Synthesis and Antimycobacterial Evaluation of *N´*-(*E*)-heteroaromatic-pyrazine-2-carbohydrazide Derivatives

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Abstract: A series of nine *N´*-(*E*)-heteroaromatic-pyrazine-2-carbohydrazide derivatives (**5a-f** and **6a-c**) have been synthesized and evaluated against *M. tuberculosis* ATCC 27294 using the micro plate Alamar Blue assay (MABA), being the activities expressed as the minimum inhibitory concentration (MIC) in μg/ml. Compounds **5a** and **5f** exhibited potent activities (3.12 and 50μg/mL, respectively) when compared to the first line drug pyrazinamide (MIC>100 μg/mL). Afterwards, these compounds were evaluated for their cell viabilities in non-infected and infected macrophages with *Mycobaterium bovis* Bacillus Calmette-Guerin (BCG) and **5f** was not cytotoxic to host cells in the effective concentration to inhibit the growth of *M. tuberculosis.*

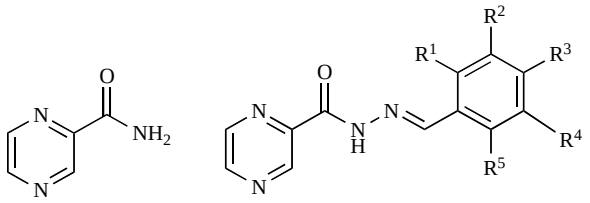
Keywords: Pyrazine, tuberculosis, drugs.

1. INTRODUCTION

Tuberculosis is a disease caused by *Mycobacterium tuberculosis* that infects one-third of global population, leading to almost two million deaths each year. [1,2]. The main problem in tuberculosis treatment is the emergence of resistant strains that can be classified in two types: multidrug-resistant tuberculosis (MDR-TB, resistant to at least isoniazid or rifampicin) and extensively drug-resistant TB (XDR-TB, is MDR-TB that is also resistant to three or more second-line drugs). World Health Organization (WHO) estimated that in 2008 there were 440.000 cases of MDR-TB and 58 countries reported at least one case of XDR-TB until March 2010 [2].

Considering the high impact of resistant strains in tuberculosis treatment, there is an urgent requirement of new drugs to treat it efficiently. A strategy commonly used in drug discovery is to synthesize analogs of drugs in current use to improve biological activity or pharmacokinetic parameters. In this context, pyrazinamide (PZA) could be considered a good start point to the development of new drugs against TB, due to its excellent sterilizing effect on semi-dormant tubercle bacilli [3]. This characteristic is responsible to reduce the time of treatment from twelve to six months [4,5]. Considering that, we have reported in our previous works two series of mono and disubstituted pyrazinecarbohydrazide derivatives, including some derivatives that showed better *in vitro* antitubercular activities than PZA (Fig. **1**) [6,7]. Hence, in our continuous research program in TB drug discovery, we propose the synthesis and antimycobacterial evaluation of nine heteroaromatic 2-pyrazinecarbohydrazide derivatives **5a-f** and **6a-c,** also designed by molecular hybridization (Scheme **1**).

The criteria used to select the five-membered heterocyclic nucleus was based on isosteric replacements: i) substitution of oxygen atom of furane ring (**3a**) by sulfur (**3d**) or nitrogen (**3e**); ii) substitution of –CH= by –N= in pyrrole ring **(3e)** to furnish an imidazole ring (**3f**). The six-membered heterocyclic nucleus (**4a-c**) was also chosen with the aim to analyze the influence of these structural modifications on the biological activity of this series.



Pyrazinamide
MIC >100 μg/ml

R_2= Cl; R_1=R_3 =R_4 = R_5 = H MIC = 50 μg/ml
R_2= CN; R_1=R_3 =R_4 = R_5 = H MIC = 50 μg/ml
R_1= NO_2; R_2=R_3 =R_4 = R_5 = H MIC = 50 μg/ml
R_2= NO_2; R_1=R_3 =R_4 = R_5 = H MIC = 100 μg/ml
R_2= F; R_1=R_3 =R_4 = R_5 = H MIC = 100 μg/ml

Fig. (1). Pyrazinamide and some pyrazinecarbohydrazide derivatives previously synthesized by our group with their respective antimycobacterial activities.

