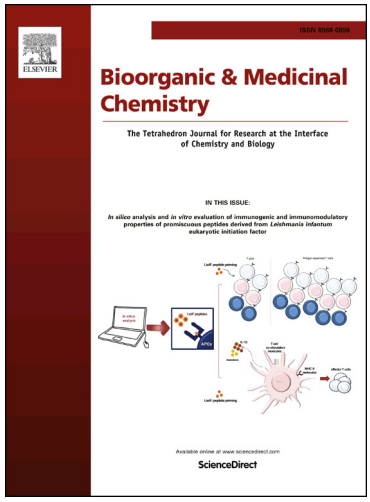
Accepted Manuscript



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PII: S0968-0896(17)31809-6
DOI: https://doi.org/10.1016/j.bmc.2017.12.047
Reference: BMC 14152

To appear in: *Bioorganic & Medicinal Chemistry*

Received Date: 13 September 2017
Revised Date: 20 December 2017
Accepted Date: 28 December 2017

Please cite this article as: Kumar, M., Singh, K., Ngwane, A.H., Hamzabegovic, F., Abate, G., Baker, B., Wiid, I., Hoft, D.F., Ruminski, P., Chibale, K., Reversed Isoniazids: Design, synthesis and evaluation against *Mycobacterium tuberculosis*, *Bioorganic & Medicinal Chemistry* (2017), doi: https://doi.org/10.1016/j.bmc.2017.12.047

Reversed Isoniazids: Design, synthesis and evaluation against *Mycobacterium tuberculosis*

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Abstract:

Novel reversed isoniazid (RINH) agents have been synthesized by covalently linking isoniazid with various efflux pump inhibitors (EPIs) cores and their structural motifs. These RINH agents were evaluated for anti-mycobacterial activity against sensitive, isoniazid mono-resistant and MDR clinical isolates of *M. tuberculosis* and a selected number of compounds were also tested *ex vivo* for intracellular activity as well as in the ethidium

bromide (EB) assay for efflux pump inhibition efficacy. The potency of some compounds against various strains of *M. tuberculosis* (**4a-c**, **7** and **8**; H37Rv-$MIC_{99} \leq 1.25$ μM, R5401-$MIC_{99} \leq 2.5$ μM, X_61- $MIC_{99} \leq 5$ μM) demonstrated the viability of reversed anti-TB agent strategy towards the development of novel anti-mycobacterial agents to address the rapidly growing issue of resistance. Further, macrophage activity with >90% inhibition by **1a-c** and **3b** ($MIC_{90} \leq 13.42$ μM) and inhibition of EB efflux demonstrated by these compounds are encouraging.

KEYWORDS: efflux pumps, chemosensitizers, macrophages, efflux pump inhibitors, *M. tuberculosis,* ethidium bromide.

1. Introduction

Tuberculosis (TB) caused by *Mycobacterium tuberculosis* (*M. tuberculosis*) is one of the most fatal infectious diseases. It is more prevalent in poor and under developed countries, especially in Africa and the South East Asian Region, and is responsible for 1.5 million deaths annually.[1] The rapid and widespread emergence of multi-drug resistant (MDR) and extremely-drug resistant (XDR) strains have hampered TB control and its eradication.[2,3] The emergence of resistance against first line anti-TB drugs (isoniazid, rifampicin, pyrazinamide and ethambutol) has complicated treatment both financially as well as socially.[4] Therefore, urgent efforts are required to counter this challenge.

Efflux pumps (EPs) are membrane bound active transporters, which decrease the antibacterial activity of unrelated drug families by reducing the cytoplasmic concentration of the drugs to sub-inhibitory levels. The survival of *M. tuberculosis* in the presence of such sub-inhibitory drug concentrations is facilitated and results in the development of resistance.[5,6] A number of measures to counter EP-mediated resistance have been adopted in the past including (i) engineering of the native drug molecule to by-pass EPs,[7] and (ii) cocktail use of drugs and efflux pump inhibitors (EPIs).[8] These approaches have shown promising results and prospects for the development of anti-TB drugs. Recently reported semisynthetic analogues of spectinomycin demonstrated potent anti-tubercular properties by evading EPs.[9] A number of efflux pump genes (*mmpL7*, *p55*, *efpA*, *mmr*, *Rv1258c* and *Rv2459*) are responsible for low to high levels of isoniazid (INH) resistance in *M. tuberculosis.*[10] The over expression of these genes is known to be countered by EPIs leading to increased susceptibility of MDR strains towards INH.[11] However, the combination of anti-TB drugs with EPIs as adjunctive agents presents additional challenges from a pharmacological perspective, which hinder the further

development of this combination strategy in clinical phases. Therefore, covalently linking an EPI or chemosensitizer moiety to INH to generate hybrid compounds, herein referred to as reversed INH agents, presents good prospective to overcome this liabilities and may reverse EP-mediated resistance and revitalize its anti-mycobacterial activity against M(/X)DR-TB.

Reversed hybrid compounds as antibacterial agents have already been reported.[12] In this regard, the dual action antibacterial agent **SS14,** developed by covalently linking the antibacterial agent berberine (a substrate of NorA-EP) and an EPI, **INF55** (5-nitro-2-phenylindole) showed enhanced accumulation of berberine in bacteria leading to superior potency as compared to a cocktail of these two agents (Figure 1). [12,13]

On the other hand, the concept of resistance reversal by reversed agents in malaria has been explored by Peyton and co-workers by developing reversed chloroquine (RCQ) agents through covalently linking chloroquine (CQ) and its analogues with various chemosensitizer cores.[14,15] When evaluated against CQ resistant *Plasmodium falciparum* strains, the RCQ agents showed resistance reversal.[16] In an analogous fashion, the concept of reversed anti-TB agents (RATAs) can also be explored in TB drug discovery (Figure 1) using various anti-TB drugs and chemosensitizers. This may present a viable strategy to mitigate the emergence of drug tolerance and/or resistance mediated by EPs.

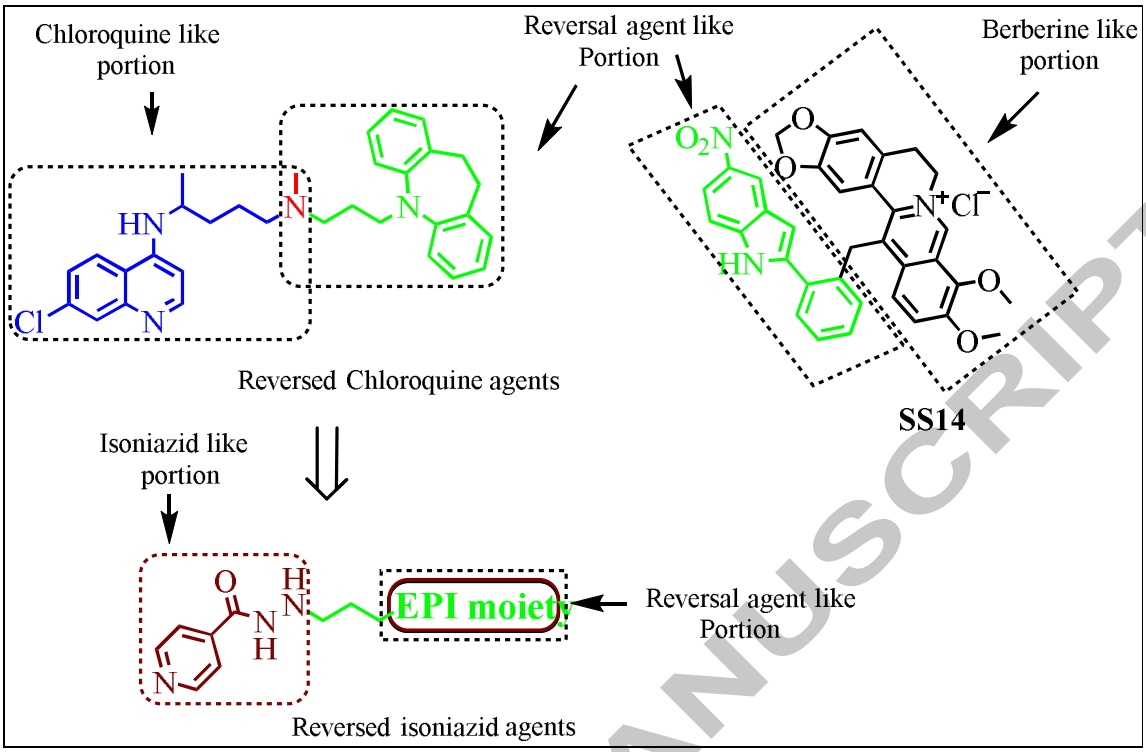


Figure 1: The concept of RCQs, **SS14** and design of RINH agents.

In this paper, we report the design, synthesis and biological evaluation of reversed isoniazid (RINH) agents as novel anti-mycobacterial agents. These RINH agents were designed by covalently linking INH with various EPIs or chemosensitizer moieties or their structural motifs derived from phenothiazines (**1a**,**b**, **2a,b** and **3a,b**), iminodibenzyl (**1c**), thioxanthene (**4a,b**), cycloheptadiene (**4c**), dibenzosuberyl (**5**), diphenylmethane (**6** and **7**) and triphenylmethane (**8**) *via* an alkyl chain linker (Figure **2**).[17,18] A propyl linker was chosen for initial studies towards establishing a proof-of-concept based on its promise in RCQs. [14,15] .

