.0 μg/mL; MIC-RDRTB = 2.0 μg/mL). Further attempts to introduce fluoro attached at meta position of the pyridine ring (**6g-1−4**). Similar results have been also obtained. The modifications

lead to the identification of an active compound **6g-3** (MIC-MDRTB = 4.0 μg/mL; MIC-RDRTB = 2.0 μg/mL) in the series.

Assessment of the collocation of these data within the SAR is difficult, but an analysis of antimycobacterial data clearly shows that antitubercular activity of the newly synthesized heterocyclic compounds, containing 1,2,4-triazole moiety fused with 1,3,4-thiadiazine ring depends on the substituents rather than the basic skeleton of the molecule. In general, both electronic and steric factors actively modulate the antitubercular activity and the presented triazolothiadiazines are proved to be better antitubercular candidates. And then these data are an important observation to follow up a rational design of more potent antitubercular candidates.

Table 1 Antibacterial activity of triazolothiadiazines **6** against *Mtb* H37Rv strain (MIC, μg/mL)

Compound	R^1	R^2	*Mtb* H37Rv MIC	Compound	R^1	R^2	*Mtb* H37Rv MIC
6a-1			<8	**6d-3**			>8
6a-2			>8	**6d-4**			>8
6a-3			>8	**6e-1**			>8
6a-4			>8	**6e-2**			>8
6b-1			>8	**6e-3**			<8

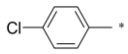
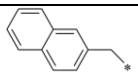
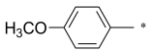
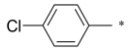
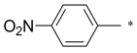
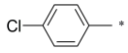
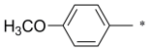
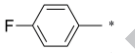
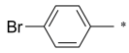
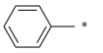
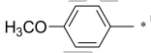
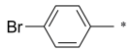
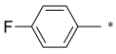
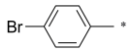
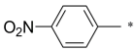
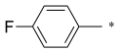
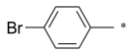
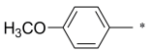
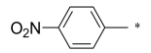
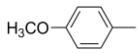
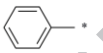
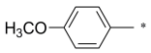
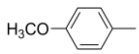
6b-2			>8	**6e-4**			>8
6b-3			8	**6f-1**			8
6b-4			>8	**6f-2**			>8
6c-1			<8	**6f-3**			<8
6c-2			>8	**6g-1**			>8
6c-3			<8	**6g-2**			>8
6c-4			8	**6g-3**			<8
6d-1			>8	**6g-4**			8
6d-2			>8				

Table 2 Antibacterial (MIC) activity against H37Rv, MDRTB and RDRTB and inhibition of *Mt* SD activity of 6 promising triazolothiadiazines

compound	*Mtb* H37Rv MIC(μg/mL)	*Mtb* MDRTB MIC(μg/mL)	*Mtb* RDRTB MIC(μg/mL)	Inhibition of *Mt* SD IC_{50}(μg/mL)
1	8.0	8.0	8.0	23.00±1.18

6a-1	2.0	4.0	2.0	28.38±1.22
6c-1	0.25	2.0	1.0	252.5±2.26
6c-3	0.25	2.0	0.25	86.39±1.21
6e-3	4.0	4.0	4.0	11.68±1.08
6f-3	2.0	4.0	2.0	20.99±1.23
6g-3	1.0	4.0	2.0	73.57±1.62
RFP[a]	0.25	≥16.0	16.0	
INH[b]	0.12	4.0	1.0	

IC_{50} values are indicated as means ± SD of three independent experiments; RFP[a], Rifampin; INH[b], Isoniazid.

3.2. *In vitro Mt* SD inhibitory activity

In order to further demonstrate that this series of compounds actually target *Mt* SD, the 6 potent compounds were selected for further evaluation of their *in vitro Mt* SD inhibitory activity (Table 2). As a consequence, the results account for the extent of inhibitory effect on *Mt* SD correlates to the extent of antitubercular activity. The *Mt* SD inhibitory assay reveals that most of the triazolothiadiazines (barring **6c-1**) possess at least a low to moderate inhibitory property. However, a weak correlation between inhibition of mycobacterial growth and *Mt* SD inhibitory activity is observed. For example, **6c-1** is very efficient in killing mycobacterial cells on a par with the control drug INH, but it displays almost no effect on *Mt* SD. Within this context the potent derivatives, **6c-3**, **6f-3** and **6g-3** reveal potential inhibitory activity on *Mt* SD (**6c-3**, *Mt*

SD-IC_{50} = 86.39 μg/mL; **6f-3**, *Mt* SD-IC_{50} = 20.99 μg/mL; **6g-3**, *Mt* SD-IC_{50} = 73.57 μg/mL). These findings confirm that specific endogenous targets for these triazolothiadiazines are still elusive; however, the results indicate that they may have pleiotropic modes of action and potential to reverse antitubercular resistance. As TB treatment regimens always comprise of a combination of complementary drugs, this off-target effect of the triazolothiadiazines could have a positive impact on the effectiveness of drug treatment regimens. In fact, the potent mycobacterial growth inhibitors showing moderate *Mt* SD inhibitory properties may also prove to be prospective leads in a multidrug therapy owing to synergistic combinations, should that arise. Thus, **6c-3** and **6g-3** could be used in combination with standard antitubercular drugs such isoniazid or rifampin to reverse multidrug resistance in tuberculosis.

