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Preliminary communication

Synthesis and anti-mycobacterial activity of (*E*)-*N*′-(monosubstituted-benzylidene)isonicotinohydrazide derivatives

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Abstract

A series of 22 (*E*)-*N*′-(monosubstituted-benzylidene)isonicotinohydrazide derivatives have been synthesized and evaluated for their in vitro antibacterial activity against *Mycobacterium tuberculosis* $H_{37}Rv$ using Alamar Blue susceptibility test and the activity expressed as the minimum inhibitory concentration (MIC) in μg/mL. Compounds **2f**, **2g**, **2j**, **2k** and **2q** exhibited a significant activity (0.31–0.62 μg/mL) when compared with first line drugs such as isoniazid (INH) and rifampicin (RIP) and could be a good starting point to develop new lead compounds in the fight against multi-drug resistant tuberculosis.

Keywords: Isoniazid; Derivatives; Anti-mycobacterial; *Mycobacterium tuberculosis*

1. Introduction

Tuberculosis (TB) is a chronic bacterial infection, spread through the air, and caused by a bacterium called *Mycobacterium tuberculosis*, first identified in 1882 by Robert Koch, which can mainly attack the lungs, although can affect other organs as well. Unfortunately, nowadays TB is becoming again a world-wide problem, declared in 1993 by the World Health Organization (WHO), a global health emergency. The resurgence of TB became a serious world-wide problem during the period 1985–1992, particularly in people infected with the HIV virus. However, there are also other problems that contribute to the increasing incidence of TB nowadays, such as immigration, war, famine, the lack of new drugs, and multi-drug-resistant tuberculosis (MDR TB) that arise from inconsistent or partial treatment [1–7]. At present, according to statistics, TB kills four people every minute somewhere in the world and accounts about two million deaths per year. It is estimated that one-third of the world's population is currently infected with the TB bacillus and 30 million people will die in the next 10 years [8]. In this context, there is an urgent need for new drugs to fight against this disease. Considering that there are three basic objectives involved in the development of new tuberculosis drugs; to reduce the total duration of treatment, to improve the MDR TB and to provide more effective treatment of latent tuberculosis infection.

In the search of new compounds, isoniazid derivatives have been found to possess potential tuberculostatic activities [4,9–15]. Studies suggest that isoniazid, a prodrug which is converted into its active form by mycobacterial catalase–peroxidase, acts on the mycobacterial cell wall by preventing the FAS-II (fatty acid synthetase II) system

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from producing long chain fatty acid precursors for mycolic acid synthesis [16,17].

As part of a current work of synthesis, anti-mycobacterial investigation and crystallographic studies of isoniazid derivatives [18–20], the aim of this article is to present a series of 22 (*E*)-*N*′-(monosubstituted-benzylidene)isonicotinohydrazide derivatives, which have been synthesized, see Scheme 1, and evaluated for their in vitro antibacterial activity against *M. tuberculosis* (Table 1).

