Letter

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Identification of Pyrazolo[1,5-a]pyridine-3-carboxamide Diaryl Derivatives as Drug Resistant Anti-tuberculosis Agents

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KEYWORDS. Anti-tuberculosis agents, INH-resistant Mtb strain, RMP-resistant Mtb strains, Structure activity relationship.

ABSTRACT: A series of pyrazolo[1,5-a]pyridine-3-carboxamide (PPA) derivatives bearing diaryl side chain were designed and synthesized as new anti-tuberculosis agents, aiming to improve the efficacy toward drug resistant *Mycobacterium tuberculosis* (*Mtb*) strains. Most of the substituted diphenyl and heterodiaryl PPAs exhibited excellent *in vitro* potency against the drug susceptive H37Rv strain (MIC: <0.002-0.381 μg/mL) and drug resistant *Mtb* strains (INH-resistant (rINH), MIC: < 0.002-0.465 μg/mL; RMP-resistant (rRMP), MIC: <0.002-0.004 μg/mL). Noticeably, some compounds also showed very low cytotoxicity against Vero cells. Further, compound **6j** displayed good pharmacokinetic profiles with oral bioavailability (F) of 41%, and significantly reduced the bacterial burden in an autoluminescent H37Ra infected mouse model.

Tuberculosis (TB), caused by *Mycobacterium tuberculosis* (*Mtb*), remains the leading infectious cause of death worldwide. It was estimated that nearly 10.4 million people fell ill with TB and 1.7 million died of TB in 2016.[1] A standard 6-9 months regimen recommended by World Health Organization (WHO) results in multidrug-resistant TB (MDR-TB) and extensively drug-resistant TB (XDR-TB) due to lack of patient adherence.[2,3] At present, MDR-TB usually has to be treated with a combination of five to seven drugs lasting up to 18-24 months.[4] Therefore, new drugs with the potential to shorten the duration of treatment or overcome drug-resistant TB are urgently needed. Encouragingly, bedaquiline (TMC207, **1**)[5,6] and delamanid (OPC67683, **2**)[7] were approved for the treatment of MDR-TB by FDA in 2012 and by EMA in 2014, respectively (Figure 1), even though the wide-scale use of **1** is restricted for safety risks.[8] It is still imperative to identify new molecules with alternative scaffolds as effective agents to banish the scourge of TB.

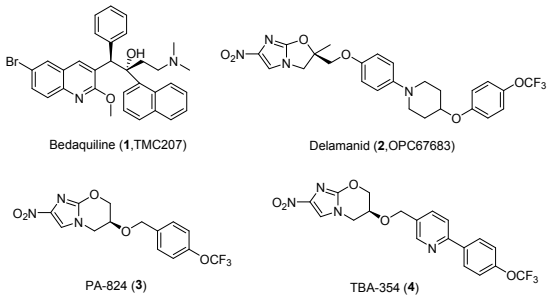


Figure 1. The chemical structures of anti-tubercular drugs bedaquiline, delamanid, PA-824, and TBA-354.

The other two nitroimidazole drugs PA-824 (**3**) and TBA-354 (**4**) are currently in clinical studies for drug-sensitive and drug-resistant TB (Figure 1).[9,10] TBA-354, only with a 4-trifluoromethoxyphenyl-pyridine group to replace the 4-trifluoromethoxyphenyl in PA-824, is promising to shorten TB treatment as part of a regimen superior to that of PA-824 in terms of anti-TB potency and improved pharmacokinetic properties.[11] Our previous studies had identified a series of pyrazolo[1,5-a]pyridine-3-carboxamides (PPAs) as anti-tuberculosis agents (Figure 2), which exhibited good activities with low nanomolar MIC values against H37Rv strain (Figure 2).[12,13] Considering PPAs linked with the N-benzylic moiety as crucial for the anti-tubercular activity, we firstly envisioned to make structural modifications on the side chain of lead **5** by introducing substituted diaryl (diphenyl or heterodiaryl) groups, which was similar to the optimization strategy of TBA354 and would possibly target drug resistant *Mtb* strains (Figure 2). In addition, our previous SAR study had suggested that the 2-methyl-5-methoxy-pyrazolo[1,5-a]pyridine scaffold-based compound displayed better potency than the related 2-methyl-5-methyl-pyrazolo[1,5-a]pyridine against drug resistant clinical *Mtb* isolates.[12] Thus, a series of 2-methyl-5-methoxy-pyrazolo[1,5-a]pyridine-3-carboxamide diaryl derivatives were designed and synthesized.

