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Benzylsulfanyl benzo-heterocycle amides and hydrazones as new agents against drug susceptible and resistant *Mycobacterium tuberculosis*†

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ABSTRACT: A series of benzylsulfanyl benzo-heterocycle amides and hydrazones were synthesized and evaluated for anti-tubercular activities. The isonicotinyl hydrazones derivatives **12d**, **12e** and **12f** exhibited good anti-tubercular activity against *Mycobacterium tuberculosis* $H_{37}Rv$ (ATCC # 27294) with MIC values of 0.23, 0.24 and 0.24 μM, respectively, and were also active against SDR-TB, MDR-TB and XDR-TB. More importantly, compound **12e** also showed low cytotoxicity, good metabolic stability, and could significantly reduce the mycobacterial burden in a mouse model infected with autoluminescent H37Ra strain, which may serve as a lead compound for further development.

KEYWORDS: Benzylsulfanyl benzo-heterocycle amides and hydrazones, Drug resistant, MDR-TB, Molecular hybridization, Anti-tubercular

1. Introduction

Tuberculosis (TB), a highly contagious and air-borne disease caused by *Mycobacterium tuberculosis* (*Mtb*), emerged with multi-drug resistant (MDR-TB), extensively drug-resistant (XDR-TB) strains and acquired immune deficiency syndrome (AIDS) in recent years.[1] The World Health Organization (WHO) 2016 *"Global Tuberculosis Report"* estimated that nearly 1.8 million people died from TB and 10.4 million new TB cases were notified to national authorities in 2015.[2] In spite of the increasing worldwide incidence of TB, only bedaquiline (SIRTURO®) and delamanid were conditionally approved by FDA in 2012 [3-4] and EMEA in 2014 for treatment of MDR-TB, respectively. However, bedaquiline possessed serious adverse effects such as cardiac arrhythmias and displayed higher death rates than that of the placebo group in a clinical investigation,[5] which limited its wide application in clinical practice. Therefore, it is an imperative need to develop novel anti-tubercular drugs that can be equally effective against *Mtb* and MDR-TB without any toxic side effects, and also can reduce the duration of therapy.

To pursue this goal, our research efforts were directed to discover new chemical classes of anti-tubercular agents. It was indicated that the benzylsulfanyl derivatives of benzoxazole/benzothiazole/benzimidazole have significant antimycobacterial activity.[6-7] Additionally, some scientists carried out on pyrazinecarboxamides derivatives and hydrazones of isoniazid as anti-tubercular pharmacophores to reduce the toxicity of isoniazid.[8-9] Under these medicinal chemistry advances, two novel classes of benzylsulfanyl benzo-heterocycle amides **7a-7f** and hydrazones **12a-12f** were designed by molecular hybridization between pyridyl amide or hydrazone and benzylsulfanyl benzo-heterocycle, respectively. Some of them showed good anti-tubercular activity against H37Rv, single-drug resistant strains (SDR-TB), MDR-TB and XDR-TB *in vitro*. Here, we report the synthesis and evaluation of benzylsulfanyl benzo-heterocycle amides and hydrazones derivatives as novel anti-tubercular agents.

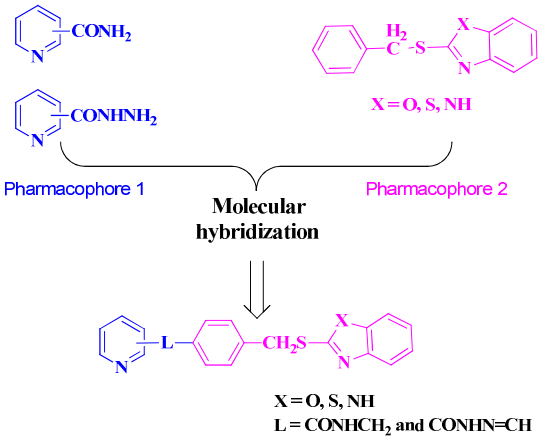

Fig. 1 The design of anti-tubercular compounds by molecular hybridization.