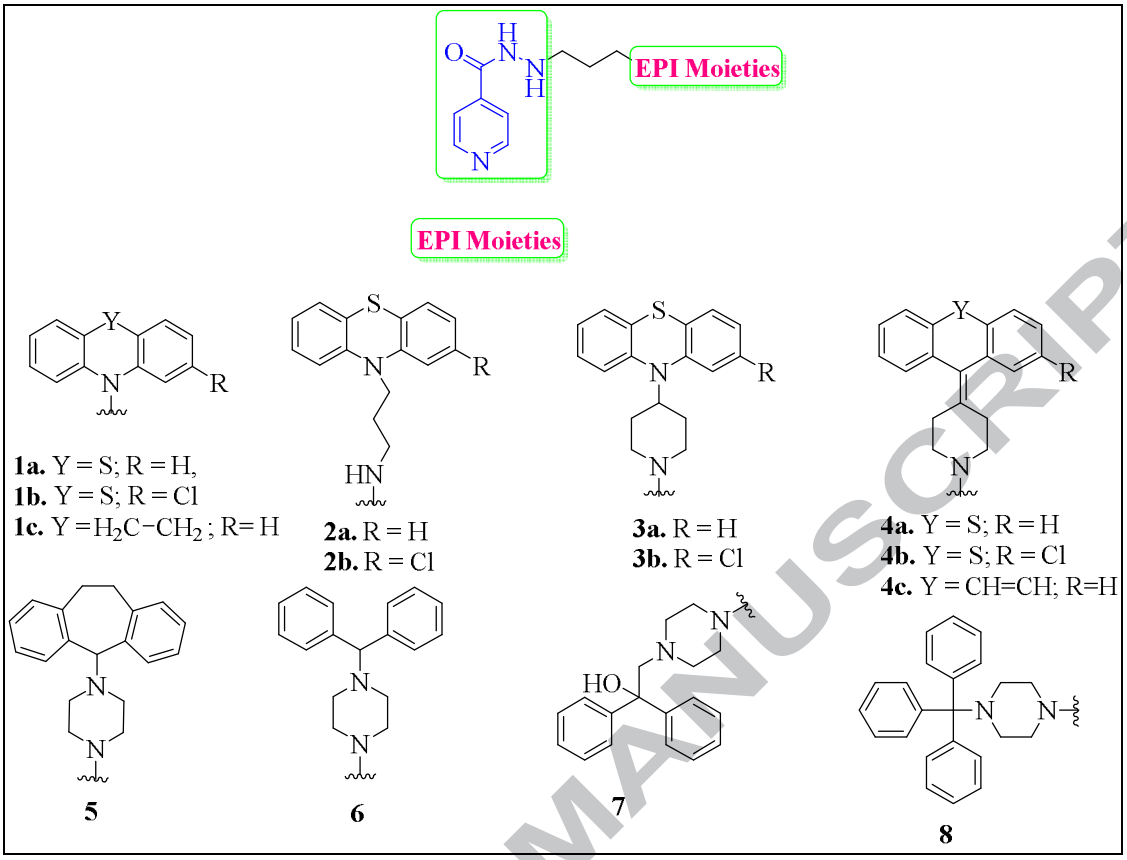
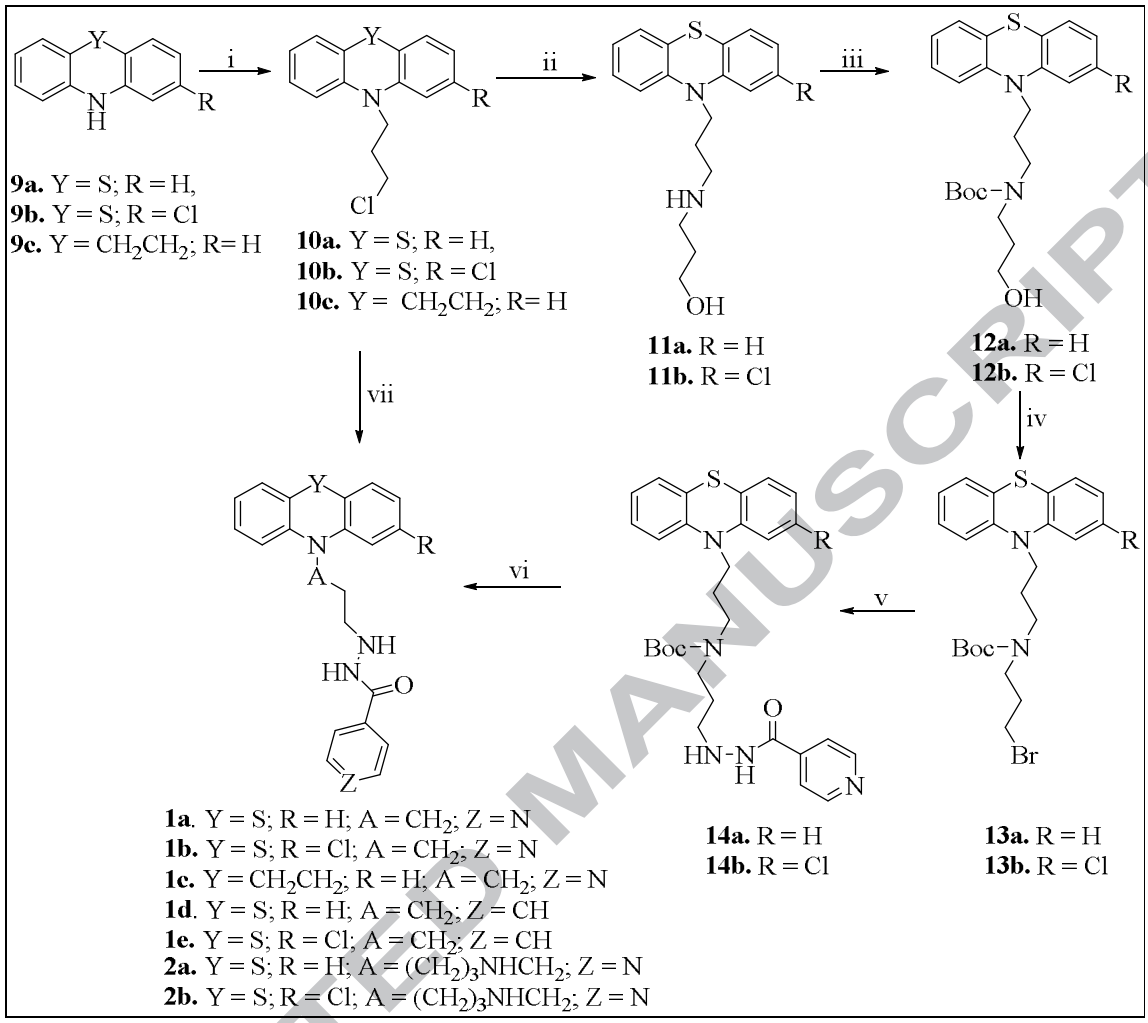


Figure 2: Designed RINH agents by covalently linking INH with various EPIs/chemosensitizers or their structural motifs.

2. Chemistry:

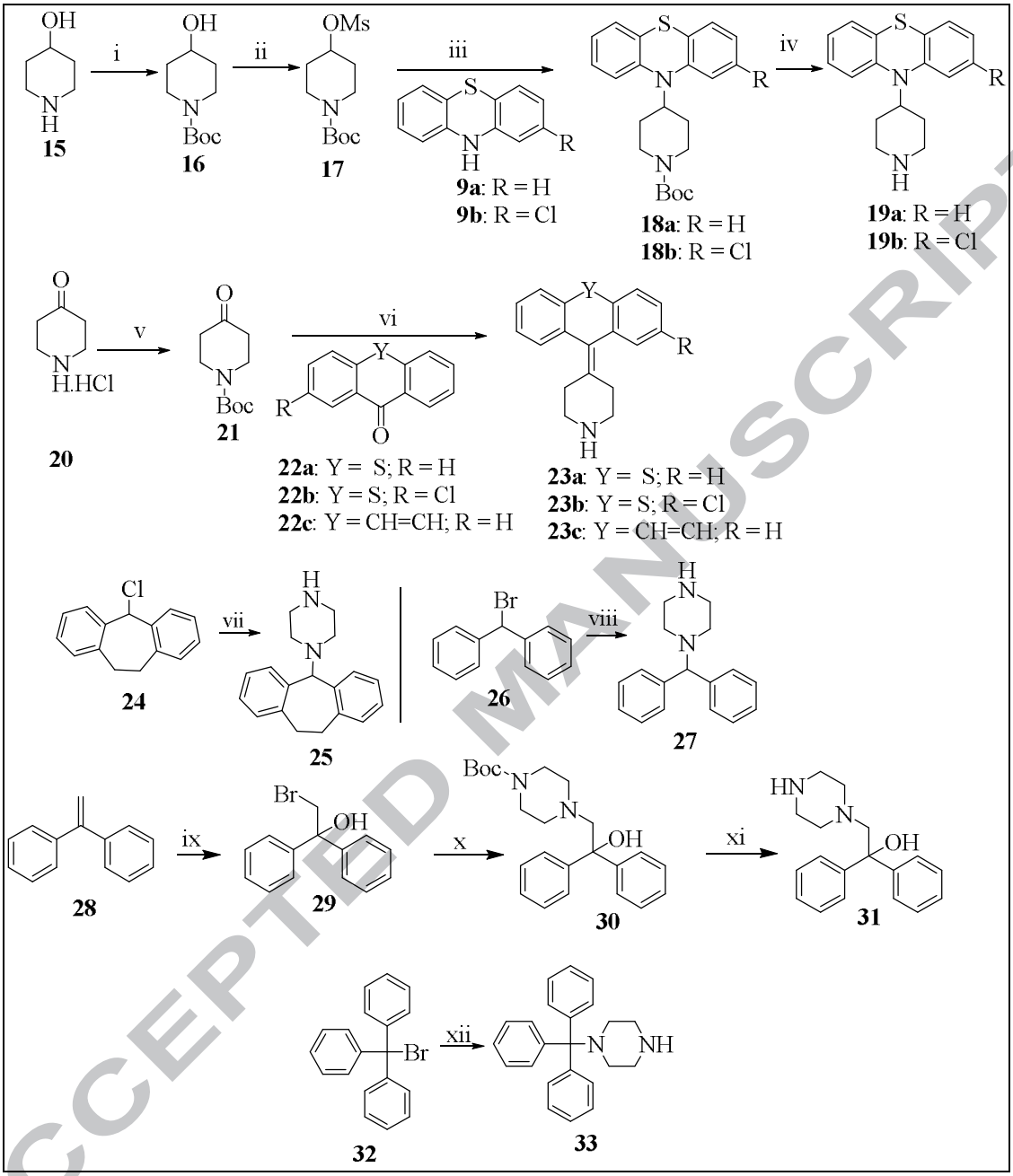
Schemes 1-3 summarize the synthesis of RINH agents **1-8**. The synthesis of compounds **1a-c** and **2a,b** commenced with the reaction of phenothiazines **9a,b** and iminodibenzyl **9c** with 1-bromo-3-chloropropane using NaH in DMF and $NaNH_2$ in toluene respectively, to obtain the corresponding intermediates **10a-c**, which upon reaction with INH and benzhydrazide afforded target compounds **1a-e**. To accomplish the synthesis of RINH agents **2a,b**, intermediates **10a,b** were reacted with 3-amino propanol using K_2CO_3 in DMF to afford the hydroxy intermediate **11a,b**, which upon reaction with Boc anhydride furnished intermediate **12a,b**. The Appel reaction on compound **12a,b** furnished the key intermediate **13a,b**, which was reacted with INH in DMF using Et_3N at 55 ºC to obtain compound **14a,b** in low yield due to over alkylation at the hydrazine NH of the target compounds. Thereafter, *N*-Boc deprotection of compound **14a,b** provided the target compounds **2a**,**b**.

Scheme 1: Synthesis of RINH agents **1a-e** and **2a,b**.