3.3. Cytotoxicity

The 4 potent derivatives synthesized were tested to assess their apparent cytotoxicity toward Vero and HepG2 cells (Table 3). In general, the selectivity index (SI), that in this case is the ratio between IC_{50} toward Vero or HepG2 cells and the MIC toward *Mtb* H37Rv, for a compound to be considered a valuable lead has usually to be > 10. As Table 3 shows, we are pleased to notice that most compounds are apparently not toxic (SI>10), while maintaining good activity compared with the hit compound **1**. Unfortunately, modifications of **6e-3** leading to an enhancement of activity, also results in a counterproductive improvement of cytotoxicity. Summarizing, the preliminary investigation around the SAR for these antitubercular triazolothiadiazines leads to the synthesis of more active compounds, meanwhile, devoid of apparent cytotoxicity. Thus, **6c-3** and **6g-3** are emerged as advanced lead compounds for further preclinical drug development.

Table 3 Cytotoxicity (IC_{50} in μM) and SI of 4 promising triazolothiadiazines against Vero, and HepG2 cell lines

compound	*Mtb* H37Rv MIC(μM)	Vero Cytotoxicity IC_{50}(μM)	Vero SI*	HepG2 Cytotoxicity IC_{50}(μM)	HepG2 SI*
1	24.54	71.05±2.53	2.90	46.58±2.28	1.90
6c-1	0.67	58.50±1.11	87.31	234.66±1.56	350.25
6c-3	0.60	13.92±1.18	23.21	10.41±1.66	17.34
6e-3	9.95	69.71±1.23	7.01	72.57±1.21	7.29
6g-3	2.80	354.57±1.56	126.63	165.78±1.58	59.21

SI* is the ratio of cytotoxicity (IC_{50} in μM) to in vitro activity against *M. tuberculosis* H37Rv (ATCC 25618 strain) expressed as MIC in μM.

4. Conclusions

In conclusion, following up the SAR study of triazolothiadiazoles for their antitubercular activities targeting *Mt* SD in our previous study [9], on the principle of scaffold hopping, the preliminary SAR study on triazolothiadiazines for their antitubercular activities against *Mtb* H37Rv, MDRTB and RDRTB strains were performed, building upon the identification of promising early lead compounds **1** obtained from an high-throughput screening campaign. Similarly, the purpose of this SAR study was to optimize the aromatic or hetero-aromatic substituents attached at 3-position and 6-position of triazolothiadiazine through systematic modifications to determine its potential for progression as a drug candidate. It has been found that the nature of the substitute group on the para site of 3-phenyl exerts noteworthy effects on the antitubercular activity. For example, electron-donating methoxy and weak electron-withdrawing chloro groups at the para-position of the phenyl ring attached at 3-position are detrimental to the activity. On the contrary, bromo substituent (electron-withdrawing group) at this position dramatically increases the potency. This breakthrough finding in this SAR study has led to the discovery of **6c-1** and **6c-3** with exceptional potency (MIC-H37Rv = 0.25 μg/mL), which possess a *p*-bromophenyl substituent attached at 3-position along with a phenyl or a *p*-nitrophenyl attached at 6-position of triazolothiadiazine core. Also, 4 other compounds were found to possess comparable potencies (MIC-H37Rv ≤4.0 μg/mL), including **6g-3** (MIC-H37Rv = 1.0 μg/mL), bearing a *p*-nitrophenyl group at the 6 position and a 3-fluoropyridyl group at the 3 position. These advanced lead compounds do not show appreciable cytotoxicity against Vero and HepG2 cells (SI > 10). The advanced leads **6c-3** and **6g-3** exhibit the similar potencies against drug-resistant *Mtb* clinical isolates, as anticipated. Other important findings in this SAR study include the fact that besides **6a-3** and **6d-3**, most compounds bearing 4-nitrophenyl moiety at the 6 position prove to be very potent against *Mtb* H37Rv, MDRTB and RDRTB strains

whereas the introduction of 4-methoxyphenyl attached at 3-position abolishs the activity (MIC > 8.0 μg/mL). *In vitro Mt* SD inhibitory activity by the most potent compounds, demonstrates the extent of inhibitory effect on *Mt* SD correlates to the extent of antitubercular activity. In general, compounds **6c-3** and **6g-3** show a better drug profile in terms of cytotoxicity toward Vero and HepG2 cells and potency against MDRTB and RDRTB clinical isolates, turning out to be two excellent lead candidates for preclinical trials. The pharmacokinetics parameters as well as *in vivo* efficacy of **6c-3** and **6g-3** will be evaluated in due course in order to prove their efficacy and to progress toward preclinical trials.

