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Chemistry & Drug Discovery

Supporting Information

Pyrido[1,2-*a*]benzimidazole-Based Agents Active Against Tuberculosis (TB), Multidrug-Resistant (MDR) TB and Extensively Drug-Resistant (XDR) TB

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Supporting Information

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1. HPLC purity determinations for the target compounds 3a–w, 4–6

Method 1. Flow rate = 1.4 mL/min; gradient elution over 20 minutes, from 30% CH_3CN–H_2O to 100% CH_3CN with 0.05% TFA.

Table 1(SI). HPLC purity of the target compounds

Compd	HPLC				Compd	HPLC			
	Gradient Method	WL (nM)	t_R (min)	Purity (%)		Gradient Method	WL (nM)	t_R (min)	Purity (%)
3a	1	280	11.9	98.1	**3n**	1	280	10.2	97.8
3b	1	280	13.7	98.0	**3o**	1	254	5.13	99.3
3c	1	280	13.7	98.1	**3p**	1	280	4.88	99.9
3d	1	280	13.6	98.6	**3q**	1	254	12.0	99.4
3e	1	280	13.2	98.4	**3r**	1	280	13.4	97.5
3f	1	280	13.1	98.2	**3s**	1	280	11.4	99.4
3g	1	280	13.9	98.1	**3t**	1	280	13.1	98.0
3h	1	280	11.9	97.8	**3u**	1	280	8.56	99.1
3i	1	280	13.3	97.9	**3v**	1	280	11.6	97.9
3j	1	280	13.0	97.7	**3w**	1	280	12.9	97.6
3k	1	280	12.7	99.1	**4**	1	280	9.12	100
3l	1	280	11.9	99.1	**5**	1	280	10.5	99.8
3m	1	280	13.0	99.1	**6**	1	280	12.9	99.1

2. Physicochemical properties of target compounds 3a–w, 4–6

Table 2(SI). ClogP, predicted aqueous solubility (AlogpS), and total polar surface area (TPSA) of the target compounds **3a**–**w**, **4**–**6**.

compd	ClogP[a]	AlogpS[b]	TPSA[c]	compd	ClogP	AlogpS	TPSA
3a	4.8	–4.08	61.1	**3n**	2.6	–3.47	74.0
3b	4.8	–4.08	61.1	**3o**	2.6	–3.47	74.0
3c	4.8	–4.08	61.1	**3p**	2.6	–3.47	74.0
3d	5.0	–4.72	61.1	**3q**	3.4	–3.78	74.1
3e	4.0	–3.72	70.3	**3r**	5.9	–4.47	69.2
3f	5.1	–4.74	70.3	**3s**	3.9	–3.69	61.1
3g	4.6	–3.81	61.1	**3t**	5.2	–4.21	61.1
3h	4.0	–3.72	70.3	**3u**	4.7	–4.34	74.1
3i	4.2	–4.01	61.1	**3v**	4.1	–3.67	61.1
3j	4.7	–4.05	61.1	**3w**	2.5	–2.44	61.1
3k	4.4	–4.73	61.1	**4**	2.5	–3.27	78.14
3l	4.4	–4.33	61.1	**5**	2.5	–3.89	95.2
3m	5.5	–4.33	61.1	**6**	3.4	–3.40	81.3

[a]Calculated with ChemDraw Ultra 7.0, CambridgeSoft®; [b]predicted water solubility calculated by using ALOGPS 2.1 (www.vcclab.org/lab/alogps/);[26] [c]total polar surface area calculated by using Molinspiration (http://www.molinspiration.com/services/psa.html).[27]

