Synthesis and antimycobacterial activity of some *N,N′*-disubstituted isonicotinohydrazide derivatives

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Dedicated to Dr. Nitya Anand on the occasion of his 80th birthday

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Abstract

A new series of antimycobacterial agents **2a**–**j**, **3a**–**d**, **6b**–**f**,**j**, and **7b**,**f** was designed, synthesized and evaluated for antimycobacterial activity against different mycobacterium species i.e. *M. tuberculosis*, *M. avium*, and *M. intracellulare* in an agar dilution method. Some of these compounds (**2e**, **3a**–**d**) were highly potent with *in vitro* activity against *M. tuberculosis* H_{37}Rv and clinical isolates (sensitive strains), equivalent to that of Isoniazid.

Keywords: Tuberculosis, antimycobacterial activity, isonicotinohydrazide derivatives, pyrazine-2-carbonyl substituent

Introduction

Tuberculosis (TB) is one of the leading causes of death due to a single infectious organism in the world. As per survey reported by Global Alliances, Geneva, there are eight to ten million new active cases of TB and approximately three million deaths each year.[1–4] In spite of the fact that TB is treatable; TB is predicted to an increase of an alarming rate every year. Treatment of TB infection that has been caused by multi drug resistant (MDR), *Mycobacterium (M.) tuberculosis* has become major concern world over. The term MDR TB is used to describe strains that are resistant to one or more anti tuberculosis drugs.[5–7] Furthermore, immunocompromised patients such as observed with AIDS, or after transplantation, are easily infected by pathogenic fungi, protozoa and mycobacteria, leading rapidly to death.[8] This pathogenic synergy of TB with HIV is what makes the resurgence of TB especially alarming. TB is therefore a leading cause of death among people who are HIV positive. Since in the last 40 years there has not been a new drug for

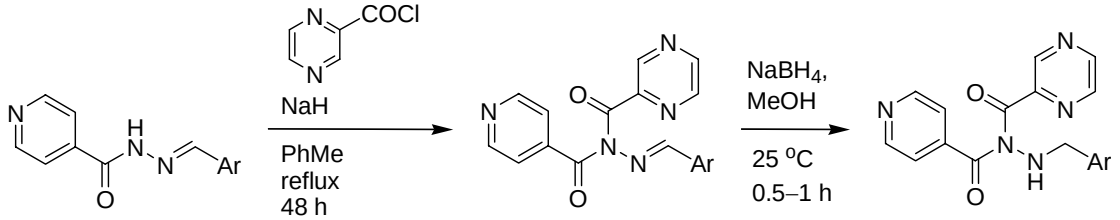
TB introduced to the market, there is an unmet need to discover new synthetic lead molecules and drugs.

In view of the above discussion, we have designed, synthesized and evaluated antimycobacterial activity of new series of *N*,*N*′-disubstituted isonicotinohydrazide derivatives **2**, **3, 6b**–**f**,**j**, and **7b**,**f**, which we wish to report in this paper.

Results and Discussion

Chemistry

Compounds **2a**–**j** were prepared in good yields by the reaction of *N*′-(arylmethylene)-isonicotinohydrazides (**1a**–**j**) with pyrazine-2-carbonyl chloride[9] in the presence of sodium hydride in toluene at reflux temperature (Scheme 1). The hydrazones **1a**–**j** were prepared by the condensation of isonicotinic hydrazide with various mono- or di-substituted arylaldehydes according to the procedure described in literature.[10] Further, reduction of these compounds **2a**–**d** by sodium borohydride in methanol at ambient temperature afforded their corresponding reduced analogs **3a**–**d** (Scheme 1).

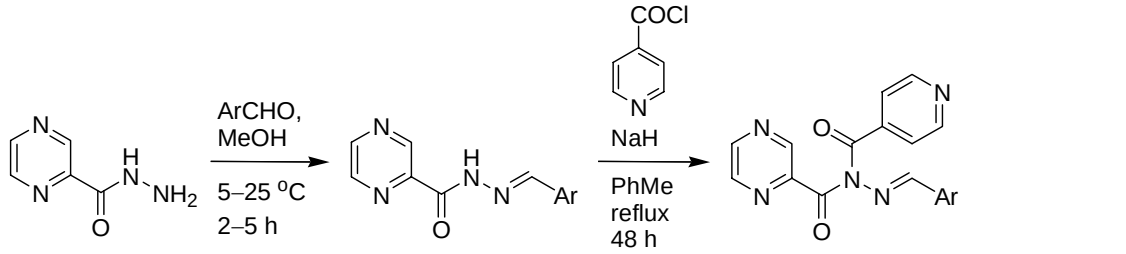


	Ar
a	3-ClC_6H_4
b	4-ClC_6H_4
c	3-$O_2NC_6H_4$
d	4-$MeOC_6H_4$
e	4-ClC_6H_4
f	2,4-$Cl_2C_6H_3$
g	2-furyl
h	3-MeO-4-HOC_6H_3
i	2-HO-4-$MeOC_6H_3$
j	3-$PhOC_6H_4$