5. Experiments

Methods and materials

Methods. ^{1}H and ^{13}C NMR spectra were recorded on Bruker or Varian 400 or 500 MHz NMR spectrometers operating at the frequencies indicated. Chemical shifts (δ) are in ppm, referenced to tetramethylsilane. Coupling constants (J) are reported in hertz and rounded to 0.5 Hz. Splitting patterns are abbreviated as follows: singlet (s), doublet (d), triplet (t), quartet (q), multiplet (m), broad (br) or some combination of them. Melting points were measured on a Mettler Toledo capillary melting point apparatus and are uncorrected. Reactions were monitored by thin-layer chromatography (TLC, silica gel 60 F254) and spots were observed by UV-lamp. APCI high-resolution mass spectra (HRMS) was recorded on an Autospec Ultima-TOF spectrometer. MB/BacT ALERT 3D system, which includes a computerized database management system. Carbon dioxide released into the medium by actively growing mycobacteria is detected through a gas-permeable sensor containing a colorimetric indicator embedded at the bottom of culture vials. Ensprire^{TM} enzyme-labelling measuring instrument (Perkin Elmer) was used in vitro *Mt SD* inhibitory activity and cell cytotoxicity experiments.

Materials. Modified Middlebrook 7H9 broth, MB reconstituting fluid, and the buffers were purchased from Biomerieux. RFP and INH were purchased from National Institute for Food and Drug Control.

5.1. Synthetic procedure and analytical data of aryl or hetero-aryl acid hydrazides 3a-g, potassium dithiocarbazinate 4a-g and 4-amino-5-substituted -3-mercapto-1,2,4-triazoles 5a-g

Hydrazides **3a-g**, potassium dithiocarbazinate **4a-g** and 1,2,4-triazoles **5a-g** were synthesized as described in the literature. [9]

5.2. Synthetic procedure and analytical data of 3,6-disubstituted triazolothiadiazines (6a-g)

5.2.1. 3,6-biphenyl-7H-[1,2,4]triazolo[3,4-b][1,3,4]thiadiazine (6a-1). A mixture of **5a** (0.05 g, 0.26 mmol) and 2-bromoacetophenone (0.057 g, 0.29 mmol) were dissolved in absolute alcohol (2.5 mL), and the resulting mixture was heated at 95 °C in the microwave for 30 min. The mixture was allowed to stand overnight, separated solid was filtered, washed thoroughly with cold alcohol and ether, and dried to obtain **6a-1** (0.071 g, 93% yield) as a white solid: mp 226 – 227 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.04 – 7.99 (m, 4H), 7.64 – 7.55 (m, 6H), 4.46 (s, 2H); ^{13}C NMR (101 MHz, DMSO-d_6) δ156.05, 151.65, 142.61, 133.47, 131.97, 130.24, 129.15, 128.78, 127.93, 127.56, 125.97, 22.76. HRMS (APCI) m/z calcd. for $C_{16}H_{12}N_4SH^+$: 293.0855. Found: 293.0846.

The same procedure was followed for the synthesis of **6a-2−4**, **6b-1−4**, **6c-1−4**, **6d-1−4**, **6e-1−4**, **6f-1−3** and **6g-1−4**.

5.2.2. 6-(4-Fluorophenyl)-3-phenyl-7H-[1,2,4]triazolo[3,4-b][1,3,4]thiadiazine (6a-2). White solid (95% yield); mp 244 – 245 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.12 – 8.06 (m, 2H), 8.05 – 7.96 (m, 2H), 7.62 – 7.54 (m, 3H), 7.49 – 7.40 (m, 2H), 4.45 (s, 2H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 165.33, 154.82, 151.45, 142.16, 130.03, 129.96, 128.50, 127.73, 125.83, 116.11, 115.89, 22.66. HRMS (APCI) m/z calcd. for $C_{16}H_{11}FN_4SH^+$: 311.0761. Found: 311.0763.

5.2.3. 6-(4-Nitrophenyl)-3-phenyl-7H-[1,2,4]triazolo[3,4-b][1,3,4]thiadiazine (6a-3). Light yellow powder (91% yield); mp 247 – 248 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.41 (dd, J = 6.8, 2.0 Hz, 2H), 8.25 (dd, J = 6.8, 2.0 Hz, 2H), 8.01 – 7.98 (m, 2H), 7.62 – 7.55 (m, 3H), 4.52

(s, 2H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 154.39, 151.88, 149.16, 142.47, 139.41, 130.40, 128.97, 128.84, 128.03, 125.73, 124.16, 22.89. HRMS (APCI) m/z calcd. for $C_{16}H_{11}N_5O_2SH^+$: 338.0706. Found: 338.0713.

