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MEDICINAL CHEMISTRY RESEARCH

ORIGINAL RESEARCH

Novel lead generation of an anti-tuberculosis agent active against non-replicating mycobacteria: exploring hybridization of pyrazinamide with multiple fragments

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Abstract The key to shortening tuberculosis (TB) drug regimen lies in eliminating the reservoir of non-replicating persistent (NRP) *Mycobacterium tuberculosis* (Mtb). Pyrazinamide (PZA) is the only known drug used as part of a combination therapy that is believed to kill NRP Mtb and achieve sterilization. PZA is active only under low pH screening conditions. Screening and identification of NRP-active anti-TB compounds are severely limited because compounds are usually inactive under regular assay conditions. In an effort to design novel NRP-active anti-TB compounds, we used pyrazinamide as a core and hybridized it with the fragments derived from marketed drugs. One of these designs, compound **8,** was a hybrid with fluoroquinolone. This compound exhibited >10 fold improvement in NRP activity under low pH condition as compared to pyrazinamide and a modest activity (0.8 $\log_{10}$ kill) under nutritionally starved NRP condition. Furthermore, compound **8** was active against fluoroquinolone-resistant strains and did not show any activity in a DNA supercoiling assay (gyrase inhibition), suggesting that its mechanism of action is not that of the parent fluoroquinolone. These results provide a novel avenue in the exploration of new chemotypes that are active against non-replicating Mtb.

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Keywords Tuberculosis (TB) · Non-replicating (NRP) · Pyrazinamide (PZA) · Fluoroquinolone (FQ) · Hybridization

Tuberculosis (TB) causes approximately 2 million deaths per year. An estimated one-third of the world's population is thought to be infected with dormant or latent forms of *Mycobacterium tuberculosis* (Dye and Williams, 2010; Russel *et al.*, 2010). Infections with multidrug-resistant (MDR) and extensively drug-resistant (XDR) mycobacterial strains as well as co-infection with HIV create a global health challenge (Gandhi *et al.*, 2010; Mandavilli *et al.*, 2007). Currently, treatment of TB involves administration of a combination of isoniazid, rifampin, pyrazinamide and ethambutol antibiotics for the first 2 months, followed by an additional 4-month treatment with isoniazid and rifampin (Mitchison, 2005). In the case of MDR tuberculosis, the antibacterial chemotherapy is extended to 12–24 months. This long treatment duration coupled with poor patient compliance contributes directly to the emergence of MDR and XDR strains of *M. tuberculosis* limiting the efficacy of standard therapy (Check, 2007; Dye, 2009). Screening of new antimycobacterial agents against actively replicating bacteria is more popular and high throughput than non-replicating bacteria. However, it is generally believed that a non-replicating persistence (NRP) is responsible for antimicrobial tolerance. In TB, the key to shortening the 6-month regimen lies in targeting the NRP subpopulation (Cho *et al.*, 2007). Herein, we present a novel approach to target NRP Mtb for the development of new anti-TB drugs. Pyrazinamide (PZA), one of the frontline anti-TB drugs, is known to be highly effective against experimental murine TB in combination with isoniazid (McCune *et al.*, 1956). PZA is effective against NRP in vivo, but under in vitro conditions it shows anti-TB

