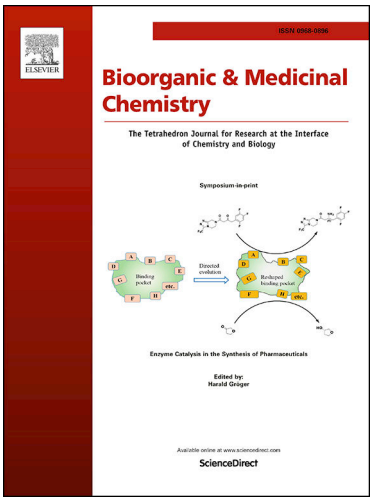
Journal Pre-proofs



Discovery of Thienothiazolocarboxamide Analogues as Novel Anti-tubercular Agent

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Discovery of Thienothiazolocarboxamide Analogues as Novel Anti-tubercular Agent

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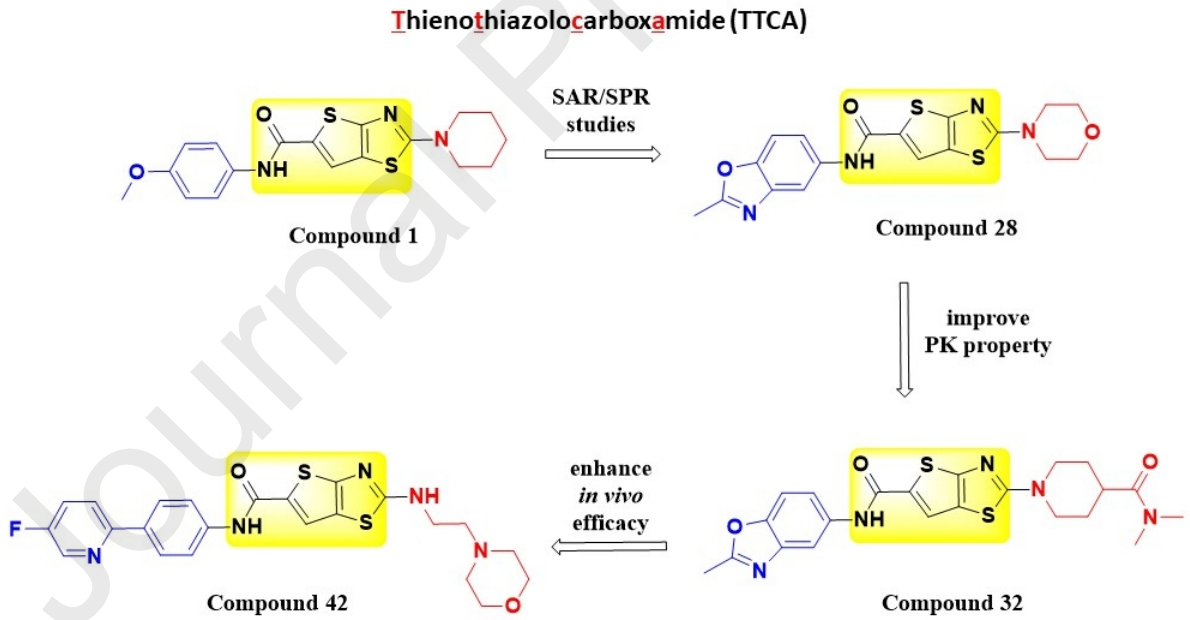
Highlights

- Thienothiazolocarboxamide analogues were prepared and evaluated as anti-tubercular agents.
- Compound **42** showed activities against *M. tuberculosis* H37Rv replicating outside and inside macrophages (extracellular IC_{50} = 0.76 μM, intracellular IC_{50} = 0.19 μM).
- Compound **42** showed favorable *in vivo* PK profile and efficacy in a murine model of tuberculosis which proposed it as a potential lead compound for further evaluation.

ABSTRACT

In order to identify anti-tubercular agents with a novel scaffold, commercial libraries of small organic compounds were screened against a fluorescent strain of *Mycobacterium tuberculosis* H37Rv, using a dual phenotypic assay. Compounds were assessed against bacteria replicating in broth medium, as well as inside macrophages, and thienothiazolocarboxamide (TTCA) scaffold was identified as hit in both assays, with submicromolar inhibitory concentrations. Derivatives of TTCA were further synthesized and evaluated for their inhibitory effects on *M. tuberculosis* H37Rv. In the present study we report the structure-activity relationship of these TTCA derivatives. Compounds **28**, **32** and **42** displayed good anti-tubercular activities, as well as favorable ADME and PK properties. Compound **42** exhibited excellent oral bioavailability in mice with high distribution to lungs, within 1 hour. It was found to be efficacious in a dose dependent manner in a murine model of *M. tuberculosis* infection. Hence, compound **42** is now under evaluation as a potential lead candidate for treatment of tuberculosis.

Graphical abstract



Keywords: Tuberculosis (TB); Thienothiazolocarboxamide (TTCA); Structure-activity relationship (SAR); Structure-property relationship (SPR); Pharmacokinetics (PK); *in vivo* efficacy.

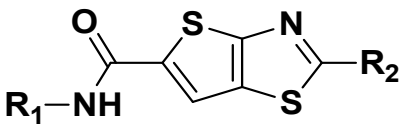
1. Introduction

Tuberculosis (TB), mostly caused by *Mycobacterium tuberculosis* in humans, remains one of the top 10 causes of death worldwide and is the leading cause of death from a single infectious agent [1]. TB typically affects the lungs, but may also affect other parts of the body. According to the WHO report published in 2019, 10 million people fell ill with TB in 2018, while nearly 1.5 million people died from the disease, including about 250,000 patients co-infected with the human immunodeficiency virus (HIV) [2]. The current standard regimen against TB involves a combination of four drugs, isoniazid (INH), rifampicin (RIF), pyrazinamide (PZA) and ethambutol (EMB) [3]. Treatment of an infected individual requires at least six months of chemotherapy. The emergence of drug-resistant strains, including multidrug-resistant (MDR) and extremely drug-resistant (XDR) strains, threatens the goal of TB elimination worldwide [4]. During the last decade, several compounds reached pre-clinical and clinical trials, and three new drugs – bedaquiline, delamanid, and pretomanid – have recently been approved [5–7]. However, treatment for drug-resistant TB consists in lengthy regimens with drugs that are more expensive and more toxic [2]. Therefore, there is an urgent need for new therapeutic strategies that could not only shorten the treatment regimen but also help control the spread of drug-resistant forms of the disease.

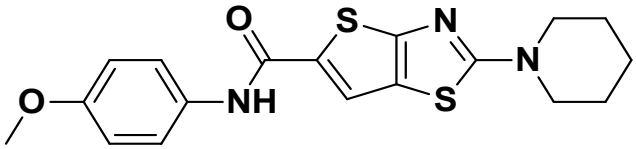
In order to search for novel chemotypes to be developed as potential anti-tubercular agents, a library of 30,270 small molecules was subjected to high-throughput screening using a conventional phenotypic approach featuring *M. tuberculosis* strain H37Rv, constitutively expressing a green fluorescent protein (GFP), replicating in broth medium (extracellular assay). In parallel, compounds were also tested against the same mycobacterial strain replicating inside macrophages, or intracellular assay [8,9]. Among the hit compounds for this dual screening, compound **1**, containing thienothiazolocarboxamide (TTCA) scaffold (**Fig 1A**), exhibited submicromolar inhibitory concentrations with IC_{50} values less than 1 μM in both extracellular and intracellular assays. This promising scaffold, which is structurally different from known anti-tubercular drugs (**Fig 1B**), was further investigated through chemical modifications focused on R1 and R2 position. Compounds from this TTCA series showed higher potency against *M. tuberculosis* replicating within macrophages, as compared to broth culture medium. Herein, we describe the synthesis, structure-activity relationship (SAR) and structure-property relationship (SPR) analysis of these TTCA compounds, along with their *in vitro* biological

evaluation. Selected compounds with good pharmacokinetics (PK) profiles were tested for *in vivo* efficacy in a murine mode of TB.

A.



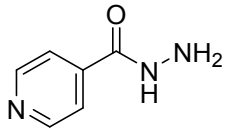
TTCA



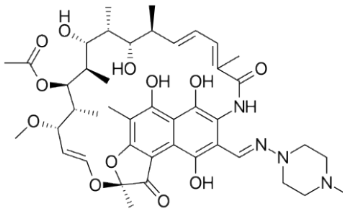
1

B.

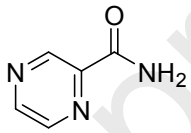
First line



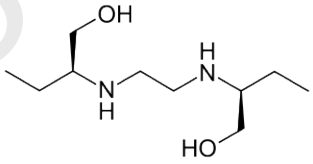
Isoniazid (INH, H)



Rifampicin (RIF, R)

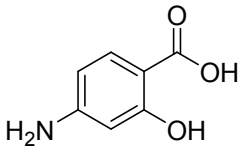


Pyrazinamide (PZA, Z)

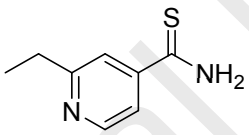


Ethambutol (EMB, E)

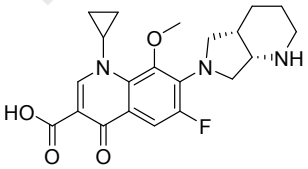
Second line



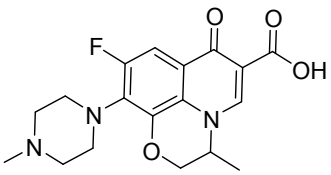
p-aminosalicylic acid (PAS)



Ethionamide (ETH or Eto)

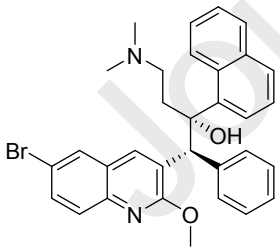


Moxifloxacin (MXF, Mox or Mfx)

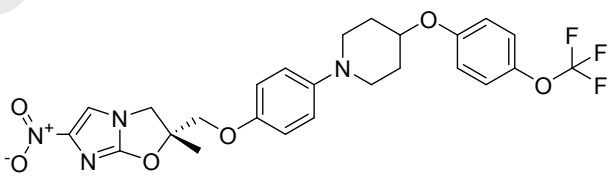


Ofloxacin (Ofx)

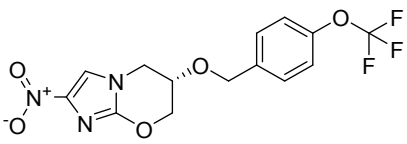
Recently approved



Bedaquiline (BDQ)



Delamanid



Pretomanid

Fig. 1. A. Generic structure of the thienothiazolocarboxamide (TTCA) scaffold (left) and chemical structure of initial hit compound **1** (right). B. Structures of representative first and second line as well as recently approved anti-tubercular drugs.

