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Design, Synthesis and Biological Evaluation of Pyrazolo[1,5a]pyridine-3-carboxamides as Novel Anti-tubercular Agents

Jian Tang,^{†, §, ||} Bangxing Wang,^{†,‡, ||} Tian Wu,[†] Junting Wan,[†] Zhengchao Tu,[†] Moses Njire,^{†, §} Baojie Wan,^{\perp} Scott G. Franzblauc,^{\perp} Tianyu Zhang,^{$\dagger,*$} Xiaoyun Lu,^{$\dagger,*$} and Ke Ding^{$\dagger,*$}

†State Key Laboratory of Respiratory Diseases, Guangzhou Institutes of Biomedicine and Health, Chinese Academy of Science, #190 Kai Yuan Avenue, Guangzhou 510530, China.

College of Life Science, Anhui University, 111 Jiulong Road, Hefei 230601, China.

§ University of Chinese Academy of Sciences, #19 Yuquan Road, Beijing 100049, China.

L Institute for Tuberculosis Research, College of Pharmacy, University of Illinois at Chicago, 833 South Wood Street, Chicago, IL 60612, USA.

KEYWORDS: Anti-tubercular agent, H37Rv, pyrazolo[1,5-a]pyridine, structure-activity relationship, tuberculosis

ABSTRACT: A series of pyrazolo[1,5-a]pyridine-3-carboxamide derivatives were designed and synthesized as new anti-*Mycobacterium tuberculosis* (Mtb) agents. The compounds exhibit promising *in vitro* potency with nanomolar MIC values against the drug susceptive H37Rv strain and a panel of clinically isolated multidrug-resistant Mtb (MDR-TB) strains. One of the representative compounds (5k) significantly reduces the bacterial burden in an autoluminescent H37Ra infected mouse model, suggesting its promising potential to be a lead compound for future anti-tubercular drug discovery.

Tuberculosis (TB) remains one of the world's deadliest pandemic diseases with over 9.0 million new cases and 1.5 million deaths estimated by the World Health Organization (WHO) in 2013.¹ Despite the forty-year success of the inexpensive quadruple-drug therapy [a combination of isoniazid (INH), rifampicin (RIF), pyrazinamide and ethambutol], development and dissemination of multidrugresistant (MDR) and extensively drug-resistant (XDR) Mycobacterium tuberculosis (Mtb) strains, together with co-infection with Human Immunodeficiency Virus (HIV), have intensified an urgent need for new anti-TB drug discovery.^{2,3} Encouragingly, for the first time since 1970s, an ATP synthase inhibitor bedaquiline⁴ (1, also known as TMC207) was approved by the US FDA as a novel active ingredient of combinational therapies for clinical management of adult patients with MDR pulmonary TB in 2012.⁵ However, the drug possesses serious adverse effects such as cardiac arrhythmias,⁵ and displayed higher death rates than that of the placebo group in a clinical investigation,⁶ which may limit its wide application in clinical practice. Several other anti-TB molecules were also identified with different modes of action.² For instance, a bicyclic nitroimidazofuran pro-drug PA-824 (2) was reported to kill both replicating and hypoxic nonreplicating Mtb through a Ddn-mediated activation and has been advanced to phase II clinical trial.^{7,8} Imidazo[1,2-a]pyridine amide (IPA)^{9,10,11,12,13} analogues [e.g. Q203 (3)^{9,10} and compound 4ⁿ] were also discovered to demonstrate strong inhibitory potencies against a panel of drug-susceptible

and drug-resistant Mtb strains by targeting the QcrB subunit of the menaquinol cytochrome c oxidoreductase (bc1 complex), which is a critical component of mycobacterial energy metabolism¹⁴. Clinical outcomes of the compounds, particularly their capability against MDR and XDR Mtb strains in patients, are eagerly awaited. However, given the fact of only one singular FDA approval in forty years and the high attrition rate of drug development, it is still highly valuable to identify new molecules with alternative scaffolds as effective anti-TB drug candidates.



Figure 1. Representatives of the anti-tubercular agents and the new designed molecules.

Pyrazolo[1,5-a]pyridine moiety is a drug-like scaffold which is frequently observed in FDA approved or clinically investigating drugs including antiallergic agent Ibudilast¹⁵, platelet aggregation inhibitor KC-764¹⁶, and dopa-ACS Paragon Plus Environment

mine D4 antagonist FAUC213¹⁷ etc. (Supporting Information). It shares highly similar 3-dimensional conformation and electronic property to that of imidazo[1,2a]pyridine which is the pharmaceutical core of Q203 (3) and compound 4. Therefore, a series of pyrazolo[1,5a]pyridine-3-carboxamide derivatives (5a-5v) were designed as new anti-TB agents by using a scaffold hopping strategy, in which 2, 5-dimethyl groups were introduced at first because of the synthetic feasibility and keeping the geometric similarity to that of compound 4, (Figure 1).