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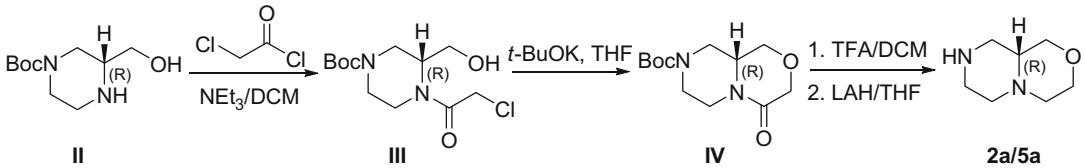
activity only under acidic pH conditions (Heifets and Lindholm-Levy, 1992; Heifets and Sanchez, 2000). Several attempts have been made to make pharmacologically superior analogs of PZA without success (Zimhony *et al.*, 2007). Recently, we have shown Fragmentation Intelligent Re-combination Enumeration (F.I.R.E.) to synthesize virtual libraries and in silico screening to generate hits (Iyer and Panda, 2014). In this approach, marketed drugs and clinical candidates were fragmented into cores and daughters, and recombined in various permutation and combinations to regenerate the full molecules. These molecules could potentially contribute to the biological activity. In the present study, we have used PZA as a core and designed a set of compounds by hybridizing it with specific fragments. Each of these compounds was screened on Mtb under NRP conditions (acidic stressed and nutrient starvation) to find a potential lead candidate.

Scheme 2a Synthesis of fragment **2a/5a**

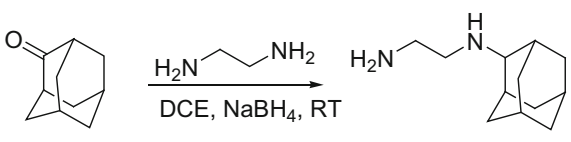


Syntheses of fragments

The required fragments have been synthesized by following literature procedures.

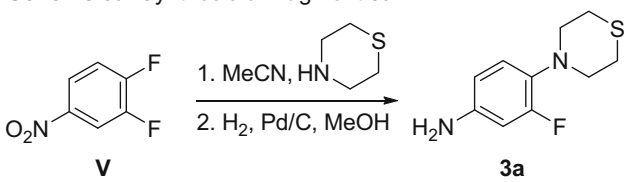
1. The selective reductive amination of 2-adamantanone **I** with ethylenediamine using sodium borohydride ($NaBH_4$) in 1,2-dichloroethane (DCE) provided the required fragment **1a** (Meng *et al.*, 2007) of SQ-109 (Scheme 1a).

Scheme 1a Synthesis of fragment **1a**



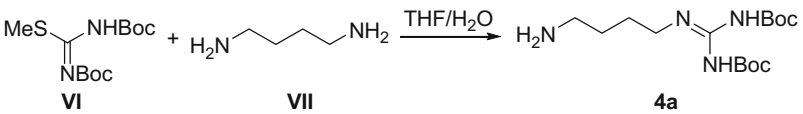
2. (*R*)-1-Boc-3-(hydroxymethyl)piperazine **II** was treated with chloroacetyl chloride to give an amide **III** which was cyclized to intermediate **IV** under basic conditions. *Tert*-butyloxycarbonyl (Boc) deprotection of intermediate **IV** was carried out by using trifluoroacetic acid (TFA) followed by reduction with lithium aluminum hydride (LAH) to give the fragment **2a/5a** (Safina *et al.*, 2012) of GSK-13222322 (Scheme 2a).
3. The nucleophilic aromatic substitution (S_NAr) of 3,4-difluoronitrobenzene **V** with thiomorpholine followed by palladium catalyzed hydrogenation afforded the required fragment **3a** (Ryukou *et al.*, 2007) of Sutezolid (Scheme 3a).

Scheme 3a Synthesis of fragment **3a**



4. The nucleophilic substitution reaction of 1,3-bis(*tert*-butoxycarbonyl)-2-methyl-2-thiopseudourea **VI** with 1,4-diaminobutane in tetrahydrofuran (THF)/water provided the required fragment **4a** (Carmignani *et al.*, 2001) of PMX-30063 (Scheme 4a).