5.2.4. 6-(4-Methoxyphenyl)-3-phenyl-7H-[1,2,4]triazolo[3,4-b][1,3,4]thiadiazine (6a-4). White solid (96% yield); mp 240 – 241 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.07 – 7.94 (m, 4H), 7.65 – 7.52 (m, 3H), 7.14 (d, J = 8.8 Hz, 2H), 4.43 (s, 2H), 3.86 (s, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 162.35, 155.71, 151.37, 142.71, 130.26, 129.48, 128.77, 127.91, 125.91, 125.44, 114.61, 55.59, 22.53. HRMS (APCI) m/z calcd. for $C_{17}H_{14}N_4OSH^+$: 323.0961. Found: 323.0975.

5.2.5 3-(4-Chlorophenyl)-6-phenyl-7H-[1,2,4]triazolo[3,4-b][1,3,4]thiadiazine (6b-1). White solid (97% yield); mp 273 – 274 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.03 (m, 4H), 7.68 (d, J = 8.4 Hz, 2H), 7.65 – 7.56 (m, 3H), 4.46 (s, 2H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 156.26, 150.75, 142.90, 135.04, 133.37, 132.06, 129.60, 129.17, 128.99, 127.64, 124.80, 22.78. HRMS (APCI) m/z calcd. for $C_{16}H_{11}ClN_4SH^+$: 327.0466. Found: 327.0469.

5.2.6. 3-(4-Chlorophenyl)-6-(4-fluorophenyl)-7H-[1,2,4]triazolo[3,4-b][1,3,4]thiadiazine (6b-2). White solid (95% yield); mp 284 – 285 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.09 (dd, J = 8.8, 5.2 Hz, 2H), 8.03 (dd, J = 6.4, 2.0 Hz, 2H), 7.67 (dd, J = 6.8, 2.0 Hz, 2H), 7.46 – 7.42 (m, 2H), 4.45 (s, 2H). HRMS (APCI) m/z calcd. for $C_{16}H_{10}ClFN_4SH^+$: 345.0371. Found: 345.0368.

5.2.7 3-(4-Chlorophenyl)-6-(4-nitrophenyl)-7H-[1,2,4]triazolo[3,4-b][1,3,4]thiadiazine (6b-3). Light yellow powder (99% yield); mp 259 – 260 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.40 (dd, J = 6.8, 2.0 Hz, 2H), 8.26 (dd, J = 6.8, 2.0 Hz, 2H), 8.02 (dd, J = 6.4, 2.0 Hz, 2H), 7.68 (dd, J = 6.8, 2.0 Hz, 2H), 4.53 (s, 2H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 154.58, 150.98, 149.19,

142.76, 139.28, 135.21, 129.73, 129.06, 129.04, 124.53, 124.14, 22.93. HRMS (APCI) m/z calcd. for $C_{16}H_{10}ClN_5O_2SH^+$: 372.0316. Found: 372.0301.

5.2.8. 3-(4-Chlorophenyl)-6-(4-methoxyphenyl)-7H-[1,2,4]triazolo[3,4-b][1,3,4]thiadiazine (6b-4). White solid (90% yield); mp 247 – 248 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.05 (dd, *J* = 6.8, 2.0 Hz, 2H), 8.00 (dd, *J* = 6.8, 2.0 Hz, 2H), 7.67 (dd, *J* = 6.8, 2.0 Hz, 2H), 7.13 (dd, *J* = 7.2, 2.0 Hz, 2H), 4.42 (s, 2H), 3.86 (s, 3H). HRMS (APCI) m/z calcd. for $C_{17}H_{13}ClN_4OSH^+$: 357.0571. Found: 357.0561.

5.2.9. 3-(4-Bromophenyl)-6-phenyl-7H-[1,2,4]triazolo[3,4-b][1,3,4]thiadiazine (6c-1). White solid (98% yield); mp 266 – 267 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.04 – 8.00 (m, 2H), 7.97 (dd, *J* = 6.8, 2.0 Hz, 2H), 7.81(dd, *J* = 6.8, 2.0 Hz, 2H), 7.65 – 7.56 (m, 3H), 4.46 (s, 2H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 156.25, 150.83, 142.93, 133.35, 132.05, 131.90, 129.78, 129.16, 127.64, 125.14, 123.86, 22.76. HRMS (APCI) m/z calcd. for $C_{16}H_{11}BrN_4SH^+$: 372.9940. Found: 372.9936.