Reagents and reaction conditions: (i) 1-bromo-3-chloropropane (1.2 eq.), NaH (1.2 eq.), DMF, 0-25 °C, 11-12 h (**10a** and **10b**), 63-75%; 1-bromo-3-chloropropane (2 eq.), $NaNH_2$ (2.5 eq.), toluene, reflux (115 °C), 14 h, (**10c),** 40%; (ii) 3-aminopropanol (1.5 eq.), K_2CO_3 (1.3 eq.), DMF, 80 °C, 10-12 h; 65-68%; (iii) $(Boc)_2O$ (1.3 eq.), Et_3N (1.2 eq.), DCM, 0-25 °C, 30-40 min, 93-95%; (iv) Ph_3P (1.5 eq.), CBr_4 (1.5 eq.), DCM, 25 °C, 1.5-2 h, 82-88%; (v) isoniazid (5 eq.), Et_3N (3 eq.), DMF, 25-55 °C, 10-12 h, 30-35%; (vi) TFA/DCM (10% v/v), 25 °C, 1-1.5 h, 66-83% (**2a,b**); (vii) isoniazid (1.2 eq.), NaH (1.2 eq.), DMF, 0-25 °C, 5-6 h, (**1a**,**b**), 18-24%; isoniazid (4 eq.), Et_3N (4 eq.), *iso*-propanol, reflux (90 °C), 12-24 h, (**1c**), 15%; benzhydrazide (4 eq.), Et_3N (4 eq.), *iso*-propanol, reflux (90 °C), 12 h, (**1d** and **1e**), 20-25%.

Scheme 2: Synthesis of compounds **19a,b**, **23a-c**, **25**, **27**, **31** and **33**.



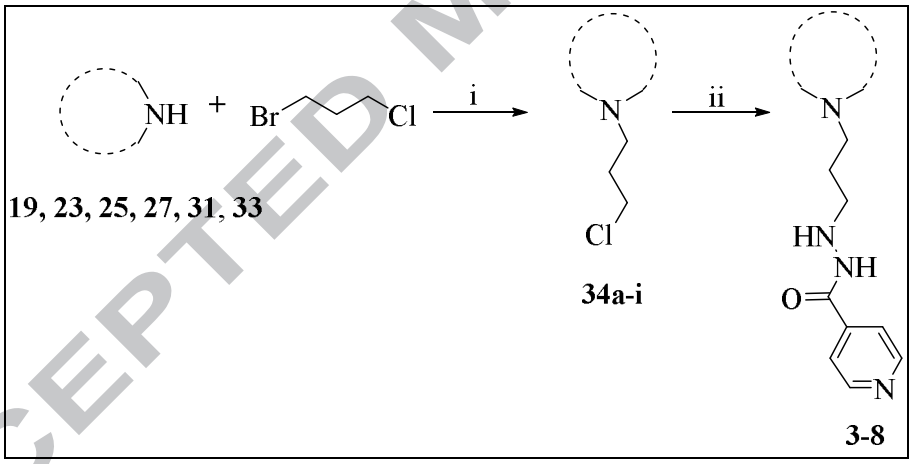
Reagents and reaction conditions: (i) $(Boc)_2O$ (1.3 eq.), Et_3N (1.3 eq.), DCM, 0-25 °C, 1.5 h, 90%; (ii) methanesulfonyl chloride (1.2 eq.), Et_3N (1.2 eq.), DCM, 0-25 °C, 1 h, 97%; (iii) NaH (1.1 eq.), DMSO, 25-70-100 °C, 18-20 h, 30-32%; (iv) TFA (10% v/v in DCM), 1*N*-NaOH, 1-1.5 h, 87-95%; (v) $(Boc)_2O$ (1.3 eq.), Et_3N (1.5 eq.), DMAP (0.01 eq.), DCM, 0-25 °C, 20 h, 95%; (vi) Zn (4.5 eq.), $TiCl_4$ (1M in toluene) (2 eq.), 1,4-dioxane, 60 °C-reflux, 10-12 h, 40-55%; (vii) piperazine (1.1 eq.), DBU (0.25 eq.), MS-4 A°, 0-25 °C, toluene, 10 h, 67%; (viii) piperazine (2 eq.), K_2CO_3 (1 eq.), NaI (0.2 eq.), acetonitrile, reflux (90 °C), 8 h, 65%; (ix) NBS (1.1 eq.), H_2O Acetone (1:5), 25 °C, 2 h,

90%; (x) 1-Boc-piperazine (1.1 eq.), DMSO, Cs_2CO_3 (2 eq.), 60 °C, 8 h, 40%; (xi) TFA/DCM (10% v/v), DCM, 25 °C, 1 h, 97%; (xii) piperazine (10 eq.), DCM, 0-25 °C, 1 h, 73.5%.

Scheme 2 outlines the synthesis of various EPI moieties based on phenothiazines (**19a,b** and **23a,b**), dibenzosuberenone **23c**, dibenzosuberyl **25** and diphenylmethane **27** , using reported methods.[19] The synthesis of analogue **31** commenced with the treatment of commercially available diphenylethene **28** with *N*-bromosuccinimide (NBS) in a mixture of acetone and water (5:1) to afford intermediate **29** (Scheme 2).[20] This intermediate was further reacted with 1-Boc-piperazine in DMSO in the presence of Cs_2CO_3 to afford **30**, which, upon *N*-Boc-deprotection, yielded compound **31**.The triphenylpiperazine derivative **33** was obtained by reacting trityl bromide **32** with piperazine in DCM.

The piperazine and piperidine containing EPI moieties (**19a,b**, **23a-c**, **25**, **27**, **31** and **33**) were further reacted with 1-bromo-3-chloropropane to generate key intermediates **34a-i** (Scheme 3), which upon reaction with INH and Et_3N in *iso*-propanol provided target compounds **3-8**.

Scheme 3: Synthesis of RINH agents **3-8**



Reagents and reaction conditions: (i) Et_3N (2.5 eq.), toluene, 65 °C, 8-12 h, 60-90%; (ii) isoniazid (4eq.), *iso*-propanol, Et_3N (4 eq.), 95 °C, 12-16 h, 30-45%.

3. Results and Discussions:

A total of 14 RINH agents were synthesized along with two benzhydrazide-based compounds to explore the role of the pyridine nitrogen in RINHs. Primarily, anti-mycobacterial activity of all the compounds was evaluated against the drug sensitive strain (H37Rv) of *M. tuberculosis* (Table 1) using the BACTEC 960 system. The compounds with good potency against H37Rv ($MIC_{99} \leq 5$ μM) were further evaluated against various low to high level

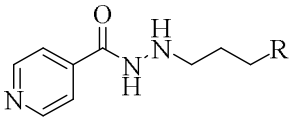
isoniazid mono-resistant strains (R73, R4965 and R5401; experimental section, Table 3) and four MDR clinical isolates (TT149, X_3, X_60 and X_61; experimental section, Table 3) of *M. tuberculosis* to investigate reversal of resistance. The promising compounds with potency against H37Rv and R5401 strains ($MIC_{99} \leq 5$ μM), were also tested against Chinese Hamster Ovary (CHO) cell lines to determine their cytotoxicity profile. INH was used as a positive control for all the *in vitro* evaluations. A selected number of compounds were further tested for intracellular *bacillus Calmette-Guerin* (BCG) bacilli (an attenuated *Mycobacterium bovis* vaccine strain) inhibition in macrophages and for EP inhibitory activity in the ethidium bromide (EB) assay.

3.1. *In vitro* activity against *Mycobacterium tuberculosis*

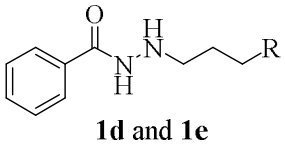
All the RINH agents were found to be active against the H37Rv strain of *M. tuberculosis* ($MIC_{99} \leq 5$ μM), compounds **1a-c**, **3b**, **4a-c**, **7** and **8** ($MIC_{99} \leq 1.25$ μM) showed activity comparable to INH ($MIC_{99} = 0.73$ μM) (Table 1). Compounds **1d** and **1e**, which are analogues of **1a** and **1b**, respectively, did not display activity up to the highest tested concentration (10 μM), demonstrating the importance of the pyridyl nitrogen for anti-mycobacterial activity. All the compounds with $MIC_{99} \leq 5$ μM were further tested against clinical isolates of *M. tuberculosis* with low (R5401; 0.73 μM ≤ INH-MIC_{99} ≤ 2.93 μM) to high (R73 and R4965; INH-$MIC_{99} \geq 2.43$ μM) levels of INH mono-resistance.

All compounds were found to be inactive up to the highest tested concentration (10 μM) against high level INH-resistant strains R73 (INH-$MIC_{99} > 10$ μM**;** mutation in *inhA* promoter and *katG* gene; Table 3) and R4965 (INH-$MIC_{99} > 10$ μM; mutation in *katG* gene). However, compounds **3a,b**, **4a-c**, and **6-8** demonstrated activity against a low level INH mono-resistant strain R5401 ($MIC_{99} \leq 2.5$ μM; mutation in *inhA* promoter) (Table **1**). Compounds **3a** and **6** displayed 2-fold superior anti-mycobacterial activity against the clinical isolate R5401 as compared to the H37Rv strain. However, compound **3b** displayed analogous activity against both strains (H37Rv and R5401). On the other hand, compounds **4a-c**, **7** and **8** demonstrated a two-fold reduction in potency against the R5401 strain as compared to H37Rv strain of *M. tuberculosis*. These results revealed that chemosensitizers when covalently linked to INH are able to circumvent low level INH resistance but failed to do so in strains with high levels of resistance. This is in line with the known fact that EPs contribute toward low level INH resistance but are dominated by mutations in strains with high levels of resistance.[10]

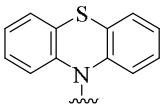
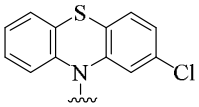
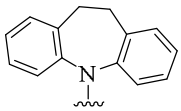
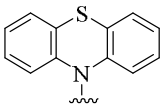
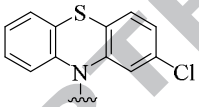
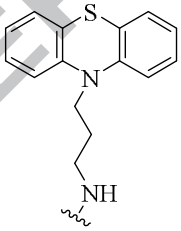
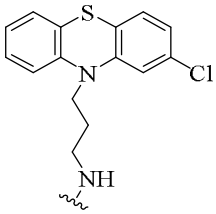
Table 1: Anti-mycobacterial activity, cytotoxicity and selectivity index of RINH agents.