3. Structure of compound 3b

Figure 1(SI) Tautomers for compound 3b

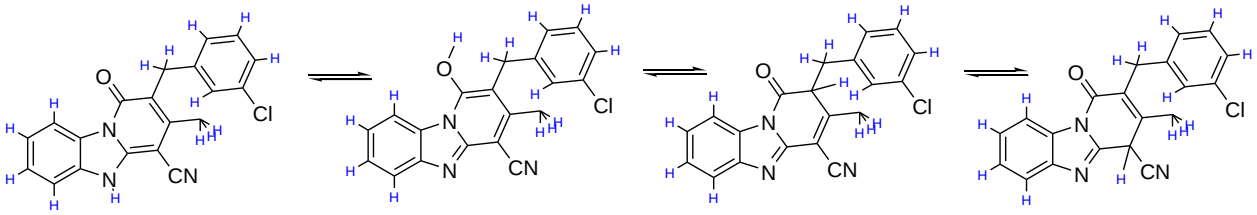
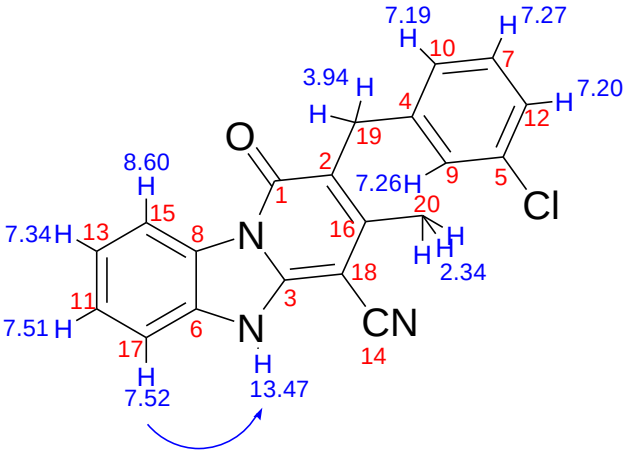


Table 3(SI) Assignment of ^{1}H and ^{13}C resonances.

Carbon atoms	^{13}C peak	^{1}H peak
1	159.1	–
2	147.8	–
3	146.0	–
4	143.6	–
5	133.4	–
6	132.1	–
7	130.5	7.27
8	128.2	–
9	128.1	7.26
10	127.1	7.19
11	127.0	7.51
12	126.2	7.20
13	122.6	7.34
14	117.2	–
15	116.7	8.60
16	114.0	–
17	111.6	7.52
18	70.2	–
19	31.3	3.94
20	18.7	2.34
?		**13.47**

The assignment of protons to the respective carbon atoms was accomplished through a combination of COSY and HMBC experiments at 900 MHz. Lower field used initially (400 MHz) was unable to resolve resonance overlap present in both ^{1}H and ^{13}C spectra. In addition to standard HMBC experiment, CIGAR-HMBC variant was applied to eliminate peak overlap from unfiltered one bond correlations. Once the assignments were made (Table 3SI), selective 1D NOESY methodology was used to determine what were the protons proximal to the exchangeable proton at δ_H **13.47**. This methodology utilizes shaped pulse to selectively irradiate the resonance at δ_H **13.47**, so as to reveal in the spectrum only those protons proximal to δ_H **13.47**. The only peak detected had a chemical shift at δ_H **7.52**, thus establishing that the exchangeable proton was attached at the nitrogen of the benzimidazole (Figure 2SI).

Figure 2SI. Resonances of protons and their assignment to the carbons



4. Brief description of the biological assays

Mycobacterium tuberculosis H37Rv and the KZN strains were cultured in Middlebrook 7H9 broth supplemented with 0.05% (v/v) Tween 80, 0.2% Glycerol (v/v) and 10% (v/v) OADC enrichment (Becton Dickinson). Initial MIC screening was done with microplate Alamar Blue assay (MABA). Candidates were verified by broth dilution methods for MIC and MBC determination. For MABA assay, briefly, *Mtb* strains were grown to mid-log phase and cultures were diluted to OD_{600}=0.001 with 7H9 without Tween 80. 100 µL of diluted culture containing about 10^4 colony forming units (CFUs) was added to each well containing 100 µL of 2-fold serially diluted compounds or isoniazid control. The 96-well plates were incubated at 37^{o}C for 7 days and 35 µL of Alamar Blue/Tween 80 (2:1.5, v/v) were added to each well and incubated for 16 hours. Plates were read at 544ex/590em with the Optima microplate reader (BMG). Data were analyzed and minimum concentration that yields 90% of inhibition was defined as MIC. Broth dilution MIC and MBC verification was done with 15 mL conical tubes and with total volume of 2.5 mL with 7H9 media without Tween 80. 10^5 CFUs of bacilli were added to each tube containing 2-fold serially diluted compounds or isoniazid control. Test tubes were incubated at 37 ^{o}C. Pellet formation was recorded at day 7 and day 14, with the lowest concentration that did not form pellet defined as MIC. All tubes without pellet were plated out on 7H10 agar plates and CFUs were enumerated. The lowest concentration that killed 99% of the inoculum was defined as MBC.