5.2.10. 3-(4-Bromophenyl)-6-(4-fluorophenyl)-7H-[1,2,4]triazolo[3,4-b][1,3,4]thiadiazine (6c-2). White solid (97% yield); mp 269 – 270 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.09 (dd, *J* = 8.8, 5.2 Hz, 2H), 7.95 (dd, *J* = 6.8, 2.0 Hz, 2H), 7.81 (dd, *J* = 6.8, 2.0 Hz, 2H), 7.44 (t, *J* = 8.8 Hz, 2H), 4.45 (s, 2H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 155.28, 150.84, 142.84, 131.89, 130.38, 130.29, 129.81, 125.10, 123.86, 116.38, 116.16, 22.76. HRMS (APCI) m/z calcd. For $C_{16}H_{10}BrFN_4SH^+$: 390.9846. Found: 390.9852.

5.2.11. 3-(4-Bromophenyl)-6-(4-nitrophenyl)-7H-[1,2,4]triazolo[3,4-b][1,3,4]thiadiazine (6c-3). Light yellow powder (96% yield); mp 254 – 255 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.40 (dd, *J* = 7.2, 2.0 Hz, 2H), 8.26 (dd, *J* = 6.8, 2.0 Hz, 2H), 7.95 (dd, *J* = 6.8, 2.0 Hz, 2H), 7.81 (dd,

J = 6.8, 2.0 Hz, 2H), 4.52 (s, 2H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 154.58, 151.09, 149.20, 142.80, 139.28, 131.96, 129.92, 129.07, 124.89, 124.15, 124.05, 22.91. HRMS (APCI) m/z calcd. for $C_{16}H_{10}BrN_5O_2SH^+$: 417.9792. Found: 417.9794.

5.2.12. 3-(4-Bromophenyl)-6-(4-methoxyphenyl)-7H-[1,2,4]triazolo[3,4-b][1,3,4]thiadiazine (6c-4). White solid (99% yield); mp 253 – 254 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.02 – 7.95 (m, 4H), 7.81 (dd, *J* = 6.8, 2.0 Hz, 2H), 7.13 (dd, *J* = 6.8, 2.0 Hz, 2H), 4.42 (s, 2H), 3.86 (s, 3H). ^{13}C NMR (101 MHz, DMSO-d_6)) δ 162.36, 155.74, 150.63, 142.90, 131.86, 129.72, 129.55, 125.37, 125.26, 123.74, 114.59, 55.58, 22.51. HRMS (APCI) m/z calcd. for $C_{17}H_{13}BrN_4OSH^+$: 403.0046. Found: 403.0044.

5.2.13. 3-(4-Methoxyphenyl)-6-phenyl-7H-[1,2,4]triazolo[3,4-b][1,3,4]thiadiazine (6d-1). White solid (91% yield); mp 202 – 203 °C; ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.03 – 8.00 (m, 2H), 7.98 (dd, *J* = 7.0, 2.0 Hz, 2H), 7.64 – 7.57 (m, 3H), 7.14 (dd, *J* = 7.0, 2.0 Hz, 2H), 4.44 (s, 2H), 3.84 (s, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 160.73, 155.85, 151.48, 142.01, 133.55, 131.91, 129.46, 129.14, 127.53, 118.36, 114.26, 55.36, 22.72. HRMS (APCI) m/z calcd. for $C_{17}H_{14}N_4OSH^+$: 323.0961. Found: 323.0982.

5.2.14. 6-(4-Fluorophenyl)-3-(4-methoxyphenyl)-7H-[1,2,4]triazolo[3,4-b][1,3,4]thiadiazine (6d-2). White solid (93% yield); mp 242 – 243 °C; ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.08 (dd, *J* = 9.0, 5.0 Hz, 2H), 7.96 (dd, *J* = 6.5, 2.0 Hz, 2H), 7.43 (dd, *J* = 9.0, 2.0 Hz, 2H), 7.14 (dd, *J* = 7.0, 2.0 Hz, 2H), 4.43 (s, 2H), 3.84 (s, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 160.74, 154.90, 151.48, 141.93, 130.25, 130.16, 129.48, 118.33, 116.37, 116.16, 114.27, 55.36, 22.72. HRMS (APCI) m/z calcd. for $C_{17}H_{13}FN_4OSH^+$: 341.0867. Found: 341.0865.

5.2.15. 3-(4-Methoxyphenyl)-6-(4-nitrophenyl)-7H-[1,2,4]triazolo[3,4-b][1,3,4]thiadiazine (6d-3). Light yellow powder (95% yield); mp 237 – 238 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.41 (dd, J = 7.2, 2.0 Hz, 2H), 8.25 (dd, J = 6.8, 2.0 Hz, 2H), 7.95 (dd, J = 6.8, 2.0 Hz, 2H), 7.15 (dd, J = 6.8, 2.0 Hz, 2H), 4.51 (s, 2H), 3.85 (s, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 160.89, 154.28, 151.71, 149.14, 141.94, 139.47, 129.64, 128.97, 124.16, 118.00, 114.35, 55.40, 22.86. HRMS (APCI) m/z calcd. for $C_{17}H_{13}N_5O_3SH^+$: 368.0812. Found: 368.0805.