Scheme 4a Synthesis of fragment **4a**

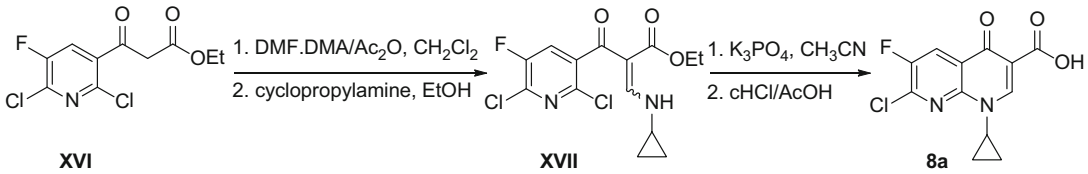


5. The commercially available 2,4-dinitroimidazole **VIII** was treated with (*S*)-glycidol to afford the intermediate **IX**. The resulting secondary hydroxyl group of the intermediate **IX** was protected with the tetrahydropyranyl (THP) ether by using dihydropyran (DHP) and pyridinium *p*-toluenesulfonate (PPTS) to give the intermediate **X**. The selective deprotection of

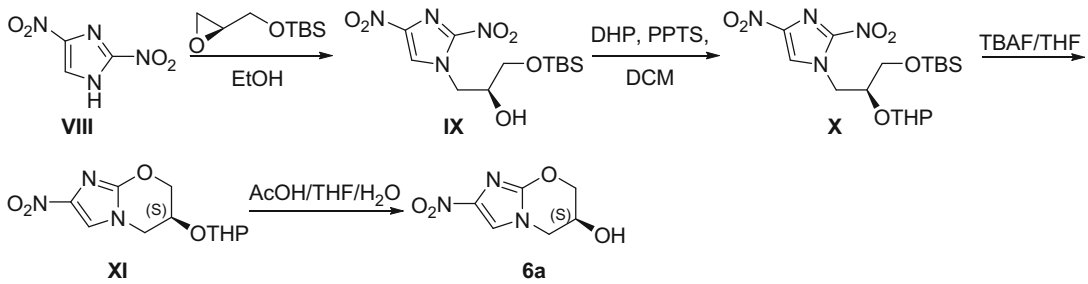
tert-butyldimethyl (TBS) ether of intermediate **X** with tetra-n-butyl ammonium fluoride (TBAF) followed by cyclization gave the intermediate **XI**. Finally, the THP deprotection was carried out by using aqueous acetic acid to give the required fragment **6a** (Orita *et al.*, 2007) of PA-824 (Scheme 6a).

7. The commercially available β-ketoester **XVI** was treated with *N*,*N*-dimethylformamide dimethyl acetal (DMF.DMA) followed by cyclopropylamine to give the intermediate **XVII**. The base-mediated cyclization of intermediate **XVII** followed by ester hydrolysis under acidic conditions afforded the required fragment **8a** (Matsumoto *et al.*, 1986) of Moxifloxacin.

Scheme 8a Synthesis of fragment **8a.**



Scheme 6a Synthesis of Fragments **6a**



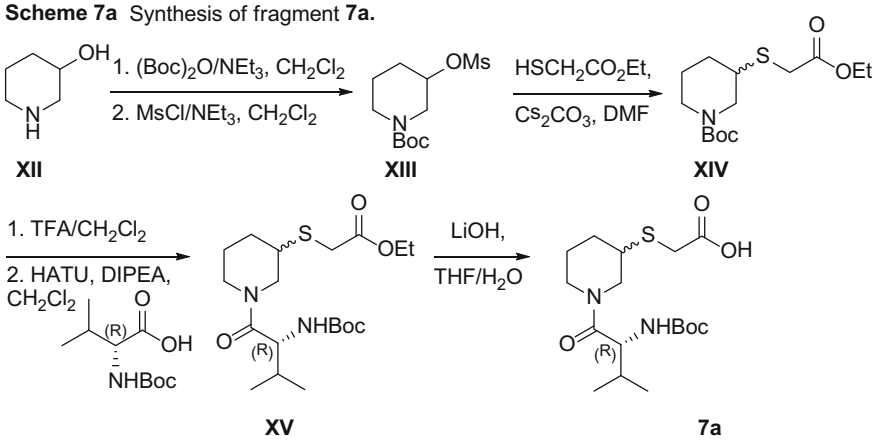
6. The free hydroxyl group of piperidin-3-ol **XII** was activated by mesylation after protecting the free amine with *tert*-butyloxycarbonyl (Boc) under standard conditions to give the intermediate **XIII**. The mesyloxy (OMs) group of intermediate **XIII** was displaced with ethyl thioacetate to give the intermediate **XIV**. *Tert*-butyloxycarbonyl (Boc) deprotection using trifluoroacetic acid (TFA) followed by coupling with Boc-L-valine gave amide **XV**. Finally, the ethyl ester was hydrolyzed to give the corresponding acid **7a** (Berner and Kerber, 2004) of BC-3205 (Scheme 7a).