Compounds 5a-5v were readily synthesized using a straightforward amidation of pyrazolo[1,5-a]pyridine-3carboxylic acids 8 or 11 with different primary amines (Scheme 1). Briefly, an N-amination of substituted pyridines 6 or 9 with O-(2,4-dinitrophenyl)hydroxylamine (DNPH) or O-mesitylenesulfonylhydroxylamine (MSH), followed by a 1,3-bipolar cycloaddition with substituted ethyl propiolate, produced the pivotal intermediate pyrazolo[1,5-a]pyridine-3-carboxylates 7 or 10.¹⁸ Compounds 7 were hydrolyzed to yield the carboxylic acids 8 which were coupled with different commercially available or self-prepared amines (Supporting Information) to produce the designed compounds **5a-50**, **5q** and **5r** with good yields (Route A). While due to the difficulty of direct Namination of halogen-substituted pyridine, additional removal of Boc protecting group and diazotization reaction of compounds 10 were needed to convert NHBoc group to Chlorine substitution for the synthesis of compounds **5p** and **5s-5v** (Route B).

Scheme 1. Synthesis of Compounds 5a-v^a



^aReagents and conditions: (a) DNPH, MeCN, 40°C, 18h for all compounds expect **5q**, or MSH, DCM, 0°C, 2h for compound **5q**, (b) K_2CO_3 , DMF, rt, 18h, 32-57%; (c) NaOH, EtOH, H₂O, 60°C, 100%; (d) amines, EDCI, HOBt, Et₃N, DMF, 80°C, overnight, 40-87%. (e) (i) TFA, DCM, rt, 2h, 100%. (ii) CuCl, con. HCl, NaNO₂ (aq, 0.4M), 0-80°C, 45min, 46-86%.

Anti-TB activities of compounds **5a-5v** were preliminarily screened by using a cost-efficient in vitro assay against a selectable marker-free avirulent autoluminescent H37Ra Mtb strain, in which the bacteria growth was conveniently monitored by the bioluminescence intensity without adding any substrates.^{19,20} The minimum inhibitory concentration (MIC) values of the active compounds were further determined by a well-established microplate alamar blue assay (MABA) against H₃₇Rv.²¹ The MICs are interpolated values obtained by using an in-house curvefitting program. The VERO cell growth inhibition was also tested using the Cell Counting Kit-8 (CCK-8) assay to assess the compounds' potential cytotoxicity. All of the 3 reference compounds (i.e. INH, RIF and Q203) displayed comparable MIC values to the reported data,¹⁰ supporting the reliability of our screen conditions (Table 1).

We were pleased to find the first designed molecule 5a, which carries an identical *p*-trifluoromethylbenzyl group to that in compound 4, exhibited strong antimycobacterial activity against Mtb strain H37Rv and the avirulent Mtb strain H37Ra with MIC values of 69.1 and 287.9 nM, respectively. Further investigation revealed the 4-CF₃ group could be replaced by a dimethylamino moiety (5b) without obviously affecting the anti-TB potency. When a 1piperidinyl (5c) or 4-(trifluoromethyl)piperidin-1-yl (5d) was introduced at the R₃ position, the anti- H₃₇Rv activity was improved 5-6 folds. The investigation also suggested that R₃ position is well tolerated with large hydrophobic substituent. For instance, trifluoromethoxyphenyl (5e) and 4-(4-fluorophenyl)piperazin-1-yl (5f) derivatives displayed comparable anti-TB activities to that of 5d. Encouragingly, compound 5g, which harbors the identical lipophilic tail to that of Q203, demonstrated the strongest antimycobacterial activity against both H37Rv and H37Ra Mtb strains, with MIC values of 7.7 and 5.7 nM, respectively. Moreover, compound 5g did not display obvious cytotoxity with an IC₅₀ value of > 100 μ M in a VERO cell growth inhibition assay, supporting it as a new promising starting point for further structure-activity relationship study.