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1573-4064/11 $58.00+.00

2. MATERIALS AND METHODS

2.1. General Procedures

Melting points were determined on a Buchi apparatus and are uncorrected. Infrared spectra were recorded on a Thermo Nicolet Nexus 670 spectrometer as potassium bromide pellets and frequencies are expressed in cm^{-1}. Mass spectra (ESI assay in solution of ammonium chloride) were recorded on Micromass ZQ Waters mass spectrometer. NMR spectra were recorded on a Bruker Avance 400 operating at 400.00 MHz (^1H) and 100.0 MHz (^{13}C) and Bruker Avance 500 spectrometer operating at 500.00 MHz (^1H) and 125.0 MHz (^{13}C), in deuterated dimethylsulfoxide. Chemical shifts are reported in ppm (δ) relative to tetramethylsilane and *J*-coupling in Hertz (Hz). Proton and carbon spectra were typically obtained at room temperature. For TLC plates coated with silica gel were run in chloroform/methanol mixture and spots were developed in ultraviolet and solution of ninhidrine (0.2% p/v in ethanol).

2.1.2. Synthesis of pyrazine-2-carbohydrazide 2

Pyrazine-2-carboxihydrazide **2** was prepared by addition of $N_2H_4.H_2O$ (25%, 20 equiv., 144 mmol) in 36 mL ethanolic solution of methyl pyrazine-2-carboxylate **1** (1.0g, 7.2 mmol) and the mixture was refluxed for 2 hours. The solvent was removed under reduced pressure and the residue was purified by washing cold Et_2O (30mL) to afford the compound **2** as solid. **Yield:** (0.81g, 80%), **mp:**158°C (158-159°C)[8].

2.1.3. General Procedures of Synthesis of **N'-*[(E)-(heteroaromatic)]*-pyrazine-2-carbohydrazide 5a-f and 6a-c**

N'-(*E*)-(heteroaromatic)-pyrazine-2-carbohydrazide **4a-f** and **6a-c** were prepared by reaction between pyrazine-2-carbohydrazide (1.0 equiv.) and the appropriate heteroaromatic aldehyde (1.2 equiv.) in a mixture of ethanol and water (1:1, 10 ml). The reaction mixture was stirred for 4-16 hours at room temperature. After that, the excess of solvent was concentrated under reduced pressure and the residue was purified by washing with cold Et_2O (3 X 10 ml), leading the pure derivatives **5a-f** and **6a-c** as solids in 50-86% yields, except for **5d** and **6c**, which were recrystallized in ethanol.

2.1.3.1. **N'-*[(E)-((5-nitrofuran-2-yl)methylene)pyrazine-2-carbohydrazide (5a)***

Yield: **58**%; **mp**: 217-219°C

^{1}H NMR (500 MHz, DMSO-d_6) δ: 12.77(1H; s; NH); 9.29(1H; s; H_3); 8.95(1H; s; H_6); 8.81(1H; s; H_5); 8.61(1H; s; N=CH); 7.80 (1H; d; *J*=3.4 Hz; H- nitrofuranyl); 7.30 (1H; d; *J*=3.4 Hz; H- nitrofuranyl) ppm;

^{13}C NMR (125 MHz, DMSO-d_6) δ: 159.4; 153.1; 151.5; 149.3; 147.8; 144.5; 144.1; 143.3; 114.6; 112.3 ppm;

ESI/MS: [M-H]: 260

IR: (KBr pellets, cm^{-1}): 3415 (NH); 1675 (CO)

2.1.3.2. **N'-*[(E)-((furan-2-ylmethylene)pyrazine-2-carbohydrazide (5b)***

Yield: 50 %; **mp**: 190-192°C; (192°C)**[9]**

2.1.3.3. **N'-*[(E)-((5-nitrothiophen-2-yl)methylene)pyrazine-2-carbohydrazide (5c)***

Yield: **62**%; **mp**: 263-265°C

^{1}H NMR (500 MHz, DMSO-d_6) δ: 12.76(1H; s; NH); 9.27(1H; s; H_3); 8.95(1H; s; H_6); 8.84(1H; s; N=CH); 8.80(1H; s; H_5); 8.13 (1H; d; *J*=4.0 Hz; H - nitrothiophenyl); 7.58 (1H; d; *J*= 4.0 Hz; H - nitrothiophenyl) ppm;

^{13}C NMR (125MHz, DMSO-d_6) δ: 159.9; 151.1; 148.1; 146.3; 144.3; 144.2; 143.4; 142.9; 130.4; 130.0 ppm;

ESI/MS: [M-H]: 276

IR: (KBr pellets, cm^{-1}): 3483 (NH); 1682 (CO).