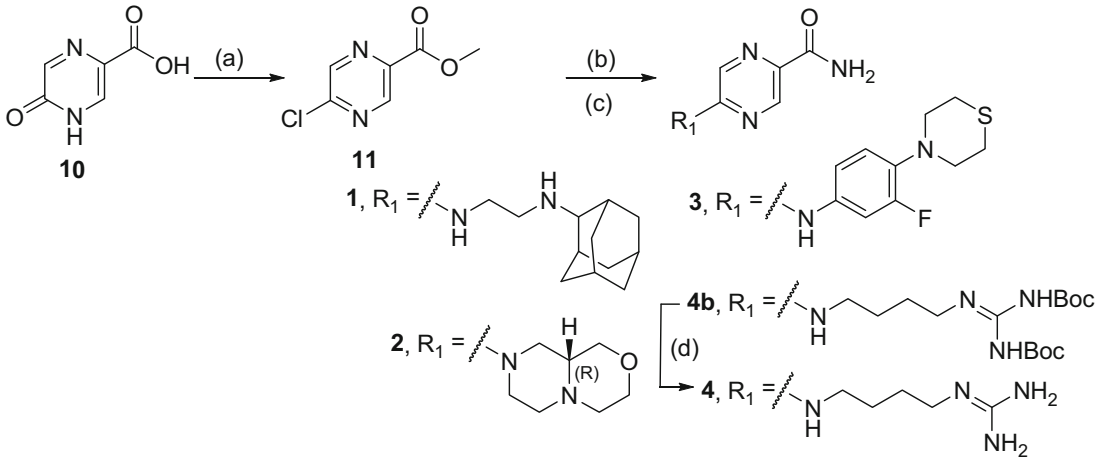
Synthesis of pyrazinamide derivatives

The chlorination followed by esterification of 5-oxo-4,5-dihydropyrazine-2-carboxylic acid **10** by using literature procedure gave the methyl 5-chloropyrazine-2-carboxylate **11** (Coe *et al.*, 2013) in 55 % yield. The chloro substitution of compound **11** was displaced with various fragments **1a**, **2a**, **3a** and **4a** followed by ammonolysis to give compounds **1**, **2**, **3** and **4**, respectively (Scheme 1).

In order to prepare 6-substituted pyrazinamide derivatives, methyl pyrazine-2-carboxylate **12** was treated with