2. Chemistry

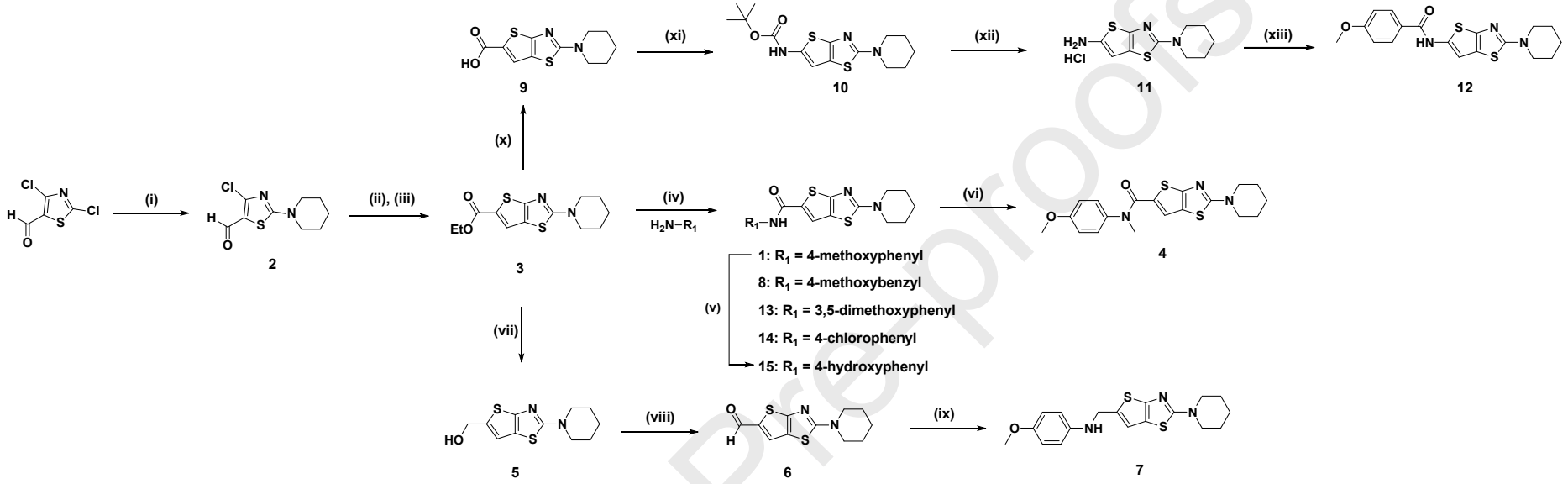
Analogues of the hit compound **1** with modified linkers were synthesized as outlined in **Scheme 1**. The commercially available 2,4-dichlorothiazole-5-carbaldehyde was alkylated with piperidine in the presence of trimethylamine (TEA) in acetonitrile to afford compound **2**. Ethyl thioglycolate was alkylated with **2** followed by cyclization in the presence of TEA in DMSO and heating. The key intermediate **3** was finally obtained after reaction with sodium ethoxide. The ester **3** was further coupled with *p*-anisidine in the presence of trimethylaluminum in toluene to provide hit compound **1**. *N*-methylated compound **4** was obtained by treating compound **1** with sodium hydride and iodomethane. Ester reduction of compound **3** using lithium aluminium hydride afforded the primary alcohol **5**, which was in turn oxidized by Dess-Martin periodinane to afford aldehyde **6**. Reductive amination using $NaBH(OAc)_2$ with *p*-anisidine finally afforded the amine linker compound **7**. Compound **8** was prepared from the ester **3** by coupling with 4-methoxybenzylamine. Hydrolysis of ester intermediate **3** gave the carboxylic acid **9**. A Curtius rearrangement was used to directly convert **9** into carbamate **10**, involving diphenylphosphoryl azide (DPPA) and TEA in *tert*-butanol. Deprotection with Conc. HCl afforded amine **11**, which was coupled with 4-methylbenzoic acid in the presence of EDCi, HOBt and TEA to obtain the reversed amide linker compound **12**. Additionally, the ester intermediate **3** was coupled with 3,5-dimethoxyaniline or 4-chloroaniline to give compounds **13** and **14**, respectively. Finally, compound **1** was further demethylated by BBr_3 in THF to yield compound **15**.

As shown in **Scheme 2**, compounds **18-23** were synthesized using methods similar to those described in **Scheme 1**. Commercially available amines were alkylated with 2,4-dichlorothiazole-5-carbaldehyde to give compounds **16a-e**, which then afforded **17a-e** through alkylation and cyclization. Amines selected included *N*-boc-piperazine, morpholine, 1-methylpiperazine, 4-difluoropiperidine and dimethylamine. Compounds **17a-e** were further coupled with *p*-anisidine to provide compounds **18, 19, 21, 22** and **23**. Compound **20** was prepared by de-protection of compound **18**.

The synthesis of amine **25** and TTCA analogues **28-35** is described in **Scheme 3**. *N*-boc-piperidine-4-carboxylic acid was coupled with dimethylamine in the presence of EDCi, HOBt and TEA to produce amide **24**, subsequently de-protected in TFA to give amine **25**. Using the same synthetic route as described in **Scheme 2**, 2,4-dichlorothiazole-5-carbaldehyde was coupled with various amines (including **25**) to provide intermediate aldehydes **26a-g**, which were converted into esters **27a-g**. The 2-methylbenzo[*d*]oxazol-5-amine was coupled with **17b**, **27a-g** to provide the desired compounds **28-35**.

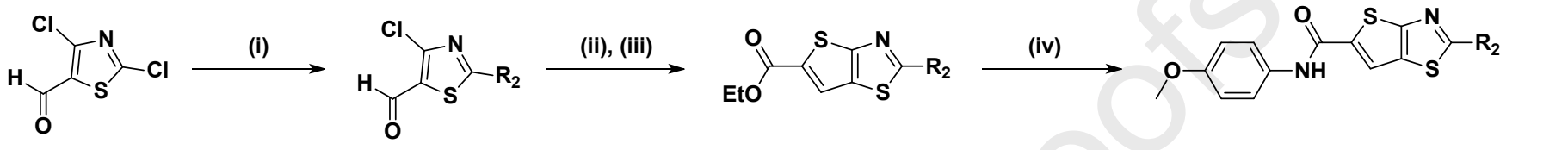
Synthesis of the biaryl amines **37a-h** and TTCA analogues **38-45** is described in **Scheme 4**. Suzuki coupling of substituted pyridines **36a-f** and 4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)aniline using Pd(dppf)Cl_2 and sodium carbonate gave the desired amines **37a-f**. In addition, **37g** and **37h** were synthesized from 4-fluorophenylboronic acid by Suzuki coupling with 3-amino-6-bromopyridine (**36g**) and 2-amino-5-bromopyridine (**36h**), respectively. The ester **27g** was coupled with amines **37a-h** using trimethylaluminum in toluene to yield compounds **38-45**.

Scheme 1. Scheme for the synthesis of TTCA analogues with modified linkers.



Reagents and conditions: (i) piperidine, TEA, CH_3CN, rt, 2h; (ii) ethyl thioglycolate, TEA, DMSO, 120°C, 3h; (iii) NaOEt, EtOH, rt, 2h; (iv) amine, $AlMe_3$, toluene, 100°C, overnight; (v) BBr_3, THF, 0°C; (vi) NaH, MeI, 0°C to rt, 2h; (vii) LAH, THF, 0°C, 2h; (viii) Dess-Martin periodinane, MC, rt, 1h; (ix) *p*-anisidine, $NaBH(OAc)_2$, MC, rt, overnight; (x) LiOH, $MeOH/H_2O$ (3:1, v/v), 50°C, 3h; (xi) DPPA, TEA, *t*-BuOH, reflux, 95°C, 4h; (xii) Conc. HCl, 1,4-dioxane, rt, 2h; (xiii) 4-methoxybenzoic acid, EDCi, HOBt, TEA, DMF, rt, overnight.

Scheme 2. Scheme for the synthesis of TTCA derivatives 18-23.



16a: R_2 = *N*-boc-piperazine
16b: R_2 = morpholine
16c: R_2 = 1-methylpiperazine
16d: R_2 = 4-difluoropiperidine
16e: R_2 = dimethylamine

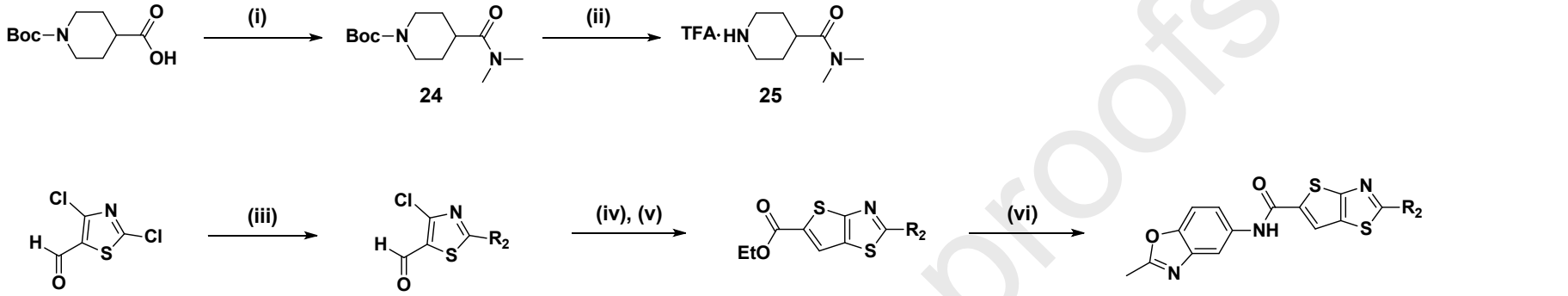
17a: R_2 = *N*-boc-piperazine
17b: R_2 = morpholine
17c: R_2 = 1-methylpiperazine
17d: R_2 = 4-difluoropiperidine
17e: R_2 = dimethylamine

(v)

18: R_2 = *N*-boc-piperazine
19: R_2 = morpholine
20: R_2 = piperazine
21: R_2 = 1-methylpiperazine
22: R_2 = 4-difluoropiperidine
23: R_2 = dimethylamine

Reagents and conditions: (i) *N*-boc-piperazine or morpholine or 1-methylpiperazine or 4-difluoropiperidine or dimethylamine, TEA, CH_3CN, rt, 2h; (ii) ethylthioglycolate, TEA, DMSO, 120°C, 3h; (iii) NaOEt, EtOH, rt, 2h; (iv) *p*-anisidine, $AlMe_3$, toluene, 100°C, overnight; (v) TFA, dichloromethane, 4h.

Scheme 3. Scheme for the synthesis of TTCA derivatives **28**-**35**.



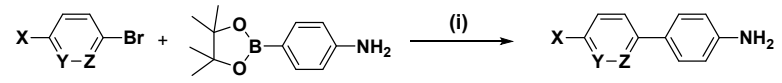
16b: R_2 = morpholine
26a: R_2 = 4-hydroxypiperidine
26b: R_2 = 4-(hydroxymethyl)piperidine
26c: R_2 = 2,6-dimethylmorpholine
26d: R_2 = 4-(dimethylcarbamoyl)piperidine
26e: R_2 = 2-(methylamino)ethan-1-ol
26f: R_2 = 3-(methylamino)propan-1-ol
26g: R_2 = 2-morpholinoethanamine

17b: R_2 = morpholine
27a: R_2 = 4-hydroxypiperidine
27b: R_2 = 4-(hydroxymethyl)piperidine
27c: R_2 = 2,6-dimethylmorpholine
27d: R_2 = 4-(dimethylcarbamoyl)piperidine
27e: R_2 = 2-(methylamino)ethan-1-ol
27f: R_2 = 3-(methylamino)propan-1-ol
27g: R_2 = 2-morpholinoethanamine

28: R_2 = morpholine
29: R_2 = 4-hydroxypiperidine
30: R_2 = 4-(hydroxymethyl)piperidine
31: R_2 = 2,6-dimethylmorpholine
32: R_2 = 4-(dimethylcarbamoyl)piperidine
33: R_2 = 2-(methylamino)ethan-1-ol
34: R_2 = 3-(methylamino)propan-1-ol
35: R_2 = 2-morpholinoethanamine

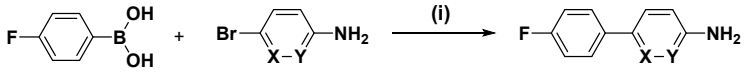
Reagents and conditions: (i) dimethylamine, EDCi, HOBt, TEA, dichloromethane, rt, overnight; (ii) TFA, dichloromethane, 4h; (iii) TEA, CH_3CN, rt, 2h; (iv) ethylthioglycolate, TEA, DMSO, 120°C, 3h; (v) NaOEt, EtOH, rt, 2h; (vi) 2-methylbenzo[*d*]oxazol-5-amine, $AlMe_3$, toluene, 100°C, overnight.

Scheme 4. Scheme for the synthesis of biaryl amines **37a-h** and TTCA analogues **38-45**.



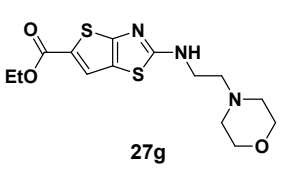
36a X = Me, Y = CH, Z = N
36b X = OMe, Y = CH, Z = N
36c X = CF_3, Y = CH, Z = N
36d X = Cl, Y = CH, Z = N
36e X = F, Y = CH, Z = N
36f X = F, Y = N, Z = CH

37a X = Me, Y = CH, Z = N
37b X = OMe, Y = CH, Z = N
37c X = CF_3, Y = CH, Z = N
37d X = Cl, Y = CH, Z = N
37e X = F, Y = CH, Z = N
37f X = F, Y = N, Z = CH

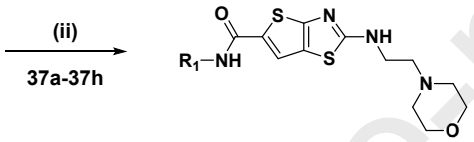


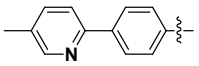
36g X = N, Y = CH
36h X = CH, Y = N

37g X = N, Y = CH
37h X = CH, Y = N



27g



38: R_1 =


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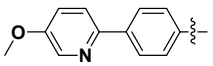

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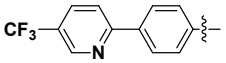

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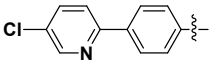

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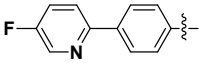
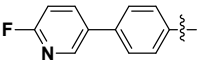


43: R_1 =


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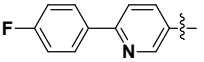
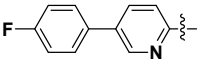


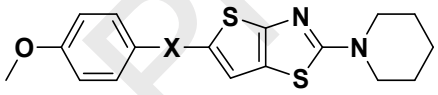
45: R_1 =


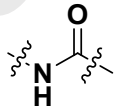
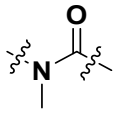
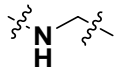
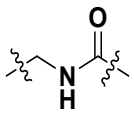
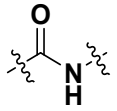
Reagents and conditions: (i) $Pd(dppf)Cl_2$, Na_2CO_3, DME/H_2O (3:1, v/v), 150°C, 1h; (ii) $AlMe_3$, toluene, 100°C, overnight.