5.2.16. 3,6-Bis(4-methoxyphenyl)-7H-[1,2,4]triazolo[3,4-b][1,3,4]thiadiazine (6d-4). White solid (91% yield); mp 228 – 229 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.03 – 7.95 (m, 4H), 7.17 – 7.11 (m, 4H), 4.41 (s, 2H), 3.86 (s, 3H), 3.85 (s, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 162.32, 160.80, 155.62, 151.17, 142.20, 129.50, 129.47, 125.50, 118.15, 114.60, 114.28, 55.59, 55.39, 22.48. HRMS (APCI) m/z calcd. for $C_{18}H_{16}N_4O_2SH^+$: 353.1067. Found: 353.1077.

5.2.17. 3-(β-naphthylmethyl)-6-phenyl-7H-[1,2,4]triazolo[3,4-b][1,3,4]thiadiazine (6e-1). White solid (93% yield); mp 230 – 231 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.30 (d, J = 8.0 Hz, 1H), 8.00 – 7.92 (m, 3H), 7.85 (d, J = 7.6 Hz, 1H), 7.63 – 7.53 (m, 5H), 7.52 – 7.44 (m, 2H), 4.75 (s, 2H), 4.39 (s, 2H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 154.76, 152.35, 140.48, 133.43, 131.96, 131.93, 131.50, 129.04, 128.49, 127.59, 127.51, 127.46, 126.25, 125.83, 125.53, 124.10, 27.76, 22.76. HRMS (APCI) m/z calcd. for $C_{21}H_{16}N_4SH^+$: 357.1168. Found: 357.1155.

5.2.18. 6-(4-Fluorophenyl)-3-(β-naphthylmethyl)-7H-[1,2,4]triazolo[3,4-b][1,3,4]thiadiazine (6e-2). White solid (91% yield); mp 223 – 224 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.29 (d, J = 8.0 Hz, 1H), 8.07 – 8.02 (m, 2H), 7.96 – 7.91 (m, 1H), 7.85 (dd, J = 7.2, 2.0 Hz, 1H), 7.59 – 7.51 (m, 2H), 7.50 – 7.39 (m, 4H), 4.74 (s, 2H), 4.38 (s, 2H). HRMS (APCI) m/z calcd. For $C_{21}H_{15}FN_4SH^+$: 375.1074. Found: 375.1072.

5.2.19. 3-(β-Naphthylmethyl)-6-(4-nitrophenyl)-7H-[1,2,4]triazolo[3,4-b][1,3,4]thiadiazine (6e-3). Light yellow powder (92% yield); mp 235 – 236 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.39 (dd, *J* = 7.2, 2.0 Hz, 2H), 8.29 (d, *J* = 8.0 Hz, 1H), 8.21 (dd, *J* = 6.8, 2.0 Hz, 2H), 7.97 – 7.93 (m, 1H), 7.86 (d, *J* = 7.6 Hz, 1H), 7.60 – 7.46 (m, 4H), 4.76 (s, 2H), 4.46 (s, 2H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 152.97, 152.57, 149.12, 140.31, 139.38, 133.44, 131.81, 131.50, 128.83, 128.51, 127.65, 127.63, 126.29, 125.85, 125.58, 124.10, 124.05, 27.81, 22.82. HRMS (APCI) m/z calcd. for $C_{21}H_{15}N_5O_2SH^+$: 402.1019. Found: 402.1010.

5.2.20. 6-(4-Methoxyphenyl)-3-(β-naphthylmethyl)-7H-[1,2,4]triazolo[3,4-b][1,3,4]thiadiazine (6e-4). White solid (95% yield); mp 217 – 218 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.29 (d, *J* = 8.0 Hz, 1H), 7.98-7.42 (m, 3H), 7.86 (d, *J* = 7.6 Hz, 1H), 7.61 – 7.53 (m, 2H), 7.52 – 7.44 (m, 2H), 7.11 (d, *J* = 8.8 Hz, 2H), 4.76 (s, 2H), 4.37 (s, 2H), 3.85 (s, 3H). HRMS (APCI) m/z calcd. for $C_{22}H_{18}N_4OSH^+$: 387.1274. Found: 387.1281.

5.2.21. 6-Phenyl-3-(pyridin-4-yl)-7H-[1,2,4]triazolo[3,4-b][1,3,4]thiadiazine (6f-1). Light yellow powder (92% yield); mp 275 – 276 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.06 – 9.00 (m, 2H), 8.51 – 8.40 (m, 2H), 8.12 – 8.07 (m, 2H), 7.70 – 7.61 (m, 3H), 4.53 (s, 2H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 157.13, 155.75, 148.37, 145.52, 145.33, 133.02, 132.44, 129.24, 127.93, 123.13, 23.00. HRMS (APCI) m/z calcd. for $C_{15}H_{11}N_5SH^+$: 294.0808. Found: 294.0807.