Scheme 1

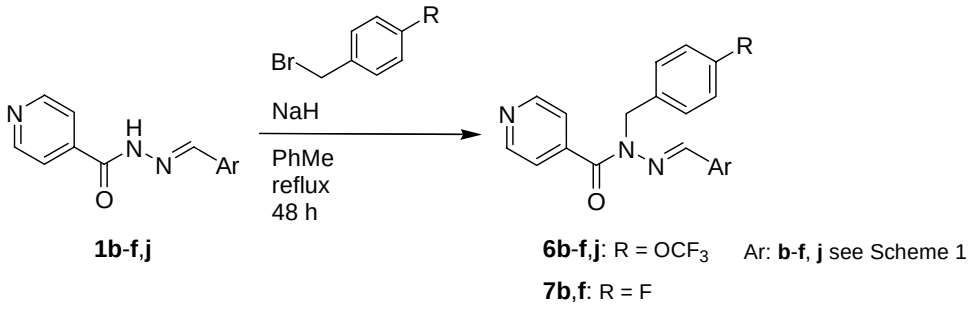
Compounds **2a**–**j** can be prepared by an alternative route also as exemplified by the, the synthesis of compounds **2a** and **2b** (Scheme 2) starting from pyrazine-2-carbo-hydrazide (**4**).[11]

Condensation of **4** with the appropriate arylaldehydes provided the corresponding *N*′-(arylmethylene)pyrazine-2-carbohydrazides **5a**–**b** in good yields, which were subsequently reacted with isonicotinoyl chloride[12] in the presence of sodium hydride in refluxing toluene to provide the compounds **2a**,**b** (Scheme 2). Compounds **2a**,**b** prepared by this procedure match in all aspects (^{1}H NMR, MS and elemental analysis) those obtained by the method given in Scheme 1; yields of compounds **2a,b** were almost the same by either method.



Scheme 2

Additionally, various arylmethyl-substituted analogs **6b**–**f**,**j** and **7b**,**f** were prepared by alkylation of selected *N*′-(arylmethylene)isonicotinohydrazides **1b**–**f**,**j** with 4-(trifluoromethoxy)benzylbromide and 4-fluorobenzylbromide, respectively, in the presence of sodium hydride in refluxing toluene (Scheme 3).



Scheme 3

Antimycobacterial activity

The antimycobacterial activity of the compounds was determined with the objective to identify the compounds having inhibitory activity against susceptible (sensitive strains; inhibited by the two front line anti TB drugs viz. Isoniazid, Rifampicin) and resistant strains (not inhibited by either Isoniazid or Rifampicin or by both) of *M. tuberculosis* (causative agent of human tuberculosis). In addition to *M. tuberculosis*, the antimycobacterial activity was also evaluated against *M. avium* and *M. intracellulare* which are primary causative agents for avian tuberculosis but are also associated with the disease in humans in the developed countries in AIDS patients

and immunocompromised individuals for the selection of the compounds possessing broad-spectrum activity. Since the resistant strains of *M. tuberculosis*, *M. avium* and *M. intracellulare* are not inhibited by the two front line Anti TB drugs Isoniazid and Rifampicin (control drugs), it was our objective to develop molecules having activity primarily against *M. tuberculosis* and additionally against the opportunistic pathogens (*M. avium* and *M. intracellulare*).

The preliminary antimycobacterial activity of all the compounds was evaluated by the agar dilution assay[13] at three different concentrations (50, 25, and 12.5 μg/mL) against three reference strains of mycobacterium i.e. *M. tuberculosis* H_{37}Rv ATCC 27294, *M. avium* ATCC 49601, and *M. intracellulare* ATCC 13950. All compounds inhibited the mycobacterial species.

The active compounds were then assayed for determination of minimum inhibitory concentration (MIC) against a panel of mycobacterial cultures consisting of appropriate reference strains of three mycobacterial species, eighteen clinical isolates representing the sensitive and resistant (either Isoniazid/Rifampicin or by both) strains of *M. tuberculosis* were included in the study. Control drugs Isoniazid and Rifampicin were included in each batch of test.

Interesting results were obtained from these assays and data is reported in Table 1. Among the tested compounds, **2a**–**e** and **3a**–**d** were exhibited very good activity against *M. tuberculosis* H_{37}Rv and clinical isolates (sensitive strains) and in particular **2e**, **3a**–**d** were identified as the most active compounds (Table 1). The *in vitro* antimycobacterial activities of these compounds (**2e**, **3a**–**d**) were equivalent to that of Isoniazid against *M. tuberculosis* H_{37}Rv and sensitive clinical isolates. Further, the compounds **2e**, **3a**–**d** had either little or no activity (4 – >16 μg/mL) against resistant strains of *M. tuberculosis*. However, none of the compounds showed activity against *M. avium* or *M. intracellulare* suggesting that compounds possess specific anti tuberculosis activity. This could be probably due to mutation in the binding region in the resistant strains of *M. tuberculosis* and no activity against *M. avium* and *M. intracellulare* may be because of absence of binding receptor.

