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# Letter

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# Nitrofuranyl Methyl Piperazines as New Anti-TB agents: Identification, Validation, Medicinal Chemistry and PK Studies

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*KEYWORDS: Mycobacterium tuberculosis, MTB*  $H_{37}Rv$ , *Multidrug resistant-TB, 6-nitro-2,3-dihydroimidazooxazole, Structure activity relationship.* 

**ABSTRACT:** Whole-cell screening of 20,000 drug-like small molecules led to the identification of nitrofuranyl methylpiperazines as potent anti-TB agents. In the present study, validation followed by medicinal chemistry has been used to explore the structure-activity relationship. Ten compounds demonstrated potent MIC in the range of  $0.17 - 0.0072 \mu$ M against H<sub>37</sub>Rv *Mycobacterium tuberculosis* (MTB) and were further investigated against non-replicating and resistant (Rif<sup>R</sup> and MDR) strains of MTB. These compounds were also tested for cytotoxicity. Among the 10 tested compounds, five showed sub-micromolar to nanomolar potency against non-replicating and resistant (Rif<sup>R</sup> and MDR) strains of MTB along with a good safety index. Based on their overall *in vitro* profiles, the solubility and pharmacokinetic properties of five potent compounds were studied, and two analogs, **14f** and **16g**, were found to have comparatively better solubility than others tested and acceptable pharmacokinetic properties. This study presents the re-discovery of a nitrofuranyl class of compounds with improved aqueous solubility and acceptable oral PK properties, opening a new direction for further development.

The emergence of resistant tuberculosis has been a serious concern worldwide that has reinvigorated drug discovery efforts in search of novel candidates that are effective against both susceptible and resistant strains as well as safe and potentially faster-acting, with the aim of shortening lengthy TB treatments.<sup>1-3</sup> Whole cell screening is an attractive approach to the fast identification of novel compounds active against TB.<sup>4</sup> The success of the whole cell screening-driven approach to tuberculosis is best illustrated by the discovery of bedaquiline (TMC207, diarylquinoline derivative).<sup>5,6</sup> Similar success has been shown by the discovery of other pre-clinical candidates such as benzothiazoles TCA-1<sup>7</sup> and imidazopyridine amides Q-203.<sup>8</sup> Considering the high attrition rate in clinical trials, further enrichment of the clinical pipeline is greatly needed.

To discover novel and potent anti-TB agents, a whole-cell screening approach was adapted and 20,000 small drug-like compounds were procured and screened.<sup>9</sup> The aim of this approach was to find drug-like compounds in this collection and then to chemically modify these compounds to improve their PK/PD behaviors. The library was initially screened against sensitive ( $H_{37}Rv$ ) and rifampicin resistant-(Rif<sup>R</sup>) strains of MTB at the concentration of 16 µg/ml, and 707 molecules demonstrated >90% growth inhibition. A minimum inhibitory concentration (MIC) determination for these

compounds yielded 233 molecules with  $\leq 8 \ \mu g/ml$  MIC against sensitive and resistant strains of MTB. The chemical clustering of these hits revealed nitrofuranyl methylpiperazine as one of the most potent scaffolds (Fig 1). The identified nitrofuranyl methylpiperazine cluster included six compounds **1a-f** with a MIC in the range of 0.2 – 25.3  $\mu$ M against H<sub>37</sub>Rv and Rif<sup>R</sup> strains of MTB (Fig. 1).

A literature survey revealed that nitrofuran containing compounds are known to possess anti-TB potential. Lee and co-workers<sup>10-16</sup> have extensively studied nitrofuranylamides 2 and also generated several leads {3 (Lee-562), 4 (Lee-878) and 5 (Lee-1106)} as shown in Fig. 2. Despite excellent in vitro profiles, these molecules possessed poor in vivo efficacy because of their low oral bio-availability and aqueous solubility, indicating that further effort is needed to utilize these compounds effectively. Our whole-cell screening program resulted in the identification of nitrofuranyl methyl piperazine 1, wherein a nitrofuranyl ring is directly attached to a piperizine ring through a methylene instead of an amide linkage. The high potency of these compounds, along with their comparatively simpler structure and likeliness of increased aqueous solubility indicated the need for further investigation. A medicinal chemistry program was initiated on nitrofuranyl methyl piperazine 1 using the strategy shown in Fig. 3.