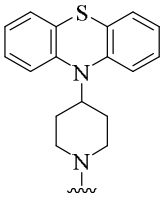
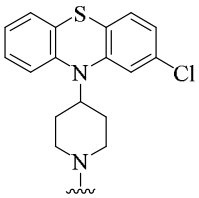
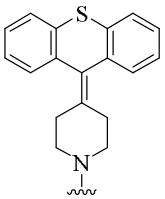
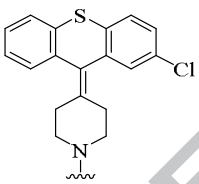
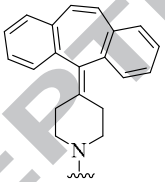
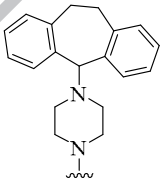
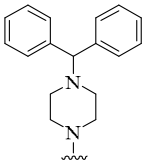
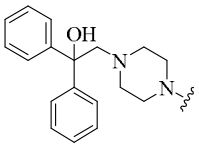


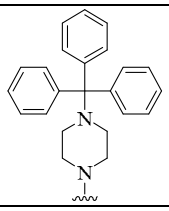
1a-c and **2-8**



1d and **1e**

Compounds	R	MIC_{99} (µM)[X]				IC_{50} (µM)	SI_{R5401}
		H37Rv	R5401	X_61	X_60	CHO Cell lines	
INH		0.73	2.5	2.5	> 10	>100	>35
1a		1.0	> 10	[a]ND	[a]ND	[a]ND	[a]ND
1b		0.25	> 10	[a]ND	[a]ND	[a]ND	[a]ND
1c		1.0	> 10	[a]ND	[a]ND	[a]ND	[a]ND
1d		> 10	[b]ND	[b]ND	[b]ND	[b]ND	[b]ND
1e		> 10	[b]ND	[b]ND	[b]ND	[b]ND	[b]ND
2a		5.0	>10	[a]ND	[a]ND	[a]ND	[a]ND
2b		5.0	> 10	[a]ND	[a]ND	[a]ND	[a]ND

Compounds	R	MIC$_{99}$ (µM)^{X}				IC$_{50}$ (µM)	SI$_{R5401}$
		H37Rv	R5401	X_61	X_60	CHO Cell lines	
3a		5.0	2.5	2.5	> 10	122	48.8
3b		1.0	1.0	> 10	> 10	37.4	37
4a		1.25	2.5	2.5	> 10	152	60.8
4b		0.625	1.25	2.5	> 10	296	236.8
4c		1.25	2.5	5.0	> 10	148	59.2
5		5.0	> 10	[a]ND	[a]ND	[a]ND	[a]ND
6		5.0	2.5	5	> 10	48.1	19.3
7		1.25	2.5	5	> 10	174	139.2

Compounds	R	MIC_{99} (μM)[X]				IC_{50} (μM)	SI_{R5401}
		H37Rv	R5401	X_61	X_60	CHO Cell lines	
8		1.25	2.5	2.5	2.5	97.6	78.1

MIC_{99} (μM)[X]: No significant activity was observed against R73, R4965 (MIC_{99} > 10 μM) , TT149 and X-3 clinical isolates up to the highest tested concentration (10 μM) of compounds; [a]ND: Not determined as MIC_{99} > 10 μM against R5401 strain of *M. tuberculosis*; [b]ND: Not determined as MIC_{99} > 10 μM against H37Rv strain of *M. tuberculosis*; SI_{R504}: Selectivity index of compounds with respect to MIC_{99} of *M. tuberculosis* strain R5401 and IC_{50} against CHO cell lines.

Compounds **3a-b**, **4a-c**, **6**, **7** and **8** with good potency against R5401 strain ($MIC_{99} \leq 2.5$ μM) were further evaluated for cytotoxicity against the CHO cell line and were found to have low cytotoxicity (IC_{50} > 10 μM) with good selective index values (SI > 10) with respect to the R5401 strain. These RINH agents were further evaluated against four MDR clinical isolates TT149 (INH-MIC_{99} > 10 μM), X_3 (INH-MIC_{99} > 10 μM), X_61 (INH-MIC_{99} < 5 μM) and X_60 (INH-MIC_{99} > 10 μM). All the compounds were found to be inactive against TT149 and X_3 strains up to the highest tested concentration (10 μM). However, some potency was displayed by compounds **3a**, **4a-c, 6, 7** and **8** against X_61 ($MIC_{99} \leq 5$ μM), and analogue **8** was also potent against X_60 strains (MIC_{99} = 2.5 μM). The activity of most of these compounds against the X_61 strain ($MIC_{99} \leq 5$ μM) is comparable to their potency against the R5401 strain ($MIC_{99} \leq 2.5$ μM). R5401 and X_61 harbours low level INH resistance ($0.73 \leq MIC_{99} \leq 2.93$ μM) against INH, therefore, it was unsurprising that compounds **3a**, **4a** and **8** showed analogous activity against both strains (MIC_{99} = 2.5 μM), while two-fold lower potency was exhibited by compounds **4b-c**, **6** and **7** (MIC_{99} = 5 μM) against X_61 as compared to the R5401 (MIC_{99} = 2.5 μM) strain.

Only one RINH agent, **8** showed potency (MIC_{99} = 2.5 μM) against the X_60 clinical isolate with high levels of INH resistance (MIC_{99} > 2.93 μM). This is an encouraging result and may lay a foundation for the development of RINH agents to counter both low as well as high levels of INH resistance.

In general, phenothiazine- (**1a,b, 2a,b** and **3a,b**), thioxanthene- (**4a,b**), tricyclic- (**1c**), dibenzosuberyl- (**5**), diphenylmethane (**6** and **7**) and triphenylmethane- (**8**) based EPI compounds showed good to moderate potency against the H37Rv strain of *M. tuberculosis* ($MIC_{99} \leq 5$ μM). Chloro-substituted phenothiazine (**1b** and **3b**) and thioxanthene (**4b**) based compounds showed superior activity as compared to their unsubstituted analogues (**1a**, **3a** and **4a**). The introduction of piperidine (**3a,b** and **4a-c**) and piperazine (**6**, **7** and **8**) moieties in EPI structural motifs enhanced activity against drug sensitive (H37Rv), INH mono resistant (R5401) and MDR clinical isolates (X_61 and X_60) of *M. tuberculosis*. The nitrogen atom in the pyridyl moiety of RINH agents was found to be necessary for anti-mycobacterial activity as a drastic loss of anti-mycobacterial potency was observed with the replacement of the INH moiety with a benzhydrazide group (**1a**-**b** vs **1d-e)** (Table **1**).

3.2. *Ex vivo* cytotoxicity and anti-mycobacterial evaluation of RINH agents

It is well known that *M. tuberculosis* induces a number of EPs during and post macrophage infection, which assist in the development of EP-mediated tolerance. This tolerance leads to the emergence of various forms of high to low level resistance both in active as well as in persistent (non-replicating) forms of *M. tuberculosis*.[21,22] The synthesized RINHs were hypothesized to reverse macrophage induced tolerance due to the attached EPI or chemosensitizer moiety or structural motif. Therefore, a subset of compounds (**1a-e** and **3a**) was evaluated against intracellular BCG in macrophages for proof-of-concept studies. Amongst these selected compounds, **3b** was chosen for the demonstration of retention of *in vitro* potency in macrophages while **1a**, **1b**, **1c**, **1d** and **1e** were selected to explore the significance of the pyridine nitrogen and sulphur atoms in the anti-mycobacterial activity both *in vitro* and in macrophages. All compounds were first evaluated for cytotoxicity against THP-1 cell lines as THP-1 monocytes were used for macrophage development.

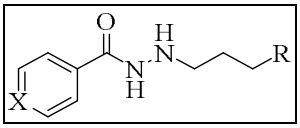
Chlorpromazine (CPZ), a known chemosensitizer and EPI,[17,23] and INH were used as positive controls in these experiments. A low cytotoxicity cut-off IC_{20} (inhibitory concentration of 20% of THP-1 cell lines) was used to achieve higher selectivity against intracellular BCG with a minimum effect on the viability of THP-1 macrophages. Therefore, compounds were evaluated against intracellular BCG at a concentration $\leq IC_{20}$.

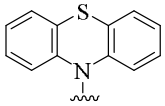
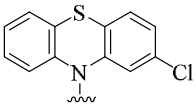
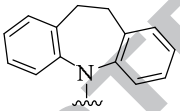
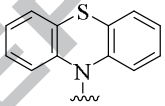
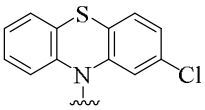
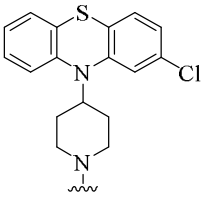
INH and compounds **1a**, **1c**, **1d** and **1e** demonstrated a low cytotoxicity profile with $IC_{20} > 100$ μM, while compounds **1b**, **3b** and CPZ demonstrated IC_{20} values of 24.4, 2.2 and

4 μM, respectively (Table **2**). It appears that piperidine-based compound **3b** is more toxic to THP-1 cells compared to other compounds (Table 2). However, because no other piperidine-compounds were tested on THP-1 cells, it is unclear whether or not the piperidinyl moiety is responsible or for the observed THP-1 cytotoxicity of **3b**. The results summarized in Table 1 showed that compounds **3b** and **6** were more toxic on CHO cell lines than compounds **4a**, **4b**, **7** and **8** despite all incorporating a piperidinyl moiety, suggesting that this moiety may not be the reason for cytotoxicity. INH and CPZ displayed 90% and 40% inhibition of intracellular BCG at a minimum inhibitory concentration (MIC) value of 0.015 and 4 μM (Table **2**), respectively. All the compounds significantly inhibited (> 90%) the growth of intracellular BCG in macrophages (Table **2**) at a concentration ≤ IC_{20} except **1d** and **1e** (40% inhibition). Two RINH agents **1b** and **3b** displayed MIC $_{90}$ inhibition of intracellular BCG at much lower concentrations of 2.44 and 1 μM, respectively as compared to **1a** (13.30 μM) and **1c** (13.42 μM), but exhibited lower potency as compared to INH. However, structural modifications, such as the introduction of a piperidine linker in the EPI moiety (**1b** vs **3b)** improves the potency by 2-fold. This indicates that the potency of RINH agents can be enhanced with structural modifications, which may afford compounds with superior activity than INH both *in vitro* and in macrophages.

A comparison of activities (**1a,b** vs **1d,e)** demonstrated that the pyridyl nitrogen is required for the intracellular anti-mycobacterial activity in macrophages as was previously established during *in vitro* studies. Additionally, inhibition of intracellular BCG by **1d** and **1e** is possibly due to inhibition of EPs as these compounds lack activity against axenic *M. tuberculosis* (H37Rv).[20] The 2-chloro substituted phenothiazine containing compounds (**1b** and **1e**) showed superior activity as compared to unsubstituted ones (**1a** and **1d**). All these compounds were further evaluated in an ethidium bromide assay to assess their efflux pump inhibition efficacy.

Table 2: Cytotoxicity against THP-1 cell lines and intracellular inhibition of BCG by CPZ, INH, and selected compounds **1a-e** and **3b**.