2. Results and discussion

Two series of benzylsulfanyl benzo-heterocycle amides and hydrazones derivatives are shown in Table 1, and the synthetic procedures used for their preparation are demonstrated in Schemes 1-2. In Scheme 1, synthesis of compounds **7a-7f** started from preparation of 4-(bromomethyl) benzonitrile **2** by bromination of α-H in 4-methylbenzonitrile **1** using $KBrO_3/NaHSO_3$ as bromination agents and under an incandescent light illumination. Mercaptomethyl benzonitrile benzo-heterocycle derivatives **4a-4c** were prepared by nucleophilic substitution reaction in *N*, *N*-dimethylformamide (DMF) in the presence of potassium carbonate at 20 °C or sodium methoxide at room temperature. Reduction of **4a-4c** with lithium aluminium hydride in dry THF gave the mercaptomethyl aniline benzo-heterocycle derivatives **5a-5c**. Condensation derivatives **5a-5c** with pyridine carboxylic acid **6** gave the desired amide analogues **7a-7f**, respectively.

The benzylsulfanyl benzo-heterocycle hydrazone derivatives **12a-12f** were synthesized as shown in Scheme 2. Selectivity sin-

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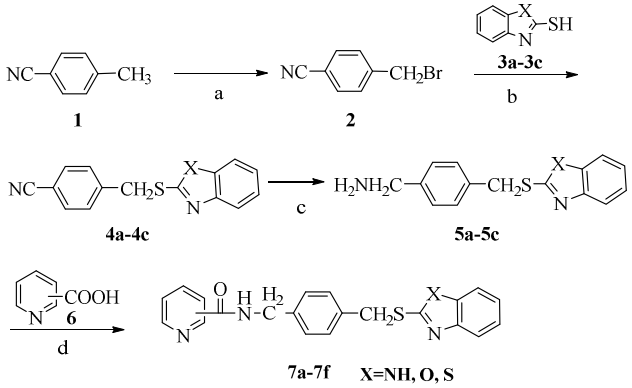
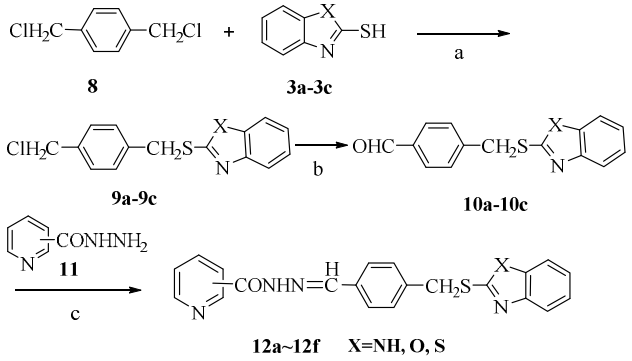
gle nucleophilic substitution of commercially available 1, 4-bis(chloromethyl)benzene **8** with thiols **3a-3c** in the presence of methanol solutions of sodium hydroxideled to the formation of derivatives **9a-9c**. Then, mercaptomethyl benzaldehyde benzoheterocycle derivatives **10a-10c** were obtained by sommelet name reaction under intermediates **9a-9c**. The desired hydrazone derivatives **12a-12f** were prepared by the condensation of hydrazides **11** in ethanol solution catalyzed by acetic acid.



Scheme 1 Reagents: (a) $KBrO_3$, $NaHSO_3$, illumination, 50-55 °C; (b) K_2CO_3, DMF, 20 °C or Na, CH_3OH, DMF; (c) $LiAlH_4$, dry THF, N_2, 0 °C; (d) i) $SOCl_2$, ref; ii) CH_2Cl_2, NEt_3, rt or 47 °C.

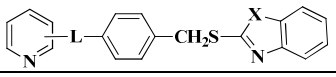


Scheme 2 Reagents: (a) NaOH, H_2O, CH_3OH, 0-20 °C; (b) Hexamethylenamine, 60%C_2H_5OH; (c)CH_3COOH, C_2H_5OH, reflux.

All newly synthesized compounds were evaluated for their *in vitro* anti-tubercular activity against *Mtb* strain H37Rv (ATCC # 27294) in Middlebrook 7H12 using the Microplate Alamar Blue Assay (MABA) [10, 11], which are summarized in Table 1. For the sake of comparison, the values of MICs of positive drugs (isoniazid (INH), rifampicin (RIF), moxifloxacin (Mox), streptomycin (SM) and PA-824) were also included.