3. Results and discussion

The *in vitro* activity of synthetic TTCA analogues was evaluated against *M. tuberculosis* H37Rv expressing GFP (H37Rv-GFP) replicating in liquid broth culture medium (extracellular IC_{50}), but also inside macrophages (intracellular IC_{50}) using already established assays [7,8]. The initial structure-activity relationship (SAR) studies evaluated a set of analogues that contained replacement of the amide linker in an attempt to affect potency of hit compound **1** (Table 1). Replacement of hydrogen with methyl in the amide linker yielded compound **4**, which showed no activity against H37Rv-GFP replicating outside and inside macrophages. Extension of the length of the amide linker by one carbon towards the phenyl ring (**8**) did not yield any activities as well. Finally, compounds with an amine (**7**) or a reversed amide (**12**) linker showed no potency in any of the two assays. Therefore, this set of linker modifications revealed that the carboxamide linked to a phenyl ring was a critical feature to maintain anti-mycobacterial activity.

Table 1. Anti-tubercular activity of TTCA analogues with linker modification.

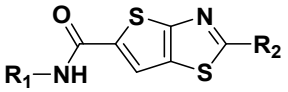


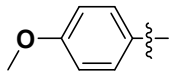
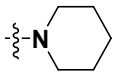
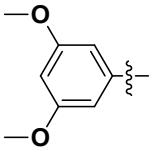
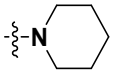
Compound	X	Anti-tubercular activity	
		Extracellular IC_{50} (μM)	Intracellular IC_{50} (μM)
1		0.83	0.17
4		> 20	> 20
7		> 20	> 20
8		> 20	> 20
12		> 20	> 20
INH		0.40	0.35

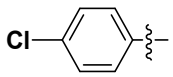
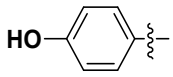
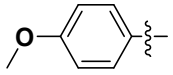
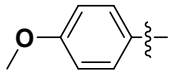
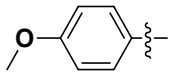
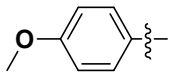
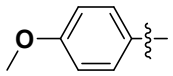
RIF	0.01	0.06

Next, modifications were focused on R1 and R2 positions, as shown in Table 2. In position R1, the anti-tubercular activity was lost when substituting the phenyl ring with two methoxy groups in meta position (**13**), and it showed poor metabolic stability. This suggested that the para position on the phenyl ring might be important for the compound potency. A chloro substitution in para position (**14**) showed similar potency and metabolic stability compared with compound **1**. Replacement of methoxy with hydroxyl, as in compound **15**, reduced the activity against H37Rv-GFP replicating outside and inside macrophages by approximately 7 and 14 fold, respectively. Replacement of the piperidine ring at R2 position with morpholine gave compound **19**, which showed similar potency and increased metabolic stability as compared to compound **1**. With a piperazine substituent (**20**), IC_{50} values for both assays were increased. Substitution of the piperazine with a methyl in -NH position, as in compound **21**, restored the overall potency while decreasing the metabolic stability. Compound **22**, presenting a 4-difluoropiperidine substituent at position R2, showed activities and metabolic stabilities very similar to that of compound **1**. Opening the ring at position R2, as in the dimethylamine substituted compound **23**, slightly decreased the potency but reduced the metabolic stability. Overall, R2 substitutions were found to affect mostly the metabolic stability, while R1 position was more critical for the activity.

Table 2. Biological evaluation of TTCA derivatives.



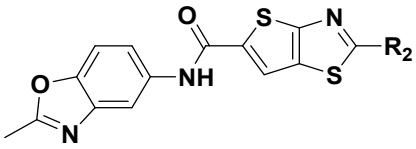
Compound	R_1	R_2	Anti-tubercular activity		Metabolic stability Phase I (min)	
			Extracellular IC_{50} (μM)	Intracellular IC_{50} (μM)	Human	Mouse
1			0.83	0.17	> 60	13.9
13			> 20	> 20	24.5	33.6

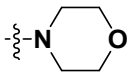
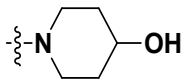
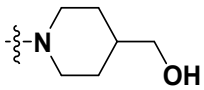
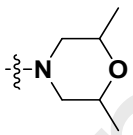
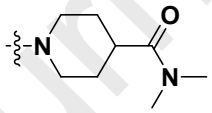
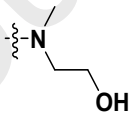
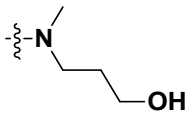
14			1.1	0.11	> 60	22.5
15			4.8	2.75	–	–
19			1.1	0.21	57.6	> 60
20			13.7	2.1	–	–
21			1.3	0.11	16.2	1.4
22			1.1	0.33	> 60	35.9
23			1.6	0.27	23.6	13.7
INH			0.40	0.35		
RIF			0.01	0.06		

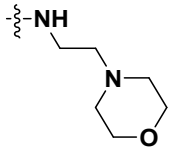
Overall increase in activity was obtained when R1 was changed from 4-methoxy to benzoxazole (Table 3). Various substitutions at position R2 were explored with several saturated ring and aliphatic chain substituents. Compound **28**, displaying a morpholine substituent at position R2, exhibited good metabolic stability with high potency. With a hydroxyl group in para position of the piperidine ring (**29**), the activity was not enhanced in both extracellular and intracellular assays, while high metabolic stabilities were maintained. Moving the hydroxyl further away from the piperidine ring using a one carbon linker (**30**) did not improve extracellular and intracellular activities, as compared to **28**. Substitution of the morpholine ring with two methyl (**31**) reduced the anti-mycobacterial activity and was not favorable for the stability in mouse liver microsome. Presenting a dimethylamine carbonyl group substituted at para position of the piperidine ring (**32**) resulted in similar extracellular

and intracellular activity as compared to compound **28**, solubility and metabolic stability were similarly maintained as well.

Table 3. Biological evaluation of TTCA derivatives.



Compound	R_2	Anti-tubercular activity		Solubility (ug/mL)		Metabolic stability Phase I (min)	
		Extracellular IC_{50} (μM)	Intracellular IC_{50} (μM)	pH 7.4	pH 2.1	Human	Mouse
28		0.42	0.20	0.82	5.3	> 60	> 60
29		0.64	0.46	2.4	9.7	> 60	> 60
30		0.50	0.23	0.87	7.9	–	–
31		1.1	0.25	1.1	1.9	> 60	27.2
32		0.50	0.51	1.2	4.0	> 60	> 60
33		0.88	1.3	4.5	20.6	> 60	37.7
34		1.0	0.70	1.3	10.6	> 60	> 60

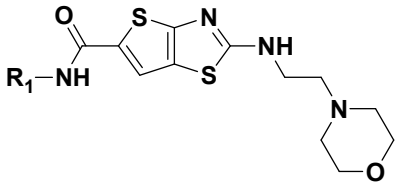
35		1.35	0.47	2.1	37.1	> 60	> 60
INH		0.40	0.35				
RIF		0.01	0.06				

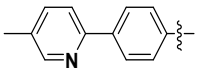
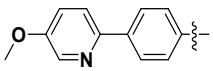
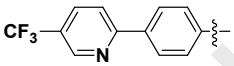
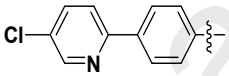
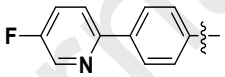
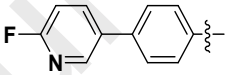
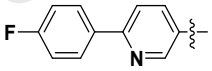
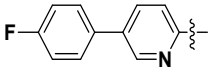
In order to improve the solubility, the polar substituents and linear alkyl chain were presented. Therefore, aliphatic linkers with hydroxyl group were prepared and evaluated. Compound **33**, mimicking an opened morpholine ring through a two carbon linker, exhibited enhanced solubility but slightly decreased potency and metabolic stability in mouse liver microsome. A three carbon linker (**34**) showed high metabolic stability with slightly increased solubility, but no enhancement of the potency. Spacing the morpholine from the core by a two carbon linker (**35**) resulted in a moderate increase in solubility with high metabolic stability, but a slight decrease in activity. These results revealed that compounds with aliphatic linker formation exhibited better physicochemical properties as compared to the saturated ring formation at position R2.

In an effort to enhance the potency outside and inside macrophages, the R2 position was fixed with the ethyl-morpholine substituent, while R1 position was further varied with biaryl substituents presenting both pyridine and phenyl rings (Table 4). The methyl pyridine compound **38** showed moderate anti-mycobacterial activity and increased solubility but lower metabolic stability. Additionally, methoxy, trifluoromethyl and chloro pyridine rings led to higher extracellular activities (**39-41**). However, substitution with 5-fluoro pyridine (compound **42**) showed strong activity against H37Rv-GFP replicating outside as well as inside macrophages (extracellular IC_{50} = 0.76 μM, intracellular IC_{50} = 0.19 μM), with enhanced solubility and good metabolic stability. Maintaining this fluoro substitution, different positions of the nitrogen atom on the biaryl rings were also investigated (**43-45**). The 6-fluoro pyridine compound **43** showed the highest potency against extracellular and intracellular mycobacteria but showed poor solubility and decreased metabolic stability. Lastly, when switching the position of phenyl and pyridine rings (**44** and **45**), around 3 to 5 fold reduction of both extracellular and intracellular anti-tubercular activities was observed. Compound **42** has also

shown promising inhibition against tested ten MDR and one XDR clinical isolates with MIC less than 2 to 4 uM (Supplementary Table 1). We have successfully synthesized TTCA derivatives with anti-mycobacterial activities which are comparable or similar to the known drug, INH, used as the control with RIF.

Table 4. Biological evaluation of TTCA derivatives.



Compound	R_1	Anti-tubercular activity		Solubility (ug/mL)		Metabolic stability Phase I (min)	
		Extracellular IC_{50} (μM)	Intracellular IC_{50} (μM)	pH 7.4	pH 2.1	Human	Mouse
38		1.35	0.31	1.3	52.3	20.9	27.1
39		1.35	0.19	0.20	>50	<30	<30
40		4.5	0.21	9.8	44.3	–	–
41		3.2	0.21	0.20	43.4	–	–
42		0.76	0.19	0.76	43.6	> 60	45.5
43		0.61	0.10	1.1	1.5	25.1	45.6
44		4.6	0.37	0.57	>50	–	–
45		4.7	0.36	0.56	44.2	–	–
INH		0.40	0.35				
RIF		0.01	0.06				

3.1 In vitro ADME evaluation

On the basis of the SAR study, we selected compounds **28**, **32** and **42** for *in vitro* ADME evaluation, as shown in Table 5. In the drug discovery process, metabolic stability, plasma stability and plasma protein binding are important parameters to consider before initiating further *in vivo* PK studies. Generally, results from Phase I metabolic stability studies highlights susceptibility to oxidation/reduction, while results from Phase II indicates glucuronidation. The metabolic stability of the three compounds was studied in microsomes from human and mouse. All three exhibited good stability for both phase I and II. These compounds also showed good plasma stability for both human and mouse. Compounds **28** and **42** showed high rates of binding to plasma proteins, but these were more moderate for compound **32**. Drug-drug interaction is another critical factor to consider for an anti-tubercular agent, due to combination therapy with other medications for treatment. Therefore, we also evaluated the cytochrome P450 inhibition with 5 different isozymes for compounds **32** and **42**, as shown in Table 6. Compound **32** did not show inhibition tested in 5 CYP isozymes. However, compound **42** was found to be non-inhibitor for 1A2 and 2C19, and it inhibited moderately in rest of the isoforms. All selected compounds were stable in the plasma, and free unbound fraction of compounds suggested that these compounds are suitable for further PK studies.

Table 5. ADME evaluation of selected compounds **28**, **32** and **42**.

Compound	Metabolic stability Phase I ($t_{1/2}$, min)		Metabolic stability Phase II ($t_{1/2}$, min)		Plasma Stability (at 4h, %)/(at 2h, %)*		Plasma protein binding (%)	
	Human	Mouse	Human	Mouse	Human	Mouse	Human	Mouse
28	>60	>60	>60	>60	86.8	74.7	98.8	99.9
32	>60	>60	99.7	124	86.1*	95.7*	95.1	96.4
42	>60	45.5	>60	>60	98.2*	>100*	99.8	99.8

Table 6. CYP inhibition profile for compounds **32** and **42**.