Comp	R	X	IC_{20} (µM) THP-1 cell line	% Inhibition of BCG in macrophages	MIC (µM)
CPZ		-	4	40	4
INH		-	>100	>90	0.015
1a		N	>100	>90	13.3
1b		N	24.4	>90	2.44
1c		N	>100	>90	13.42
1d		C	>100	40	26.6
1e		C	>100	40	12.2
3b		N	2.2	>90	1

Comp: compound; MIC: Minimum inhibitor concentration corresponding to maximum

inhibition of BCG in macrophages.

3.3. Ethidium bromide evaluation of selected RINH agents

The short-listed compounds (Table **2**) were also evaluated for their EP inhibition activity in the Ethidium bromide (EB) assay. The recorded increase in mean fluorescence intensity in the presence of a compound as compared to the negative control signifies the EP inhibition efficiency of a compound leading to the higher concentration of EB in the BCG-EB assay. Therefore, a higher intensity indicates superior inhibition of EPs responsible for EB efflux in BCG in macrophages.

CPZ, **1b** and **3b** were tested at concentrations of 25, 25 and 10 µM, respectively, due to their low IC_{20} values against the THP-1 cell lines, while other RINH agents (**1a**, **1c**, **1d**, **1e** and INH) were evaluated at 50 µM. A 40-90% increase in fluorescent intensity was demonstrated by most of the RINH agents (**1b**, **1d**, **1e** and **3b**), which is two-fold higher than the increase in intensity demonstrated by CPZ while **1a** and **1c** showed fluorescence intensity slightly less than CPZ. The negligible effect on EB efflux by INH was demonstrated by very low increase in fluorescent intensity, and a higher increase in intensity exhibited by RINHs (**1b**, **1c**, **1d**, **1e** and **3b**). This signifies superior EP inhibition efficiency towards EPs responsible for EB efflux as compared to the known EP inhibitor CPZ (Figure 3).

These observations highlighted the dual action nature of RINH agents as inhibitors of EPs (as suggested by the EB assay, Figure 3) and anti-mycobacterial agents per se (demonstrated by *in vitro* and macrophage anti-mycobacterial data in table **1** and **2**, respectively). The enhanced EP inhibition activity of **1a** and **1b** and their analogues **1d** and **1e**, respectively, as compared to INH is exclusively due to the phenothiazine core, albeit loss in anti-mycobacterial activity was observed with **1d** and **1e** due to the absence of a pyridyl nitrogen. This implies that the pyridyl nitrogen may not be required for EP inhibition activity but necessary for anti-mycobacterial potency both *in vitro* and in macrophages.

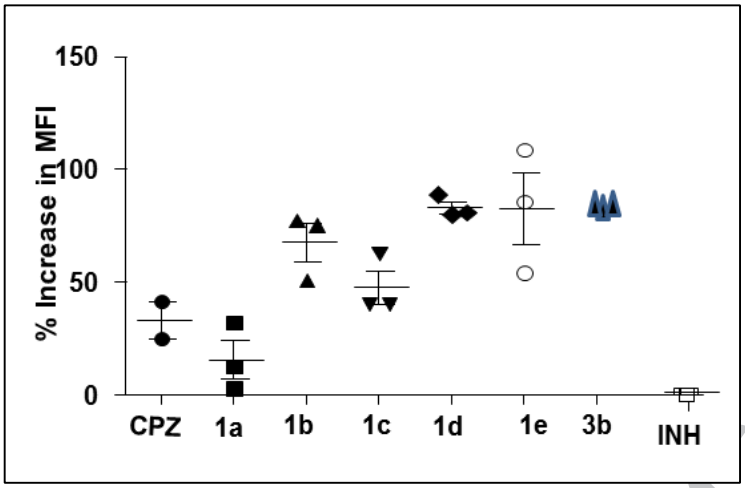


Figure 3: EP inhibition evaluations of selected RINH agents in the EB assay. (MFI: Mean fluorescence intensity)

4. Summary and conclusions

A total of 14 RINH agents and two benzhydrazide-based compounds were synthesized and evaluated against sensitive and resistant strains of *M. tuberculosis* (Table **1**). A selected number of compounds were also tested *ex vivo* (Table **2**) for intracellular activity, and in the ethidium bromide assay for efflux pump inhibition efficacy (Figure **3**).

The potency of RINH agents against both sensitive and resistant (INH mono-resistant and MDR) strains of *M. tuberculosis* demonstrated the viability of the strategy of reversed anti-TB agents for the development of novel anti-mycobacterial compounds to address the rapidly growing issue of resistance. In addition to phenothiazine (**3a,b**) and thioxanthene (**4a,b**) based RINH agents, demonstration of good to moderate potency by compounds containing tricyclic (iminodibenzyl **1c**, cyproheptadine **4c** and trityl **8**) and bicyclic (diphenylmethane, **6** and **7**)-based EPI/chemosensitizer moieties further expanded the set of chemosensitizer motifs, which can also be explored for further development of reversed anti-TB agents.

Macrophage activity and inhibition of EB efflux in the EB-assay by selected compounds (**1a-c** and **3b**) demonstrated proof of the dual acting nature of RINH agents. Preliminary structure–activity relationship (SAR) studies (**1a,b** vs **1d,e**) showed the significance of the pyridyl nitrogen for anti-mycobacterial activity both *in vitro* and in macrophages. This SAR also demonstrated proof of retention of EPI characteristics in hybrid compounds by attached EPI moieties as exemplified by inhibition of intracellular BCG and EB efflux by **1d** and **1e**.

In conclusion, we have demonstrated the potential of dual acting RINH agents as anti-mycobacterial agents effective against MDR and XDR strains of *M. tuberculosis*. These encouraging anti-mycobacterial and resistance reversal activities displayed by RINH agents lay a foundation for further exploration the concept of reversed anti-TB agents (RATAs) to counter EP-mediated resistance.

5. Experimental section:

5.1. Chemistry

All commercially available chemicals were purchased from either Sigma-Aldrich or Combi Blocks. All solvents were dried by appropriate techniques. Unless otherwise stated, all solvents used were anhydrous. Purification of compounds were carried out by either by recrystallization or column chromatography on silica gel 60 (Fluka), particle size 0.063–0.2 mm (70–230 mesh), as the stationary phase. All target compounds and intermediates were characterized by ^{1}H NMR and LC-MS. NMR spectra were recorded on either a Varian Mercury-300 (^{1}H 300.1 MHz, ^{13}C 75.5 MHz) or Bruker-400 (^{1}H 400.2 MHz, ^{13}C 100.6 MHz) instrument using $CDCl_3$, CD_3OD, and DMSO-d_6 as solvents. Liquid chromatograph with mass spectrometer (LC-MS) analysis was performed using an Agilent® 1260 Infinity binary pump, Agilent® 1260 Infinity diode array detector (DAD), Agilent® 1290 Infinity column compartment, Agilent®1260 Infinity standard autosampler, and a Agilent® 6120 quadrupole (single) mass spectrometer, equipped with APCI and ESI multimode ionization source. Purities were determined by Agilent® LC-MS using a Kinetex Core C18 2.6 µm column (50 mm × 3 mm). Mobile phase B: 0.4% acetic acid, 10 mM ammonium acetate in a 9:1 ratio of HPLC grade methanol, and type 1water. Mobile phase A: 0.4% acetic acid in 10 mM ammonium acetate in HPLC grade (type 1) water, with flow rate = 0.9 mL/min; detector, diode array (DAD), and all final compounds were confirmed to have ≥95% purity.

*5.1.1. General Procedure for the synthesis of compounds **1a** and **1b***: NaH (1.2 equiv.) was added to a cooled solution (0 °C) of compound **10a** or **b** (1.0 equiv) and isoniazid (1.2 equiv) in anhydrous DMF (3 ml). The resulting reaction mixture was stirred at room temperature (25 °C) for 5-6 h. After completion of reaction (TLC), DMF was removed *in vacuo* and the residue was taken in EtOAc (25 ml). The organic phase was washed with brine (3×15 ml), dried over anhydrous sodium sulfate and concentrated *in vacuo*. Purification by flash chromatography on silica gel using MeOH-DCM as eluent afforded product **1a** or **1b.**

5.1.1.1. N'-(3-(10H-phenothiazin-10-yl)propyl)isonicotinohydrazide **(1a).** Yield (0.24 g, 18%) as an oil. ^{1}H-NMR (400 MHz, $CDCl_3$) δ 8.71 (d, *J* = 6.0 Hz, 2H), 7.45 (d, *J* = 6.4 Hz, 2H), 7.19 (m, 4H), 6.98 (dd, *J* = 7.2 and 1.2 Hz, 2H), 6.94 (dd, *J* = 7.2 and 1.2 Hz, 2H), 4.08 (t, *J* = 6.4 Hz, 2H), 3.13 (t, *J* = 6.4 Hz, 2H), 2.06 (m, H-6,); ^{13}C-NMR (101 MHz, $CDCl_3$) δ 165.2, 150.2 (2C), 145.2 (2C), 140.1, 127.7 (2C), 127.4 (2C), 125.5 (2C), 122.8 (2C), 120.8 (2C), 115.7 (2C), 49.6, 44.7 and 25.3; MS (EI+) *m/z* calculated for $C_{21}H_{20}N_4OS$: 376.1; found, 377.3 (M+1). HPLC purity: 96%.

5.1.1.2. N'-(3-(2-chloro-10H-phenothiazin-10-yl)propyl)isonicotinohydrazide **(1b).** Yield (0.32 g, 24%) as an oil. ^{1}H-NMR (400 MHz, $CDCl_3$) δ 8.70 (d, *J* = 6.0 Hz, 2H), 7.44 (d, *J* = 6.0 Hz, 2H), 7.16 (m, 2H), 7.02 (d, *J* = 8.0 Hz, 1H), 6.92 (td, *J* = 7.6 and 0.8 Hz, 1H), 6.89 (dd, *J* = 8.4 and 2.4 Hz, 2H), 6.83 (d, *J* = 2.0 Hz, 1H), 4.01 (t, *J* = 6.4 Hz, 2H), 3.09 (t, *J* = 6.4 Hz, 2H), 2.03 (quin, *J* = 6.4 Hz, 2H). ^{13}C-NMR (101 MHz, $CDCl_3$) δ 165.1, 150.5 (2C), 144.9 (2C), 142.1, 133.4, 128.5, 127.9 (2C), 125.9, 123.8 (2C), 120.8 (3C), 115.6 (2C), 49.5, 44.6 and 25.1; MS (EI+) *m/z* calculated for $C_{21}H_{19}ClN_4OS$: 410.10; found, 411.5 (M+1), 412.5 (M+2) and (M+3); HPLC purity: 96%.

*5.1.2. General Procedure for the synthesis of compounds **14a** and **14b**:* A solution of compound **13a** or **13b** (1 equiv.) in DMF (1 ml) was added to a mixture of isoniazid (5 equiv) and triethylamine (3 equiv) under an atmosphere of nitrogen at room temperature (25 °C). The reaction mixture was continuously stirred for an additional hour, after which it was heated to 55 °C and stirred for another 10-12 hours. After completion of reaction (TLC), DMF was removed *in vacuo*, dried over sodium sulfate and concentrated *in vacuo*. The crude product was purified by biotage flash chromatography.