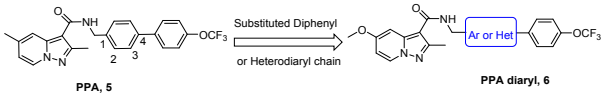
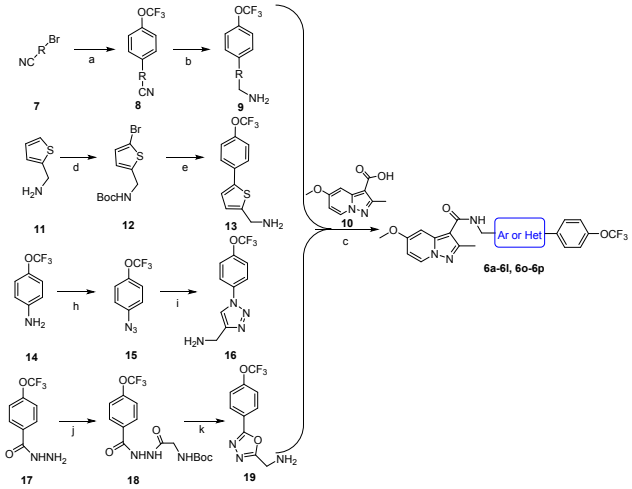


Figure 2. Design of pyrazolo[1,5-a]pyridine-3-carboxamide diaryl derivatives.

Compounds **6a-6l** and **6o-6p** were readily synthesized using a straightforward amidation of pyrazolo[1,5-a]pyridine-3-carboxylic acid **10**[12] with different self-prepared primary amines **9**, **13**, **16** and **19** (Scheme 1). Different strategies were used to synthesize these amines. Briefly, the amines **8** were synthesized by Suzuki coupling bromo-substituted materials **7** and (4-(trifluoromethoxy)phenyl)boronic acid followed by nitrile reduction with lithium aluminium hydride (supporting information). The synthesis of (5-(4-(trifluoromethoxy)phenyl)thiophen-2-yl)methanamine **13** was started by Boc protection and bromination of thiophen-2-ylmethanamine **11** to give bromothiophene **12**, which was followed by Suzuki coupling and deprotection reactions. The (1-(4-(trifluoromethoxy)phenyl)-1H-1,2,3-triazol-4-

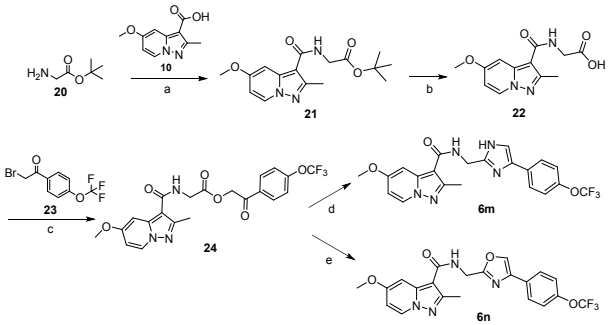
yl)methanamine **16** was obtained by diazo and click reactions starting from 4-(trifluoromethoxy)aniline **14**. The (5-(4-(trifluoromethoxy)phenyl)-1,3,4-oxadiazol-2-yl)methanamine **19** was synthesized starting from **17** that was condensed with BOC-glycine, then followed by cyclization and deprotection reactions based on the published procedures.[14]



Scheme 1. Synthesis of compounds **6a-6l** and **6o-6p**. Reagents and conditions: (a) (4-(Trifluoromethoxy)phenyl)boronic acid, $Pd(PPh_3)_4$, Na_2CO_3, toluene, 110 ℃ , overnight, 83-92%; (b) $LiAlH_4$, THF, -40 ℃ , 3h, 48-53%; (c) HATU, DIPEA, DCM, rt, overnight, 49-53%; (d) i. $NaHCO_3$, Boc_2O, THF, rt, 3h, 97%; ii. NBS, DMF, rt, 5h, 82%; (e) i. 4-(Trifluoromethoxy)phenylboronic acid, K_2CO_3, $Pd(PPh_3)_4$, DME, 80 ℃, 4h, 87%; ii. TFA, DCM, rt, 5h, 82%; (h) Hydrochloric acid, $NaNO_2$, NaN_3, H_2O, 0 ℃ →rt, 2.5h, 92%; (i) 2-propynylamine, CuI, THF, DIPEA, rt, overnight, 70%; (j) BOC-glycine, HATU, DIPEA, DMF, rt, overnight, 85%; (k) i. Et_3N, PPh_3, CCl_4, DMF, rt, overnight, 68%; ii TFA, DCM, rt, 2h, 98%.