Compound	CYP Inhibition (IC_{50}, μM)				
	3A4	2D6	1A2	2C9	2C19
32	>50	>50	>50	>50	>50
42	6.95	6.85	>100	7.41	>100

3.2 Toxicity evaluation

Cytotoxicity of compounds **32** and **42** was evaluated against a panel of four human-cancer cell lines, as seen in Table 7. Tested compounds were found to be non-cytotoxic to all these cells, at concentrations up to 50 μM. As bedaquiline and delamanid were reported to have potential cardiotoxicity issues [10,11], we used the hERG potassium channel patch-clamp assay to test compounds **32** and **42**. Results suggested almost no risk for cardiotoxicity. In addition, compounds **32** and **42** showed no genetic toxicity in a mini-Ames mutagenicity test.

Table 7. Cytotoxicity and mutagenicity evaluations for compounds **32** and **42**.

Compound	Cytotoxicity (IC_{50}, μM)				hERG (μM)	Mini-Ames	
	A549	HEK-293	HepG2	Jurkat		S9(-) TA98/TA100	S9(+) TA98/TA100
32	>50	>50	>50	>50	>30	negative/negative	negative/negative
42	>50	>50	>50	>50	27.6	negative/negative	negative/negative

3.3 In vivo pharmacokinetic (PK) studies

Based on their acceptable *in vitro* drug-like ADME properties, we evaluated compounds **28**, **32** and **42** for *in vivo* PK in mice, after intravenous (i.v.) and oral (p.o.) administration (Table 8). Compound **28** exhibited short half-life and high clearance with low volume of distribution. In contrast, the two other compounds displayed moderate volume of distributions and low clearance. Compounds **32** and **42** showed highest AUC with low clearance and

excellent bioavailability. Additionally, compound **42** ($T_{1/2}$ = 2.3 h) had longer half-life than compound **32** ($T_{1/2}$ = 0.99 h). The distribution of compounds **32** and **42** in the target organ – the lungs – was assessed at T_{max} 1 hour, as shown in Table 9. Compounds **32** and **42** showed good distribution to the lung. Especially, compound **42** showed good distribution to the lung as compared to plasma, with a ratio of 4.8.

Table 8. *In vivo* pharmacokinetic evaluation of compounds **28**, **32** and **42**.

Compound	Pharmacokinetics (i.v.)				Pharmacokinetics (p.o.)				
	$t_{1/2}$ (h)	Cl (mL/min/kg)	Vd_{ss} (mL/kg)	Dose (mg/kg)	$t_{1/2}$ (h)	C_{max} (ng/mL)	AUC_{last} (ng·h/mL)	F (%)	Dose (mg/kg)
28	0.33	121	626	2	ND	313	ND	ND	10
32	0.92	11.8	701	2	0.99	7010	10005	71.2	10
42	1.2	14.9	1520	3	2.3	2937	10369	102	10

ND: PK parameters were not calculated due to the limited number of data points.

Table 9. Plasma and lung distribution for compound **32** and **42**.

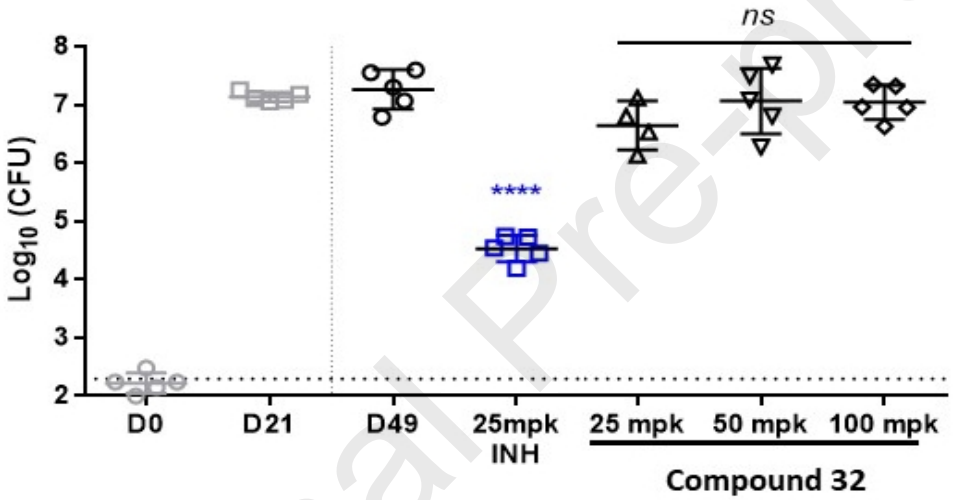
Compound	Pharmacokinetics (p.o.)				
	Plasma conc. (ng/mL)	Lung conc. (ng/mL)	Lung/Plasma ratio	T_{max} (h)	Dose (mg/kg)
32	4400	2986	0.7	1	10
42	3380	16180	4.8	1	10

3.4 In vivo efficacy

Based on its desirable *in vivo* PK profile, we conducted an *in vivo* efficacy study in mice for compounds **32** and **42** (**Fig. 2**). Compound treatment was initiated three weeks after infection of BALB/c female mice with 300 to 600 CFU (colony forming units) of *M. tuberculosis* H37Rv by the intranasal route (5 mice per groups). Compounds **32**, **42** and

positive control isoniazid (INH) were administered by oral gavage for 28 days, five times per week. Bacterial loads were enumerated in the lung of infected mice at the end of the 28 days of treatment. Compound **32** was used at 25, 50 and 100 mg/kg, compound **42** was used at 20, 50 and 100 mg/kg dose and INH was used as the positive control at a 25 mg/kg dose. Compound **32** showed no effect at any dose. In contrast, compound **42** displayed a decrease in CFU that was proportional to the administered dose. It had a better activity against *M. tuberculosis* at 50 mg/kg compared to the control INH, but was not effective at 20 mg/kg. At 100 mg/kg, compound **42** showed very high reduction of the bacterial burden in the lungs of infected mice (4 to 5 logs reduction) and the number of CFU fell below the detection limit (2.3 logs).

A.



B.

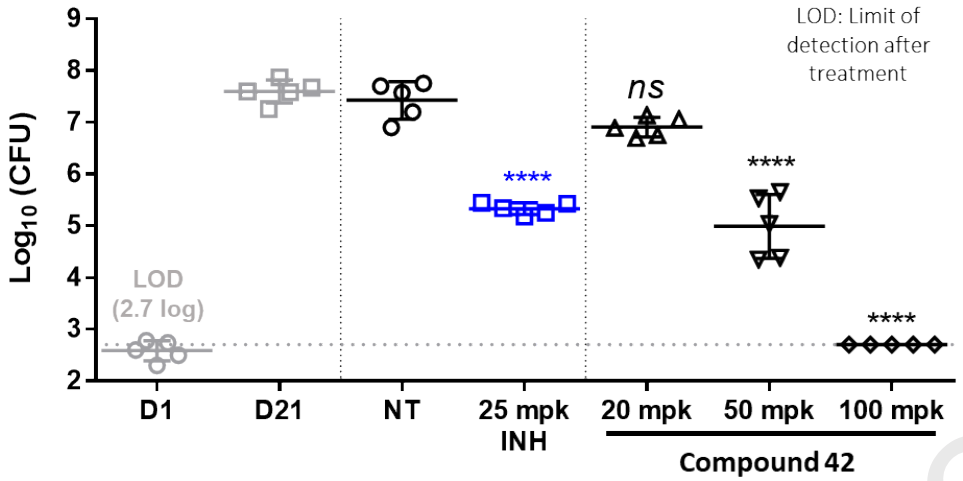


Fig 2. A. *In vivo* efficacy of compound **32**; B. *In vivo* efficacy of compound **42**. Groups of 5 Balb/C mice were infected intranasaly with ~300 to 600 colony forming units (CFU) of *M. tuberculosis* H37Rv. A control group was sacrificed one day after infection (D1) to ascertain the required amounts of CFU were administered. Another control group was sacrificed 3 weeks post-infection (D21) to validate the progression of the infection. The remaining mice were treated from D21 by daily gavage (5 times a week), for a period of four weeks, with TTCA compounds or the control drug isoniazid (INH). A control group was left untreated and mice received only the vehicle used for TTCA compound (NT). Animals were sacrificed one day after the treatment completion and lungs were collected. One lobe was homogenized and serial-diluted in PBS to enumerate the CFU. The limit of detection (LOD, below which no CFU could be counted) was approximately 200 CFU per mice (2.3 logs). Statistical significance for the number of CFU in treated against untreated mice (NT) was calculated using a Bonferroni's multiple comparison test. Adjusted P values are indicated on the graph. ****: < 0.0001; ns: not significant.

4. Conclusions

In summary, a new series of thienothiazolocarboxamide analogues were designed and synthesized as potential anti-tuberculosis agents. Preliminary SAR and SPR studies on the scaffold revealed that the carboxamide linker with a phenyl group was critical for the anti-mycobacterial activity. In addition, biaryl substituents at position R1 led to increased anti-

tubercular potency, while varying the substituents at R2 position allowed tuning of the physicochemical properties with minor influence on the activity. The compound **42** showed good *in vitro* activity and drug-like ADME properties. It also demonstrated absence of toxicity in various human-cancer cells, low risk of cardiotoxicity and no genotoxicity. In addition, compound **42** exhibited a favorable *in vivo* pharmacokinetic profile, with an oral bioavailability over 100% in mice (10 mg/kg). Compound **42** was efficacious in a murine model of TB, in a dose-dependent manner. Hence, **42** was selected as a new lead compound for further evaluation as a potential clinical candidate to combat tuberculosis.

5. Experimental section

5.1 Chemistry

5.1.1 General information

All commercially available chemicals were of reagent grade and were used without further purification. Solvents were dried with standard procedures. All reactions were carried out under argon atmosphere in flame-dried glassware with magnetic stirring. The proton nuclear magnetic resonance (^{1}H-NMR) spectra and carbon nuclear magnetic resonance (^{13}C-NMR) spectra were determined on a Varian (400 MHz, 100 MHz) or Bruker (400 MHz, 100 MHz) spectrometer. Melting points (mp) were measured on an electro thermal melting point apparatus, M-565 (BÜCHI). LC/MS data were obtained using a Waters ZQ2000 platform. The purity of all biologically tested compounds were determined as ≥ 90 % by HPLC. All of the reactions were purified by flash column chromatography using silica gel 60 (Merck, 230-400 mesh). Additionally, thin-layer chromatography was performed on 0.25 mm silica gel 60-F254 plates (Merck).

*5.1.2. Procedure for the synthesis of compound **2**.*

To a stirred solution of 2,4-dichlorothiazole-5-carbaldehyde (1.0 equiv.) in CH_3CN (10 mL) was added piperidine (1.2 equiv.) and triethylamine (3.0 equiv.) and the mixture was stirred for 4 hours at room temperature. After the reaction was completed, as determined by TLC, the reaction mixture was poured to the cold water and then extracted with EtOAc (20 mL), washed with sat. $NaHCO_3$ (10 mL), dried over $MgSO_4$ and concentrated. The crude

residue was purified by flash column chromatography (methylene chloride:MeOH = 50:1) to give the desired compound **2**.

*5.1.3. Procedure for the synthesis of compound **3**.*

A mixture of **2** (1.0 equiv.), ethylthioglycolate (1.2 equiv.) and triethylamine (2.0 equiv.) in DMSO (10 mL) was heated at 120 ℃ for 3 hours. The reaction mixture was diluted with EtOAc (30 mL), washed with water (10 mL), dried over $MgSO_4$ and concentrated. The resulting residue was dissolved in EtOH (20 mL) and a sodium ethoxide solution (21% in EtOH, 2.0 equiv.) was added. The reaction mixture was stirred for one hour at room temperature. After completion, the reaction was quenched with water (5 mL) and the organic solvent was evaporated. The reaction mixture was extracted with EtOAc (20 mL), washed with water, dried over $MgSO_4$ and concentrated. The crude residue was purified by flash column chromatography (*n*-hexane:EtOAc = 7:3) to give **3**.

*5.1.4. General procedure for the preparation of compounds **1**, **8**, **13** and **14**.*

To a cooled (ice bath), stirred solution of **3** (1.0 equiv.) and the desired amine (1.0 equiv.) in toluene was added trimethylaluminum (2.0 M in toluene, 1.0 equiv.). The resulting mixture was allowed to reach room temperature and then heated to 100 ℃ overnight. After reaction completion, the reaction mixture was cooled down to room temperature, washed with CH_2Cl_2/MeOH (20:1, v/v) and the solvent evaporated. The crude was purified by flash column chromatography (methylene chloride:MeOH = 20:1) to obtain compounds **1**, **8**, **13** and **14**.

