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Letter

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Identification of N-benzyl 3,5-Dinitrobenzamides Derived from PBTZ169 as Antitubercular Agents

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KEYWORDS Antitubercular agents, N-benzyl 3,5-dinitrobenzamides, structure-activity relationships, pharmacokinetics

ABSTRACT: A series of benzamide scaffolds were designed and synthesized by the thiazinone ring opening of PBTZ169, and *N*-benzyl 3,5-dinitrobenzamides were finally identified as anti-TB agents in this work. 3,5-Dinitrobenzamides **D5, 6, 7, 12** exhibit excellent *in vitro* activity against the drug susceptive *Mycobacterium tuberculosis* H37Rv strain (MIC: 0.0625 μg/mL) and two clinically isolated multidrug-resistant strains (MIC: < 0.016-0.125 μg/mL). Compound **D6** displays acceptable safety and better pharmacokinetic profiles than PBTZ169, suggesting its promising potential to be lead compound for future antitubercular drug discovery.

Tuberculosis (TB) is an airborne infectious disease mainly caused by *Mycobacterium tuberculosis* (MTB).[1] The World Health Organization (WHO) reported that MTB still caused an estimated approximately 10.4 million infections and 1.3 million deaths in 2016.[2] Recently, the increasement of multidrug-resistant (MDR) TB and even the emergence of extensively drug-resistant (XDR) TB, together with coinfection with Human Immunodeficiency Virus (HIV), have challenged us seriously.[3-5] Two drugs with new mechanisms of action, Bedaquiline [6] and Delamanid (Figure 1)[7], were approved (in 2012 and 2014, respectively) for the treatment of MDR-TB, but some adverse events have been noted.[3] Therefore, it is urgently needed to develop novel anti-TB agents active against MDR- and XDR-TB.[8]

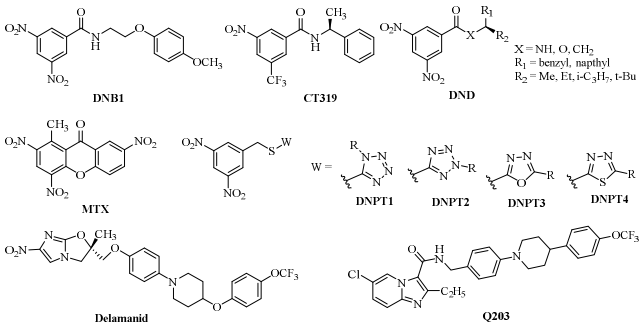


Figure 1. Structures of selected dinitrobenzene derivatives, Q203 and Delamanid.

Recently, 8-nitro-6-(trifluoromethyl)-1,3-benzothiazin-4-ones (BTZs), a novel class of TB agents targeting DprE1,[9-11] have been extensively studied, and candidate PBTZ169 (Figure 2) is in Phase II clinical trials at present.[2] Meanwhile, some nitroaromatic compounds that contain an electron-withdrawing substituent (NO_2, CF_3) in the position *meta* to the nitro group were also identified as DprE1 inhibitors, such as dinitrobenzamide DNB1[12] and its analogue CT319 [13], dinitrobenzene derivatives (here abbreviated as DNDs) [14], and xanthone derivative 1-methyl-2,4,7-trinitroxanthone (MTX) (Figure 1)[13, 15]. Moreover, 3,5-dinitrophenylmethanethiol derivatives DNPT1-4 (Figure 1) [16-18] targeting the synthesis of mycobacterial nucleic acids, were found to have considerable antimycobacterial activity.

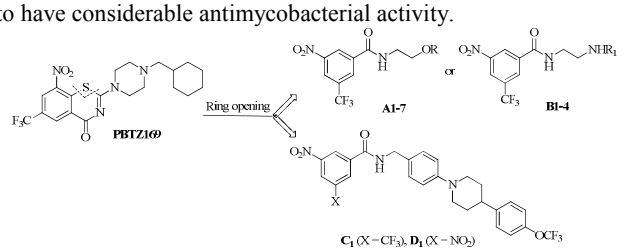


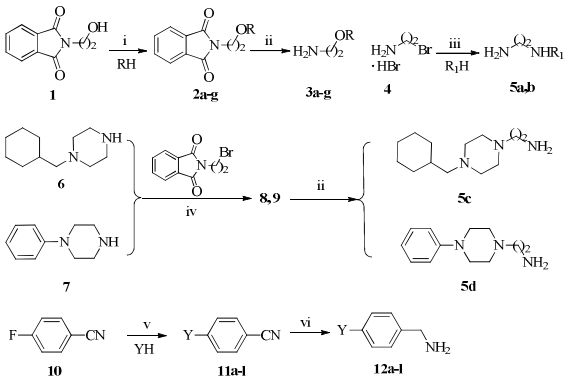
Figure 2. Design of the new molecules.