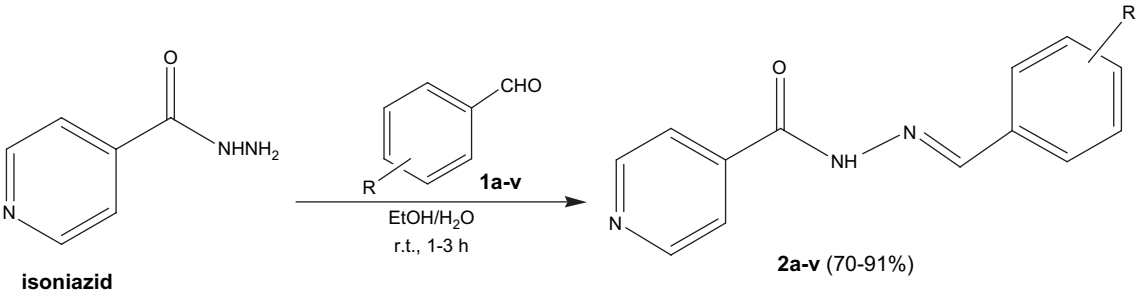
2. Results and discussion

2.1. Chemistry

The synthesis of (monosubstituted-benzylidene)isonicotinohydrazide derivatives involved the reaction between appropriate monossubstituted benzaldehydes (**1a–v**) and isoniazid, as described in the general procedure. Reaction mixtures were maintained at room temperature, leading to the desired compounds **2a–v** in 70–91% yields. All the compounds were identified by spectral data. In general, IR spectra showed the C=O peak at 1651–1703 and the NH stretching vibrations at 3013–3213 cm^{-1}. In the nuclear magnetic resonance spectra (^1H NMR) the signals of the respective protons of the synthesized compounds were verified on the basis of their chemical shifts, multiplicities and coupling constants. The spectra showed the hydrazide (NH) proton as a singlet at 12.42–11.94 ppm and the imine proton (N=C–H) at 8.89–8.40 ppm. The ^{13}C NMR spectrum showed the C=O signals at 162.0–161.4 and C=N signals at 140.6–140.0 ppm. The lipophilicities of the synthesized compounds **2a–v** and the standard drugs (INH and RIP), which were expressed as log *P* values, were determined through CSlogP method, a commercially available program (Table 1).

2.2. Anti-mycobacterial activity

The anti-mycobacterial activities of compounds **2a–v** were assessed against *M. tuberculosis* ATTC 27294 [21] using the micro plate Alamar Blue assay (MABA) [22] (Table 1). This methodology is nontoxic, uses a thermally-stable reagent and shows good correlation with proportional and BACTEC radiometric methods [23,24]. Briefly, 200 μL 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 100 μL of the Middlebrook 7H9 broth (Difco laboratories, Detroit, MI, USA) and a serial dilution of the compounds **2a–v** was made directly on the plate. The final drug concentrations tested were 0.01–20.0 μg/mL. Plates were covered and sealed with parafilm and incubated at 37 °C for five days. After this time, 25 μL of a freshly prepared 1:1 mixture of Alamar Blue (Accumed International, Westlake Ohio) reagent and 10% tween 80 was added to the plate and incubated for 24 h. 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 blue to pink.



2a	R = H	**2l**	R = 3-CN
2b	R = 2-Br	**2m**	R_3 = 4-CN
2c	R = 3-Br	**2n**	R = 2-NO_2
2d	R = 4-Br	**2o**	R = 3-NO_2
2e	R = 2-Cl	**2p**	R = 4-NO_2
2f	R = 3-Cl	**2q**	R = 2-OCH_3
2g	R = 4-Cl	**2r**	R = 3-OCH_3
2h	R = 2-F	**2s**	R = 4-OCH_3
2i	R = 3-F	**2t**	R = 2-OCH_2CH_3
2j	R = 4-F	**2u**	R_2 = 3-OCH_2CH_3
2k	R = 2-CN	**2v**	R = 3-OH

Scheme 1. Synthetic route used for the preparation of isoniazid derivatives **2a–v**.

Table 1
The in vitro activity of compounds **2a–v** against *M. tuberculosis* $H_{37}Rv$ strain (ATCC 27294, susceptible both to rifampin and isoniazid)

Number	Compound	MIC (μg/mL)	log P[a]
2a	R = H	3.12	2.34
2b	R = 2-Br	5.0	3.00
2c	R = 3-Br	2.5	3.11
2d	R = 4-Br	5.0	3.12
2e	R = 2-Cl	1.25	2.91
2f	R = 3-Cl	*0.31*	3.05
2g	R = 4-Cl	*0.31*	2.86
2h	R = 2-F	3.12	2.15
2i	R = 3-F	3.12	2.48
2j	R = 4-F	*0.31*	2.34
2k	R = 2-CN	*0.62*	2.22
2l	R = 3-CN	1.25	2.26
2m	R_3 = 4-CN	5.0	2.28
2n	R = 2-NO_2	5.0	2.01
2o	R = 3-NO_2	5.0	1.95
2p	R = 4-NO_2	1.25	2.00
2q	R = 2-OCH_3	*0.31*	2.38
2r	R = 3-OCH_3	5.0	2.44
2s	R = 4-OCH_3	5.0	2.49
2t	R = 2-OCH_2CH_3	1.25	2.88
2u	R_2 = 3-OCH_2CH_3	1.25	2.96
2v	R = 3-OH	1.25	2.54
INH	–	0.2	−0.58
RIP	–	1.0	−2.38

[a] Calculated using online www.logp.com site.