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Fig 1: Schematic representation of whole-cell screening of 20,000 molecules

#### Results and Discussion:

#### **Chemistry:**

Initially, the potent hit **1a** was prepared by the reductive amination of 5-nitrofuraldehyde **6** with 4-phenyl piperazine **7** (Scheme 1). Next, to ascertain the role of the nitro group, the des-nitro derivative **13** was prepared using the synthetic strategy shown in Scheme 2. The role of the furan ring was also investigated by synthesizing analogs **14a-c**, in which the furan ring was replaced with a thiophene ring using the same synthetic strategy. The effect of ring C was also studied, and analogs **14d-l** were synthesized with varying substituents. Through further modification, analogs **16a-h** were prepared by replacing the phenyl ring (ring C) with alkyl/aryl sulfonyl groups as per the synthetic strategy shown in Scheme 3. In another modification, the piperazine ring (ring B) was replaced with piperidine and morpholine rings using the synthetic scheme shown in Scheme 4.



Fig 2: Nitrofuranyl containing anti-TB molecules



Fig 3: Medicinal chemistry approach

Scheme 1: Synthesis of identified hit 1a



#### **Biological evaluation:**

The synthesized analogs **1a**, **13**, **14a-l**, **15**, **16a-g** and **18a-b** were screened for *in vitro* activity against MTB  $H_{37}Rv$  (ATCC27294 strain) using the microbroth dilution method. The MIC was determined as the minimum concentration of the compound required to inhibit 90% of bacterial growth. The MIC values of all of the synthesized compounds are summarized in Table 1.

#### Scheme 2: Synthesis of ring A and C modified nitrofuranyl methyl piperazines



Reagents and Conditions: a) Pd(OAc)<sub>2</sub>, Cs<sub>2</sub>(CO)<sub>3</sub>, rac-BINAP, toluene, reflux, 4 h, 60-65%; b) TFA, DCM, rt, 2 h; c) Na(OAc)<sub>3</sub>BH AcOH, DCM, rt, 12 h, 80-90%.





**Reagents and conditions:** a) Na(OAc)<sub>3</sub>BH, AcOH, DCM, rt, 12 h, 80%; b) TFA, DCM, rt, 15 min; c) R-SO<sub>2</sub>CI, TEA, DMAP, DCM, rt, 12 h, 90-95%.

Scheme 4: Synthesis of nitrofuranyl analogs



#### Reagents and Conditions: a) Na(OAc)3BH, AcOH, DCM, rt, 12 h, 75-80%.

Synthesized compound **1a** exhibited an MIC value of 0.2  $\mu$ M against the H<sub>37</sub>Rv strain of MTB, similar to that observed during the library screen. The removal of nitro group destroyed the activity of the compound **13**, which revealed that the presence of the nitro group is essential. The replacement of furan with a thiophene ring was also unfavorable, and none of the thiophene ring-containing analogs **14a-c** demonstrated any inhibition at concentrations up to 10  $\mu$ M. The results suggested that the nitro-furan moiety is essential for activity.

The effect of ring C and its substituents were also investigated. The nature and position of the substituents greatly influenced activity. The presence of substituents at *para*- and *meta*-positions on ring C was found to be favorable. Among the different *para*-groups, compounds with bulkier groups **14f** (4-*ter*-butyl), **14 h** (4-morpholinyl) demonstrated comparative-

ly better MIC values of 0.0072 and 0.02  $\mu$ M against H<sub>37</sub>Rv strain of MTB, respectively. Among the *meta*-groups, compounds with methoxy group **14j** and cyano group **14k** demonstrated the most potent MIC values of 0.047 and 0.019  $\mu$ M, respectively (Table 1). The activity results suggested that both the nature and position of substituents influenced activity, and the presence of bulkier groups at the *para*-position and smaller groups at the *meta*-position led to enhanced potency.

In further modifications, the phenyl ring (ring C) was replaced by alkyl/aryl sulfonyl groups. Compound 16a, with a methane sulfonyl group on the piperazine ring, demonstrated decreased activity. However, nitrofuranyl methyl piperazines with un/substituted phenyl sulfonyl groups 16b-h showed good to excellent activity (Table 1). Moreover, due to the presence of a sulfonyl group between ring B and ring C, analogs with an even smaller group at the para-position of ring C exhibited good activity. Overall, these results suggest that replacement of the phenyl ring with an arylsulfonyl moiety is acceptable. The replacement of the piperazine ring with piperidine in compound 18a led to a MIC of 0.59 µM, but replacement with morpholine in compound 18b led to a complete loss of activity. These results indicate that the piperazine ring is preferred over piperidine or morpholine rings. A brief summary of SAR is shown in Fig. 4.