In our previous studies, many BTZs containing various cyclic ketoxime，spiro-heterocycle and piperidine moieties were identified as potential antitubercular agents.[19-21] Moreover, replacement of the *N*-benzyl group on the amide linker of Q203 (Figure 1) with a *N*-(2-phenylamino)ethyl or *N*-(2-phenoxy)ethyl moiety maintains potent activity.[22-23] Given that above compounds belong to aromatic carboxamides, we intended to simplify the structure of PBTZ169 to benzamide scaffolds by the thiazinone ring opening in this work. Thus, a series of 3-nitro-5-(trifluoromethyl)benzamides bearing *N*-oxyethyl (**A1-7**), *N*-

aminoethyl **(B1-4)** and *N*-benzyl **(C1)**, as well as *N*-benzyl-3,5-dinitro-benzamide **(D1)**, were first designed to identify the optimal benzamide cores (Figure 2), and then the effect of substituents on the amide linker was further investigated. Our primary objective was to find optimized benzamides with potent antimycobacterial activity. A preliminary structure-activity relationship (SAR) study was also explored.

Detailed synthetic pathways to side chains **3**, **5** and **12** and target compounds **A-D** are outlined in Schemes 1 and 2, respectively. Coupling of different phenols (RH) with *N*-(2-hydroxyethyl)phthalimide **1** in the presence of diethyl azodicarboxylate (DEAD) and PPh_3 (**2a-g**) followed by treatment with hydrazine hydrate in ethanol at 50°C yielded desired amines **3a-g**. Treatment of anilines (R_1H) with 2-bromoethanamine hydrobromide **4** in toluene under reflux condition gave the corresponding 1,2-diamines **5a,b**. Nucleophilic substitution of 1-(cyclohexylmethyl)piperazine **6** and 1-phenylpiperazine **7** with 2-(2-bromoethyl)isoindoline-1,3-dione in acetonitrile in the presence of potassium carbonate, gave intermediate compounds **8** and **9**, which were treated with hydrazine hydrate in ethanol yielded side chains **5c** and **5d**, respectively. 4-Fluorobenzonitrile **10** was treated with various nitrogen heterocyclic amines (YH) in DMSO in the presence of K_2CO_3, and the resulting intermediates **11a-l** were subsequently reduced with $LiAlH_4$ in THF to produce the desired benzylamines **12a-l** (Scheme 1).

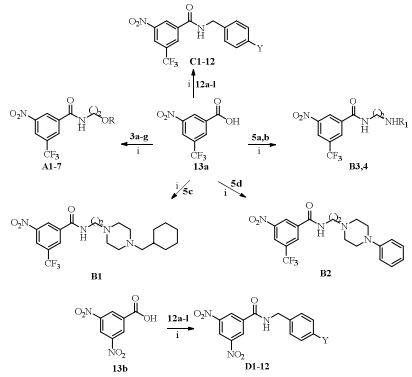
Scheme 1. Synthesis of side chain compounds **3**, **5** and **12**.



Reagents and conditions: (*i*) DEAD, PPh_3, THF, 0-5°C; (*ii*) hydrazine hydrate, EtOH, 50°C; (*iii*) toluene, reflux, then NaOH, rt. (*iv*) K_2CO_3, MeCN, reflux; (v) K_2CO_3, DMSO; (*vi*) $LiAlH_4$, THF.

Target compounds **A-D** were easily obtained by amidation of acids **13a, b** with the above side chain compounds **3a-g, 5a-d** and **12a-l** in the presence of triethylamine (TEA) and condensating agent bis(2-oxo-3-oxazolidinyl)phosphonic chloride (BOP-Cl) (Scheme 2).

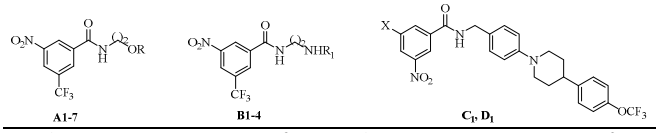
Scheme 2. Synthesis of target compounds **A-D** (See Tables 1, 2).