*5.1.4.1. N-(4-Methoxyphenyl)-2-(piperidin-1-yl)thieno[2,3-d]thiazole-5-carboxamide (**1**).* Ivory solid; mp = 235 °C; ^{1}H-NMR (400 MHz, DMSO-d_6) δ 10.01 (s, 1H), 8.08 (s, 1H), 7.59 (d, J = 8.8 Hz, 2H), 6.91 (d, J = 8.8 Hz, 2H), 3.73 (s, 3H), 3.52 - 3.56 (m, 4H), 1.61 - 1.65 (m, 6H); LCMS (electrospray) m/z 373 $[M + H]^+$.

*5.1.4.2. N-(4-methoxybenzyl)-2-(piperidin-1-yl)thieno[2,3-d]thiazole-5-carboxamide (**8**).* Yellow solid; mp = 142 °C; ^{1}H-NMR (400 MHz, DMSO-d_6) δ 8.84 (t, J = 8.0 Hz, 1H), 7.87 (s, 1H), 7.24 (d, J = 8.8 Hz, 2H), 6.89 (d, J = 8.8 Hz, 2H), 4.36 (d, J = 8.8 Hz, 2H), 3.72 (s, 3H), 3.49 - 3.55 (m, 4H), 1.60 - 1.65 (m, 6H); ^{13}C-NMR (100 MHz, DMSO-d_6) δ 173.35, 160.87, 157.64, 156.95, 130.97, 130.86, 128.05, 121.20, 119.20, 113.10, 54.47, 48.49, 41.38, 24.09, 22.90; LCMS (electrospray) m/z 388 $[M + H]^+$.

*5.1.4.3. N-(3,5-Dimethoxyphenyl)-2-(piperidin-1-yl)thieno[2,3-d]thiazole-5-carboxamide (**13**).* Yellow solid; mp = 148 °C; ^{1}H-NMR (400 MHz, DMSO-d_6) δ 10.02 (s, 1H), 8.11 (s, 1H), 6.99 (s, 1H), 6.98 (s, 1H), 6.23 (s, 1H), 3.73 (s, 6H), 3.52 - 3.56 (m, 4H), 1.61 - 1.65 (m, 6H); ^{13}C-NMR (100 MHz, DMSO-d_6) δ 178.68, 159.81, 159.68, 158.07, 140.05, 130.56, 121.38, 120.49, 97.65, 94.89, 54.50, 48.58, 24.12, 22.89; LCMS (electrospray) m/z 404 $[M + H]^+$.

*5.1.4.4. N-(4-chlorophenyl)-2-(piperidin-1-yl)thieno[2,3-d]thiazole-5-carboxamide (**14**).* Pale yellow solid; mp = 275.2 °C; ^{1}H-NMR (400 MHz, acetone-d_6) δ 9.48 (brs, 1H), 7.96 (s, 1H), 7.79 (d, J = 8.0 Hz, 2H), 7.35 (d, J = 8.0 Hz, 2H), 3.58 - 3.63 (m, 4H), 1.68 - 1.74 (m, 6H); ^{13}C-NMR (100 MHz, DMSO-d_6) δ 173.75, 159.72, 158.18, 137.37, 130.12, 127.97, 126.38, 121.43, 120.93, 120.78, 48.57, 24.12, 22.88; LCMS (electrospray) m/z 378, 380 $[M + H]^+$ (Cl^- isotope pattern).

*5.1.5. Procedure for the synthesis of compound **4**.*

To a stirred solution of **1** (1.0 equiv.) in *N*,*N*-dimethylformamide (0.5 mL) was added sodium hydride (1.2 equiv.) at 0 °C. After 20 min of stirring, iodomethane (2.0 equiv.) was added to the solution and the mixture was further stirred at room temperature for 2 hours. After reaction completion, the reaction mixture was poured in water and extracted with methylene chloride (10 mL × 3). The organic layer was washed with brine (5 mL), dried over anhydrous $NaSO_4$ and concentrated *in vacuo*. The resulting crude residue was purified by flash column chromatography (methylene chloride:MeOH = 20:1) to give **4**.

*5.1.5.1. N-(4-Methoxyphenyl)-N-methyl-2-(piperidin-1-yl)thieno[2,3-d]thiazole-5-carboxamide (**4**).* Ivory solid; mp = 195 °C; ^{l}H-NMR (400 MHz, acetone-d_6) δ 7.29 (d, J = 7.6 Hz, 2H), 7.02 (d, J = 7.6 Hz, 2H), 6.72 (s, 1H), 3.85 (s, 3H), 3.46 - 3.52 (m, 4H), 3.18 (s, 3H), l.61 - 1.69 (m, 6H); ^{13}C-NMR (100 MHz, DMSO-d_6) δ 174.62, 162.23, 159.39, 159.26, 136.89, 129.94, 129.61, 124.26, 121.86, 115.46, 55.88, 49.57, 25.11, 23.94; LCMS (electrospray) m/z 388 $[M + H]^+$.

*5.1.6. Procedure for the synthesis of compound **5**.*

To a stirred solution of **3** (3.4 mmol) in tetrahydrofuran (20 mL) was added lithium aluminum hydride (5.1 mmol) at 0 °C and the reaction mixture was stirred for 2 hours while maintaining the temperature below 4 °C. The reaction was quenched by addition of water with stirring and the insoluble solid was filtered off. The filtrate was extracted with methylene

chloride (50 mL × 2), dried over anhydrous $MgSO_4$ and concentrated *in vacuo*. The crude product was purified by flash column chromatography (*n*-hexane:EtOAc = 3:1) to give **5**.

*5.1.6.1. (2-(Piperidin-1-yl)thieno[2,3-d]thiazol-5-yl)methanol (**5**).* ^{1}H-NMR (400 MHz, DMSO-d_6) δ 7.02 (s, 1H), 5.39 (t, J = 5.6 Hz, 1H, OH), 4.56 (d, J = 5.6 Hz, 2H), 3.42 - 3.45 (m, 4H), 1.58 - 1.60 (m, 6H); LCMS (electrospray) m/z 255 $[M + H]^+$.

5.1.7. Procedure for the synthesis of compound 7.

To a stirred solution of **5** (0.87 mmol) in methylene chloride (20.0 mL) was added Dess-Martin periodinane (0.95 mmol) and the resulting mixture was stirred at room temperature for one hour. The reaction mixture was diluted with methylene chloride (10.0 mL), washed with brine (20 ml × 2), dried over anhydrous $MgSO_4$ and concentrated *in vacuo*. The crude product was purified by flash column chromatography (n-hexane:EtOAc = 5:1) to give **6**. To the aldehyde **6** (0.20 mmol) in methylene chloride (10.0 mL) was then added *p*-anisidine (0.22 mmol) and sodium triacetoxyborohydride (0.40 mmol) and the resulting mixture was stirred at room temperature overnight. After reaction completion, the mixture was diluted with methylene chloride (10 mL), washed with brine (10 mL × 2), dried over anhydrous $MgSO_4$ and concentrated *in vacuo*. The crude residue was purified by flash column chromatography (n-hexane:EtOAc = 4:1) to give **7**.

*5.1.7.1. 4-Methoxy-N-((2-(piperidin-1-yl)thieno[2,3-d]thiazol-5-yl)methyl)aniline (**7**).* Yellow solid; mp = 105 °C; ^{1}H-NMR (400 MHz, acetone-d_6) δ 7.07 (s, 1H), 6.72 (d, J = 8.8 Hz, 2H), 6.69 (d, J = 8.8 Hz, 2H), 5.09 (brs, 1H), 4.47 (d, J = 5.6 Hz, 2H), 3.67 (s, 3H), 3.48 - 3.50 (m, 4H), 1.65 - 1.68 (m, 6H); LCMS (electrospray) m/z 360 $[M + H]^+$.

*5.1.8. Procedure for the synthesis of compound **10**.*

To a stirred suspension of ester compound **3** in MeOH (6.0 mL) was added an aqueous solution of lithium hydroxide (6.32 mmol in 2.0 mL of water) and the mixture was heated to 50 °C for 3 hours. The organic solvent was evaporated and 1*N* HCl was added until the pH reached 4. The residual white solid was collected by filtration, washed with water and dried *in vacuo* to give the desired acid **9** as a white solid. To a solution of **9** (1.11 mmol) in *tert*-buthanol (8 mL) was added diphenylphosphorylazide (1.34 mmol). The reaction mixture was stirred at 95 °C for 4 hours. The reaction mixture was diluted with methylene chloride (5 mL) and

washed with brine (5 mL). The organic layer was dried over anhydrous $MgSO_4$ and concentrated *in vacuo*. The crude product was purified by flash column chromatography (n-hexane: EtOAc = 10:1) to give the desired product **10**.

*5.1.8.1. tert-Butyl (2-(piperidin-1-yl)thieno[2,3-d]thiazol-5-yl)carbamate (**10**)*. Ivory solid; mp = 157 °C; ^{1}H-NMR (400 MHz, $CDCl_3$) δ 6.62 (s, 1H), 6.61 (brs, 1H), 3.45 - 3.49 (m, 4H), 1.66 -1.71 (m, 6H), 1.50 (s, 9H); LCMS (electrospray) m/z 339 $[M + H]^+$.

*5.1.9. Procedure for the synthesis of reversed amide compound **12.***

To a stirred solution of carbamate **10** (0.58 mmol) in 1,4-dioxane (4 mL) was added 35% hydrogen chloride (0.7 mL). The reaction mixture was stirred at room temperature. After 2 hours, the reaction mixture was concentrated *in vacuo* and the crude product containing the intermediate product **11** was used without further purification. To a stirred solution of 4-methoxybenzoic acid (0.16 mmol) in anhydrous DMF (2.0 mL) were added l-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (0.24 mmol), 1-hydroxybenzotriazole (0.082 mmol), triethylamine (0.33 mmol) and amine **11** (0.15 mmol) at room temperature. The resulting solution was stirred at room temperature overnight. The reaction mixture was concentrated, and the resulting crude residue purified by flash column chromatography to obtain the desired product **12**.

*5.1.9.1. 4-Methoxy-N-(2-(piperidin-1-yl)thieno[2,3-d]thiazol-5-yl)benzamide (**12**)*. Green solid; mp = 92 °C; ^{1}H-NMR (400 MHz, DMSO-d_6) δ 11.33 (s, 1H), 7.96 (d, J = 8.8 Hz, 2H), 7.08 (d, J = 8.8 Hz, 2H), 6.97 (s, 1H), 3.84 (s, 3H), 3.41 - 3.44 (m, 4H), 1.59 - 1.63 (m, 6H); ^{13}C-NMR (100 MHz, DMSO-d_6) δ 170.84, 161.81, 161.50, 146.68, 133.57, 128.88, 124.48, 117.88, 113.21, 102.28, 54.86, 48.56, 24.03, 23.00; LCMS (electrospray) m/z 374 $[M + H]^+$.

*5.1.10. Procedure for the synthesis of compound **15**.*

To a solution of compound **1** (1.0 equiv.) in methylene chloride was added borontribromide (1.0 M in methylene chloride) at 0 °C. The reaction mixture was stirred at room temperature for 2 hours. After reaction completion, the reaction mixture was quenched with cold water and extracted with methylene chloride (10 mL × 2). The organic layer was dried over anhydrous $MgSO_4$ and concentrated *in vacuo*. The crude product was purified by flash column chromatography (methylene chloride:MeOH = 50:1) to give **15**.

*5.1.10.1. N-(4-Hydroxyphenyl)-2-(piperidin-1-yl)thieno[2,3-d]thiazole-5-carboxamide (**15**).* Yellow solid; mp = 246 °C; ^{1}H-NMR (400 MHz, DMSO-d_6) δ 9.91 (s, 1H), 8.05 (s, 1H), 7.44 (d, J = 8.8 Hz, 2H), 6.72 (d, J = 8.8 Hz, 2H), 3.51 - 3.55 (m, 4H), 1.60 - 1.64 (m, 6H); ^{13}C-NMR (100 MHz, DMSO-d_6) δ 173.47, 159.22, 157.41, 153.01, 131.16, 129.78, 121.50, 121.26, 119.81, 114.44, 48.54, 24.11, 22.89; LCMS (electrospray) m/z 360 $[M + H]^+$.

*5.1.11. General procedure for the preparation of compounds **16a-e**.*

To a stirred solution of 2,4-dichlorothiazole-5-carbaldehyde (1.0 equiv.) in CH_3CN (10 mL) were added the desired amine (1.2 equiv.) and triethylamine (3.0 equiv.) and the mixture was stirred for 4 hours at room temperature. After the reaction completion, the reaction mixture was poured in cold water and extracted with EtOAc (20 mL), washed with sat. $NaHCO_3$ (10 mL), dried over $MgSO_4$ and concentrated. The crude residue was purified by flash column chromatography (methylene chloride:MeOH = 50:1) to give desire compounds **16a-e**.