Among all of the tested compounds, the 10 most active nitrofuranyl methyl piperazine analogs with activities of  $\leq 0.2 \ \mu M$  (Table 1) were further screened against nonreplicating (streptomycin starved *M. tuberculosis* 18b) and resistant (Rif<sup>R</sup> & MDR) strains of MTB. The cytotoxic potential of these compounds was also investigated in a HepG2 cell line. The results are shown in Table 2. Five analogs *viz.*, **14f**, **14h**, **14k**, **15** and **16 g** demonstrated potent MIC values against non-replicating and resistant strains of MTB. None of the tested compounds were toxic in HepG2 cell lines, and all have acceptable safety indices.

Table 1: In vitro activity of all synthesized compounds<sup>a</sup>

Compound	MIC (H <sub>37</sub> Rv) (μM)	Compound	MIC (H <sub>37</sub> Rν) (μΜ)
13	>16.5	15	0.004 ± 0
14a	>13.1	16a	23.07 ± 7.9
14b	>11.8	16b	0.17 ± 0
14c	>11.5	16c	0.16 ± 0
14d	13.2 ± 6.8	16d	0.31 ± 0
14e	0.12 ± 0.4	16e	0.15 ± 0
14f	0.0072 ± 0.03	16f	0.5 ± 0.1
14g	0.37 ± 0.18	16g	0.08 ± 0
14h	0.02 ± 0	16h	1.3 ± 0
14i	0.3 ± 0.1	18a	0.59 ± 0.2
14j	0.047 ± 0	18b	31.4 ± 10.8
14k	0.019 ± 0.006	Rifampicin	0.07 ± 0.03
141	25.2 ± 7.6		

<sup>a</sup>Values reported are the average of three individual measurements ± SD.



Fig 4: Brief SAR of nitrofuranyl methylpiperzine series

Based on the measured activity levels against all of the tested strains, the five most active compounds, 14f, 14h, 14k, 15 and 16g were further tested for solubility and pharmacokinetic activity. Previous studies have reported that nitrofuran based compounds suffer from poor absorption, which might be caused by their poor aqueous solubility. We studied the solubility of potent compounds in water and in other media (basic and acidic), and all of the tested compounds except 14h possess good to excellent aqueous solubility. The oral *in vivo* pharmacokinetic properties of these compounds were also studied in mice at a dose of 10 mg/kg, and the results are summarized in Table 3 (detail given in SI).

 Table 2: Activity against non-replicating and resistant strains of MTB, and cytotoxicity studies<sup>a</sup>

Compound	NRP <sup>6</sup> μM	MIC (Rif <sup>R</sup> ) MIC (MDR) μΜ μΜ		CC₅₀ <sup>c</sup> µM	Selectivity index (SI) <sup>d</sup>	
14e	12.6 ± 0	1.32±0.45	>25.2	>63	>700	
14f	1.4 ± 0	$0.072 \pm 0.02$	0.029 ± 0.01	>58	>11,600	
14h	0.56 ± 0.2	0.08 ± 0	0.08 ± 0	>53	>2650	
14j	2.11±0.9	0.37 ± 0	0.09 ± 0	>63	>1575	
14k	1.34 ± 0.4	0.19±0	0.06 ± 0.02	>64	>3200	
15	1.6±0	$0.32 \pm 0.1$	0.12 ± 0.05	>64	>1600	
16b	>22.7	$2.13 \pm 0.8$	>22.7	>56	>329.4	
16c	>21.9	0.68 ± 0	$14.59 \pm 6.32$	>54	>337.5	
16e	20.1 ± 0	0.63 ± 0	4.19±1.46	>50	>333.3	
16g	>21.6	1.35 ± 0	1 ± 0.47	>54	>675	
Rifampicin	2.4 ± 1.4	311.07±0	155.5 ±0			
Gatifloxacin	2.66 ± 0	2.66 ± 1.5	1.33 ± 1.5			

<sup>a</sup>Values reported are the average of three individual measurements  $\pm$  SD. <sup>b</sup>Non-Replicating Phase of *M.tb*, <sup>c</sup>Cytotoxicity (concentration causing death of 50% of cells; CC<sub>50</sub>) to HepG2 cells.<sup>d</sup>Selectivity index (CC<sub>50</sub>(µM)/MIC(µM).