5.2.22. 6-(4-Fluorophenyl)-3-(pyridin-4-yl)-7H-[1,2,4]triazolo[3,4-b][1,3,4]thiadiazine (6f-2). Light yellow powder (90% yield); mp 275 – 276 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.99 (d, *J* = 5.2 Hz, 2H), 8.39 (d, *J* = 4.8 Hz, 2H), 8.17 (dd, *J* = 8.8, 5.2 Hz, 2H), 7.47 (t, *J* = 8.8 Hz, 2H), 4.51 (s, 2H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 165.78, 163.28, 156.09, 148.48, 145.93, 145.12,

137.68, 130.71, 130.62, 129.60, 129.57, 123.02, 116.47, 116.25, 22.99. HRMS (APCI) m/z calcd. for $C_{15}H_{10}FN_5SH^+$: 312.0713. Found: 312.0707.

5.2.23. 6-(4-Methoxyphenyl)-3-(pyridin-4-yl)-7H-[1,2,4]triazolo[3,4-b][1,3,4]thiadiazine (6f-3). Light yellow powder (99% yield); mp 276 – 277 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.02 (dd, J = 5.6, 1.6 Hz, 2H), 8.45 (d, J = 6.4 Hz, 2H), 8.08 (dd, J = 6.8, 2.0 Hz, 2H), 7.16 (dd, J = 7.2, 2.0 Hz, 2H, 4.48 (s, 2H), 3.88 (s, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 162.69, 156.60, 148.14, 145.50, 145.32, 138.25, 129.93, 124.98, 123.03, 114.67, 55.67, 22.70. HRMS (APCI) m/z calcd. for $C_{16}H_{13}N_5OSH^+$: 324.0914. Found: 324.0921.

5.2.24. 3-(3-Fluoropyridin-4-yl)-6-phenyl-7H-[1,2,4]triazolo[3,4-b][1,3,4]thiadiazine (6g-1). Light yellow powder (90% yield); mp 223 – 224 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.87 (d, J = 2.0 Hz, 1H), 8.69 (d, J = 4.8 Hz, 1H), 7.97 – 7.94 (m, 2H), 7.89 – 7.84 (m, 1H), 7.65 – 7.60 (m, 1H), 7.59 – 7.54 (m, 2H), 4.52 (s, 2H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 156.26, 154.24, 146.32, 146.27, 143.74, 139.39, 139.16, 133.11, 132.20, 129.15, 127.61, 124.65, 121.24, 121.13, 23.15. HRMS (APCI) m/z calcd. for $C_{15}H_{10}FN_5SH^+$: 312.0713. Found: 312.0705.

5.2.25. 6-(4-Fluorophenyl)-3-(3-fluoropyridin-4-yl)-7H-[1,2,4]triazolo[3,4-b][1,3,4]thiadiazine (6g-2). Light yellow powder (92% yield); mp 226 – 227 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.87 (d, J = 2.0 Hz, 1H), 8.68 (d, J = 4.8 Hz, 1H), 8.03 (dd, J = 8.8, 5.2 Hz, 2H), 7.90 – 7.82 (m, 1H), 7.45 – 7.39 (m, 2H), 4.51 (s, 2H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 155.27, 154.22, 146.32, 146.26, 143.63, 139.38, 139.15, 130.36, 130.27, 129.65, 124.66, 121.21, 116.41, 116.19, 23.13. HRMS (APCI) m/z calcd. for $C_{15}H_9F_2N_5SH^+$: 330.0619. Found: 330.0612.

5.2.26. 3-(3-Fluoropyridin-4-yl)-6-(4-nitrophenyl)-7H-[1,2,4]triazolo[3,4-b][1,3,4]thiadiazine (6g-3). Light yellow powder (94% yield); mp 193 – 194 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.87 (s, 1H), 8.69 (d, *J* = 4.4 Hz, 1H), 8.39 (d, *J* = 8.8 Hz, 2H), 8.19 (d, *J* = 8.8 Hz, 2H), 7.88 – 7.86 (m, 1H), 4.58 (s, 2H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 154.57, 154.21, 149.28, 146.34, 143.62, 139.42, 139.18, 139.01, 129.03, 124.68, 124.15, 121.03, 23.27. HRMS (APCI) m/z calcd. for $C_{15}H_9FN_6O_2SH^+$: 357.0564. Found: 357.0548.