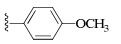
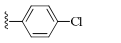
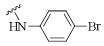
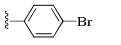
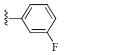
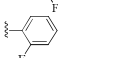
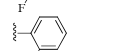
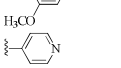
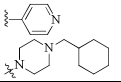


Reagents and conditions: (i) BOP-Cl, TEA, CH_2Cl_2

The target compounds **A1-7**, **B1-4** and **C1** bearing different kinds of substituents on the amide linker to ensure side chain flexibility and structure diversity, and *N*-benzyl 3,5-dinitro-benzamide **(D1)** were first synthesized. They were preliminarily examined for *in vitro* activity against drug sensitive MTB strain (H37Rv ATCC27294), using the Microplate Alamar Blue Assay (MABA).[24] The minimum inhibitory concentration (MIC) is defined as the lowest concentration effecting a reduction in fluorescence of >90% relative to the mean of replicate bacterium-only controls. The MIC values of the compounds along with PBTZ169, isoniazid (INH), and rifampicin (RFP) as references were obtained in Table 1.

Table 1. Structures and activity of compounds **A-D** against *MTB H37Rv*



Cpds.	R or NHR_1	MIC[a]	Cpds.	NHR_1 or X	MIC[a]
A1		1	B2		1
A2		1	B3		8
A3		1	B4		>8
A4		1	C1	CF_3	0.5
A5		1	D1	NO_2	0.0625
A6		2	PBTZ 169	/	0.0625
A7		8	INH	/	0.0781
B1		4	RFP	/	0.0781

[a]MIC determined in μg/mL; Cpds., compounds; INH, isoniazid; RFP, rifampicin.

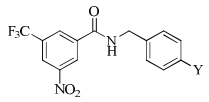
Synthesized compound **A1** exhibits an MIC value of 1 μg/mL against the H37Rv strain of MTB, much less than that (75 ng/mL)[12] of DNB1 (a bioisostere of **A1**), which reveals that replacement of the nitro group of DNB1 with trifluoromethyl is detrimental. Except for *N*-(2-pyridin-4-yloxy)ethyl compound **A7** (MIC: 8 μg/mL), activity of the other *N*-phenoxyethyl analogues (**A2-6**) is, as expected, comparable to **A1**. On the other hand, compound **B1**, wherein a 4-(cyclohexylmethyl)piperazine ring (the same side chain of PBTZ169) is directly attached to the amide through an ethylene, displays obviously decreased activity

(MIC: 4 μg/mL) than PBTZ169 (MIC: 0.0625 μg/mL), but introduction of a benzene ring on the piperazine instead of the alkyl group is beneficial to the activity (**B1** *vs* **B2**). Furthermore, *N*-phenoxyethyl group is preferred over *N*-phenylaminoethyl one (**A3** *vs* **B3**).

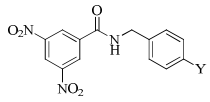
Interestingly enough, compound **C1** having the same side chain as Q203, shows better activity (MIC: 0.5 μg/mL) than **A1-7** and **B1-4**. Replacement of the trifluoromethyl group of **C1** with another electron-withdrawing one (NO_2) in compound **D1** leads to a MIC of 0.0625 μg/mL (Table 1). These results indicate that the 3,5-dinitrobenzamide core and *N*-benzyl group are preferred over the corresponding 3-nitro-5-(trifluoromethyl)benzamide and *N*-phenoxyethyl or *N*-aminoethyl, respectively.