Structure activity relationship

The antimycobacterial activity data in Table 1 clearly show that the compounds **2a,b,e** having a chloro substituent in *m-, p-, o*-position of the phenyl ring exhibited significant activity against susceptible clinical isolates of *M. tuberculosis*. Compounds **2a**,**b**,**e** had an almost similar range of MIC values; they differ only by one or two fold dilution, in spite of having a chloro substituent at different positions. The replacement of the chloro substituent by a nitro or a methoxy group in phenyl ring as in **2c** and **2d**, respectively, caused a reduction of activity. Interestingly, 2,4-dichloro-, 3-phenoxy-, and 2-hydroxy-4-methoxy-substitution as in **2f**, **2j**, and **2i**, respectively, abolished any activity indicating that specific steric requirements are needed for activity. This suggests that a bulky group or disubstitution on the phenyl ring are not favourable for antimycobacterial activity.

Table 1. Range of MIC values (μg/mL) of compounds 2a-j and 3a-d against clinical isolates of *Mycobacterium tuberculosis*

Com-pound	Ar	*M. tb.* $H_{37}Rv$ ATCC 27294	*M. tb.* Clinical isolates		*M. a.* ATCC 49601	*M. i* ATCC 13950
			Sensitive (N = 9)[a]	Resistant (N = 9)[a]		
2a	3-ClC_6H_4	0.5	0.5–1	8–>16	>16	>16
2b	4-ClC_6H_4	0.5	0.5–2	8–>16	>16	>16
2c	3-$O_2NC_6H_4$	1	1–2	>16	>16	>16
2d	4-$MeOC_6H_4$	1	1–2	4–>16	>16	>16
2e	2-ClC_6H_4	0.25	0.25–2	4–>16	>16	>16
2f	2,4-$Cl_2C_6H_3$	>16	>16	>16	>16	>16
2g	2-furyl	4	4–8	8–>16	>16	>16
2h	4-HO-3-$MeOC_6H_3$	4	2–4	>16	>16	>16
2i	2-HO-4-$MeOC_6H_3$	>16	>16	>16	>16	>16
2j	3-$PhOC_6H_4$	>16	>16	>16	>16	>16
3a	3-ClC_6H_4	0.125	0.25	8–16	>16	8
3b	4-ClC_6H_4	0.125	0.125–0.25	8–>16	>16	8
3c	3-$O_2NC_6H_4$	0.25	0.25	8–16	>16	8
3d	4-$MeOC_6H_4$	0.25	0.125–0.25	8–>16	>16	8
	Isoniazid	0.25	0.12–0.25	8–>16	>16	8
	Rifampicin	0.125	0.03–0.5	2–>16	2	4

M. tb. = Mycobacterium tuberculosis; *M. a. = Mycobacterium avium*; *M. i. = Mycobacterium intracellulare*.

[a] N = number of clinical isolates used per group.

Furthermore, the initially synthesized hydrazones **2a**–**d** were reduced to the corresponding aryl methyl analogs **3a**–**d**, which improved in the *in vitro* activity against *M. tuberculosis* isolates. The activities of compounds **3a**–**d** were comparable to that of Isoniazid and do not follow substituent requirements for activity as observed in the hydrazones **2**. This may be due to additional bond rotational degrees of freedom available to the aryl methyl analogs, allowing for better binding to a receptor leading to inhibitory activity. It is clear from these results that the reduced compounds **3** are more active than the hydrazone analogs **2**.

Finally, for comparative purposes several *N*-(arylmethylene)-*N*-(isonicotinoyl)-araldehyde hydrazones **6b**–**f**,**j** and **7b**,**f** were prepared from **1b**–**f**,**j** with substituted benzyl bromides. All products **6b**–**f**,**j** and **7b**,**f** gave in poorer results as compared to compounds **2** and **3**; they displayed no activity against clinical isolates of *M. tuberculosis*.

In conclusion, a new series of antimycobacterial agents was designed and synthesized, that demonstrated significant activity against clinical (sensitive strains) isolates of *M. tuberculosis*. Some of these compounds were highly potent and their activity against *M. tuberculosis* was

found to be equivalent to that of Isoniazid, a well-known drug for tuberculosis. The low or no activity against resistant strains of *M. tuberculosis* may be probably due to the mutation in the binding domain of the receptor in these isolates.