The other two compounds **6m** and **6n** were synthesized in Scheme 2. The procedure was started by condensation of tert-butyl glycinate **20** and pyrazolo[1,5-a]pyridine-3-carboxylic acid **10**, followed by hydrolysis in TFA to give intermediate **22**. The intermediate **22** was then reacted with 2-bromo-1-(4-(trifluoromethoxy)phenyl)ethan-1-one **23** to give the ester **24**, and followed by cycloaddition with ammonium acetate and acetamide to produce the compounds **6m** and **6n**, respectively.

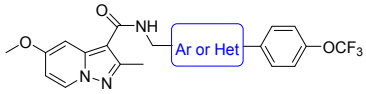
The minimum inhibitory concentration (MIC) values of all the new compounds were preliminarily screened against *Mtb* H37Rv strain in microplate alamar blue assay (MABA) and low oxygen recovery assay (LORA) with the assay concentrations ranged from 0.02 to 5 μg/mL (Table 1).[15] Compounds showing encouraging MICs (< 0.02 μg/mL) in MABA were further tested against *Mtb* H37Rv, rRMP and rINH with the assay concentrations ranged from 0.002 to 0.5 μg/mL, and were also tested against Vero Cells to assess the compounds' potential cytotoxicity (Table 2). Rifampicin (RMP), isoniazid (INH), linezolid (LIZ), and TMC207 were used as positive control drugs to support the reliability of our screening results.

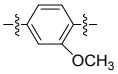
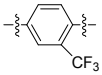


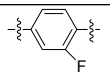
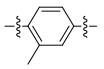
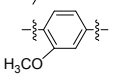
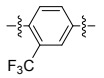
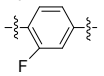
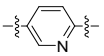
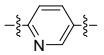
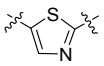
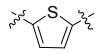
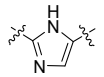
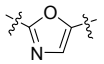
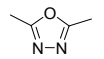
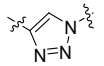
Scheme 2. Synthesis of compounds **6m** and **6n**. Reagents and conditions: (a) HATU, DIEA, DCM, rt, overnight, 70%; (b) TFA, DCM, rt, 2h, 98%; (c) Cs_2CO_3, EtOH, DMF, rt, overnight, 46%, (d) Ammonium acetate, xylene, 140 ℃, 6h, 56 %; (e) Acetamide, xylene, 140 ℃, 3h, 45 %.

The first round of modifications was to investigate the role of the substituents at 3 position of the phenyl group (Figure 2). Therefore, keeping intact the diphenyl chain, small functional groups (CH_3, OCH_3, CF_3, F) were introduced at the 3 position of the phenyl group. The resulting compounds **6a-6d** exhibited strong anti-tubercular activity against *Mtb* H37Rv with all MICs values of < 0.02 μg/mL at the first round assay with concentrations ranged from 0.02 to 5 μg/mL (Table 1). This prompted us to gain further insights into the exact MICs at lower concentrations. Under the second screening concentrations (0.002-0.5 μg/mL), the MICs values of compounds **6a-6d** were 0.013, 0.007, 0.029 and < 0.002 μg/mL, respectively, which were comparable to that of RMP, TMC207, and the lead compound **5** (MIC = 0.006 μg/mL) (Table 2).[12] These results suggested that this position is tolerated for substitution, especially for the OCH_3 and F groups. Switching these substituents from the 3 to 2 position of the phenyl group, an almost 43- and 40-fold reduction in activity was noticed for compounds **6e** (MIC = 0.57 μg/mL) and **6f** (MIC = 0.28 μg/mL), whereas the anti-tubercular activity of compound **6g** was completely lost when the CF_3 was introduced at the 2 position. Interestingly, introduction of F atom in the 2 position led to compound **6h** (MIC = 0.007 μg/mL), which kept potent anti-tubercular activity compared to compound **6d**. A plausible explanation of this result maybe that F atom does not affect the activity due to its relatively small size (similar to that of H atom).

Table 1 *In vitro* anti-tubercular activity of compounds **6a-6p** against the *Mtb* strains H37Rv in MABA and LORA with the assay concentrations ranged from 0.02 to 5 μg/mL.