We first investigated the potential impact of R₁ substituent on the anti-TB activity by altering the substituted position of a methyl group on the pyridine ring (5g-5j). It was shown that the 5-position is optimal for substitution (5g). When the methyl group was merged to 4-, 6-, or 7position, the resulting compounds (5h, 5i and 5j) were 2-149 folds less potent against Mtb H37Rv strain. Further investigation also revealed that the 5-methyl group (5g) could be replaced by a methoxyl (5k), ethyl (5m) or chloride (5p) moiety without obviously affecting the anti-TB potency. However, a large hydrophobic substituent at this position is detrimental. For instance, 5-isopropyl (5n) and 5-phenyl (5r) displayed MIC values of 47.4 and 478.9 nM against Mtb H37Rv strain, which are 6.2 and 62.2 folds less potent than 5g, respectively. Interestingly, a removal of the 5-substitutent group (51) barely affected the

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Table 1. In Vitro Anti-tubercular Activity of Compounds 5a-v against the Mtb Strain H37Rv and H37Ra, and VERO **Cellular** Toxicity



Compd.	R.	Ra	R.	MIC [nM (µg/mL)]		$IC_{5^{o}}(\mu M)$
compan	- 1	112		H37Rv ^a	H37Ra ^b	VERO ^c
5a	5-Me	Me	-CF ₃	69.1 (0.024 ^d)	287.9 (0.1)	>100
5b	5-Me	Me	a	86.8 (0.028)	310.2 (0.1)	>100
5C	5-Me	Me	b	16.6 (0.006)	27.6-82.8 (0.01-0.03)	>100
5d	5-Me	Me	с	13.9 (0.006)	2.3 (0.001)	>100
5e	5-Me	Me	d	13.7 (0.006)	6.8 (0.003)	50.74
5f	5-Me	Me	e	13.1 (0.006)	6.6 (0.003)	>100
5g	5-Me	Me	f	7.7 (0.004)	5.7 (0.003)	>100
5h	4-Me	Me	f	1148 (0.60)	>19136 (>10.0)	n.d. ^e
- 5i	6-Me	Me	f	13.4 (0.007)	574.1 (0.3)	>100
5j	7-Me	Me	f	114.8 (0.06)	5741 (3.0)	n.d.
5k	5-OMe	Me	f	11.1 (0.006)	5.6 (0.003)	>100
51	H	Me	f	9.8 (0.005)	589.9 (0.3)	>100
5m	5-Et	Me	f	11.2 (0.006)	18.6-55.9 (0.01-0.03)	>100
5n	5-i-Pr	Me	f	47.2 (0.026)	1816-5448 (1.0-3.0)	n.d.
50	5- <i>t</i> -Butyl	Me	f	>1771 (>1.0)	>17710 (>10)	n.d.
5 P	5-Cl	Me	f	7.4 (0.004)	18.4 (0.01)	>100
59	5-CF ₃	Me	f	52.0 (0.03)	1734 (1.0)	>100
5r	5-Phenyl	Me	f	478.9 (0.28)	>17105 (>10)	n.d.
5 8	5-Cl	Н	f	358.7 (0.20)	18905 (10.0)	n.d.
5t	5-Cl	Et	f	10.8 (0.006)	53.8 (o.o3)	>100
5u	5-Cl	<i>cycl</i> o-Pr	f	17.6 (0.01)	175.7 (0.1)	>100
5v	5-Cl	Phenyl	f	1547 (0.94)	>16528 (>10)	n.d.
3		-		10.8 (0.006)	5.4 (0.003)	>100
INH		-		2989 (0.41)	729 (0.10)	n.d.
RIF		-		36.4 (0.03)	3.64 (0.00 <u>3)</u>	n.d.

^aAnti-TB activity assays against H37Rv were performed using MABA. ^bAnti-TB activity assays against H37Ra were performed using the autolumilenscent assay. °VERO: African green monkey kidney cell line. The cell growth inhibition was evaluated using the CCK-8 assay. ^dThe activity data in the brackets are reported in µg/mL. ^eNot determined. Values are means of two or more independent experiments, and the variation is <20%.

suppressing function against H37Rv strain. The impact of the R₂ substituent was also investigated. It was clear that this position was well tolerated by a small hydrophobic group such as a methyl (5g), ethyl (5t) or cyclopropyl (5u) moiety. However, when it was unsubstituted (5s) or phenyl substituted (5v), the resulting compounds were significantly less potent with MIC values of 358.7 and 1547 nM against H37Rv strain, respectively.

The anti-mycobacterial activities of compounds 5f, 5g, **5k** and **5t** were further validated by determining their MIC, IC_{50} and IC_{50} values against the replication of a fluorescent reporter strain of Mtb H37Rv in liquid medium under aerobic conditions (Table 2).²² It was shown that compounds **5f** and **5k** displayed comparable antitubercular effects against H37Rv to that of RIF, whereas compounds 5g and 5t were moderately less potent. The IC_{50} and IC_{90} values of compound 5k are 2.5 and 6.7 nM, respectively.

Table 2. In Vitro MIC, IC50 and IC90 Values of Com-
pounds 5f, 5g, 5k and 5t against Fluorescent Reporter
Strain of Mtb H37Rv.