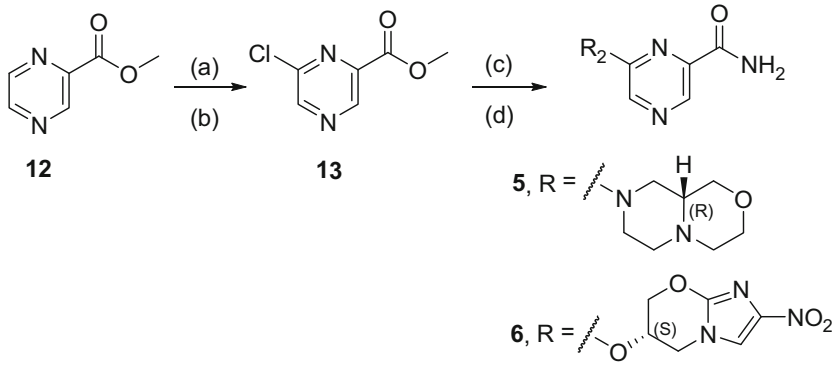
Scheme 7a Synthesis of fragment **7a.**





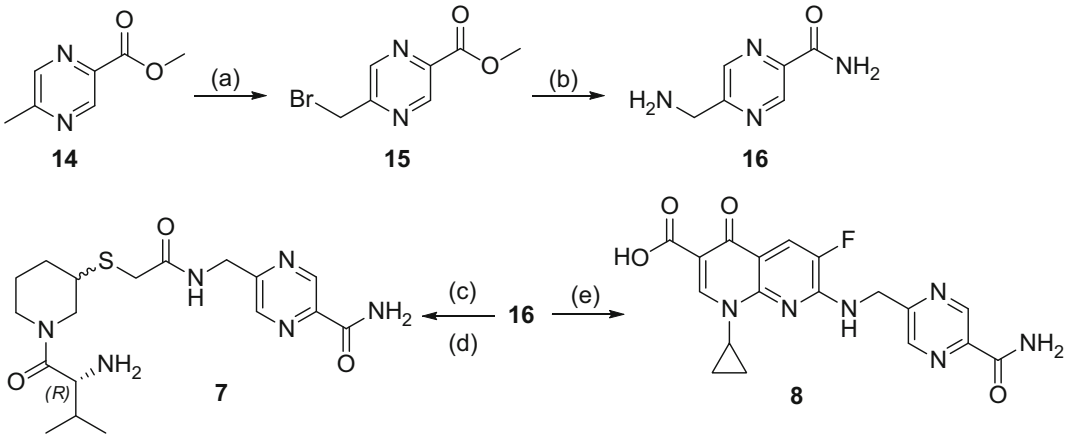
Scheme 1 Reagents and conditions. (*a*) (i) $SOCl_2$, $PhCH_3$, DMF (cat.), 110 °C, 2 h; (ii) MeOH, RT, 16 h; (*b*) RR′NH, K_2CO_3, DMF, 120 °C, MW, 1 h; (*c*) NH_3 in MeOH (8 M), 120 °C, 16 h; (*d*) HCl in dioxane (4 M), RT, 1 h

Scheme 2 Reagents and conditions: (*a*) *m*-CPBA, $ClCH_2CH_2Cl$, 60 °C, 16 h; (*b*) $SOCl_2$, 75 °C, 8 h; (*c*) K_2CO_3, DMF, 120 °C, 1 h (for 5); Cs_2CO_3, DMF, MW, 100 °C, 1 h (for 6); (*d*) NH_3 in MeOH (8 M), 80 °C, 1 h



m-CPBA followed by thionyl chloride to give methyl 6-chloropyrazine-2-carboxylate **13** (Scanio *et al.*, 2011) by using literature procedures. The chloro substitution of compound **13** was displaced with fragments **5a** and **6a** followed by ammonolysis to give compounds **5** and **6**, respectively (Scheme 2).

The required 5-(aminomethyl)pyrazine-2-carboxamide **16** was obtained from methyl 5-methylpyrazine-2-carboxylate **14**. The bromination of compound **14** by using NBS/AIBN (*N*-bromosuccinimide/azoisobutyronitrile) gave compound **15** (Aditya and Kodadek, 2012). Finally, ammonolysis of the compound **15** gave the compound **16**. The amine



Scheme 3 Reagents and conditions: (*a*) NBS, AIBN, CCl_4, 80 °C, 16 h; (*b*) NH_3 in MeOH (8 M), RT, 2 h; (*c*) **7a**, HATU, DIPEA, DCM, RT, 16 h; (*d*) HCl in dioxane (4 M); (*e*) **8a**, CH_3CN, NEt_3, 80 °C, 2 h

functionality of the compound **16** was coupled with the carboxylic acid of fragment **7a** to give the required amide which was treated with HCl to afford the compound **7**. The chloro functionality of the 7-chloro-1-cyclopropyl-6-fluoro-4-oxo-1,4-dihydro-1,8-naphthyridine-3-carboxylic acid **8a** was displaced with amine **16** to give compound **8** (Scheme 3).