5.1.2.1. tert-butyl (3-(10H-phenothiazin-10-yl)propyl)(3-isonicotinoylhydrazinyl)propyl) carbamate **(14a)**Yield (0.18 g, 35%) as a white solid. m.p. 55-58 °C; ^{1}H-NMR (400 MHz, CD_3OD) δ 8.67 (d, *J* = 6.0 Hz, 2H), 7.73 (d, *J* = 6.4 Hz, 2H), 7.18 (td, *J* = 7.2 and 1.6 Hz, 2H), 7.12 (dd, *J* = 7.6 and 1.2 Hz, 2H), 6.96 (dd, *J* = 7.6 and 0.8 Hz, 2H), 6.95 (td, *J* = 7.2 and 1.2 Hz, 2H), 3.90 (t, *J* = 6.4 Hz, 2H), 3.35 (t, *J* = 7.2 Hz, 2H), 2.81 (t, *J* = 7.2 Hz, 2H), 2.78 (t, *J* = 6.8 Hz, 2H), 2.01 (quin, *J* = 6.4 Hz, 2H), 1.80 (quin, *J* = 7.2 Hz, 2H), 1.40 (bs, 9H); ^{13}C-NMR (101 MHz, CD_3OD) δ 164.5 (2C), 156.1, 149.6 (2C), 145.4, 141.3, 127.1 (2C), 126.9 (2C), 122.6 (2C), 121.7 (2C), 121.4, 115.7 (2C), 79.8, 48.5, 44.6 (2C), 44.0, 27.3 (3C), 26.5 and 26.4; MS (EI+) *m/z* calculated for $C_{29}H_{35}N_5O_3S$: 533.2; found, 534.2 (M+1).

5.1.2.2. tert-butyl(3-(2-chloro-10H-phenothiazin-10-yl)propyl)(3-(2-isonicotinoylhydrazinyl)propyl)carbamate **(14b).** Yield (0.15 g, 30%) as a white solid; m.p. 62-64 °C; ^{1}H-NMR (400 MHz, CD_3OD) δ 7.20 (td, *J* = 7.6 and 2.0 Hz, 1H), 7.18 (dd, *J* = 8.0 and 1.6 Hz, 1H), 7.13 (d, *J* = 8.0 Hz, 1H), 7.01 (m, 3H), 6.99 (dd, *J* = 8.0 and 1.6 Hz, 1H), 3.93 (t, *J* = 6.8 Hz, 2H), 3.33 (t, *J* = 7.2 Hz, 2H), 2.84 (t, *J* = 7.2 Hz, 2H), 2.80 (t, *J* = 6.4 Hz, 2H), 2.07 (quin, *J* = 6.4 Hz, 2H), 1.70 (quin, *J* = 7.2 Hz, 2H), 1.36 (bs, 9H); ^{13}C-NMR (101 MHz, CD_3OD) δ 165.2, 156.5 148.3 (2C), 145.3 142.9, 139.1, 132.1, 126.2, 126.1, 126.0, 124.1, 123.3, 121.8, 121.0, 120.1 (2C), 114.8 and 114.7, 79.5, 48.4, 44.2 (2C), 44.0, 27.1 (3C), 26.5 and 26.4; MS (EI+) *m/z* calculated for $C_{29}H_{34}ClN_5O_3S$: 567.21; found, 568.1 (M+1) and 570.1 (M+3).

*5.1.3. General Procedure for the synthesis of compounds **2a** and **2b***: 10 ml of a 10% mixture of TFA (10 equiv) in DCM by volume was added to compound **14a** and **14b** (1 equiv) in a round bottom flask and stirred at room temperature (25 °C) for 1-1.5 hours. After completion of reaction (TLC), excess of TFA was removed *in vacuo* and residue was dissolved in 10% MeOH in DCM. The amberlyst A-21 was added to scavenger the residual TFA and stirred for one hour, after which reaction mixture was filtered through celite and washed with a 20% mixture of methanol in DCM. The combined organic layer was concentrated under reduced pressure to obtain **2 (a** and **b).**

5.1.3.1. N'-(3-((3-(10H-phenothiazin-10-yl)propyl)amino)propyl)isonicotinohydrazide **(2a).** Yield (0.40 g, 66%) as a white solid; m.p. 48-50 °C; ^{1}H-NMR (400 MHz, DMSO-d_6) δ 10.30 (s, 1H), 8.70 (d, *J* = 5.2 Hz, 2H), 7.73 (d, *J* = 6.0 Hz, 2H), 7.18 (m, 4H), 6.96 (d, *J* = 8.0 Hz, 2H), 6.95 (t, *J* = 6.8 Hz, 2H), 3.91 (t, *J* = 6.8 Hz, 2H), 3.01 (m, 4H), 2.89 (t, *J* = 6.0 Hz, 2H), 2.04 (quin, *J* = 7.2 Hz, 2H), 1.70 (quin, *J* = 6.8 Hz, 2H); ^{13}C-NMR (101 MHz, DMSO-d_6) δ 164.3, 150.2 (2C), 144.5 (2C), 140.0, 127.6 (2C), 127.2 (2C), 124.2 (2C), 122.7 (2C), 121.2 (2C), 115.9 (2C), 48.4, 45.8, 44.7, 43.6, 23.7 and 23.3; MS (EI+) *m/z* calculated for $C_{24}H_{27}N_5OS$: 433.19; found, 434.2 (M+1); HPLC purity 99%.

5.1.3.2. N'-(3-((3-(2-chloro-10H-phenothiazin-10-yl)propyl)amino)propyl)isonicotinohydrazide **(2b).** Yield (0.37 g, 83%) as a white solid; m.p. 61-64 °C; ^{1}H-NMR (400 MHz, CD_3OD) δ 8.74 (dd, *J* = 6.4 and 2.0 Hz, 2H), 7.74 (dd, *J* = 6.4 and 1.6 Hz, 2H), 7.15 (m, 2H), 7.05 (d, *J* = 8.0 Hz, 1H), 6.90 (m, 2H), 6.95 (td, *J* = 7.6 and 1.2 Hz, 1H), 6.88 (dd, *J* = 8.4 and 2.0 Hz, 1H), 4.18 (t, *J* = 6.4 Hz, 2H), 3.22 (m, 4H), 3.09 (t,

J = 6.0 Hz, 2H), 2.04 (quin, *J* = 6.4 Hz, 2H), 1.70 (quin, *J* = 6.0 Hz, 2H); ^{13}C-NMR (101 MHz, CD_3OD) δ 165.1, 148.3 (2C), 145.7, 142.9, 139.2, 132.1, 126.5, 126.1, 125.9, 124.4, 123.3, 121.9, 121.0, 120.1 (2C), 114.8, 114.7, 49.3, 44.1, 42.4 (2C), 22.9 and 21.9; MS (EI+) *m/z* calculated for $C_{24}H_{26}ClN_5OS$: 467.15; found, 468.1 (M+1), 469.1 $(M+2)^+$ and 470.1 (M+3); HPLC purity 97.1%.

*5.1.4. General Procedure for the synthesis of compounds **1c-e** and **3-8**.* Triethylamine (4 equiv.) was added to a solution of benzhydrazide (4 equiv.; **1d** and **1e**) or isoniazid (4 equivalent; **1c** and **3-8**) and corresponding chloro-intermediate (**1a-c** or **34a-i**; 1 equiv.) in *iso*-propanol, and the resulting mixture was refluxed for 12-24 hours. After completion of reaction (TLC), *iso*-propanol was removed *in vacuo* and residue was taken in DCM (40 ml), and washed with water (5 x 15 mL). The aqueous layer was extracted with DCM (5 x 10 mL) and the combined DCM extracts was washed with brine (1 x 20 ml), dried over sodium sulphate and concentrated *in vacuo*. The crude product was purified by column chromatography to afford the target compound **1c-e** and **3-8**.

5.1.4.1. N'-(3-(10,11-dihydro-5H-dibenzo[b,f]azepin-5-yl)propyl)isonicotinohydrazide **(1c).** Yield (0.11 g, 15%) as an oil. ^{1}H-NMR (400 MHz, $CDCl_3$) δ 7.16 - 7.08 (m, 4H), 7.60 (d, *J* = 7.2 Hz, 2H), 6.99 (m, 2H), 3.88 (t, *J* = 6.8 Hz, 2H), 2.99 (t, *J* = 6.4 Hz, 2H), 3.12 (s, 4H), 2.27 (quin, *J* = 6.8, Hz, 2H); ^{13}C-NMR (101 MHz, $CDCl_3$) δ 165.1, 149.4 (2C), 147.3, 140.6, 134.1, 129.4, 126.1, 122.2, 121.4, 119.6, 49.0 (2C), 31.9 (2C), 26.0; MS (EI+) *m/z* calculated for $C_{23}H_{24}N_4O$: 372.2; found, 373.1 (M+1); HPLC purity: 95.1%.

*5.1.4.2. N'-(3-(10H-phenothiazin-10-yl)propyl)benzohydrazide (**1d**):* Yield (80 mg, 20%) as an oil; ^{1}H-NMR (400 MHz, $CDCl_3$) δ 7.72 (d, *J* = 8.4 Hz, 2H), 7.52 (tt, *J* = 7.6 and 1.6 Hz, 1H), 7.43 (t, *J* = 8.0 Hz, 2H), 7.15 (m, 2H), 7.10 (dd, *J* = 7.6 and 1.2 Hz, 2H), 6.99 (dd, *J* = 7.6 and 1.2 Hz, 2H), 6.90 (td, *J* = 7.2 and 1.2 Hz, 2H), 4.05 (t, *J* = 6.4 Hz, 2H), 3.04 (t, *J* = 6.4 Hz, 2H), 2.02 (quin, *J* = 6.8 Hz, 2H); ^{13}C-NMR (101 MHz, $CDCl_3$) δ 167.8, 145.4 (2C), 132.9, 131.3 (2C), 128.2 (2C), 127.1 (2C), 126.9 (2C), 126.8 (2C), 125.3, 122.2 (2C), 115.6 (2C), 48.8, 44.4 and 25.0; MS (EI+) *m/z* calculated for $C_{22}H_{21}N_3OS$: 375.1; found, 376.1 $[M+1]^+$, HPLC purity 96.8%