5.2.27. 3-(3-Fluoropyridin-4-yl)-6-(4-methoxyphenyl)-7H-[1,2,4]triazolo[3,4-b][1,3,4]thiadiazine (6g-4). Light yellow powder (92% yield); mp 215 – 216 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.92 (d, *J* = 2.0 Hz, 1H), 8.74 (dd, *J* = 5.2, 1.2 Hz, 1H), 7.99 (dd, *J* = 6.8, 2.0 Hz, 2H), 7.92 (dd, *J* = 6.0, 5.2 Hz, 1H), 7.17 (dd, *J* = 6.8, 2.0 Hz, 2H), 4.53 (s, 2H), 3.90 (s, 3H). HRMS (APCI) m/z calcd. for $C_{16}H_{12}FN_5OS\ H^+$: 342.0819. Found: 342.0809.

5.3. Biological assays

Bacterial strains and growth

H37Rv and clinical *Mtb* strains MDRTB and RDRTB exhibiting resistant profiles to Isoniazid and Rifampin resistant strains were used. For evaluation of drug sensitivity all strains were grown in Difco 7H9 Middlebrook liquid medium (BD Biosciences, 271310) supplemented with casein, bovine albumen and catalase at 37 °C. The growth of *Mycobacterium tuberculosis* (≤36 hours, subculture, then the growth was diluted 1:1 in sterile distilled water) formed the direct growth control of approximately Mc Farland no.2 (DGC).

5.3.1. Antibacterial Activity assay

Antibacterial Activity assay was used as described previously [9] to assess the minimum inhibitory concentrations of the compounds on *M. tuberculosis* H37Rv, and MDRTB and RDRTB. Briefly, the assay was conducted in a The BacT/Alert MP bottle format. MIC of the compounds was determined by MB/BacT in triplicate. All the synthesized compounds, isonizaid and rifampcin were solubilized in DMSO to the working solution (2,560 µg/mL) and serially diluted (2-fold dilutions). Working solution (2,560 µg/mL) was added aseptically to 0.5 mL of reconstitution fluid (Tween 80, glycerol and amaranth), 0.5 mL DGC and MB Bact medium to achieve the required concentration (16, 8, 4, 2, 1, 0.5, 0.25, 0.12, 0.06 µg/mL) in a final volume of 10 mL each BacT/Alert MP bottle. To 9 mL of MB Bact medium, 0.5 mL of reconstitution fluid and 0.5 mL DGC were added to a bottle and this was the positive control. To 9.5 mL of MB Bact medium, 0.5 mL of reconstitution fluid was inoculated into another bottle formed the negative control. These bottles were incubated in the system at 35 °C for 8-9 days and monitored to detect growth.

The compound was considered as ineffective if the bottles containing it flagged positive at the same time or before the positive control. The compound was considered effective if the bottle containing it remained negative during the test period or flagged positive 2 days after the positive control. If the positive control did not flag positive in 12 days the test was invalidated and had to be repeated.

5.3.2. *In vitro Mt* SD inhibitory assay

Mtb SD Protein Preparation was carried out as described [27]. *Mt* SD inhibitory assay was carried out as described previously [9]. All spectrophotometric assays were performed at 25 °C, and the increase in NADPH was monitored at 340 nm. Briefly, the assays were conducted in a

final volume of 100 µL, containing the following components: 100 mM Tris HCl, pH 9.0, 1 mM shikimic acid, 0.5 mM $NADP^+$ and 25 U/100 µL *Mt* SD. All of the components except for the SD enzyme were premixed in a reservoir and dispensed. The reaction mixture was incubated at 25 °C for 5 min to reach a stable background. The SD enzyme was added in the end to trigger the reaction as described [8]. Stock solutions of all compounds were prepared in DMSO such that the final concentration of this co-solvent was constant at 1% v/v in a final volume 100 µL for all kinetic reactions. Negative control reactions were carried out with the same conditions as described above but without inhibitor. The inhibitory activity of each derivative was expressed as the percentage inhibition of *Mt* SD activity with respect to the negative control reaction without inhibitor. All activity assays were performed in triplicate.

5.3.3. Cytotoxicity assay

The cytotoxicity of the compounds was tested against Vero and HepG2 cells. Vero and HepG2 cells were grown without CO_2 in L15 medium supplemented with antibiotics and heat inactivated calf serum. Serial 2-fold dilutions of the drugs were performed in the 96-well microplates. The Vero and HepG2 cells, in medium containing 2× Alamar Blue, were added to the wells to a final concentration of 1.3×10^4 cells per well. The plates were incubated for 3 days at 37 °C. The IC_{50} was calculated according to manufacturer directions.

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Highlights

- Antitubercular 3,6-disubstituted triazolothiadiazines were designed and synthesized.
- Preliminary SAR of 3,6-disubstituted triazolothiadiazines was described.
- Compounds **6c-3** and **6g-3** show exceptional antitubercular potency and no cytotoxicity.
- Compounds **6c-3** and **6g-3** may be promising multidrug-resistance-reversing agents.

Graphical abstract