Compds	Ar Or Het	H37Rv MIC (μg/mL)		ClogP[a]
		MABA	LORA	
6a		<0.02	2.28	5.87
6b		<0.02	>5	5.20
6c		<0.02	>5	6.23

6d		<0.02	>5	5.50
6e		0.57	>5	5.87
6f		0.28	2.25	5.20
6g		>5	>5	6.23
6h		<0.02	>5	5.50
6i		<0.02	4.36	4.52
6j		<0.02	>5	4.22
6k		0.53	>5	4.52
6l		<0.02	>5	5.26
6m		>5	>5	3.23
6n		1.96	2.84	3.29
6o		>5	>5	2.79
6p		>5	>5	3.49
RMP	-	0.02	2.95	-
INH	-	0.48	＞128	-
LIZ	-	3.27	5.98	-
TMC207	-	0.03	-	-

[a]: calculated by MarvinSketch 5.4.1.1 version

Like TBA-354, the pyridine ring was used to replace the phenyl group. The yielded compound **6i** showed an MIC value of 0.053 μg/mL, which was better than that of the control drug INH and LIZ, and comparable to TMC207 (Table 2). Moving the N atom from the 3 to 2 position led to compound **6j**, with improved anti-tubercular activity against H37Rv to <0.002 μg/mL. Inspirited by the activities of compounds **6i** and **6j**, we further investigated five-membered heterocyclic ring instead of the phenyl group. However, only the thiophene derivative **6l** (MIC < 0.002 μg/mL) had the MIC value comparable to **6j**. The thiazole **6k** (MIC = 0.53 μg/mL) and oxazole **6n** (MIC = 1.96 μg/mL) had almost 265- and 980-fold reduction in activities compared to **6j** (Table 1). The imidazole **6m**, oxadiazole **6o** and triazole **6p** completely lost the activities with all of MICs values of > 5 μg/mL. Briefly, it was suggested that five-membered heterocyclic ring such as thiophene can substitute the phenyl, but others led to loss of activity in varying degrees.

The compounds were also tested in LORA, which is an in vitro model for the assessment of activity against persistent *Mtb*. Among the current anti-tubercular drugs, only RMP and PZA have been reported to possess activity against dormant strain. From the results (Table 1), it was very remarkable that compounds **6a** (MIC = 2.28 μg/mL), **6f** (MIC = 2.25 μg/mL) and **6n** (MIC = 2.84 μg/mL) exhibited good LORA MICs values comparable to that of RMP (MIC = 2.95 μg/mL). However, most of the other compounds were not very active in LORA with MIC values of > 5 μg/mL.

Encouraged by their strong potencies against the drug sensitive H37Rv strain, compounds **6a-6d**, **6h-6j** and **6l** were further evaluated against rRMP and rINH strains. It was shown that all of the compounds displayed excellent potencies against rRMP and rINH strains with MIC values < 0.002-0.465 and <0.002-0.004 μg/mL (Table 2). Moreover, the *in vitro* Vero cell toxicity of these compounds was further determined. As it was shown in Table 2, the compounds had low or no cytotoxicity against VERO cells with IC_{50} values from 3.15 to > 50 μg/mL. Particularly, compounds **6d**, **6j** and **6l** had no cytotoxicity against VERO cells, with all of the IC_{50} values of > 50 μg/mL and the selectivity index (SI) values of > 25000.

Table 2. *In vitro* anti-tubercular activity of compounds **6a-6d**, **6h-6j** and **6l** against the *Mtb* strains H37Rv, rRMP and rINH with the assay concentrations ranged from 0.002 to 0.5 μg/mL, and Vero cellular toxicity.

Compds	MIC (μg/mL)			VERO cell IC_{50} (μg/mL)	Selectivity index (SI) [a]
	H37Rv	rRMP	rINH		
6a	0.013	<0.002	<0.002	3.15	242
6b	0.007	<0.002	0.003	11.8	1685
6c	0.029	<0.002	0.003	11.6	402
6d	<0.002	<0.002	0.004	>50	>25000
6h	0.007	<0.002	0.004	>50	>7142
6i	0.053	0.458	0.004	28.4	535
6j	<0.002	<0.002	0.002	>50	>25000
6l	<0.002	0.465	0.002	>50	>25000
RMP	<0.008	>2	<0.008	>100	>12500
INH	0.239	0.438	>8	-	-
LIZ	1.254	1.870	0.953	-	-
TMC207	0.020	<0.016	<0.016	>10	>500

[a]SI: IC_{50}/ MIC for H37Rv

Combined with other factors such as ClogP, we selected compound **6j** for further study. The pharmacokinetic (PK) properties of compound **6j** were evaluated in rats by single intravenous injection (i.v. 2.5 mg/kg) and oral administration (p.o. 10 mg/kg). As shown in Table 3, **6j** exhibited a half-life of 5.1 h and a relative high optimal plasma exposure with AUC value of 15638 h*μg/L. It also displayed a good oral bioavailability of 41%, suggesting its good absorption in rats.

Table 3 The pharmacokinetic profile of compound **6j** in rats.