Experimental Section

General Procedures. Melting points were determined in open capillaries on a Büchi B-545 melting point apparatus. Compounds were routinely checked for their purity on silica gel 60 F_{254} TLC plates and their spots were visualized by exposing them to iodine vapour, UV light or by spraying the plates with Dragendorff's or $KMnO_4$ reagents. ^{1}H NMR spectra were recorded on Bruker Advance DRX 200 MHz instrument as solutions (in $CDCl_3$ or DMSO-d_6) using TMS as internal reference, and chemical shifts values are expressed in δ units. Mass spectra were run on Applied Biosystems API 3000 instrument using direct inlet system under positive ion electrospray ionization source. Elemental analyses were carried out with a Perkin Elmer 2400 analyzer and the values found were within ±0.4% of theoretical values.

***N′*-[(3-Chlorophenyl)methylene]-*N*-isonicotinoylpyrazine-2-carbohydrazide (2a). General procedures for the preparation of 2**

Method 1. To a stirred suspension of sodium hydride (0.26 g, 5.33 mmol) pre-washed with hexane, in toluene (30 mL) was added *N′*-[(3-chlorophenyl)methylene]isonicotino-hydrazide (1.26 g, 4.85 mmol) at 25–30 °C with stirring. The resulting reaction mixture was refluxed for 24 h. After cooling to 25–30 °C, pyrazine-2-carbonyl chloride (0.76 g, 5.33 mmol) was added and the reaction mixture was refluxed for another 24 h. Toluene was evaporated under reduced pressure; the residue was suspended in the water (30 mL) and extracted with chloroform (2×50 mL). The combined organic layer was washed with water (2×20 mL), brine (1×20 mL), dried over anhydrous Na_2SO_4 and filtered. The filtrate was concentrated under reduced pressure to obtain crude product. The crude product was purified by column chromatography over silica gel (100–200 mesh) using MeOH/$CHCl_3$ (1.5:98.5) as eluent to give **2a** as off white solid (0.775 g, 44%).

Method 2. Hexane washed sodium hydride (1.17 g, 48.56 mmol) was added into a stirred solution of **5a** (11.50 g, 44.15 mmol) in toluene (225 mL) at 25–30 °C. The resulting reaction mixture was refluxed for 24 h. After cooling to 25–30 °C, isonicotinoyl chloride hydrochloride (6.87 g, 48.56 mmol) and potassium carbonate (3.05 g, 22.10 mmol) were added to the above reaction mixture at 25–30 °C and the reaction mixture was refluxed for another 24 h. Toluene was evaporated under reduced pressure; the residue was suspended in water (100 mL) and extracted with chloroform (2×150 mL). The combined chloroform extracts were washed with water (2×50 mL), brine (1×50 mL), dried over anhydrous Na_2SO_4 and filtered. The filtrate was concentrated under reduced pressure to obtain crude product. The crude product was purified by column chromatography over silica gel (100–200 mesh) using MeOH/$CHCl_3$ (1.5:98.5) as eluent

to give **2a** as off white solid (7.70 g, 47%); mp 152–154 °C. ^{1}H NMR ($CDCl_3$): δ 7.30–7.48 (m, 4H), 7.67 (s, 1H), 8.21 (s, 1H), 8.92–9.32 (m, 5H), 10.36 (s, 1H). MS: *m/z* (%) 366 (100) [M+1]. Anal. Calcd for $C_{18}H_{12}ClN_5O_2$ (365.77): C, 59.11; H, 3.31; N, 19.15. Found: C, 59.25; H, 3.51; N, 19.36.

***N′*-[(4-Chlorophenyl)methylene]-*N*-isonicotinoylpyrazine-2-carbohydrazide (2b).** (Methods1 and 2). Pink solid (46%); mp 129–130 °C. ^{1}H NMR (200 MHz, $CDCl_3$): δ 7.34–7.49 (m, 4H), 7.62 (s, 1H), 8.31 (s, 1H), 8.92–9.32 (m, 5H), 10.38 (s, 1H). MS: *m/z* (%) 366 (100) [M+1]. Anal. Calcd for $C_{18}H_{12}ClN_5O_2$ (365.77): C, 59.11; H, 3.31; N, 19.15. Found: C, 58.95; H, 3.19; N, 19.33.

***N′*-[(3-Nitrophenyl)methylene]-*N*-isonicotinoylpyrazine-2-carbohydrazide (2c).** (Method 1). Yellow solid (43%); mp 136–137 °C. ^{1}H NMR (200 MHz, $CDCl_3$): δ 7.64–8.03 (m, 5H), 8.53 (s, 1H), 8.92–9.32 (m, 5H), 10.39 (s, 1H). MS: *m/z* (%) 377 (100) [M+1]. Anal. Calcd for $C_{18}H_{12}N_6O_4$ (376.33): C, 57.45; H, 3.21; N, 22.33. Found: C, 57.60; H, 3.29; N, 22.07.