*5.1.12. General procedure for the preparation of compounds **17a-e**.*

A mixture of aldehyde (**16a-e**) (1.0 equiv.), ethylthioglycolate (1.2 equiv.) and triethylamine (2.0 equiv.) in DMSO (10 mL) was heated to 120 ℃ for 3 hours. The reaction mixture was diluted with EtOAc (30 mL), washed with water (10 mL), dried over $MgSO_4$ and concentrated. The resulting residue was dissolved in EtOH (20 mL) and sodium ethoxide solution (21% in EtOH, 2.0 equiv.) was added. The reaction mixture was stirred for one hour at room temperature. After completion, the reaction was quenched with water (5 mL) and the organic solvent was evaporated. The reaction mixture was extracted with EtOAc (20 mL), washed with water, dried over $MgSO_4$ and concentrated. The crude residue was purified by flash column chromatography (*n*-hexane:EtOAc = 7:3) to give compounds **17a-e**.

*5.1.13. General procedure for the preparation of compounds **18-19** and **21-23**.*

To a stirred solution of ester (**17a-e**) (1.0 equiv.) and *p*-anisidine (1.0 equiv.) in toluene was added trimethylaluminum (2.0 M in toluene, 1.0 equiv.) under cooling with an ice bath. The resulting mixture was allowed to reach room temperature and then heated at 100 ℃ overnight. After completion, the reaction mixture was cooled down to room temperature, washed with CH_2Cl_2/MeOH (20:1, v/v) and the solvent evaporated. The crude was purified by flash column chromatography (methylene chloride:MeOH = 20:1) to obtain compounds **18-19** and **21-23**.

*5.1.13.1. N-(4-Methoxyphenyl)-2-morpholinothieno[2,3-d]thiazole-5-carboxamide (**19**).* Pale brown solid; mp = 248.8 °C; ^{1}H-NMR (400 MHz, acetone-d_6) δ 9.32 (s, 1H), 7.96 (s, 1H), 7.66 (d, J = 8.8 Hz, 2H), 6.91 (d, J = 8.8 Hz, 2H), 3.79 - 3.81 (m, 4H), 3.78 (s, 3H), 3.57 - 3.59 (m, 4H); ^{13}C-NMR (100 MHz, DMSO-d_6) δ 173.99, 159.28, 157.05, 154.88, 131.72, 131.29, 121.70, 121.22, 120.07, 113.23, 64.73, 54.58, 47.43; LCMS (electrospray) m/z 376 $[M + H]^+$.

*5.1.13.2. N-(4-Methoxyphenyl)-2-(4-methylpiperazin-1-yl)thieno[2,3-d]thiazole-5-carboxamide (**21**).* White solid; mp = 219.8 °C; ^{1}H-NMR (400 MHz, DMSO-d_6) δ 10.03 (s, 1H), 8.11 (s, 1H), 7.63 (d, J = 8.8 Hz, 2H), 6.96 (d, J = 8.8 Hz, 2H), 3.73 - 3.78 (m, 4H), 3.58 (s, 3H), 3.53 (s, 3H), 2.23 - 2.78 (s, 4H); ^{13}C-NMR (100 MHz, DMSO-d_6) δ 174.74, 160.38, 158.35, 155.95, 132.52, 132.39, 122.74, 122.28, 121.12, 114.31, 55.66, 54.06, 48.39, 46.11; LCMS (electrospray) m/z 389 $[M + H]^+$.

*5.1.13.3. 2-(4,4-Difluoropiperidin-1-yl)-N-(4-methoxyphenyl)thieno[2,3-d]thiazole-5-carboxamide (**22**).* Pale yellow solid; mp = 263.5 °C; ^{1}H-NMR (400 MHz, DMSO-d_6) δ 10.06 (s, 1H), 8.11 (s, 1H), 7.58 (d, J = 8.8 Hz, 2H), 6.91 (d, J = 8.8 Hz, 2H), 3.74 (s, 3H), 3.69 - 3.72 (m, 4H), 2.09 - 2.19 (m, 4H); ^{13}C-NMR (100 MHz, DMSO-d_6) δ 174.10, 160.33, 158.04, 155.97, 132.99, 132.36, 123.33, 122.89, 122.29, 121.13, 114.32, 55.66, 45.68 (t, J = 5.0 Hz), 33.01 (t, J = 24.0 Hz); LCMS (electrospray) m/z 410 $[M + H]^+$.

*5.1.13.4. 2-(Dimethylamino)-N-(4-methoxyphenyl)thieno[2,3-d]thiazole-5-carboxamide (**23**).* Pale brown solid; mp = 192.3 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.04 (s, 1H, NH), 8.12 (s, 1H), 7.63 (d, J = 8.8 Hz, 2H), 6.95 (d, J = 9.2 Hz, 2H) 3.78 (s, 3H), 3.18 (s, 6H); LCMS (electrospray) m/z 334 $[M + H]^+$.

*5.1.14. Procedure for the synthesis of compound **20**.*

To a stirred solution of **18** in methylene chloride (10.0 mL) was added excess trifluoroacetic acid at room temperature. The mixture was stirred at room temperature for 2-4 hr. The solvent was evaporated *in vacuo* to give compound **20** without further purification.

*5.1.14.1. N-(4-Methoxyphenyl)-2-(piperazin-1-yl)thieno[2,3-d]thiazole-5-carboxamide (**20**).* Pale yellow solid; ^{1}H-NMR (400 MHz, acetone-d_6) δ 9.32 (s, 1H), 7.96 (s, 1H), 7.67 (d, J = 8.8 Hz, 2H), 6.91 (d, J = 8.8 Hz, 2H), 3.79 (s, 3H), 3.58 - 3.59 (m, 4H), 3.00 - 3.01 (m, 4H); LCMS (electrospray) m/z 375 $[M + H]^+$.

*5.1.15. Procedure for the synthesis of compound **25**.*

To a stirred solution of 1-(tert-butoxycarbonyl)piperidine-4-carboxylic acid (4.11 mmol) in methylene chloride (20.0 mL) was added dimethylamine hydrochloride (4.93 mmol), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (6.17 mmol), 1-hydroxybenzotriazole hydrate (6.17 mmol) and triethylamine (13.2 mmol) at room temperature. The mixture was stirred at room temperature overnight. The reaction mixture was evaporated, extracted with EtOAc (30 mL) and washed with water (20 mL). The organic layer was dried over anhydrous $MgSO_4$ and concentrated *in vacuo*. The resulting crude residue was purified by flash column chromatography (methylene chloride:MeOH = 50:1) to give **24**. To a mixture of compound **24** (3.12 mmol) in methylene chloride (10.0 mL) was added trifluoroacetic acid (2.0 mL) at room temperature. The mixture was stirred at room temperature for 2-4 hr. The solvent was evaporated *in vacuo* to give compound **25** without further purification.

*5.1.16. General procedure for the preparation of aldehydes **26a-g**.*

To a stirred solution of 2,4-dichlorothiazole-5-carbaldehyde (1.0 equiv.) in CH_3CN was added the desired amine (1.2 equiv.), triethylamine (3.0 equiv.) and the mixture was stirred for 4 hours at room temperature. After the reaction completion, the reaction mixture was poured in cold water and extracted with EtOAc, washed with sat. $NaHCO_3$, dried over $MgSO_4$ and concentrated. The crude residue was purified by flash column chromatography (methylene chloride:MeOH = 50:1) to give the desired compounds **26a-g**.

*5.1.17. General procedure for the preparation of esters **27a-g**.*

A mixture of aldehyde (**26a-e**) (1.0 equiv.), ethylthioglycolate (1.2 equiv.) and triethylamine (2.0 equiv.) in DMSO (10 mL) was heated to 120 ℃ for 3 hours. The reaction mixture was diluted with EtOAc (30 mL), washed with water (10 mL), dried over $MgSO_4$ and concentrated. The resulting residue was dissolved in EtOH (20 mL) and sodium ethoxide solution (21% in EtOH, 2.0 equiv.) was added. The reaction mixture was stirred for one hour at room temperature. After completion, the reaction was quenched with water (5 mL) and the organic solvent evaporated. The reaction mixture was extracted with EtOAc (20 mL), washed with water, dried over $MgSO_4$ and concentrated. The crude residue was purified by flash column chromatography (*n*-hexane:EtOAc = 3:1) to give compounds **27a-g**.

*5.1.18. General procedure for synthesis of compounds **28-35**.*

To a stirred solution of ester (**17b**, **27a-g**) (1.0 equiv.) and *p*-anisidine (1.0 equiv.) in toluene was added trimethylaluminum (2.0 M in toluene, 1.0 equiv.) under cooling with an ice bath. The resulting mixture was allowed to reach room temperature and then heated at 100 °C overnight. After reaction completion, the reaction mixture was cooled down to room temperature, washed with CH_2Cl_2/MeOH (20:1, v/v) and the solvent evaporated. The crude was purified by flash column chromatography (methylene chloride:MeOH = 20:1) to obtain compounds **28-35**.

*5.1.18.1. N-(2-methylbenzo[d]oxazol-5-yl)-2-morpholinothieno[2,3-d]thiazole-5-carboxamide (**28**)*. Pale yellow solid; mp = 268.3 °C; ^{1}H-NMR (400 MHz, DMSO-d_6) δ 10.27 (s, 1H), 8.17 (s, 1H), 8.05 (d, J = 0.8 Hz, 1H), 7.64 - 7.59 (m, 2H), 3.75 (t, J = 4.8 Hz, 4H), 3.55 (t, J = 5.0 Hz, 4H), 2.61 (s, 3H); ^{13}C-NMR (100 MHz, DMSO-d_6) δ 175.19, 165.13, 160.73, 158.43, 147.21, 141.74, 135.93, 132.40, 122.85, 121.56, 118.05, 111.19, 110.52, 65.81, 48.52, 14.62; LCMS (electrospray) m/z 401 $[M + H]^+$; HRMS (ESI) calculated for $C_{18}H_{17}N_4O_3S_2$ $[M + H]^+$ 401.0742, found 401.0739.

*5.1.18.2. N-(2-Methylbenzo[d]oxazol-5-yl)-2-(piperazin-1-yl)thieno[2,3-d]thiazole-5-carboxamide (**29**)*. Pale yellow solid; mp = 238.5 °C; ^{1}H-NMR (400 MHz, DMSO-d_6) δ 10.23 (s, 1H), 8.13 (s, 1H), 8.05 (s, 1H), 7.60 - 7.63 (m, 2H), 4.85 (d, J = 4.0 Hz, 1H), 3.79 - 3.82 (m, 3H), 3.36 - 3.39 (m, 2H), 2.60 (s, 3H), 1.84 - 1.87 (m, 2H), 1.46 - 1.51 (m, 2H); ^{13}C-NMR (100 MHz, DMSO-d_6) δ 173.48, 164.03, 159.69, 157.84, 146.10, 140.66, 134.91, 130.67, 121.61, 120.44, 116.93, 110.06, 109.43, 64.31, 45.33, 32.53, 13.55; LCMS (electrospray) m/z 415 $[M + H]^+$.

*5.1.18.3. 2-(4-(Hydroxymethyl)piperidin-1-yl)-N-(2-methylbenzo[d]oxazol-5-yl)thieno[2,3-d]thiazole-5-carboxamide (**30**)*. White solid; mp = 196.4°C; ^{1}H-NMR (400 MHz, acetone-d_6) δ 9.61 (brs, 1H, NH), 8.13 (s, 1H), 8.00 (s, 1H), 7.65 (d, J = 9.2 Hz, 1H), 7.49 (d, J = 9.2 Hz, 1H), 4.08 - 4.11 (m, 2H), 3.80 - 4.90 (m, 1H), 3.46 (s, 2H), 3.15 - 3.21 (m, 2H), 2.59 (s, 3H), 1.79 - 1.90 (m, 2H), 1.28 - 1.39 (m, 2H); LCMS (electrospray) m/z 429 $[M + H]^+$.

*5.1.18.4. 2-(2,6-Dimethylmorpholino)-N-(2-methylbenzo[d]oxazol-5-yl)thieno[2,3-d]thiazole-5-carboxamide (**31**)*. White solid; mp = 274°C; ^{1}H-NMR (400 MHz, DMSO-d_6) δ 10.27 (s, 1H), 8.17 (s, 1H), 8.06 (s, 1H), 7.60-7.62 (m, 2H), 3.84 - 3.87 (m, 2H), 3.70 - 3.72 (m, 2H), 2.81 - 2.87 (m, 2H), 2.60 (s, 3H), 1.17 (d, J = 5.6 Hz, 6H); ^{13}C-NMR (100 MHz, DMSO-d_6) δ

174.72, 165.13, 160.73, 158.48, 147.20, 141.74, 135.95, 132.25, 122.71, 121.58, 118.01, 111.15, 110.52, 71.07, 53.44, 18.87, 14.61; LCMS (electrospray) m/z 429 $[M + H]^+$.