5.1.4.3. N'-(3-(2-chloro-10H-phenothiazin-10-yl)propyl)benzohydrazide (**1e**). Yield (75 mg, 25%) as an oil; ^{1}H-NMR (400 MHz, $CDCl_3$) δ 7.72 (d, *J* = 7.2 Hz, 2H), 7.52 (t, *J* = 7.6 Hz, 1H), 7.43 (t, *J* = 7.2 Hz, 2H), 7.12 (td, *J* = 8.0 and 1.6 Hz, 1H), 7.11 (dd, *J* = 8.0 and 1.6 Hz, 1H), 7.05-7.01 (m, 2H), 7.00 (d, *J* = 2.0, 2H), 6.95 (td, *J* = 7.6 and 1.2 Hz, 1H), 6.90 (dd, *J* =

8.4 and 2.4 Hz, 1H), 4.04 (t, *J* = 6.4 Hz, 2H), 3.05 (t, *J* = 6.4 Hz, 2H), 2.02 (quin, *J* = 6.8 Hz, 2H); ^{13}C-NMR (101 MHz, $CDCl_3$) δ 167.8, 146.8, 144.6, 133.1, 132.9, 131.4, 128.1 (2C), 127.6, 127.2, 126.9, 126.8 (2C), 125.0, 124.1, 122.7, 121.9, 116.0, 115.7, 48.7, 44.6 and 24.9; MS (EI+) *m/z* calculated for $C_{22}H_{20}ClN_3OS$: 409.1; found: 410.1 $[M+1]^+$, 411.1 $[M+2]^+$, 412.1 $[M+3]^+$; HPLC purity 97%.*5.1.4.4.*

5.1.4.4. N'-(3-(4-(10H-phenothiazin-10-yl)piperidin-1-yl)propyl)isonicotinohydrazide **(3a).** Yield (0.136 g, 30%) as a white solid**.** m.p. 54-56 °C; ^{1}H-NMR (400 MHz, DMSO-d_6) δ 8.74 (d, *J* = 6.0 Hz, 2H), 7.75 (d, *J* = 6.0 Hz, 2H), 7.20 (m, 2H), 7.19 (m, 2H), 7.15 (d, *J* = 7.0 Hz, 2H), 7.03 (m, 2H), 3.75 (t, *J* = 12.8 Hz, 1H), 3.00 (bd, *J* = 9.2 Hz, 2H), 2.87 (t, *J* = 6.8 Hz, 2H), 2.44 (t, *J* = 7.2 Hz, 2H), 2.18 (m, 4H), 2.1 (bd, *J* = 10.0 Hz, 2H), 1.65 (quin, *J* = 6.8 Hz, 2H); ^{13}C-NMR (101 MHz, DMSO-d_6) δ 163.8, 150.4 (2C), 145.3 (2C), 140.7, 130.0 (2C), 127.8 (2C), 127.5 (2C), 123.8 (2C), 121.5 (2C), 120.8 (2C), 62.5, 56.2, 53.2 (2C), 50.2, 31.6 (2C) and 25.4; MS (EI+) *m/z* calculated for $C_{26}H_{29}N_5OS$: 459.2; found, 460.2 (M+1); HPLC purity 99.8%.

5.1.4.5. N'-(3-(4-(2-chloro-10H-phenothiazin-10-yl)piperidin-1-yl)propyl)isonicotinohydrazide **(3b)**. Yield (0.17 g, 30%) as a white solid; m.p. 85-87 °C; ^{1}H-NMR (400 MHz, CD_3OD) δ 8.74 (d, *J* = 6.4 Hz, 2H), 7.75 (d, *J* = 6.0 Hz, 2H), 7.19 (m, 2H), 7.17 (d, *J* = 2.0 Hz, 1H), 7.13 (d, *J* = 7.2 Hz, 1H), 7.09 (d, *J* = 7.6 Hz, 1H), 7.02 (m, 1H), 6.99 (dd, *J* = 8.4 and 2.4 Hz, 1H), 3.79 (m, 1H), 3.10 (bd, *J* = 10.0 Hz, 2H), 3.00 (t, *J* = 6.4 Hz, 2H), 2.61 (t, *J* = 6.8 Hz, 2H), 2.32 (m, 4H), 2.05 (m, 4H), 2.05 (quin, *J* = 7.2 Hz, 2H); ^{13}C-NMR (101 MHz, CD_3OD) δ 164.5, 149.6 (2C), 146.3, 144.6, 141.3, 132.5, 129.0, 128.4, 127.6, 127.1, 126.9, 123.7, 123.2, 121.4 (2C), 121.3, 121.0, 62.5, 56.1, 52.8 (2C), 50.0, 31.1 (2C) and 24.5; MS (EI+) *m/z* calculated for $C_{26}H_{28}ClN_5OS$: 493.17; found, 494.1 (M+1), 495.1 (M+2) and 496.1 (M+3), HPLC purity 99.5%.

5.1.4.6. N'-(3-(4-(9H-thioxanthen-9-ylidene)piperidin-1 yl)propyl)isonicotinohydrazide **(4a).** Yield (70 mg, 37%) as a white solid; m.p. 105-107 °C; ^{1}H-NMR (400 MHz, DMSO-d_6) δ 8.76 (bs, 2H), 7.67 (d, *J* = 5.6 Hz, 2H), 7.49 (dd, *J* = 7.6 and 1.2 Hz, 2H), 7.29 (dd, *J* = 7.6 and 1.6 Hz, 2H), 7.25 (td, *J* = 7.6 and 1.6 Hz, 2H), 7.18 (td, *J* = 7.6 and 1.6 Hz, 2H), 3.02 (d, *J* = 5.6 Hz, 2H), 2.92 (m, 2H), 2.82-2.60 (m, 4H), 2.55 (t, *J* = 5.6 Hz, 2H), 2.18 (m, 2H), 1.80 (quin, *J* = 6.4 Hz, 2H); ^{13}C-NMR (101 MHz, $CDCl_3$) δ 164.2, 150.6 (2C), 140.5, 136.6 (2C), 135.3 (2C), 134.1, 130.9, 128.7 (2C), 127.3 (2C), 126.3 (2C), 125.9 (2C), 121.0 (2C), 56.6,

54.7 (2C), 51.1, 30.1 (2C) and 25.5; MS (EI+) *m/z* calculated for $C_{27}H_{28}N_4OS$: 456.20; found, 457.2 (M+1); HPLC purity 96.5%.

5.1.4.7. N'-(3-(4-(2-chloro-9H-thioxanthen-9-ylidene)piperidin-1-yl)propyl)isonicotinohydrazide **(4b)**. Yield (0.06 g, 32%) as a white solid; m.p. 99-102 °C; ^{1}H-NMR (400 MHz, $CDCl_3$) δ 8.50 (d, *J* = 5.2 Hz, 2H), 7.70 (d, *J* = 5.6 Hz, 2H), δ 7.48 (d, *J* = 8.0 Hz, 1H), 7.43 (d, *J* = 8.4 Hz, 1H), 7.30 (m, H-5', 3H), 7.21 (m, 1H), 7.28 (dd, *J* = 8.4 and 2.0 Hz, 1H), 3.02 (t, *J* = 6.0 Hz, 2H), 2.97 (m, 2H), 2.76 (m, 4H), 2.62 (t, *J* = 6.4 Hz, 2H), 2.23 (m, 1H), 1.82 (quin, *J* = 6.4 Hz, 2H); ^{13}C-NMR (101 MHz, $CDCl_3$) δ 164.5, 150.6 (2C), 140.3, 138.2, 136.0, 135.0, 134.6, 133.9, 131.9, 130.4, 128.8, 128.5, 128.4, 127.3, 126.7, 126.4, 126.2, 121.0 (2C), 56.3, 54.6, 54.4, 52.0, 50.7, 29.8 and 22.0; MS (EI+) *m/z* calculated for $C_{27}H_{27}ClN_4OS$: 490.2; found, 491.2 (M+1), 492.2 (M+2) and 493.2 (M+3); HPLC purity 96.8%.

5.1.4.8. N'-(3-(4-(5H-dibenzo[a,d][7]annulen-5-ylidene)piperidin-1-yl)propyl)isonicotinohydrazide **(4c)**. Yield (90 mg, 40%) as a white solid; m.p. 120-122 °C; ^{1}H-NMR (400 MHz, CD_3OD) δ 8.70 (dd, *J* = 6.0 and 1.6 Hz, 2H), 7.66 (dd, *J* = 6.4 and 1.6 Hz, 2H), 7.34-7.39 (m, 4H), 7.27-7.30 (m, 2H), 7.22 (dd, *J* = 8.0 and 1.2 Hz, 2H), 6.95 (s, 2H), 2.99 (t, *J* = 6.8 Hz, 2H), 2.80 (m, 2H), 2.59 (t, *J* = 6.8 Hz, 2H), 2.45 (m, 4H), 2.23 (m, 1H), 1.82 (quin, *J* = 6.8Hz, 2H); ^{13}C-NMR (101 MHz, $CDCl_3$) δ 164.2, 150.5 (2C), 140.3, 138.7 (2C), 134.7 (2C), 134.3, 134.1, 131.0 (2C), 128.3 (4C), 127.6 (2C), 126.4 (2C), 121.0 (2C), 56.4, 55.0 (2C), 50.8, 29.4 (2C) and 25.3; MS (EI+) *m/z* calculated for $C_{29}H_{30}N_4O$: 450.24; found, 451.2 (M+1); HPLC purity 95.5%.

5.1.4.9. Isonicotinic acid N'-{3-[4-(10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5-yl)-piperazin-1-yl]-propyl}-hydrazide **(5).** Yield (0.2 g, 42%) as a white solid; m.p. 155-157 °C; ^{1}H-NMR (400 MHz, $CDCl_3$) δ 8.68 (d, *J* = 5.6 Hz, 2H), 7.67 (d, *J* = 5.6 Hz, 2H), 7.02 (m, 8H), 3.87 (s, 1H), 3.85 (m, 2H), 2.91 (t, *J* = 5.6 Hz, 2H), 2.68 (m, 2H), 2.65-2.49 (two br s, 8H), 2.65 (t, *J* = 6.4 Hz, 2H), 1.76 (quin, *J* = 6.4 Hz, 1H); ^{13}C-NMR (101 MHz, $CDCl_3$) δ 164.3, 150.5 (2C), 140.5, 139.6, 138.5, 130.9 (2C), 130.8 (3C), 128.10 (3C), 125.7 (2C), 121.2 (2C), 56.1, 53.3 (2C), 50.6 (2C), 50.2, 31.8 (2C), 29.6 and 25.0; MS (EI+) *m/z* calculated for $C_{28}H_{33}N_5O$: 455.27; found, 456.20 (M+1); HPLC purity 99.8%.