***N′*-[(4-Methoxyphenyl)methylene]-*N*-isonicotinoylpyrazine-2-carbohydrazide (2d).** (Method 1). Light green solid (43%); mp 102–104 °C. ^{1}H NMR (200 MHz, $CDCl_3$): δ 3.58 (s, 3H), 6.82–7.20 (m, 4H), 7.64 (s, 1H), 8.30 (s, 1H), 8.90–9.23 (m, 5H), 10.37 (s, 1H). MS: *m/z* (%) 362 (100) [M+1]. Anal. Calcd for $C_{19}H_{15}N_5O_3$ (361.35): C, 63.15; H, 4.18; N, 19.38. Found: C, 63.01; H, 4.35; N, 19.57.

***N′*-[(2-Chlorophenyl)methylene]-*N*-isonicotinoylpyrazine-2-carbohydrazide (2e).** (Method 1). Off white solid (31%); mp 141–142 °C. ^{1}H NMR (200 MHz, $CDCl_3$): δ 7.16–7.30 (m, 4H), 7.65 (s, 1H), 8.85–9.32 (m, 6H), 10.38 (s, 1H). MS: *m/z* (%) 366 (100) [M+1]. Anal. Calcd for $C_{18}H_{12}ClN_5O_2$ (365.77): C, 59.11; H, 3.31; N, 19.15. Found: C, 59.17; H, 3.61; N, 18.91.

***N′*-[(2,4-Dichlorophenyl)methylene]-*N*-isonicotinoylpyrazine-2-carbohydrazide (2f).** (Method 1). Off white solid (41%); mp 175–177 °C. ^{1}H NMR (200 MHz, $CDCl_3$): δ 7.21–7.40 (m, 2H), 7.65–7.92 (m, 2H), 8.86–9.34 (m, 6H), 10.40 (s, 1H). MS: *m/z* (%) 399 (100) [M+1]. Anal. Calcd for $C_{18}H_{11}Cl_2N_5O_2$ (400.22): C, 54.02; H, 2.77; N, 17.50. Found: C, 53.72; H, 3.13; N, 17.37.

***N′*-[2-Furylmethylene]-*N*-isonicotinoylpyrazine-2-carbohydrazide (2g).** (Method 1). Thick syrup (39%). ^{1}H NMR (200 MHz, $CDCl_3$): δ 6.56 (s, 1H), 6.76 (s, 1H), 7.63–7.74 (m, 3H), 8.92–9.32 (m, 5H), 10.38 (s, 1H). MS: *m/z* (%) 322 (100) [M+1]. Anal. Calcd for $C_{16}H_{11}N_5O_3$ (321.29): C, 59.81; H, 3.45; N, 21.80. Found: C, 60.09; H, 3.27; N, 21.89.

***N′*-[(4-Hydroxy-3-methoxyphenyl)methylene]-*N*-isonicotinoylpyrazine-2-carbohy-drazide (2h).** (Method 1). Yellow solid (47%); mp 285–286 °C (dec.). ^{1}H NMR (200 MHz, $CDCl_3$): δ 3.83 (s, 3H), 5.01 (br s, 1H), 6.87–7.22 (m, 3H), 7.64 (s, 1H), 8.25 (s, 1H), 8.92–9.32 (m, 5H), 10.38 (s, 1H). MS: *m/z* (%) 378 (100) [M+1]. Anal. Calcd for $C_{19}H_{15}N_5O_4$ (377.35): C, 60.47; H, 4.01; N, 18.56. Found: C, 60.86; H, 4.21, N, 18.39.

***N′*-[(2-Hydroxy-4-methoxyphenyl)methylene]-*N*-isonicotinoylpyrazine-2-carbohy-drazide (2i).** (Method 1). Yellow solid (39%); mp 223–224 °C (dec.). ^{1}H NMR (200 MHz, DMSO-d_6): δ 3.93 (s, 3H), 6.62–6.85 (m, 3H), 7.65 (s, 1H), 8.34 (s, 1H), 8.94–9.32 (m, 5H), 10.36 (s, 1H), 12.30 (br s, 1H). MS: *m/z* (%) 378 (100) [M+1]. Anal. Calcd for $C_{19}H_{15}N_5O_4$ (377.35): C, 60.47; H, 4.01; N, 18.56. Found: C, 60.29; H, 4.17; N, 18.83.

***N′*-[(3-Phenoxyphenyl)methylene]-*N*-isonicotinoylpyrazine-2-carbohydrazide (2j).** (Method 1). Off white solid (45%); mp 190–191 °C. ^{1}H NMR (200 MHz, $CDCl_3$): δ 6.53 (s, 1H), 7.01–7.15 (m, 5H), 7.30–7.48 (m, 3H), 7.64 (s, 1H), 8.25 (s, 1H), 8.92–9.34 (m, 5H), 10.37 (s, 1H). MS: *m/z* (%) 424 (100) [M+1]. Anal. Calcd for $C_{24}H_{17}N_5O_3$ (423.42): C, 68.08; H, 4.05; N, 16.54. Found: C, 68.33; H, 3.89; N, 16.91.