*5.1.18.5. 2-(4-(Dimethylcarbamoyl)piperidin-1-yl)-N-(2-methylbenzo[d]oxazol-5-yl)thieno[2,3-d]thiazole-5-carboxamide (**32**).* White solid; mp = 284.2 °C; ^{1}H-NMR (400 MHz, DMSO-d_6) δ 10.25 (s, 1H), 8.15 (s, 1H), 8.05 (s, 1H), 7.60 - 7.61 (m, 2H), 3.99 - 4.02 (m, 2H), 3.06 (s, 3H), 2.97 - 2.99 (m, 2H), 2.82 (s, 3H), 2.60 (s, 3H), 1.75 - 1.78 (m, 2H), 1.59 - 1.63 (m, 3H); ^{13}C-NMR (100 MHz, DMSO-d_6) δ 174.64, 173.74, 165.13, 160.76, 158.83, 147.18, 141.73, 135.97, 131.84, 122.67, 121.53, 118.02, 111.15, 110.51, 48.27, 37.17, 37.09, 35.50, 27.83, 14.62; LCMS (electrospray) m/z 470 $[M + H]^+$; HRMS (ESI) calculated for C22H24N5O3S2, $[M + H]^+$ 470.1321, found 470.1321.

*5.1.18.6. 2-((2-Hydroxyethyl)(methyl)amino)-N-(2-methylbenzo[d]oxazol-5-yl)thieno[2,3-d]thiazole-5-carboxamide (**33**).* Yellow solid; mp = 226.3 °C; ^{1}H-NMR (400 MHz, DMSO-d_6) δ 10.22 (s, 1H), 8.14 (s, 1H), 8.06 (s, 1H), 7.57 - 7.63 (m, 2H), 4.90 (t, J = 5.2 Hz, 1H), 3.64 - 3.70 (m, 2H), 3.57 - 3.63 (m, 2H), 3.17 (s, 3H), 2.60 (s, 3H); ^{13}C-NMR (100 MHz, DMSO-d_6) δ 174.61, 165.08, 160.80, 159.23, 147.12, 141.70, 136.01, 131.04, 122.31, 121.50, 117.94, 111.06, 110.49, 58.42, 55.61, 14.61; LCMS (electrospray) m/z 389 $[M + H]^+$.

*5.1.18.7. 2-((3-Hydroxypropyl)(methyl)amino)-N-(2-methylbenzo[d]oxazol-5-yl)thieno[2,3-d]thiazole-5-carboxamide (**34**).* White solid; mp = 213.8 °C; ^{1}H-NMR (400 MHz, DMSO-d_6) δ 10.23 (s, 1H), 8.14 (s, 1H), 8.06 (s, 1H), 7.60 - 7.61 (m, 2H), 4.58 - 4.61 (m, 1H), 3.59 - 3.60 (m, 2H), 3.46 - 3.50 (m, 2H), 3.13 (s, 3H), 2.60 (s, 3H), 1.78 - 1.81 (m, 2H); ^{13}C-NMR (100 MHz, DMSO-d_6) δ 174.29, 165.08, 160.80, 159.26, 147.12, 141.71, 136.01, 131.08, 122.32, 121.49, 117.94, 111.06, 110.48, 58.55, 50.61, 38.59, 30.11, 14.60; LCMS (electrospray) m/z 403 $[M + H]^+$.

*5.1.18.8. N-(2-Methylbenzo[d]oxazol-5-yl)-2-((2-morpholinoethyl)amino)thieno[2,3-d]thiazole-5-carboxamide (**35**).* Ivory solid; mp = 196 °C; ^{1}H-NMR (400 MHz, DMSO-d_6) δ 10.19 (s, 1H), 8.35 (d, J = 5.4 Hz, 1H), 8.06 (s, 1H), 8.05 (d, J = 0.8 Hz, 1H), 7.63 - 7.58 (m, 2H), 3.59 (t, J = 4.6 Hz, 4H), 3.48 (q, J = 6.1 Hz, 2H), 2.60 (s, 3H), 2.54 (t, J = 6.6 Hz, 2H), 2.43 (t, J = 4.4 Hz, 4H); ^{13}C-NMR (100 MHz, DMSO-d_6) δ 173.45, 165.09, 160.82, 158.65, 147.14, 141.73, 136.04, 131.22, 122.03, 121.49, 118.00, 111.12, 110.48, 66.63, 57.31, 53.77, 41.96, 14.62; LCMS (electrospray) m/z 444 $[M + H]^+$.

*5.1.19. General procedure for the preparation of biaryl amines **37a-f**.*

To a stirred solution of pyridine **36 (a-f)** in 1,2-dimethoxyethane/H_2O (3:1,v/v) solvent mixture was added 4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)aniline (1.2 equiv.), 1,1'-bis(diphenylphosphino)ferrocene)-dichloropalladium(II) (0.1 equiv.) and Na_2CO_3 (2.0 equiv.). The mixture was stirred at 150 ℃ for 1 hour and the mixture cooled down to room temperature. The palladium catalyst was filtered out and the clarified mixture extracted with EtOAc (30 mL), washed with brine (10 mL), dried over $MgSO_4$ and concentrated. The resulting residue was purified by flash column chromatography (*n*-hexane:EtOAc = 7:3) to give **37a-f**.

*5.1.20. General procedure for the preparation of biaryl amines **37g-h**.*

To a solution of 3-amino-6-bromopyridine (**36g**) or 2-amino-5-bromopyridine (**36h**) (1.0 equiv.) in 1,2-dimethoxyethane/H_2O (3:1,v/v) solvent mixture was added 4-fluorophenylboronic acid (1.0 equiv.), 1,1'-bis(diphenylphosphino)ferrocene)-dichloropalladium(II) (0.1 equiv.) and Na_2CO_3 (2.0 equiv.). The mixture was stirred at 150 ℃ for 1 hour and the mixture cooled down to room temperature. The palladium catalyst was filtered out and the clarified mixture extracted with EtOAc (30 mL), washed with brine (10 mL), dried over $MgSO_4$ and concentrated. The resulting residue was purified by flash column chromatography (*n*-hexane:EtOAc = 7:3) to give **37g** and **37h**.

*5.1.21. Procedure for the synthesis of compound **38-45**.*

To a stirred solution of compound **27g** (1.0 equiv.) and amine **37a-h** (1.0 equiv.) in toluene was added trimethylaluminum (2.0 M in toluene, 1.0 equiv.) under cooling in an ice bath. The resulting mixture was allowed to reach room temperature and then heated at 100 ℃ overnight. After reaction completion, the mixture was cooled down at room temperature, washed with CH_2Cl_2/MeOH (20:1, v/v) and the solvent evaporated. The crude was purified by flash column chromatography (methylene chloride:MeOH = 20:1) to obtain compounds **38-45**.

*5.1.21.1. N-(4-(5-Methylpyridin-2-yl)phenyl)-2-((2-morpholinoethyl)amino)thieno[2,3-d]thiazole-5-carboxamide (**38**).* Yellow solid; mp = 124.1 °C; ^{1}H-NMR (400 MHz, DMSO-d_6) δ 10.21 (s, 1H), 8.47 (d, J = 2.0 Hz, 1H), 8.37 (s, 1H), 8.10 (s, 1H), 8.06 (d, J = 8.4 Hz, 2H), 7.84 (d, J = 8.4 Hz, 1H), 7.80 (d, J = 8.4 Hz, 2H), 7.67 (dd, J = 8.4, 2.0 Hz, 1H), 3.56 - 3.58 (m, 4H), 3.46 - 3.50 (m, 2H), 2.50 - 2.55 (m, 2H), 2.40 - 2.44 (m, 4H), 2.32 (s, 3H); ^{13}C-NMR (100 MHz, DMSO-d_6) δ 173.50, 160.80, 158.83, 153.47, 150.12, 140.06, 137.92, 134.00,

131.70, 131.19, 127.02, 122.08, 121.70, 120.38, 119.51, 66.60, 57.28, 53.74, 41.91, 18.06; LCMS (electrospray) 480 m/z $[M + H]^+$.

*5.1.21.2. N-(4-(5-Methoxypyridin-2-yl)phenyl)-2-((2-morpholinoethyl)amino)thieno[2,3-d]thiazole-5-carboxamide (**39**).* Yellow solid; mp = 238.4°C; ^{1}H-NMR (400 MHz, DMSO-d_6) δ 10.19 (s, 1H), 8.35 - 8.37 (m, 2H), 8.10 (s, 1H), 8.00 (d, J = 8.0 Hz, 2H), 7.88 (d, J = 8.8 Hz, 1H), 7.80 (d, J = 8.8 Hz, 2H), 7.45 (d, J = 7.6 Hz, 1H), 3.87 (s, 3H), 3.584 (s, 4H), 3.47 - 3.49 (m, 2H), 2.54 - 2.56 (m, 2H), 2.43 (s, 4H); ^{13}C-NMR (100 MHz, DMSO-d_6) δ 173.47, 160.77, 158.77, 154.90, 148.73, 139.56, 137.44, 133.89, 131.26, 126.66, 122.09, 121.87, 121.65, 120.53, 120.44, 66.55, 57.25, 56.09, 53.71, 41.88; LCMS (electrospray) m/z 496 $[M + H]^+$.

*5.1.21.3. 2-((2-Morpholinoethyl)amino)-N-(4-(5-(trifluoromethyl)pyridin-2-yl)phenyl)thieno[2,3-d]thiazole-5-carboxamide (**40**).* Yellow solid; mp = 253.3 °C; ^{1}H-NMR (400 MHz, DMSO-d_6) δ 10.30 (s, 1H), 9.00 (s, 1H), 8.40 - 8.38 (m, 1H), 8.26 - 8.23 (m, 1H), 8.19 - 8.16 (m, 3H), 8.12 - 8.11 (m, 1H), 7.91 - 7.88 (m, 2H), 3.59 (d, J = 4.0 Hz, 4H), 3.49-3.47 (m, 2H), 2.55-2.53 (m, 2H), 2.43 (s, 4H); ^{13}C-NMR (100 MHz, DMSO-d_6) δ 173.62, 160.96, 159.83, 159.08, 146.64 (d, J = 5.0 Hz), 141.60, 135.02 (d, J = 4.0 Hz), 132.27, 130.97, 128.15, 124.48 (q, J = 270.0 Hz), 123.51 (q, J = 32.0 Hz), 122.17, 121.97, 120.40, 120.08, 66.61, 57.29, 53.76, 41.96; LCMS (electrospray) m/z 534 $[M + H]^+$.

*5.1.21.4. N-(4-(5-Chloropyridin-2-yl)phenyl)-2-((2-morpholinoethyl)amino)thieno[2,3-d]thiazole-5-carboxamide (**41**).* Yellow solid; mp = 239.5 °C; ^{1}H-NMR (400 MHz, DMSO-d_6) δ 10.26 (s, 1H), 8.67 (s, 1H), 8.38 (brs, 1H), 8.07 - 8.11 (m, 3H), 7.98 (s, 2H), 7.85 (d, J = 8.0 Hz, 2H), 3.56 (s, 4H), 3.49 (s, 2H), 2.50 - 2.52 (m, 2H), 2.44 (s, 4H); ^{13}C-NMR (100 MHz, DMSO-d_6) δ 173.57, 160.89, 158.96, 154.71, 148.35, 140.80, 137.32, 132.66, 131.10, 129.81, 127.47, 122.15, 121.85, 121.34, 120.42, 66.57, 57.25, 53.72, 41.92; LCMS (electrospray) m/z 500 $[M + H]^+$.

*5.1.21.5. N-(4-(5-Fluoropyridin-2-yl)phenyl)-2-((2-morpholinoethyl)amino)thieno[2,3-d]thiazole-5-carboxamide (**42**).* Ivory solid; mp = 237.9 °C; ^{1}H-NMR (400 MHz, DMSO-d_6) δ 10.24 (s, 1H), 8.62 (d, J = 3.2 Hz, 1H), 8.37 (t, J = 5.4 Hz, 1H), 8.10 (s, 1H), 8.05 (dd, J = 7.0, 1.8 Hz, 2H), 8.01 (t, J = 4.4 Hz, 1H), 7.84 (dd, J = 6.8, 2.0 Hz, 2H), 7.79 (td, J = 8.8, 3.2 Hz, 1H), 3.59 (t, J = 4.6 Hz, 4H), 3.48 (q, J = 6.0 Hz, 2H), 2.54 (t, J = 6.6 Hz, 2H), 2.43 (t, J = 4.4 Hz, 4H); ^{13}C-NMR (100 MHz, DMSO-d_6) δ 173.55, 160.87, 158.92, 157.56, 152.87, 152.84,

140.36, 137.85, 137.61, 133.04, 131.12, 127.34, 124.66, 124.48, 122.11, 121.78, 121.50, 121.46, 120.44, 66.63, 57.31, 53.77, 41.97; LCMS (electrospray) m/z 484 $[M + H]^+$; HRMS (ESI) calculated for $C_{23}H_{23}FN_5O_2S_2$, $[M + H]^+$ 484.1277, found 470.1280.