2.1.3.4. **N'-*[(E)-(thiophen-2-ylmethylene)pyrazine-2-carbohydrazide (5d)***

Yield: 50 %; **mp**: 185-186°C; (187°C) **[9]**

2.1.3.5. **N'-*[(E)-((1H-pyrrol-2-yl)methylene)pyrazine-2-carbohydrazide (5e)***

Yield: 50 %; **mp**: 176-178°C; (179°C) **[9]**

2.1.3.6. **N'-*(E) -((1H-imidazol-2-yl)methylene)pyrazine-2-carbohydrazide (5f)***

Yield: **58**%; **mp**: 217-219°C

^{1}H NMR (500 MHz, DMSO-d_6) δ: 12.88(1H; s; NH-imidazolyl); 12.42(1H; s; NH - pyrazine) 9.27(1H; s; H_3); 8.93(1H; s; H_6); 8.79(1H; s; H_5); 8.56(1H; s; N=CH); 7.24-7.10(2H; m; H- - imidazolyl) ppm;

^{13}C NMR (125 MHz, DMSO-d_6) δ: 159.4; 152.7; 149.3; 147.8; 144.5; 143.3; 137.8; 127.8; 127.0 ppm;

ESI/MS: [M-H]: 231

IR: (KBr pellets, cm^{-1}): 3468 (NH); 1666 (CO)

2.1.3.7. **N'-*[(E)-(pyridine-2-ylmethylene)pyrazine-2-carbohydrazide (6a)***

Yield: **60**%; **mp**: 203-204°C

^{1}H NMR (500 MHz, DMSO-d_6) δ: 12.60(1H; s; NH); 9.28(1H; d; *J*= 1.5 Hz; H_3); 8.94(1H; d; *J*= 2.4 Hz; H_6); 8.80(1H; dd; *J*= 1.5 and 2.4 Hz; H_5); 8.69(1H; s; N=CH); 8.63 (1H; d; *J*= 4.5 Hz; $H_{1'}$); 8.01 (1H; d; *J*= 7.5 Hz $H_{4'}$); 7.90 (1H; ddd; *J*= 1.0, 7.5 and 8.0Hz; $H_{3'}$); 7.43 (1H; dd; *J*= 4.5 and 7.5 Hz; $H_{2'}$) ppm;

^{13}C NMR (125MHz, DMSO-d_6) δ: 158.9; 147.6; 145.0; 144.0; 143.2; 142.6; 127.0; 1123.0; 114.1; 109.4ppm;

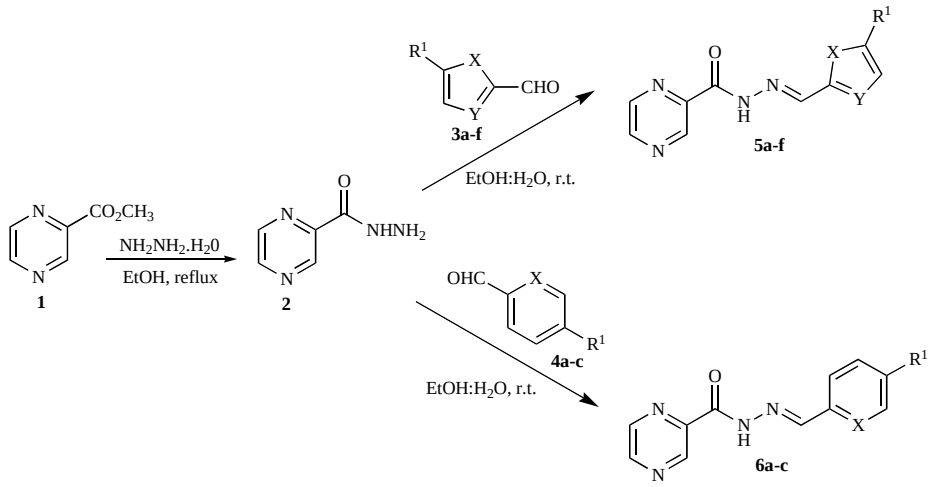
ESI/MS: [M-H]: 226

IR: (KBr pellets, cm^{-1}): 3418 (NH); 1706 (CO)

2.1.3.8. **N'-*[(E)-(4-(pyridin-2-yl)benzylidene)pyrazine-2-carbohydrazide (6b)***

Yield: **86**%; **mp**: 254-256°C

^{1}H NMR (400 MHz, DMSO-d_6) δ: 12.35(1H; s; NH); 9.28(1H; d; *J*= 1.3 Hz; H_3); 8.94(1H; d; *J*= 2.2 Hz; H_6); 8.80(1H; dd; *J*= 1.3 and 2.2 Hz; H_5); 8.71(1H; s; N=CH); 8.70 (1H; d; *J*=7.7 Hz; H- pyridinyl); 8.21 (2H; d; *J*=8.4 Hz; H- benzylidene); 8.03 (1H; d; *J*=8.0 Hz; H- pyridinyl); 7.91 (1H; ddd; *J*= 1.7; 7.7 and 8.0 Hz; H- pyridinyl); 7.85 (2H; d;