***N′*-[3-Chlorobenzyl]-*N*-isonicotinoylpyrazine-2-carbohydrazide (3a). General procedure for the preparation of 3**

Sodium borohydride (0.20 g, 5.30 mmol) was added in portion to the solution of **2** (0.20 g, 0.55 mmol) in methanol (10 mL) at 0–5 °C with vigorous stirring. Stirring was continued at 25–30 °C for 0.5 h. Methanol was evaporated at reduced pressure and the residue was suspended in water (10 mL) and then extracted with ethyl acetate (2×25 mL). The combined organic layer was washed with brine (1×20 mL), dried over anhydrous Na_2SO_4 and filtered. The filtrate was concentrated under reduced pressure to obtained crude product. The crude product was purified by column chromatography over silica gel (100–200 mesh) using MeOH/$CHCl_3$ (2:98) as eluent to give **3a** as pale yellow oil (0.091 g, 45%). ^{1}H NMR (200 MHz, $CDCl_3$): δ 3.20 (br s, 1H), 4.11 (s, 2H), 7.25–7.48 (m, 5H), 8.51–9.12 (m, 5H), 9.95 (s, 1H). MS: *m/z* (%) 368 (100) [M+1]. Anal. Calcd for $C_{18}H_{14}ClN_5O_2$ (367.79): C, 58.78; H, 3.84; N, 19.04. Found: C, 58.57; H, 3.71; N, 18.93.

***N′*-[4-Chlorobenzyl]-*N*-isonicotinoylpyrazine-2-carbohydrazide (3b).** Pale yellow oil (40%). ^{1}H NMR (200 MHz, $CDCl_3$): δ 3.22 (br s, 1H), 4.12 (s, 2H), 7.27–7.50 (m, 5H), 8.52–9.10 (m, 5H), 9.98 (s, 1H). MS: *m/z* (%) 368 (100) [M+1]. Anal. Calcd for $C_{18}H_{14}ClN_5O_2$ (367.79): C, 58.78; H, 3.84; N, 19.04. Found: C, 59.07; H, 4.09; N, 19.36.

***N′*-[3-Nitrobenzyl]-*N*-isonicotinoylpyrazine-2-carbohydrazide (3c).** Off white solid (46%); mp 222–223 °C. ^{1}H NMR (200 MHz, $CDCl_3$): δ 3.12 (br s, 1H), 4.30 (s, 2H), 7.50–8.07 (m, 5H), 8.53–9.30 (m, 5H), 10.01 (s, 1H). MS: *m/z* (%) 379 (100) [M+1]. Anal. Calcd for $C_{18}H_{14}N_6O_4$ (378.34): C, 57.14; H, 3.73; N, 22.21. Found: C, 57.49; H, 3.94; N, 22.56.

***N′*-[4-Methoxybenzyl]-*N*-isonicotinoylpyrazine-2-carbohydrazide (3d).** Thick oil (45%). ^{1}H NMR (200 MHz, $CDCl_3$): δ 3.18 (br s, 1H), 3.56 (s, 3H), 4.28 (s, 2H), 7.32–7.47 (m, 4H), 7.60 (s, 1H), 8.90–9.30 (m, 5H), 10.37 (s, 1H). MS: *m/z* (%) 364 (100) [M+1]. Anal. Calcd for $C_{19}H_{17}N_5O_3$ (363.37): C, 62.80; H, 4.72; N, 19.27. Found: C, 62.73; H, 4.93; N, 19.31.

***N′*-[(3-Chlorophenyl)methylene]pyrazine-2-carbohydrazide (5a).** To a solution of pyrazine-2-carbohydrazide (**4**) (8.43 g, 61.0 mmol) in methanol (80 mL), 3-chlorobenzaldehyde (8.58 g, 61.0 mmol) was added dropwise at 0 °C and allowed to stir at 25–30 °C for 2 h. Solid precipitated out was filtered and dried under vacuum to give **5a** as white solid (11.50 g, 72%); mp 209–211 °C. ^{1}H NMR (200 MHz, $CDCl_3$): δ 7.22–7.51 (m, 5H), 8.03 (s, 1H), 8.74 (s, 1H), 9.83 (s, 1H), 12.08 (br s, 1H). MS: *m/z* (%) 261 (100) [M+1]. Anal. Calcd. For $C_{12}H_9ClN_4O$ (260.68): C, 55.29; H, 3.48; N, 21.49. Found: C, 55.51; H, 3.66; N, 21.67.