Among the compounds, the isonicotinyl hydrazones derivatives **12d**, **12e** and **12f** exhibited significant activity against *Mtb* H37Rv with MIC values of 0.23 μM, 0.24 μM and 0.24 μM, respectively, better than INH, Mox, SM and PA-824 (Table 1) and less potent than RIF. While most of benzylsulfanyl benzazole amides compounds possessed poor antimycobacterial activity, with MIC values of 0.9-128 μM. Only one amide derivative **7e** exhibited the moderate antimycobacterial activity with MIC value of 0.9 μM, which was equivalent to isoniazid. From the results, it was concluded that the activities of hydrazones derivatives are better than that of amide derivatives, which suggested that the isonicotinic moiety may serve as a key anti-tubercular pharmacophore. For benzylsulfanyl benzo-heterocycle moiety, the preliminary SAR suggested that benzoxazole ring had a positive effect on the anti-tubercular activity compared to benzimidazole and benzothiazole (Table 1).

Table 1 *In vitro* anti-tubercular activity of title compounds against H37Rv using MABA method.



Compds	L	X	MIC (μM)
7a	m-$CONCH_2$	NH	60.3
7b	m-$CONCH_2$	O	58.2
7c	m-$CONCH_2$	S	>128
7d	t-$CONCH_2$	NH	120.5
7e	t-$CONCH_2$	O	0.9
7f	t-$CONCH_2$	S	>128
12a	m-CONHN=CH	NH	114.4
12b	m-CONHN=CH	O	24.8
12c	m-CONHN=CH	S	>128
12d	t-CONHN=CH	NH	0.23
12e	t-CONHN=CH	O	0.24
12f	t-CONHN=CH	S	0.24
INH	-	-	0.81
RIF	-	-	0.08
Mox	-	-	0.5
SM	-	-	0.48
PA-824	-	-	0.44

Then, the four most active compounds (**7e**, **12d**, **12e** and **12f**) were evaluated against drug susceptible (DS), MDR and XDR clinical strains of *Mtb* (Table 2). The amide compound **7e** was only potent against MDR-TB with MIC value of 2.0 μM. The hydrazones **12d**, **12e** and **12f** exhibited good activities against DS-TB with MICs values comparable to the standard H37Rv, and were also potent against MDR-TB with the same MIC values of 6.4 μM, better than the three control drugs. More importantly, the hydrazone **12e** containing benzoxazole was **10** and **20**-fold more active against XDR-TB than ftivazide and isoniazid, respectively. The studies suggested this class of compounds may serve as lead compounds for treatment of clinical drug-resistant *Mtb*.

Table 2 The inhibitory activities against DS, MDR and XDR clinical strains of *Mtb*.

Compds	MIC (μM)		
	960 (DS-TB)	330 (MDR-TB)	431 (XDR-TB)
7e	16.0	2.0	16.0
12d	0.2	6.4	>12.8
12e	0.2	6.4	3.2
12f	0.2	6.4	>12.8
ftivazide	0.25	16	32
INH	0.5	8.0	64
RIF	0.07	>19	>19

Encouraged by the results that the three hydrazones (**12d**, **12e** and **12f**) had sub-micromolar MIC values against the H37Rv *Mtb* strain and potent anti-tubercular activities against MDR-TB, we further screened compounds **12d**, **12e** and **12f** against a panel of SDR-TB strains along with the control drug ftivazide. The three potent compounds exhibited excellent activities against a panel of ATCC SDR-TB comparable to the clinical anti-tubercular drug ftivazide (Table 3).

Table 3 The MIC results (μg/mL) against the single resistant *Mtb* strains.

SDR-TB	MIC (μg/mL)			
	12d	**12e**	**12f**	ftivazide
ATCC 35837 Ethambutol	0.156	0.156	0.312	0.312

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ATCC 35830 Ethionamide	1.25	1.25	1.25	1.25
ATCC 35827 Kanamycin	0.156	0.156	0.312	0.156
ATCC 35821 Para-aminosalicylic acid	0.312	0.312	0.625	0.312
ATCC 35820 Streptomycin	0.156	0.156	0.312	0.312
H37Rv Rifampicin	0.312	0.312	0.625	0.312

Moreover, the *in vitro* VERO cell toxicity of three compounds was determined. The cytotoxicity results were presented as percentage cell viability in Table 4. All the three derivatives **12d**, **12e** and **12f** were not cytotoxic since they did not kill more than 10% of the cells at the maximum concentration tested. It is noteworthy that **12d** and **12e** are less cytotoxic than the control drug ftivazide.

Table 4 The toxicity study results against Vero cells.