*5.1.21.6. N-(4-(6-Fluoropyridin-3-yl)phenyl)-2-((2-morpholinoethyl)amino)thieno[2,3-d]thiazole-5-carboxamide (**43**).* Ivory solid; ^{1}H-NMR (400 MHz, DMSO-d_6) δ 10.24 (s, 1H), 8.55 (d, J = 2.8 Hz, 1H), 8.39 (s, 1H), 8.24 - 8.30 (m, 1H), 8.10 (s, 1H), 7.85 (d, J = 8.4 Hz, 2H), 7.73 (d, J = 8.4 Hz, 2H), 7.27 (dd, J = 8.4, 2.8 Hz, 1H), 3.56 - 3.60 (m, 4H), 3.46 - 3.49 (m, 2H), 2.50 - 2.55 (m, 2H), 2.39 - 2.46 (m, 4H); LCMS (electrospray) m/z 484 $[M + H]^+$.

*5.1.21.7. N-(6-(4-Fluorophenyl)pyridin-3-yl)-2-((2-morpholinoethyl)amino)thieno[2,3-d]thiazole-5-carboxamide (**44**).* Yellow solid; mp = 246.3 °C; ^{1}H-NMR (400 MHz, DMSO-d_6) δ 10.37 (s, 1H), 8.92 (d, J = 2.0 Hz, 1H), 8.39 (brs, 1H), 8.20 (dd, J = 8.8, 2.4 Hz, 1H), 8.07 - 8.09 (m, 3H), 7.94 (d, J = 8.8 Hz, 1H), 7.28 (t, J = 8.8 Hz, 2H), 3.57 (t, J = 4.8 Hz, 4H), 3.44 - 3.47 (m, 2H), 2.51 - 2.54 (m, 2H), 2.41 (s, 4H); ^{13}C-NMR (100 MHz, DMSO-d_6) δ 173.71, 164.22, 161.78, 161.13, 159.20, 150.16, 141.70, 135.35, 130.38, 128.69, 128.32, 122.18, 120.30, 116.12, 115.90, 66.63, 57.30, 53.77, 41.97; LCMS (electrospray) m/z 484 $[M + H]^+$.

*5.1.21.8. N-(5-(4-Fluorophenyl)pyridin-2-yl)-2-((2-morpholinoethyl)amino)thieno[2,3-d]thiazole-5-carboxamide (**45**).* Yellow solid; mp = 202.9 °C; ^{1}H-NMR (400 MHz, DMSO-d_6) δ 10.87 (s, 1H), 8.68 (s, 1H), 8.43 (m, 1H), 8.32 (s, 1H), 8.21 (d, J = 9.2 Hz, 1H), 8.12 (dd, J = 8.8, 2.4 Hz, 1H), 7.81 – 7.77 (m, 2H), 7.35 – 7.30 (m, 2H), 3.58 (brs, 4H), 3.49 - 3.46 (m, 3H), 2.54 - 2.51 (m, 2H), 2.42 (brs, 4H); ^{13}C-NMR (100 MHz, DMSO-d_6) δ 173.65, 163.69, 161.23, 159.58, 151.89, 146.06, 136.57, 133.76, 130.66, 128.98, 122.61, 122.36, 116.49, 116.27, 114.61, 66.63, 57.28, 53.76, 41.97; LCMS (electrospray) m/z 484 $[M + H]^+$.

5.2. Biological assay

5.2.1. In vitro activity against M. tuberculosis

The aim of these experiments was to evaluate the anti-tuberculosis activities of newly synthesized TTCA derivatives. Three assays were used: *i*) a whole cell assay, or extracellular assay, where GFP-expressing bacteria were directly in contact with the compounds. Quantification was done by measuring the level of fluorescence in the well. *ii*) An intracellular assay where bacteria were first used to infect macrophages and the resulting infected cells were

incubated with the compounds. In this case, the reading was done by fluorescence microscopy and the number of GFP-expressing bacteria infecting cells was quantified through image-analysis. Lastly, *iii*) an extracellular assay using drug-resistant, clinical isolates, where bacteria were directly in contact with the compounds. The minimum inhibitory concentrations (MICs) was determined by the agar proportion method.

5.2.1.1. Extracellular assay. This assay was used to determine the concentration of compounds required to inhibit 50% of the bacterial growth (IC_{50}). The assay was performed as follows. The day of the experiment, 384-well plates (Greiner, #781091) were first prepared by automated dispensing of 0.5 µL of compound to be tested in 10 µL of PBS (EVOBird, Evotec). Two dose-responses of 10 points, starting at 20 µM and 1 µM, respectively, with a 2-fold dilution factor between each dose, were prepared. Positive controls (1 µg/mL rifampicin) together with negative controls (1% DMSO) were included in each plate to validate the performance of the assay. *Mycobacterium tuberculosis* strain H37Rv (ATCC27294) constitutively expressing the green fluorescent protein (GFP), referred to as H37Rv-GFP, was grown in 7H9 broth (Invitrogen) supplemented with 10% Oleic acid-Albumin-Dextrose-Catalase (OADC, Middlebrook), 0.05% Tween 80 (Sigma-Aldrich), 0.5% glycerol (Invitrogen) and 50 µg/mL hygromycin B (Invitrogen). Bacteria were grown for 14 days at 37°C, 5% CO_2 in ventilated Erlenmeyer flasks without shaking, with dilution at $OD_{600\ nm}$ = 0.1 using fresh medium once a week. Bacteria were harvested by centrifugation at 6,000 × *g* for 10 min and washed twice with phosphate buffered saline (PBS, Welgene) prior to infection. Absence of clumps, *i.e.* bacterial aggregates, was confirmed by light microscopy. The H37Rv-GFP bacterial suspension was diluted to $OD_{600\ nm}$ = 0.03 (~3 × 10^6 CFU/mL) using complete 7H9 medium and 40 µL were added to each well of the 384-well plates, resulting in a final volume of 50 µL containing 1% DMSO. Plates were incubated at 37°C, 5% CO_2 for 5 days. Mycobacterial growth was determined by measuring the GFP fluorescence using a multi-well plate reader (Victor 3, PerkinElmer). IC_{50} values were determined by fitting the fluorescence curves against a dose-response sigmoid by least-square regression (4 parameters) using Prism software v.5 (GraphPad).

5.2.1.2. Intracellular assay. Raw 264.7 murine macrophages were grown at 37°C, 5% CO_2 in RPMI 1640 medium (Welgene) supplemented with 10% heat-inactivated fetal bovine serum (FBS), referred to as RPMI-FBS medium. Cells were passaged every 2 or 3 days (70%

confluence) and used between passages 2 and 9. *M. tuberculosis* H37Rv-GFP was cultured in complete 7H9 medium and washed with PBS as described above for the extracellular assay. The day of the infection, macrophage cells were treated with 1× Versene (Gibco) for 10 min at 37ºC, gently detached using a cell-scraper, centrifuged at 300 × *g* for 5 min and resuspended in fresh RPMI-FBS medium. Cells were enumerated using a Thoma cell counting chamber and suspensions at 1×10^6 cells/mL were prepared. Pellets of freshly PBS-washed bacteria were resuspended in RPMI-FBS at the required concentrations to yield a multiplicity of infection (MOI) of 20 bacteria per cell. One volume of bacterial suspension was mixed with one volume of cell and the mixture was incubated in an Erlenmeyer flask for 2h at 37°C with mild shaking (100 rpm). Infected cells were washed twice with fresh medium and plated at 20,000 cells/well in 384-well plates (Greiner, #781091, 40 µL/well) containing two-fold dilutions of compounds in PBS (10 µL/well). After 5 days incubation at 37°C, 5% CO_2, 10 µL/well of a 30 µM Hoechst 33342 (Invitrogen) solution in PBS was added (final concentration 5 µM) and plates were incubated for 20 min at 37°C, 5% CO_2 before imaging by fluorescence microscopy using a 20X objective (Operetta, PerkinElmer). GFP bacteria were detected using a 488 nm laser excitation and cell nuclei using a 405 nm laser excitation. Three fields were recorded for each well and each image was processed using a dedicated in-house image analysis software, to quantify the number of cells, the total surface of bacteria (as a number of pixels), the average surface of bacteria per infected cells and the ratio of infected cells. Values per well were average of the values obtained per field. IC_{50} values were determined for all 4 parameters by fitting the curves against a dose-response sigmoid by least-square regression (4 parameters). Final values were average of the IC_{50} values found for the 4 parameters, excluding values when the fit was not successful.

5.2.1.3. Extracellular assay with drug-resistant strains. Eleven multidrug-resistant (MDR) and nine (XDR) clinical isolates with a known resistant profile (Supplementary Table 1) were acquired from the Korean Institute for Tuberculosis (KIT) in Korea. The MIC of compound **42** was determined by CLSI reference method for susceptibility testing of *M. tuberculosis* agar proportion method performed with Middlebrook 7H11 agar [12].

5.2.2. In vitro ADME and toxicity assays

All experimental methods (microsomal metabolic stability, CYP450 inhibition assays,

plasma protein binding assay, hERG patch clamp assay, mini-Ames assay, cell cytotoxicity assay, kinetic solubility) are available in the supplementary data.

5.2.3. Pharmacokinetic studies

Female BALB/c mice aged 6-8 weeks were used for the pharmacokinetic studies. Tested compounds were given at a dose of 2 and 3 mg/kg body weight intravenously 10 mg/kg body weight orally. Compounds **28** was formulated in 1% DMSO / 30% Captisol in distilled water. Compound **32** was formulated in 10% DMSO / 50% PEG400 / 40% (30% SBE-b-CD). Compound **42** was formulated in 10% DMSO / 5% Cremophor EL / 85% (30% Captisol in distilled water). All blood samples were placed in 4°C refrigerators within 1 hour following collection, before centrifugation at 3200 × *g* for 10 min at 4°C. Following centrifugation, plasma samples were transferred into appropriately labeled 1.5 mL polypropylene tube and stored in 4°C refrigerators. Compound concentrations were determined by LC-MS.

5.2.4. In vivo efficacy

For the murine model of tuberculosis infection, female BALB/c mice of 6 to 8 weeks old were acclimated for 1 week in the BSL3 facility before intranasal inoculation of ~ 300 to 600 CFU of *M. tuberculosis* H37Rv. The day of the infection, a glycerol stock of H37Rv strain was thawed at room temperature, diluted 50,000 fold in PBS to reach an estimated concentration of 10 CFU/µL, and 50 µL of the resulting solution slowly deposited in one nostril of the anesthetized mice. Groups were composed of 5 mice. A non-treated and isoniazid-treated group were included as end-point controls. Day 1 and day 21 post-inoculation control groups were used to monitor the infection. Treatments were administered by daily gavage (5 times a week) for 4 weeks. At the required time point or at the end of the experiment (day 49), animals were sacrificed and lungs harvested. One lung was kept in 1 mL formalin solution for at least 2 weeks at 4°C prior to histology studies. The second lung was added in 2 mL PBS and immediately homogenized using a gentleMACS dissociator (Miltenyi). M tubes were used with the pre-set program RNA 1. Lung homogenates were serial diluted 10-fold in PBS and 10 µL of each dilution (total 7 dilutions), together with the undiluted homogenates, were plated in duplicate, in several drops, on 7H11 agar plates, supplemented with 10% OADC, 0.05% Tween 80 and 0.5% glycerol. Agar plates were incubated at 37 °C and 5% CO_2 for 2 to 5 weeks, with regular counting of the colony forming units (CFU) at each dilution. Counts were averaged for

each mouse. Using this dilution strategy, the limit of detection for the CFU counts was around 2.3 logs, *i.e*, no colonies can be enumerated if there are less than 200 CFU in the lungs. Statistical significance for the number of CFU in treated against untreated mice (NT) was calculated by a Bonferroni's multiple comparison test using the software Prism v.5 (GraphPad).

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

The following Supplementary data are available for this article:

Experimental methods of *in vitro* ADME and toxicity assays

^{1}H and ^{13}C NMR spectrums of selected compounds **28**, **32** and **42**

Activity of compound **42** against *M. tuberculosis* clinical isolates

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Graphical abstract