***N′*-[(4-Chlorophenyl)methylene]pyrazine-2-carbohydrazide (5b).**[14] This was prepared in same manner as **5a** using 4-chlorobenzaldehyde in place of 3-chlorobenzaldehyde in same amount. White solid (74%); mp 238–239 °C (lit.[14] mp 240–242 °C). ^{1}H NMR (200 MHz,

$CDCl_3$): δ 7.30–7.51 (m, 5H), 8.13 (s, 1H), 8.75 (s, 1H), 9.84 (s, 1H), 12.10 (br s, 1H). MS: *m/z* (%) 261 (100) [M+1]. Anal. Calcd for $C_{12}H_9ClN_4O$ (260.68): C, 55.29; H, 3.48; N, 21.49. Found: C, 55.07; H, 3.31; N, 21.30.

***N′*-[(4-Chlorophenyl)methylene]-*N*-[(4-trifluoromethoxy)benzyl]isonicotinohydrazide (6b). General procedure for the preparation of 6 and 7**

To a stirred suspension of sodium hydride (1.06 g, 44.0 mmol) pre-washed with hexane, in toluene (300 mL) was added *N′*-[(4-chlorophenyl)methylene]isonicotinohydrazide (10.30 g, 40.0 mmol) at 25–30 °C with stirring. The reaction mixture was refluxed for 24 h with stirring. After cooling to 25–30 °C, substituted benzyl bromide (44.0 mmol) was added to the above reaction mixture at 25–30 °C and the resulting reaction mixture was refluxed for another 24 h. Toluene was evaporated under reduced pressure, the residue was suspended in water (300 mL) and extracted with chloroform (2×100 mL). The combined organic phase was washed with H_2O (2×50 mL), brine (1×50 mL), dried over anhydrous Na_2SO_4 and filtered. The filtrate was concentrated under reduced pressure to obtain crude product. The crude product was purified by column chromatography over silica gel (100–200 mesh) using MeOH/$CHCl_3$ (1.8:98.2) as eluent to give **6b** as brown solid (4.32 g, 25%); mp 158–159 °C. ^{1}H NMR (200 MHz, $CDCl_3$): δ 5.34 (s, 2H), 7.30–7.37 (m, 4H), 7.75–7.78 (m, 4H), 8.81–9.06 (m, 4H), 9.45 (s, 1H). MS: *m/z* (%) 434 (100) [M+1]. Anal Calcd for $C_{21}H_{15}ClF_3N_3O_2$ (433.81): C, 58.14; H, 3.49; N, 9.69. Found: C, 57.83; H, 3.23; N, 9.47.

***N′*-[(3-Nitrophenyl)methylene]-*N*-[(4-trifluoromethoxy)benzyl]isonicotinohydrazide (6c).** Yellow solid (26%); mp 178–180 °C. ^{1}H NMR (200 MHz, $CDCl_3$): δ 5.34 (s, 2H), 7.64–7.90 (m, 5H), 8.01–8.15 (m, 3H), 8.81–9.06 (m, 4H), 9.66 (s, 1H). MS: *m/z* (%) 445 (100) [M+1]. Anal. Calcd for $C_{21}H_{15}F_3N_4O_4$ (444.36): C, 56.76; H, 3.40; N, 12.61. Found: C, 56.98; H, 3.61; N, 12.97.

***N′*-[(4-Methoxyphenyl)methylene]-*N*-[(4-trifluoromethoxy)benzyl]isonicotinohydrazide (6d).** Yellow solid (26%); mp 138–140 °C. ^{1}H NMR (200 MHz, $CDCl_3$): δ 3.58 (s, 3H), 5.32 (s, 2H), 6.70–7.01 (m, 4H), 7.63–7.83 (m, 2H), 8.01–8.15 (m, 2H), 8.80–9.04 (m, 4H), 9.44 (s, 1H). MS: *m/z* (%) 430 (100) [M+1]. Anal. Calcd for $C_{22}H_{18}F_3N_3O_3$ (429.39): C, 61.54; H, 4.23; N, 9.79. Found: C, 61.88; H, 4.57; N, 10.06.

***N′*-[(2-Chlorophenyl)methylene]-*N*-[(4-trifluoromethoxy)benzyl]isonicotinohydrazide (6e).** Off white solid (26%); mp 200–201 °C. ^{1}H NMR (200 MHz, $CDCl_3$): δ 5.32 (s, 2H), 7.04–7.22 (m, 4H), 7.63–7.75 (m, 2H), 8.12–8.41 (m, 4H), 8.80–9.04 (m, 2H), 9.96 (s, 1H). MS: *m/z* (%) 434 (100) [M+1]. Anal Calcd for $C_{21}H_{15}ClF_3N_3O_2$ (433.81): C, 58.14; H, 3.49; N, 9.69. Found: C, 58.33; H, 3.61; N, 9.83.

***N′*-[(2,4-Dichlorophenyl)methylene]-*N*-[(4-trifluoromethoxy)benzyl]isonicotinohydrazide (6f).** Brown solid (25%); mp 141–142 °C. ^{1}H NMR (200 MHz, $CDCl_3$): δ 5.34 (s, 2H), 7.05–7.25 (m, 2H), 7.63–7.80 (m, 3H), 8.15–8.45 (m, 4H), 8.81–9.06 (m, 2H), 9.98 (s, 1H). MS: *m/z* (%) 468 (100) [M+1]. Anal Calcd for $C_{21}H_{14}Cl_2F_3N_3O_2$ (468.26): C, 53.86; H, 3.01; N, 8.97. Found: C, 54.05; H, 3.25; N, 9.03.