Entry	X	Y	R_1
3a	O	CH	NO_2
3b	O	CH	H
3c	S	CH	NO_2
3d	S	CH	H
3e	NH	CH	H
3f	NH	N	H
4a	N	---	---
4b	CH	---	Pyridin-2-yl
4c	CH	---	---

Scheme 1. Synthetic routes for the preparation of the *N´*-(*E*)-heteroaromatic-pyrazine-2-carbohydrazide derivatives (**5a-f** and **6a-c**).

J= 8.4 Hz; H- benzylidene); 7.39 (1H; ddd; *J*= 1.7; 7.7 and 8.0 Hz; H- pyridinyl) ppm;

^{13}C NMR (100 MHz, DMSO-d_6) δ: 159.5; 155.1; 149.3; 147.8; 144.6; 144.1; 143.3; 140.2; 137.3; 134.7; 127.6; 126.9; 122.9; 120.5 ppm;

ESI/MS: [M-H]: 302

IR: (KBr pellets, cm^{-1}): 3420 (NH); 1778 (CO)

2.1.3.9.* N'-*[(E)-phenylmethylidene]-2-pyrazinecarbohydrazide (6c)

Yield: 50%; **mp**: 224°C; (228-230°C) **[10]**

2.2. General Procedures for Biological Tests

2.2.1. Cell Viability Assay

The cellular viability for a macrophage cell line J774 (ATCC TIB-67™) was determined by Mosmans´s MTT (3-(4,5- dimethylthylthiazol-2yl)-2,5-dimethyl tetrazolium bromide; Merck) microcultured tetrazolium assay. We evaluated non infected or infected macrophages with *Mycobaterium bovis* Bacillus Calmette-Guerin (BCG) in the presence and absence of test compounds **(5a-f** and **6a-c)**. The cells were plated in flat bottom 96 well plates (2.5 X 10^6 cells/well/100µL) cultured for 24 h in a controlled atmosphere (CO_2 5% at 37 °C), and non-adherent cells were washed by gentle flushing with RPMI 1640 supplemented with fetal bovine serum (10%) and gentamicin (25µg/mL). Adherent cells were infected or not with BCG (2.5 X 10^6 UFC/well/100µL) cultured in the presence of medium alone, tween 20 (3%) (live and dead controls, respectively) or different concentrations of compounds (1.0, 10.0 and 100µg/mL) in a triplicate assay. After 48 h, stock MTT solution (5 mg/mL of saline; 20 mL/well) was added to the culture and 4 h later, the plate was centrifugate for 2 minutes at 2800 rpm, supernatant was discharged and Dimethyl sulfoxide (DMSO) (100 µL/well) was added for formazan crystals solubilization and the absorbance was read at 540 nm in a plate reader (Biorad – 450).

2.1.2. Antimycobacterial Activity

Briefly, 200µL of sterile deionized water was added to all outer-perimeter wells of sterile 96 well plates (falcon, 3072: Becton Dickinson, Lincoln Park, NJ) to minimize evaporation of the medium in the test wells during incubation. The 96 plates received 100µL of the Middlebrook 7H9 broth containing the mycobacterial cells (Difco laboratories, Detroit, MI, USA) and a serial dilution of the compounds **(5a-f** and **6a-c)** was made directly on the plate. The final drug concentrations tests were 0.01-100 µg/mL. Plates were covered and sealed with parafilm and incubated at 37ºC for five days. After this time, 25µL of a freshly prepared 1:1 mixture of Alamar Blue (Accumed International, Westlake, Ohio) reagent and 10% tween 80 was added to plate and incubated for 24h. A blue color in the well was interpreted as no bacterial growth, and a pink color was scored as growth. The MIC

(minimal inhibition concentration) was defined as the lowest drug concentration, which prevented a color change from bleu to pink.

3. RESULTS AND DISCUSSION

3.1. Chemistry

The synthetic route for preparation of *N´*-(*E*)-heteroaromatic-pyrazine-2-carbohydrazide derivatives (**5a-f** and **6a-c**) is summarized in Scheme **1**. Firstly, the pyrazine-2-carbohydrazide was synthesized by the reaction of methyl pyrazine-2-carboxylate with hydrazine hydrate under reflux. After that, compounds **5a-f** and **6a-c** were obtained from reactions of pyrazine-2-carbohydrazide and heteroaromatic aldehydes, as described in the general procedure (Scheme **1** and Table **1**). [6-11]

In general, ^{1}H NMR spectra of the compounds **5a-f** and **6a-c** showed the signals of the respective protons of the synthesized compounds, which were verified on the basis of their chemicals shifts, multiplicities and coupling constants. These spectra showed two characteristic signals about N=CH proton at 8.56-8.71 ppm and CONH protons at 12.35-12.77 ppm. The ^{13}C NMR spectra showed the C=O and C=N signals at 158.4-159.9 and 145.0-147.8 ppm, respectively.