Biological screening

Depending on macrophage activation status, intracellular Mtb is exposed to numerous stresses including reactive oxygen/nitrogen species, low pH (in the range of pH 3.0–6.5) and reduced access to micronutrients following activation with gamma interferon. This critical immunological event, results in the fusion of lysosomes with phagosomes thereby exposing intra-phagosomal Mtb to an acidic environment (ranging from pH of 3.0 to 6.5) and to numerous hydrolases that can potentially degrade microbial nucleic acids and other cellular components. During many years of evolution with the human host, Mtb has developed several mechanisms to combat the stresses present within the infected macrophage. One of the approaches to target the Mtb would be to identify compounds that work specifically under acidic pH, thereby killing bacilli that have gone into a non-replicating state following acidic stress. Recently, novel assays to screen for compounds that disrupt intrabacterial pH homeostasis have been described (Vandal *et al.*, 2009; Darby *et al.*, 2013). Therefore, in the present study, the hybridized compounds of PZA with multiple fragments along with PZA reference compound **9** as a control were screened on Mtb (*M. tuberculosis* H37Rv ATCC 27294) for their minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) using an in vitro low pH (6.3 ± 0.1) assay. The compound **8** showed MIC = 100 μM which was tenfold better than that of PZA compound **9**, MIC = 1039 μM. The other compounds turned out to be inactive at the maximum concentration tested (MIC > 100 μM) (Table 1).

To investigate whether the improved activity of compound **8** could be attributed to the fluoroquinolone core, activity against fluoroquinolone-resistant isolates was measured. Since activity was retained against fluoroquinolone-resistant isolates, the improved activity is unlikely to be mediated through the biological target of fluoroquinolones (Table 2). Fluoroquinolones are known to inhibit DNA gyrase, and therefore, it was important to understand the activity of compound **8** against DNA gyrase. In fact compound **8** was found inactive in a DNA supercoiling assay indicating novel mechanism of action (Table 3).

Compound **8** showed better MBC (~2 $\log_{10}$ reduction) results than PZA (100 vs. 2078 μM under low pH conditions on day 14 (Fig. 1), which indicated that the

Table 1 MIC and MBC under low pH conditions

Comp. No.	Mtb-MIC (μM)	Mtb-MBC (μM) 2 Log_{10} reduction
1	>100	>100
2	>100	>100
3	>100	>100
4	>100	>100
5	>100	>100
6	>100	>100
7	>100	>100
8	100	100
PZA, 9	1039	2078

Table 3 Mtu gyrase supercoiling assay

Compound	IC50 (μM)
8	>100
Moxifloxacin	7.3
Novobiocin	0.028

Table 2 MIC (μM) under low pH conditions and fluoroquinolone-resistant strains (Oflox^{res} Mtb strains[a,b] from AstraZeneca strain bank)

	Neutral pH 7.2			Low pH 6.3		
	7H9 broth			7H9-MonoKPO4		
	H37Rv	$\text{Oflox}^{®b}$	$\text{Mox}^{®a}$	H37Rv	$\text{Oflox}^{®b}$	$\text{Mox}^{®a}$
8	50	50	50	100	200	100
Moxi	1.14	1.14	>18.2	2.28	2.28	>18.2
Oflox	1.38	>11.04	1.38	2.76	>11.04	5.52
PZA	>2078	>2078	>2078	1039	1039	1039
Inh	0.23	0.23	0.23			

[a] Mutant

[b] Clinical isolate

hybridized compound of FQ and PZA looks promising for further optimization.