5.1.4.10. Isonicotinic acid N'-[3-(4-benzhydryl-piperazin-1-yl)-propyl]-hydrazide **(6).** Yield (0.2 g, 42%) as a white solid; m.p. 55-57 °C; ^{1}H-NMR (400 MHz, $CDCl_3$) δ 8.75 (d, *J* = 4.0 Hz, 2H), 7.65 (d, *J* = 5.6 Hz, 2H), 7.35 (d, *J* = 7.6 Hz, 4H), 7.30 (t, *J* = 7.2 Hz, 4H), 7.11 (t, *J* = 7.2 Hz, 2H), 4.21 (s, 1H), 3.01 (t, *J* = 6.0 Hz, 2H), 2.64 (bs, 4H), 2.47 (bs, 4H), 2.65 (t, *J* = 6.4 Hz, 2H), 1.83 (quin, *J* = 6.4 Hz, 2H); ^{13}C-NMR (101 MHz, $CDCl_3$) δ 164.1, 150.5 (2C), 142.3 (2C), 140.5, 128.5 (4C), 127.9 (4C), 127.1 (2C), 121.1 (2C), 76.0, 56.4, 53.6(2C), 51.0 (2C), 50.07, and 25.6; MS (EI+) *m/z* calculated for $C_{26}H_{31}N_5O$: 429.25; found, 430.20 (M+1); HPLC purity 98.8%.

5.1.4.11. N'-(3-(4-(2-hydroxy-2,2-diphenylethyl)piperazin-1-yl)propyl)isonicotinohydrazide **(7).** Yield (0.1 g, 35%) as a white solid; m.p.115-117 °C; ^{1}H-NMR (400 MHz, $CDCl_3$) δ 8.75 (2H, dd, *J* = 6.0 and 1.6 Hz, H-11), 7.52 (2H, dd, *J* = 6.4 and 1.6 Hz, H-10), 7.50 (4H, dd, *J* = 7.2 and 1.2 Hz, H-1), 7.32 (4H, td, *J* = 6.4 and 1.2 Hz, H-2), 7.23 (2H, tt, *J* = 7.6 and 1.2 Hz, H-3), 5.14 (1H, s, OH), 3.29 (2H, s, H-4), 2.99 (2H, t, *J* = 6.0 Hz, H-9), 2.52 (2H, t, *J* = 6.8 Hz, H-7), 2.40-2.54 (8H, m, H-5 and H-6) 1.76 (2H, quin, *J* = 6.4 Hz, H-8); ^{13}C-NMR (101 MHz, $CDCl_3$) δ 164.3, 150.6 (2C), 146.8 (2C), 140.3, 128.0 (4C), 126.7 (2C), 125.3 (4C), 120.9 (2C), 74.3, 67.5, 56.6, 54.0 (2C), 53.1 (2C), 51.0 and 25.2; MS (EI+) *m/z* calculated for $C_{27}H_{33}N_5O_2$: 459.26; found, 460.3 (M+1); HPLC purity 99.8%.

5.1.4.12. N'-(3-(4-tritylpiperazin-1-yl)propyl)isonicotinohydrazide **(8).** Yield (0.2 g, 40%) as a white solid; m.p. 199-200 °C; ^{1}H-NMR (400 MHz, $CDCl_3$) δ 8.65 (dd, *J* = 6.0 and 1.6 Hz, 2H), 7.52 (m, 8H), 7.27 (t, *J* = 7.2 Hz, 6H), 7.16 (t, *J* = 7.2 Hz, 3H), 3.37 – 2.27 (bm, 8H), 2.96 (t, *J* = 6.0 Hz, 2H), 2.64 (t, *J* = 6.4 Hz, 2H), 1.82 (quin, *J* = 6.4 Hz, 2H), ^{13}C-NMR (101 MHz, $CDCl_3$) δ 163.8, 150.4 (3C), 148.7, 140.3 (3C), 129.2 (6C), 127.6 (6C), 126.4 (2C), 120.9 (2C), 76.8, 57.1 (2C), 53.9 51.0 (2C), 47.0 and 25.6; MS (EI+) *m/z* calculated for $C_{32}H_{35}N_5O$: 505.28; found, 505.07 $(M)^+$; HPLC purity 96.3%.

5.2. Biological assays

5.2.1. *In vitro* anti-mycobacterial testing

All compounds were dissolved in 100% dimethyl sulfoxide (DMSO) and stored as frozen stocks at a concentration of 10 mM. Inocula were prepared from cultures of all strains grown in Lowestein Jensen (LJ) slants that were 2 to 5 weeks old. Stock mycobacterial cultures were prepared by inoculating frozen stocks into 10 ml 7H9 mycobacterial culture medium supplemented with Oleic Albumin Dextrose Catalase (OADC) (1%) final concentration, (BBL Microbiology Systems, Cockeysville, Maryland) and 0.05% Tween 80 For growth

evaluation in the (MGIT) 960 instrument (Becton Dickinson, Maryland, USA) was used.[24] Mycobacterial growth is monitored through changes in oxygen consumption which in turn changes fluorescence. Serially two-fold diluted compounds (0.1 ml in DMSO) were added to the 7H9 culture medium contained in Mycobacteria Growth Inhibition Tube (MGIT) tube with the final DMSO concentration not exceeding 1.2%. Five hundred microliters of a mycobacterial culture, previously cultured in a MGIT tube, was also added to the MGIT test tube (Becton Dickinson and Company**.** 1999. Bactec MGIT 960 system user's manual, catalog number 445876. Becton Dickinson and Company, Franklin Lakes, NJ). Incubation at 37°C was continued in the MGIT system and the growth units were monitored daily. For MIC_{99} evaluations, a 1% bacterial culture was prepared in a drug free MGIT tube (GC) and the MIC_{99} of the compound determined relative to the growth units (GU) of this tube. When GU of the GC reached 400 the results were interpreted and MIC_{99} of compounds was determined as GU equal or less than the GU of the GC.

Table 3: *M. tuberculosis* clinical isolates classification, genotyping and sequencing of strains used.

Clinical Isolate	**Genotype**	**Resistance Profile**	**Susceptibility Status**
R73	LCC/X	H	Mono-resistant
R4965	LAM3	H	Mono-resistant
R5401	Beijing	H	Mono-resistant
TT149	Atypical Beijing	H;R;ET;S;A;C;O;M;	XDR
X_3	Beijing	H;R;E;K;S;C;O;	XDR
X_60	Beijing	H;R;E;A;ET;O;S;C;K	XDR
X_61	Beijing	H;R;ET;A;O;K;S;	XDR

Resistance confering mutations

Clinical Isolate	*rpoB*	*katG*	*embB*	*rrs*	*inhA-promoter*	*gyrA*	*pncA*
R73	531TTG	315ACA	306ATC	ND	WT	ND	ND
R4965	WT	315ACC	WT	ND	WT	ND	WT
R5401	WT	WT	WT	ND	-15	ND	WT
TT149	516TAC	315ACC	WT	1401G	WT	94GGC	WT
X_3	531TTG	315ACC	306ATC	1401G	-15	94GGC	14CGC
X_60	531TTG	WT	306GTG	1401G	ND	94GGC	Del8G
X_61	531TTG	WT	WT	1401G	-15	94GGC	103TAG

ND: Not determined; WT: wild type; H: isoniazid; R: rifampicin; E: ethionamide; ET: ethambutol; S: streptomycin; A: amikacin; C: capreomycin; O: ofloxacin; M: moxifloxacin; Z: pyrazinamide

5.2.2. Cytotoxicity assay:

THP-1 cells were cultured for 24 hours at a concentration of 1.5 x 10^4/well in a 96 well plate in the presence of different concentrations of HEPI. After 24 hours, MTT was added to the cells and the plate was incubated at 37^{o}C for 4 hours. Solubilisation buffer was then added and the cells were incubated for an additional 30 minutes. Formazan formation was measured at an absorbance of 570 nm using an SLT Rainbow Plate reader (Tecan, U.S., Inc).

The cytotoxicity percentage was calculated using the following formula: 100 - [100 x (optical density (OD) with drug/OD without drug)].[25]

5.2.3. Intracellular drug Inhibition Assays:

Peripheral blood mononuclear cell (PBMCs) were plated in round bottom 96 well plates at a concentration of $1.5x10^4$/well. After 24 hours, non-adherent cells were gently washed away using Roswell Park Memorial Institute (RPMI) media. Adherent monocytes were cultured for 6 days and then infected with BCG for 4 hours using a multiplicity of infection (MOI) of 3. Extracellular bacteria were removed by washing three times with RPMI media. The infected monocytes were then cultured in the presence or absence of different concentrations of drugs for 72 hours. Monocytes were then lysed with a 0.2% solution of saponin in RPMI. The bacteria were quantified using an [^{3}H]-uridine incorporation assay or by culturing onto 7H10 media and counting colony forming units (CFU) weekly for 4 weeks. The [^{3}H]-uridine incorporation assay was performed by adding 1 μCi [^{3}H]-uridine in 7H9 Middlebrook broth per well and incubating for another 72 hours at 37°C. The plate was then harvested onto a filter mat using a Tomtec Mach III cell harvester (Tomtec Inc., Hamden CT). The filter mat was air dried and placed into a pouch with scintillation fluid and read using a Microbeta scintillation counter (PerkinElmer Wallac Inc.) BCG percent inhibition was calculated with the following formula: percentage inhibition = 100 − [100 × (disintegrations per min (dpm) in the drug-treated cultures/dpm in the untreated cultures)].[25]

5.2.4. Ethidium Bromide Assay:

Efflux pump activity was measured using BCG. BCG aliquots of 225ul (BCG culture turbidity in 7H9 Middlebrook broth was adjusted to the standard turbidity of McFarland 0.5) were added into 2ml tubes in the presence of ethidium bromide and verapamil at concentrations of 2.5 μM and 250 μM respectively. The bacteria were then incubated at room temperature for 1 hour. The bacteria were then centrifuged and washed with 1mL PBS and then re-suspended in PBS containing 0.4% glucose in the presence or absence of RINHs. The cultures were then incubated for an additional one hour at 37°C. Aliquots were transferred into a 96 well plate in triplicates. A minimum of 5000 events were collected via flow-cytometric acquisition using Guava easyCyte (Guava Technology, Hayward, CA). Analyses were performed using FlowJo (version 6.2.1; Tree Star) software.[25]

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Chemother. **2016**, *60*, 1216–1225.

ASSOCIATED CONTENT

Supporting Information. Additional details of synthesis and characterization of selected compounds, NMR spectra, and procedures used for the cytotoxicity, *in vitro* anti-mycobacterial and macrophage studies. Supplementary data associated with this article can be found, inthe online version.

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Notes

The authors declare no competing financial interests.

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

ACKNOWLEDGMENTS

The University of Cape Town and South African Research Chairs Initiative of the Department of Science and Technology, administered through the South African National Research Foundation, are gratefully acknowledged for support (K.C). Furthermore, we gratefully acknowledge the support of DST-NRF Centre of Excellence for Biomedical Tuberculosis Research, SAMRC Centre for TB Research, Division of Molecular Biology and Human Genetics, Faculty of Medicine and Health Sciences, Stellenbosch University.

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