2.3. Cell viability assay

Cellular viability in the presence and absence of test compounds was determined by Mosmans's MTT(3-(4,5-dimethylthylthiazol-2yl)-2,5-dimethyl tetrazolium bromide; Merck) microcultured tetrazolium assay [25]. The cells were plated in flat bottom 96 well plates (2.5×10^6 cells/mL) cultured for 1 h in a controlled atmosphere (CO_2 5% at 37 °C), and nonadherent cells were washed by gentle flushing with RPMI 1640. Adherent cells were cultured in the presence of medium alone, tween 20 (3%) (live and dead controls, respectively) or different concentrations of compounds (0.1, 1.0, 10.0 and 100 μg/mL) in a triplicate assay. After 18 h, stock MTT solution (5 mg/mL of saline; 20 μL/well) was added to the culture and 4 h later supernatant was discharged and DMSO (100 μL/well) was added for formazan crystals' solubilization and the absorbance was read at 540 nm in a plate reader (Biorad – 450).

The results were represented as percentage cell viability (Table 2). This table shows that the compounds **2a–v** were not cytotoxic to host cells at the same concentration.

3. Conclusion

The synthesis of the 22 isonicotinohydrazide derivatives (**2a–v**), among them three are unpublished (**2k**, **2l** and **2u**), was performed with good yields from commercially available materials.

In relation to the biological studies, it was found that the compounds **2f** (*m*-chlorophenyl), **2g** (*p*-chlorophenyl), **2j** (*p*-fluorophenyl)), **2q** (*o*-methoxyphenyl) (MIC = 0.31 μg/mL) and **2k** (*o*-cyanophenyl) (MIC = 0.62 μg/mL) exhibited a significant activity when compared with first line drugs such as isoniazid (INH, MIC = 0.2 μg/mL) and rifampicin (RIP, MIC = 1.0 μg/mL). These results suggest that they may be selectively targeted to *M. tuberculosis* growth, also considering that they were not cytotoxic to host cells at the same concentration. Alluding to studies involved structure, metabolism and anti-tuberculosis in vitro activity of isoniazid derivatives, these compounds can be considered original isoniazid derivatives prodrugs since hydrazides are cleaved into isonicotinic acid, that is the bioactive form of isoniazid [5].

Table 2
Data of cytotoxic effects of test compounds on murine macrophages cells 18 h after the treatment

Compound	% Cell viability/dose (μg/mL)			
	0.1	1.0	10	100
2f	100	100	100	100
2g	100	97	97	96
2j	100	100	97	96
2k	100	100	100	95
2q	100	100	100	98

In the 1950s, several 2′-monosubstituted isonicotinoylhydrazides were synthesized, however, few information about elucidation, conformation and biological activity was reported [26]. In the present work all the compounds have been also characterized through precise analytical methods.

Information about structure–activity relationship (QSAR), and their in vivo antibacterial activity test are in progress in our laboratory.

4. Experimental

4.1. Materials and methods

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 (CG/MS) were recorded on a Agilent Technologies 6890/5972A mass spectrometer. NMR spectra were recorded on a 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. 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.

4.2. General procedures for the synthesis of (*E*)-*N*′-(monosubstituted-benzylidene)isonicotinohydrazide (*2a–v*)

The isonicotinoyl hydrazide derivatives **2a–v** were prepared by reaction between the appropriate benzaldehyde **1a–v** (1.0 equiv.) with isoniazid (1.0 equiv.) in ethanol/H_2O (10 mL), initially dissolving the isoniazid in H_2O and adding the respective solution over a solution of the respective

benzaldehyde in ethanol (Scheme 1). After stirring for 1–3 h, at room temperature, the resulting mixture was concentrated under reduced pressure. The residue purified by washing with cold ethyl alcohol and ethyl ether, afforded the pure derivatives **2a–v**.