Table 1. Yields and Melting Points of *N´*-(*E*)-heteroaromatic-pyrazine-2-Carbohydrazide Derivatives (5a-f and 6a-c)

Entry	Yield (%)	m.p. (°C)
5a	58	217-219
5b	50	190-192[**9**]
5c	62	263-265
5d	50	209-210 [**9**]
5e	50	176-179 [**9**]
5f	58	217-219
6a	60	203-204
6b	86	254-256
6c	50	204 [**10**]

3.2. Antimycobacterial Activity

The antimycobacterial activities of **5a-f** and **6a-c** were assessed against *M. tuberculosis* ATCC 27294[12] using the micro plate Alamar Blue assay (MABA)[13] (Table **4)**. This methodology is nontoxic, uses a thermally-stable reagent and shows good correlation with proportional and BACTEC radiometric methods [14, 15].

Table 2. Antimycobacterial Activities and *c*logP Measurements of Pyrazinamide and heteroaromatic-pyrazine-2-carbohydrazides Derivatives (5a-f and 6a-c)

Entry	MIC [a] (μg/mL)	*c*Log*P* [b]
5a	3.12	0.71
5b	>100	0.889
5c	>100	1.613
5d	>100	1.531
5e	>100	0.786
5f	50	-0.274
6a	>100	0.461
6b	>100	2.281
6c	>100	1.631
PZA	>100	-0.711

[a] Minimum inhibitory concentration.
[b] Calculated using www.molinspiration.com

This table showed that the compounds **5a** and **5f** exhibited antimycobacterial activities of 3.12 and 50μg/mL, respectively**.** Therefore, these compounds were selected for cytotoxicity evaluation by Mosmans´s assay.

3.2. Cell Viability Assay

The cellular viability in the presence and absence of test compound (**5a-f** and **6a-c**) was determined by Mosmans´s MTT (3-(4.5-demethylthylthiazol-2-yl)-2.5-dimethyl tetrazolium bromide; Merck) microcultured tetrazolium assay [16, 17]. The results were represented as percentage cell viability (Table **3**).

This table shows that the compound **5f** did not kill more than 5% of the host cells in the minimum concentration tested. Hence, this compound was selected to be tested on macrophages infected with *Mycobaterium bovis* Bacillus Calmette- Guerin (BCG) (Table **4**).

The purpose of this test is to evaluate the action of these compounds against macrophages that show their metabolism changed after infection. Then, the derivative **5f** was not cyto-

Table 3. Data of the Cellular Viability for a Macrophage Cell Line J774 (ATCC TIB-67™) by Mosmans´s Assay

Entry	% Cell Viability/Dose (μg/ml)		
	3.12	**50**	**100**
5a	88	84	76
5f	100	96	95
Pyrazinamide	**100**	**100**	**93**

Table 4. Data of the Cellular Viability for a Macrophage Cell Line J774 Infected (ATCC TIB-67™) with BCG by Mosmans´s Assay

Entry	% Cell Viability/Dose (μg/ml)		
	3.12	**50**	**100**
5f	100	100	100
Pyrazinamide	**100**	**92**	**87**

toxic once it did not kill more than 5% of the cells at the minimum concentration tested.

4. CONCLUSION

The synthesis of nine heteroaromatic pyrazine-2-carbohydrazide derivatives (**5a-f** and **6a-c**), including five new compounds (**5a**, **5c**, **5f**, **6a** and **6b**), was performed in good yields (50-86%). All these compounds were evaluated against *M. tuberculosis* and compounds **5a** and **5f** (3.12 and 50μg/mL) exhibited antitubercular activities better than pyrazinamide (>100μg/mL) in MABA assay. These results suggest that **5a** and **5f** could be acting through a different mechanism of action of that proposed to PZA. However, only the derivative **5f** was not cytotoxic in non infected or infected macrophages with *Mycobaterium bovis* Bacillus Calmette-Guerin (BCG). Therefore, this compound could be considered a good start point to find new lead compounds in the fight against tuberculosis.

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Received: August 20, 2010 Revised: January 06, 2011 Accepted: March 24, 2011