Compd.	MIC (nM)	IC_{50} (nM)	IC_{90} (nM)
5f	6.3	2.5	6.5
5g	11.0	5.1	12.0
5k	5.6	2.5	5.7
5t	129	31.3	150
RIF	6.5	4.1	6.9

Encouraged by their strong potencies against the drug-sensitive Mtb H37Rv strain, compounds 5g, 5k, 5p and 5t were further evaluated against a panel of clinical isolated 3495, P71, 9804, 4768, P163 MDR strains²³ by using an autoluminescent assay.¹⁹ It was shown that all of the compounds displayed excellent potencies against the

resistant Mtb strains with MIC values ranged from 11.1 to 1914 nM. Particularly, compound **5k** exhibited superior inhibition against five resistant strains with similar MIC values (11.1-223 nM) to that of the wild-type Mtb H37Rv, suggesting its promising potential for both drug-sensitive and resistant Mtb strains.

Table 3. Anti-tubercular Activity of Compounds 5g, 5k, 5p and 5t against Drug-Resistant Clinical Mtb Isolates.

Strai	Re-	MIC (nM) ^b				
ns	sistance ^a	5g	5k	5P	5t	
3495	HRSZ	1914	22.3-223	n.d. ^c	n.d.	
P71	HRZ	<383	11.1-223	<184	71.8-1795	
9804	HRZ	<383	<11.1	<184	<35.9	
4768	EHRSZ	<383	11.1-223	<184	35.9-71.8	
P163	EHSZ	<383	11.1-223	<184	<35.9	
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^aH, isoniazid; R, rifampicin; S, streptomycin; E, ethambutol; Z, pyrazinamide. ^bValues are means of two independent experiments, and the variation is <20%. ^cNot Determined.

Given its promising anti-mycobacterial activity against both drug-susceptible and drug-resistant Mtb strains, the in vivo anti-tubercular efficacy of **5k** was evaluated using a modified real-time monitoring non-invasive mouse model infected with the selectable marker-free autoluminescent Mtb strain H37Ra.19,20 The animals were infected with logphase autoluminescent Mtb H37Ra via intravenous injection (2×10⁶ CFU per mouse), and then were repeatedly administrated with agent 5k once daily via oral gavage for 6 consecutive days. The bacterial burden was measured by monitoring the bioluminescence intensity (relative light unit, RLU) from the same batch of live mice every other day. As shown in Figure 2, compound 5k exhibited dose-dependent in vivo anti-tubercular activity and was well tolerated in all of the tested groups with no mortality (data not shown) observed during treatment. Administration with CMC-Na (vehicle) permitted a nearly 2.8-fold increase in RLU over six days; whereas compound 5k with the three tested doses prevented any increase over baseline, with RLU_{davn}/RLU_{davo} ratios of 0.40, 0.19 and 0.15 in 4, 20 and 100mg/kg/day treated groups, respectively. Especially, with 100mg/kg/day, compound 5k exhibited a sustained bactericidal activity against Mtb H37Ra, resulting in a 3.6-fold, 5.0-fold and 6.5-fold decrease in RLU from baseline on day2, day4 and day6, respectively. These results strongly suggest the promising potential of compound 5k to serve as a lead compound for further anti-TB drug discovery.

In summary, a series of pyrazolo[1,5-a]pyridine-3carboxamide derivatives were designed as new antitubercular agents by using a scaffold hopping strategy. The compounds exhibit excellent *in vitro* inhibitory activities with low nanomolar MIC values against both drugsensitive Mtb strain H₃₇Rv and drug-resistant clinical isolates. One of the most promising compounds **5k** displayed significant bacterial burden reduction in the Mtb H₃₇Ra infected mouse model. This compound may serve as a new promising lead compound for further antitubercular drug discovery.



Figure 2. Mean RLU count (\pm SD) assessed every other day in live, anesthetized mice and normalized to the baseline RLU value. Days post initial treatment (x-axis) is plotted against the corresponding RLU_{dayn}/RLU_{dayo} ratio (y-axis). Blue: vehicle; Purple: **5k** 100mg/kg qd; Red: **5k** 20mg/kg qd; Green: **5k** 4mg/kg qd.

ASSOCIATED CONTENT

Supporting Information. Experimental procedures for the synthesis of **5a-5v** and self-prepared amines, 1H NMR, and 13C NMR for final compounds, and details of *in vitro* and *in vivo* assays. This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author

*Tel: +86-20-32015276. Fax: +86-20-32015299. E-mail: ding_ke@gibh.ac.cn; lu_xiaoyun@gibh.ac.cn; zhang_tianyu@gibh.ac.cn.

Author Contributions

|| These authors contributed equally.

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Notes

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