Pharmacokinetics parameters of **6j**							
Route	Dose (mg/kg)	$AUC_{0-\infty}$ (h*μg/L)	C_{max} (μg/L)	T_{max} (h)	$T_{1/2}$ (h)	$MRT_{0-\infty}$(h)	F (%)
oral	10	15638	1428	4.67	5.10	8.21	41
iv	2.5	9002	4152	0.08	3.49	4.02	

Given the promising anti-tubercular activity against H37Rv and good PK properties in rats, **6j** was evaluated for its *in vivo* anti-tubercular efficacy by using the mouse model infected with the selectable marker-free autoluminescent *Mtb* strain H37Ra.[12,16] The animals were infected with log-phase autoluminescent *Mtb* H37Ra via intravenous injection (2×10^6 CFU per mouse) and then were repeatedly administered with **6j** once daily via oral gavage for 5 consecutive days. The bacterial burden was measured by monitoring the bioluminescence intensity (relative light unit, RLU) from the same batch of live mice every day. As shown in Figure 3, with the two tested doses of 10 and 50 mg/kg/day, compound **6j** exhibited dose-dependent *in vivo* anti-tubercular activity. The **6j** 50 mg/kg/day group showed a significant reduction in lung

RLU by ~1.0 $\log_{10}$ compared with the untreated CMC-Na group (Figure 3A). The **6j** 50 mg/kg/day group also reduced the spleen bacterial burdens by ~0.61 $\log_{10}$ RLU relative to that of CMC-Na group. The RMP treated group showed the decrease by ~1.42 $\log_{10}$ RLU and ~1.34 $\log_{10}$ RLU in lung and spleen, respectively. These results strongly suggest the promising potential of compound **6j** to serve as a lead compound for further anti-tubercular drug discovery.

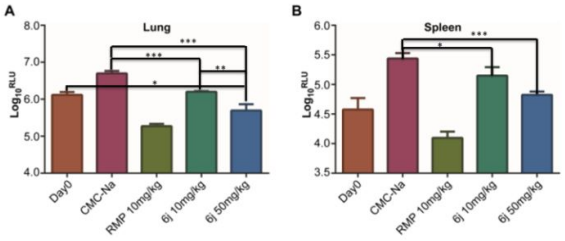


Figure 3. Mean RLU count (±SD) assessed at day 0 and the day after treatment completition. A) for lung, B) for spleen. y-axis: corresponding Log_{10}RLU/lung; x-axis: different treatment groups. (ns, not significant; *$P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.)

A series of PPA diaryl derivatives have been synthesized and evaluated for the anti-tubercular activity. Most of the derivatives exhibited excellent in vitro potency against the drug susceptive H37Rv strain (MIC: < 0.002-0.381 μg/mL) and two rINH (MIC: < 0.002-0.465 μg/mL) and rRMP (MIC: < 0.002-0.004 μg/mL) *Mtb* strains. The representative compound **6j** exhibited promising *in vitro* activities against *Mtb* H37Rv, rRMP and rINH with MIC values ≤ 0.002 μg/mL, and no toxicity against Vero cells. Further *in vivo* studies indicated that compound **6j** displayed good pharmacokinetic profiles with an oral bioavilability of 41 % and significantly reduced the mycobacterial burden in an H37Ra infected mouse model.

ASSOCIATED CONTENT

Electronic supplementary information (ESI) available: Chemistry procedures and analytical data for title compounds, and biological assay. See DOI:

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Author Contributions

# These authors contributed equally to this work.

Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

TB, tuberculosis; PPA, pyrazolo[1,5-a]pyridine-3-carboxamide; *Mtb*, *Mycobacterium tuberculosis*; WHO, World Health Organization; MDR-TB, multidrug-resistant TB; XDR-TB, extensively drug-resistant TB; MIC, minimum inhibitory concentration; MABA, microplate alamar blue assay; MRT, mean residence time; LORA, low oxygen recovery assay; RMP, rifampicin; INH, isoniazid; LIZ, linezolid; RLU, relative light unit; rINH, INH-resistant; rRMP, RMP-resistant; DIPEA, N,N-Diisopropylethylamine; HATU, 2-(7-azabenzotriazol-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate; EMA, European Medicines Agency; FDA, Food and Drug Administration.

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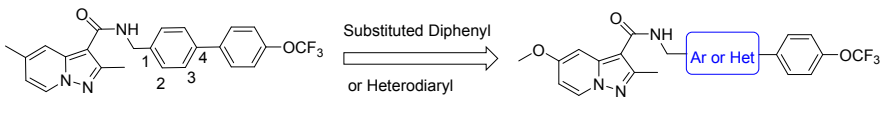

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