Compound **8** was also evaluated for its activity in the nutrient starvation model (Table 4) along with pyrazinamide as a positive control and isoniazid as a negative control. Compound **8** shows net bactericidal activity as 0.8 $\log_{10}$ cfu kill *vs*. pyrazinamide which shows 1.6 $\log_{10}$ cfu kill at 100× MIC concentrations tested (Huang *et al.*, 2007). Compound **8** exhibited a good dose response from 10×, 30× and 100× MIC concentrations tested. Isoniazid, which is known to act only on replicating bacilli, did not show any kill even at 100× MIC as expected.

In addition, the data from a panel of broad spectrum bacteria (from AstraZeneca strain bank) suggest that compound **8** is active in both gram-positive and gram-negative pathogens (Table 5). Reference antibiotic controls were used (Ofloxacin O-8757, Moxifloxacin S-1465, Isoniazid I-3377, Novobiocin N-1628, from SIGMA Chemical Co.) as appropriate in the various assays.

The in vitro DMPK data for **8** suggest that it has desirable physicochemical properties, modest aqueous solubility and human Caco2 (ATCC Catalog # HTB-37) permeability, low rat in vitro microsomal clearance and excellent PPB (Table 6).

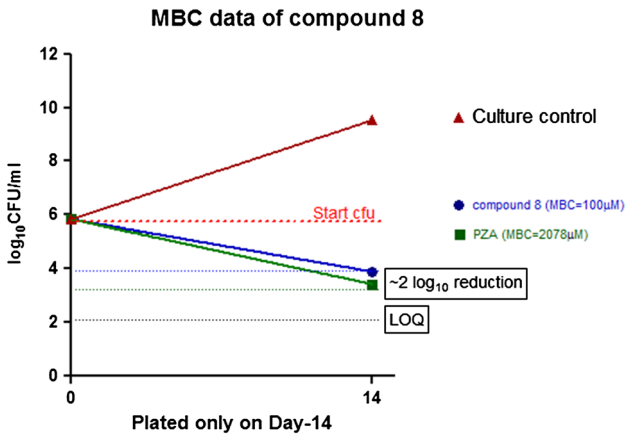


Fig. 1 MBC data of compound **8** in the low pH assay condition

Table 4 Activity of compound **8** under nutrient starvation

Activity of compound **8** in nutrient starvation model			
Control	CCntrl	$\log_{10}$ Kill D14	$\log_{10}$ Kill D28
8	10×	0.1	0.2
	30×	0.3	0.4
	100×	0.4	0.8
PZA	10×	1.0	1.4
	30×	1.1	1.6
	100×	1.2	1.6
Isoniazid	10×	0.1	−0.1
	30×	0.1	0.2
	100×	0.1	0.1

Table 5 Broad spectrum panel MIC data of compound **8**

Broad spectrum MIC data of compound **8**	
Strain	MIC (μM)
E. coli WT	50
E. coli efflux mutant	0.78
H. influenzae WT	3.12
H. influenzae efflux mutant	1.56
S. pneumoniae	100
S. aureus MRQR	>200
P. aeruginosa WT	>200
P. aeruginosa efflux mutant	12
K. pneumoniae	140

Table 6 Physicochemical properties of compound **8**

Physicochemical properties of compound **8**	
AZ LogD	−0.405
Solubility dried DMSO (pH = 7.4) (μM)	68.3
PPB (% free)	54.03
Rat Clint (μL/min/mg)	6.44
Hu Caco2 ABBA (1E − 6.cm/s)	1.01; 5.96

In conclusion, we have demonstrated a novel strategy of generating hits active against NRP mycobacteria using a hybridization approach of pyrazinamide with multiple fragments. The identified hit showed good physicochemical properties and bactericidal effect resulting in ~2 $\log_{10}$ kill at low pH and ~1 $\log_{10}$ kill under nutrient starvation condition. Furthermore, this hit was also active against a broad spectrum panel of bacteria.

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Conflict of interest None.

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