5. Metabolic stability of compounds **3a**,**3d**,**3e**,**3v**, and **3w**

Compound stock solutions were prepared in DMSO. The final concentration of DMSO in the incubation media was 0.2% (v/v). The stability of compounds **3a**, **3d**, **3e**, **3v**, **3w** in microsomes was determined in triplicate after their incubation at 1 μM with mouse liver microsomes (0.5 mg/mL) in 50 mM potassium phosphate buffer (pH 7.4) at 37 °C. The total incubation volume was 50 μL. The reaction mixture was prewarmed at 37 °C for 5 minutes before adding NADPH (1.0 mM). Reactions are quenched at 30 minutes by adding acetonitrile (150 μL) containing the generic internal standard at 0.01 μM. The samples were centrifuged at 4000 *g* for 30 minutes before liquid chromatography/tandem mass spectrometry (LC-MS-MS) analysis of the parent compound. For control experiments, NADPH was omitted from these incubations.

The half life of each compound was calculated as the formulas below:

$$\text{Half life } (t_{1/2}) = -\frac{\ln 2 \times (\text{incubation time})}{\ln(\%\text{remain ing}/100)} \quad (1)$$

$$CL_{\text{int}}' = \frac{0.693}{\text{in vitro}T_{1/2}} \bullet \frac{\text{ml incubation}}{0.5\text{mg microsomes}} \bullet \frac{45\text{ mg microsome}}{\text{gm liver}} \bullet \frac{87.5\text{gm liver}}{\text{kg b.w.}} \quad (2)$$

$$CL_{plasma} = \frac{Q \bullet CL_{\text{int}}'}{Q + CL_{\text{int}}'} \quad (3)$$

(A value of 40.5ml/min /kg was used for mouse hepatic plasma flow(Q))

$$Q_{p,h}\% = \frac{Cl_{plasma}}{Q_{p,h}} \times 100\% \quad (4)$$

LC-MS-MS assay for 3a, 3d, 3e, 3v and 3w quantitation. Quantitative assessments of compound metabolism were conducted by using an Agilent triple quadrupole mass spectrometer (Agilent, Santa Clara, CA). Reversed-phase HPLC separations during LC-MS-MS were carried out by using an XTerra MS C_{18} column (2.1 × 50 mm, 2.5 μm, Waters) connected to Agilent 1200 HPLC system. Analysis method: A binary gradient consisted of a mixture of a 0.1% formic acid in water (solvent A) and methanol (solvent B) at a flow rate of 0.5 mL/min. The LC gradient was programmed as following table for each compound:

Table 4 (SI) Gradient table of compound 3a, 3d, 3e, 3v, 3w

Comp.	Time	B%	Comp.	Time	B%
3w,3e	0	40	**3v**	0	10
	1	60		1	60
	2.5	75		5	95
3a	0	50	**3d**	0	50
	1	60		1	60
	2	70		2.5	75

The column was re-equilibrated for 3 minutes between injections. The column temperature was 40 °C, and the autosampler was maintained at 4 °C. Ionization was conducted in the positive or negative ionization mode. The electrospray voltage was 4 kV, and the capillary temperature was 300 °C. Under these conditions, compound **3a**, **3d**, **3e**, **3v**, **3w** eluted at 1.64, 1.92, 3.65, 3.7, 0.71 minutes respectively. The ion transitions with the collision energy, fragmentor and retention times during selected reaction monitoring (SRM) and selected ion monitoring (SIM) for compounds **3a**, **3d**, **3e**, **3v**, **3m** are listed in **Table 5 (SI).**

Table 5(SI) SRM or SIM transitions and retention times of analogues detected by LC-MS-MS

Comp.	**Transition(*m/z*)**	**Polarity**	**CE(eV)**	**Fragmentor (eV)**	**RT(min)**
3a	SIM: 346.1	Negative	/	200	1.64
3d	SIM: 380.1	Negative	/	200	1.92
3e	SRM: 344.1→236.1	Positive	20	200	3.65
3v	SRM: 314.1→236.1	Positive	20	200	3.7
3w	SRM: 238.1→208.2	Positive	30	200	0.71