Encouraged by the above SAR, *N*-benzyl 3-nitro-5-(trifluoromethyl) / 3,5-dinitro benzamides with various groups at the para-position of the benzene ring were further designed and synthesized. As shown in Table 2, twelve compounds show considerable activity against MTB H37Rv strain (MIC: < 1 μg/mL). Among them, six compounds **C6** and **D1, 5-7, 12** (MIC: 0.0625 μg/mL) are more active than INH / RFP (MIC: 0.0781 μg/mL), and comparable to PBTZ169. Overall, the data reveal that with a few exceptions(**C3, C4, C6, C11**), 3-nitro-5-(trifluoromethyl) benzamides are less active than the corresponding 3,5-dinitrobenzamides. Based on this, we here only discuss the effect of the substituent (Y) on the benzene ring of 3,5-dinitrobenzamide series. The nature of the substituents greatly influences activity. Compound **D2** having the same side chain as Delamanid, namely, insertion an oxygen atom between the piperidine and 4-trifluoromethoxybenzene rings of **D1**, displays less activity (MIC: 0.5 μg/mL) than **D1**. And removal of 4-trifluoromethoxybenzene ring of **D1** was found to be detrimental (**D1** *vs* **D3**). Replacement of the piperidine ring of **D3** (MIC: 2 μg/mL) with morpholine in compound **D4** leads to a complete loss of activity, but with thiomorpholine in **D5** or introduction of an electron-withdrawing group (CF_3) in **D6** leads to obviously increased activity (MIC: 0.0625 μg/mL). Interestingly, octahydro-1*H*-isoindole analogue **D7** also demonstrates the most potent MIC value of 0.0625 μg/mL. However, among the different *para*-groups on the piperazine ring, compounds with alkyl groups, **D8** (methyl) and **D9** (isopropyl), or heteroaromatic groups, **D10** (4-pyridyl) and **D11** (2-pyridyl) display comparatively less activity (MIC: 1-4 μg/mL), and the only exception is compound **D12** with an aromatic group (4-fluorophenyl) (MIC: 0.0625 μg/mL).

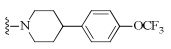
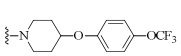
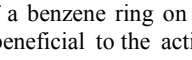
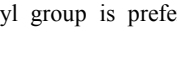
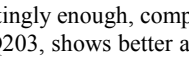
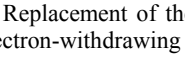
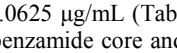
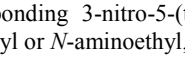
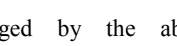
Table 2. Structures and activity of *N*-benzylated analogues **C1-12** and **D1-12**



C1-12



D1-12

Cpds.	Y	MIC (μg/mL) H37Rv	MDR-MTB1	MDR-MTB2
C1		0.5	/	/
D1		0.0625	0.210	0.474
C2		2	/	/
D2		0.5	/	/
C3		0.25	/	/
D3		2	/	/
C4		>8	/	/
D4		>8	/	/
C5		0.125	/	/
D5		0.0625	0.017	0.085
C6		0.0625	0.138	>40
D6		0.0625	<0.016	0.075
C7		0.5		
D7		0.0625	0.031	0.125
C8		>8	/	/
D8		4	/	/
C9		8	/	/
D9		4	/	/
C10		8	/	/
D10		2	/	/
C11		1	/	/
D11		1	/	/
C12		0.5	/	/
D12		0.0625	0.047	0.077
PBTZ 169		0.0625	0.116	0.232
INH		0.0781	>40	>40
RFP		0.0781	>40	>40

Cpds., compounds; INH, isoniazid; RFP, rifampicin; MDR-MTB1, MDR-MTB12525; MDR-MTB2, MDR-MTB14231.

Considering their strong potency against the drug sensitive MTB H37Rv strain (MIC: 0.0625 μg/mL), compounds **C6** and **D1, 5-7**, **12** were further evaluated against two clinical isolated MTB-MDR (12525 and14231) strains resistant to both INH and RFP. Except for **C6** and **D1**, the other four compounds exhibit better activity (MIC < 0.016-0.125 μg/mL) than PBTZ169 (Table 2), suggesting their promising potential for both drug-sensitive and resistant MTB strains (Tables 1, 2).

Table 3. Acute Toxicity and PK Profiles of Selected Compounds

Cpds.	Acute toxicity[a]	PK[b] $T_{1/2}$ (h)	T_{max} (h)	C_{max} (ng/mL)	$AUC_{0-\infty}$ (h·ng/mL)
D1	5/5	5.52	0.83	366	3262
D5	5/5	3.30	2.75	19.0	41.1
D6	5/5	3.63	0.92	1719	5605
D7	5/5	NA	NA	NA	NA
D12	5/5	13.6	1.08	15.0	219
PBTZ169	NT	2.87	0.83	1300	5478

[a]Number of animals that survived / total; [b]Dosed Orally in Mice at 50 mg/kg (n = 3); NT, not tested; NA, unavailable; Cpds., compounds.