4.3. Analytical data for compounds **2k**, **2l** and **2u**

4.3.1. (*E*)-*N*′-(2-Cyanobenzylidene)isonicotinohydrazide (**2k**)

Yield: 70%; m.p.: 160–162 °C. CG/MS: *m/z* $[M]^{+}\bullet$: 250. ^{1}H NMR [500.00 MHz (FIDRES ± 0.15 Hz), DMSO-d_6] δ: 12.43 (1H; s; N*H*); 8.86 (1H; s; N=C–H); 8.82 (2H; d; $J = 5.2$ Hz; H_2 and H_6); 8.15 (1H; d; $J = 8.0$ Hz; $H_{3'}$); 7.93 (1H; d; 7.6 Hz; $H_{6'}$); 7.88 (2H; d; $J = 5.2$ Hz; H_3 and H_5); 7.81 (1H; dd; $J = 7.2$ and 8.0 Hz; $H_{5'}$); 7.64 (1H; dd; $J = 7.6$ and 7.6 Hz; $H_{4'}$) ppm. ^{13}C NMR (125 MHz, DMSO-d_6) δ: 161.7; 150.2; 144.3; 140.2; 136.6; 133.6; 133.4; 130.7; 125.8; 121.6; 116.9; 111.1 ppm. IR ν_{max} (cm^{-1}; KBr pellets): 3198 (NH); 2225 (CN); 1703 (CO).

4.3.2. (*E*)-*N*′-(3-Cyanobenzylidene)isonicotinohydrazide (**2l**)

Yield: 79%; m.p.: 169–170 °C. CG/MS: *m/z* $[M]^{+}\bullet$: 250. ^{1}H NMR [500.00 MHz (FIDRES ± 0.15 Hz), DMSO-d_6] δ: 12.19 (1H; s; N*H*); 8.73 (2H; d; $J = 5.5$ Hz; H_2 and H_6); 8.48 (1H; s; N=C–H); 8.08 (1H; s; $H_{2'}$); 8.01 (1H; d; $J = 8.0$ Hz; $H_{4'}$); 7.82 (2H; d; $J = 5.5$ Hz; H_3 and H_5); 7.71 (1H; d; $J = 7.5$ Hz; $H_{6'}$); 7.56 (1H; dd; $J = 8.0$ and 8.0 Hz; $H_{5'}$) ppm. ^{13}C NMR (125 MHz, DMSO-d_6) δ: 161.8; 150.4; 146.7; 140.2; 135.4; 133.6; 131.1; 131.0; 130.1; 121.5; 118.3; 112.1 ppm. IR ν_{max} (cm^{-1}; KBr pellets): 3213 (NH); 2230 (CN); 1673 (CO).

4.3.3. (*E*)-*N*′-(3-Ethoxybenzylidene)isonicotinohydrazide (**2u**)

Yield: 77%; m.p.: 148–149 °C. CG/MS: *m/z* $[M]^{+}\bullet$: 269. ^{1}H NMR [500.00 MHz (FIDRES ± 0.15 Hz), DMSO-d_6] δ: 12.08 (1H; s; N*H*); 8.79 (2H; d; $J = 6.0$ Hz; H_2 and H_6); 8.43 (1H; s; N=C–H); 7.82 (2H; d; $J = 6.0$ Hz; H_3 and H_5); 7.38 (1H; dd; $J = 7.5$ and 8.0 Hz; $H_{5'}$); 7.30 (1H; d; $J = 7.5$ Hz; $H_{4'}$ or $H_{6'}$); 7.28 (1H; s; $H_{2'}$); 7.02 (1H; dd; $J = 2.0$ and 8.0 Hz; $H_{6'}$ or $H_{4'}$); 4.08 (2H; q; $J = 7.0$ Hz; OCH_2CH_3); 1.35 (3H; t; $J = 7.0$ Hz; OCH_2CH_3) ppm. ^{13}C NMR (125 MHz, DMSO-d_6) δ: 161.6; 158.8; 150.3; 148.9; 140.4; 135.4; 130.0; 121.5; 120.0; 116.9; 112.0; 63.2; 14.6 ppm. IR ν_{max} (cm^{-1}; KBr pellets): 3076 (NH); 1651 (CO).

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