***N′*-[(3-Phenoxyphenyl)methylene]-*N*-[(4-trifluoromethoxy)benzyl]isonicotinohydra-zide (6j).** Thick oil (26%). ^{1}H NMR (200 MHz, $CDCl_3$): δ 5.35 (s, 2H), 6.53–6.58 (m, 1H), 7.02–7.16 (m, 7H), 7.49–7.63 (m, 3H), 8.21–8.52 (m, 4H), 8.86–9.12 (m, 2H), 9.38 (s, 1H). MS: *m/z* (%) 492 (100) [M+1]. Anal Calcd for $C_{27}H_{20}F_3N_3O_3$ (491.46): C, 65.98; H, 4.10; N, 8.55. Found: C, 66.11; H, 4.32; N, 8.91.

***N′*-[(4-Chlorophenyl)methylene]-*N*-(4-fluorobenzyl)isonicotinohydrazide (7b).** Brown solid (27%); mp 186–188 °C. ^{1}H NMR (200 MHz, $CDCl_3$): δ 5.32 (s, 2H), 7.20–7.30 (m, 4H), 7.68–7.74 (m, 4H), 8.78–8.98 (m, 4H), 9.40 (s, 1H). MS: *m/z* (%) 368 (100) [M+1]. Anal Calcd for $C_{20}H_{15}ClFN_3O$ (367.80): C, 65.31; H, 4.11; N, 11.42. Found: C, 65.63; H, 4.37; N, 11.77.

***N′*-[(2,4-Dichlorophenyl)methylene]-*N*-(4-fluorobenzyl)isonicotinohydrazide (7j).** Brown solid (24%); mp 147–148 °C. ^{1}H NMR (200 MHz, $CDCl_3$): δ 5.37 (s, 2H), 7.20–7.36 (m, 3H), 7.74–7.78 (m, 4H), 8.80–9.04 (m, 4H), 9.34 (s, 1H). MS: *m/z* (%) 402 (100) [M+1]. Anal Calcd for $C_{20}H_{14}Cl_2FN_3O$ (402.25): C, 59.72; H, 3.51; N, 10.45. Found: C, 59.55; H, 3.69; N, 10.83.

Microbiology. The compounds **2a**–**j**, **3a**–**d**, and **6b**–**f**,**j**, and **7b**,**f** were evaluated for anti-mycobacterial activity by *in vitro* growth inhibition assay and agar dilution methods.

***In vitro* growth inhibition assay.** The ability of the compounds to inhibit the growth of mycobacterium species was determined by agar diffusion assay. Briefly, reference strains *M. tuberculosis* H_{37}Rv 27294, *M. avium* ATCC 49601 and *M. intracellulare* ATCC 13950 were grown in Middlebrook 7H9 broth containing 10% ADC supplement at 37 °C on a rotary shaker at 150 rpm for 7 days. The turbidity of the culture was adjusted to 0.5 McFarland. 0.50 mL of the individual cultures were then added to the molten Middlebrook 7H10 in 150 mm Petri plates. Uniform holes were then made in the media in which the three different concentration (50, 25 and 12.5 μg/mL) of individual compounds were added. The plates were then incubated at 37 °C for 21–28 days. Compounds showing zone of inhibition greater or equal to the control drugs were considered active.

In vitro agar dilution assay. Minimum inhibitory concentration (MIC in μg/mL) against *M. tuberculosis* strains in agar dilution assay as per the NCCLS-M24-T2 recommendations.[13] The compounds and control drugs were dissolved in DMSO and diluted twofold to obtain ten serial dilutions of each compound. Appropriate volumes of compounds were incorporated into duplicate plates of Middlebrook 7H10 agar medium supplemented with 10% Middlebrook supplement oleic acid-albumin-dextrose (OADC) enrichment at concentration of 0.03 μg/mL to 16 μg/mL. Test organisms (mycobacterium strains) were grown in Middlebrook 7H9 broth containing 0.05% Tween 80 and 10% OADC supplement. After 7 days of incubation at 37 °C the broths were adjusted to the turbidity of 1.0 McFarland standard; the organism were further diluted 10 fold in sterile water containing 0.10% Tween 80. The resulting mycobacterial suspensions were spotted (3–5 μL/spot) onto 7H10 media plates containing different dilution of compounds/control drugs. The plates were sealed and incubated at 37 °C for 3–4 weeks in upright position. The MIC was recorded as the lowest concentration/highest dilution of the compounds/control drugs that completely inhibited the growth of mycobacterial cultures.

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