Concentration (μg/mL)	% Viability			
	12d	12e	12f	ftivazide
0 (0.625%DMSO)	100	100	100	100
0.01	119	127	97	95
0.02	116	127	103	105
0.039	115	127	99	11
0.078	116	119	99	114
0.156	115	122	97	96
0.312	111	120	98	103
0.625	112	118	94	100
1.25	119	123	103	105
2.5	114	123	101	110
5	113	123	103	100
10	107	130	96	95
20	107	109	91	90

Further *in vitro* metabolic stability of three compounds was evaluated in human liver microsomes (HLM). The amount of test compounds remaining at 15, 30 and 60 minutes was summarized in Table 5. The data suggested that all of the compounds showed some metabolism following 60 minutes of incubation with HLM, ranked as **12f** > **12d** >**12e** from most metabolized to least metabolized. The studied provided the basis for *in vivo* evaluation.

Table 5 *In vitro* metabolic stability of compounds with human liver microsomes.

Compds(10 μM)	Timepoint (min)	Compunds remaining (%) [a]
12d	15	76.5 ± 1.4
	30	62.0 ± 2.4
	60	44.6 ± 4.7
12e	15	90.1 ± 2.4
	30	76.5 ± 3.5
	60	66.6 ± 2.8
12f	15	76.3 ± 4.0
	30	57.2 ± 2.5
	60	34.0 ± 2.8

[a] % of Test article remaining at T= 0 min is 100%.

The anti-tubercular activity of compound **12e** was further evaluated *in vivo* using a cost-efficient mouse model infected with the selectable marker-free autoluminescent *Mtb* strain H37Ra.[12] As shown in Fig. 2, compound **12e** exhibited a sustained anti-tubercular activity against *Mtb* H37Ra *in vivo* for 6 consecutive days. The activity of compound **12e** with 3.1 mg/kg/day is comparable to that of the positive drug RIF with 10 mg/kg/day dose. These results strongly suggest the potential of compound **12e** to serve as a lead compound for further anti-tubercular drug discovery.

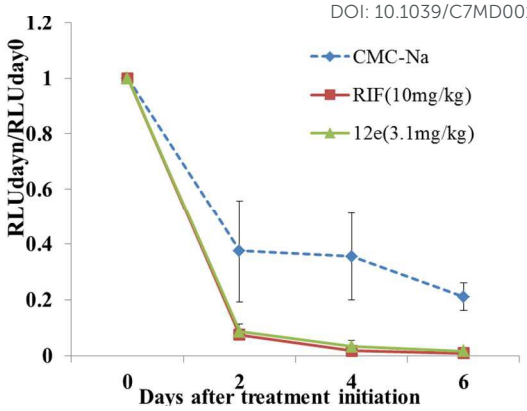

Fig. 2 Compound **12e** sustainedly inhibits the growth of *Mtb* H37Ra following 6 consecutive days of administration. Days post initial treatment (x-axis) is plotted against the corresponding RLUdayn/RLUday0 ratio (y-axis). Blue: vehicle (CMC-Na); Red: RIF 10 mg/kg qd; Green: 12e 3.1 mg/kg qd.

3. Conclusions

Two novel classes of benzylsulfanyl benzo-heterocycle amides **7a-7f** and hydrazones **12a-12f** have been designed, synthesized and evaluated for their anti-tubercular activities. The isonicotinyl hydrazones derivatives **12d**, **12e** and **12f** exhibited significant activities against *Mtb* strain H37Rv with sub-micromolar MIC values, which were better than INH, Mox, SM and PA-824. Importantly, these three compounds were also active against the resistant trains (SDR-TB, MDR-TB and XDR-TB), and exhibited low toxicity. Further metabolic stability and *in vivo* studies indicated that compound **12e** significantly reduce the mycobacterial burden in H37Ra infected mouse model, suggesting it can serve as a new lead for further anti-tubercular drug discovery.

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Notes

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ABBREVIATIONS

TB, tuberculosis; *Mtb, Mycobacterium tuberculosis*; MDR-TB, multi-drug resistant tuberculosis; XDR-TB, extensively drug-resistant tuberculosis; AIDS, acquired immune deficiency syndrome; WHO, World Health Organization; SDR-TB, single-drug resistant tuberculosis; DMF, N, N-dimethylformamide; MABA, Microplate Alamar Blue Assay; INH, isoniazid; RIF, rifampicin; Mox, moxifloxacin; SM, streptomycin; HLM, human liver microsomes.

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