Among the five tested compounds, three compounds 14f, 15 and 16g possess promising PK properties, with  $C_{max}$ values of 2352.11, 3806.90 and 3263.51 nM, respectively and AUC<sub>0-t</sub> values of 2994.63, 2709.06 and 4221.42 nM\*h, respectively. The half lives of 14f, 15 and 16 g were calculated to be 4.04, 1.69 and 1.46 h, respectively. Compound 14h had a high C<sub>max</sub> of 5342.18 and an AUC<sub>0-t</sub> of 4512.72, but a comparatively short elimination half life. Although compound 14k demonstrated good aqueous solubility, it was not detected in an in vivo oral PK experiment. This result might be due to the position and nature of the substituents present on ring C. The presence of substituents at the para-position provides stability and prevents the compound from being metabolized quickly.<sup>18</sup> In 14k, a cyano group is present at the *meta*-position, while in the other four compounds (14f, 14h, 15 and 16g), substituents are present at the para-position. Thus, compound 14k may undergo fast metabolism, preventing its detection. The PK results obtained suggest that compounds 14f and 16g demonstrate good in vivo exposure and half lives.

Table 3: Solubility and PK profiles of potent compounds

	Solubility(uM) <sup>a</sup>			PK <sup>♭</sup>				
Compound	water	PBS	SGF	, SIF	C <sub>max</sub> (nM)	T <sub>max</sub> (h)	AUC <sub>0-t</sub> (nM*h)	t <sub>1/2</sub> (h)
14f	116.5	58.2	1165.5	2.9	2352.11	0.25	2994.63	4.04
14h	2.68				5342.18	0.25	4512.72	0.88
14k	256.3	256.3	2563.1	32	nd	nd	nd	nd
15	1285.5	2571.1	2571.1	2571.1	3806.90	0.25	2709.06	1.69
16g	108.3	54.1	2167.6	13.5	3263.51	0.25	4221.42	1.46

<sup>a</sup>Solubility data were the average of three determinations (SD values <1%). <sup>b</sup>Oral dose at 10 mg/kg and the data were average of five determinations. \*nd: not detected

In conclusion, we have re-discovered nitrofuranyl methyl piperazine as a potent scaffold for compounds effective against sensitive and resistant strains of MTB. The present study demonstrates the promise of the nitrofuranyl-based class 1

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59 60 of compounds against sensitive, resistant and non-replicating strains of MTB. The reported compounds have optimal PK properties and comparatively better aqueous solubility than other reported analogs in this class. Studies to determine the *in vivo* efficacy of these compounds are currently underway.

# ASSOCIATED CONTENT

### Supporting Information

Full experimental details for the compounds synthesized, along with NMR and MS spectra and descriptions of biological assays, are provided. This material is available free of charge via the Internet at http://pubs.acs.org.

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### **Author Contributions**

 $\perp$  KRY, GM and S Singh have equally contributed to this work. KR and GM performed the chemical syntheses. SS, SK and VSR performed biological screening. AM, MT and GDS performed *in vivo* PK. SSB performed the solubility study. PPS, IAK and RAV participated in the design and execution of this study.

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	<b>→</b> 0 <sub>2</sub> N	-61	N N 14f	02	N-{}	N. 16	0   \$    N   0   5	Ú,	
Identified hit	MIC H <sub>37</sub> Rv M	тв	0.005 μM			0.0	08 μM		
	MIC Rif <sup>R</sup> MTE	Έ 0.08 μΜ 1.35 μΜ							
	MIC MDR MT	MIC MDR MTB         0.02 μM           MIC NRP MTB         1.4 μM           CC <sub>50</sub> HepG2 cell line         > 58 μM           Selectivity Index         > 11, 600		0.02 μM		1.35 μM			
Activity Profile	MIC NRP MT			>21.6			1.6 μM	.6μM	
	CC <sub>50</sub> HepG2			> 54 μM					
	Selectivity In			> 11, 600		> 675			
Solubility Prof	Solubility (uM)	water	PBS SGF	SIF	water	PBS	SGF	SIF	
		116.5	58.2 1165.5	2.9	108.3	54.1	2167.6	13.55	
	PK profile	C <sub>max</sub> AUC <sub>0</sub> . t <sub>1/2</sub>	= 2352.11 = 2994.63 = 4.04 h,	nM ⊧nM*h,	C <sub>max</sub> AUC t <sub>1/2</sub>	, = ; <sub>0-t</sub> = =	3263.51 4221.42 1.46 h,	nM 2 nM*h,	