Based on the measured activity levels against all of the tested strains, compounds **D1**, **5-7** and **12** were tested for in vivo tolerability by recording the number of survivors after a single oral dose in mice of 500 mg/ kg, followed by a 7-day observation. All of them display the low oral acute lethal toxicity (Table 3). The in vivo PK profiles of these compounds were further evaluated in mice after a single oral administration of 50 mg/kg. As shown in Table 3, compared to PBTZ169, compound **D1** has a relatively longer T1/2 of 5.52 h, but less Tmax, Cmax and $AUC_{0-\infty}$. Absorption of compounds **D5**, **7**, **12** in plasma is very poor, or not detectable. Compound **D6**, with a 4-trifluoromethylpiperidine moiety, displays better PK properties, with $T_{1/2}$ of 3.63 h, Tmax of 0.92h, Cmax of 1719 ng/mL and $AUC_{0-\infty}$ of 5605 h.ng/mL.

In conclusion, a series of various benzamide scaffolds were designed and synthesized by the thiazinone ring opening of PBTZ169, and *N*-benzyl 3,5-dinitrobenzamides were finally identified as anti-TB agents in this work. Four *N*-benzyl 3,5-

dinitrobenzamides **D5, 6, 7, 12** exhibit excellent *in vitro* inhibitory activity against both drug-sensitive MTB strain H37Rv (MIC: 0.0625 μg/mL) and drug-resistant clinical isolates (MIC < 0.016-0.125 μg/mL). Moreover, compound **D6** displays acceptable safety and better PK properties than PBTZ169, and it may serve as a promising lead compound for further antitubercular drug discovery. Studies to determine the *in vivo* efficacy of **D6** are currently underway.

ASSOCIATED CONTENT

Supporting Information

Experimental procedures and analytical data of the synthesized compounds were provided.

AUTHOR INFORMATION

Corresponding Author

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* luyu4876@hotmail.com (Y. Lu).

Author Contributions

M.L.L. and Y.L. conceived and designed the project. L.L.H., K.L. and Z.Y.T synthesized the compounds. B. W. evaluated the anti-zika virus activity. M. L.L., G.H.C., H.Y.G. and K.L. analyzed the data and prepared the manuscript.The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

‡These authors contributed equally.

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Notes

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ABBREVIATIONS

MTB, *Mycobacterium tuberculosis*; MDR-TB, multidrug-resistant tuberculosis; XDR-TB, extensively drug-resistant tuberculosis; WHO, World Health Organization; HIV, human immunodeficiency virus; BTZs, 8-nitro-6-(trifluoromethyl)-1,3-benzothiazin-4-ones; DprE1, Decaprenyl phosphoryl-β-D-ribose 2/-epimerase; DEAD, diethyl azodicarboxylate; DMSO, dimethylsulfoxide; MIC, the minimum inhibitory concentration; MABA, Microplate Alamar Blue Assay; C_{max}, the maximum concentration; T_{max}, the time to maximum concentration; $AUC_{0-\infty}$, area under curve from time zero to infinity; $t_{1/2}$, the plasma elimination half-life;.

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Identification of N-benzyl 3,5-Dinitrobenzamides Derived from PBTZ169 as Antitubercular Agents

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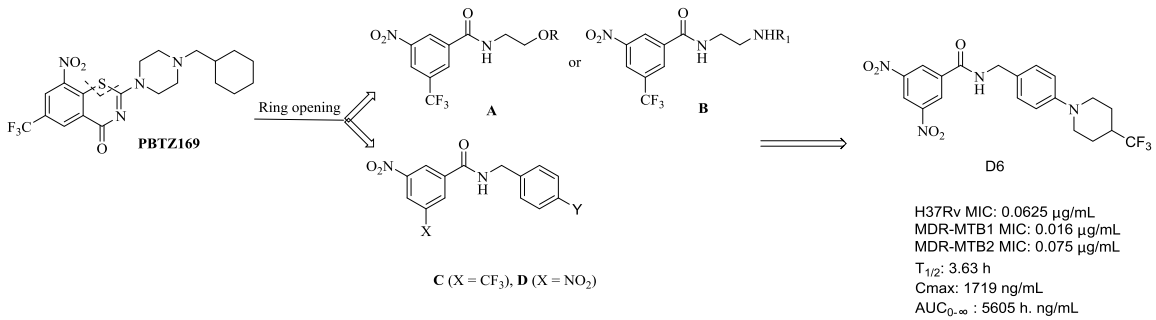

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