In vitro anti-mycobacterial activity of (*E*)-*N*´-(monosubstituted-benzylidene) isonicotinohydrazide derivatives against isoniazid-resistant strains

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Abstract

A series of twenty-three *N*-acylhydrazones derived from isoniazid (INH 1-23) have been evaluated for their *in vitro* antibacterial activity against INH- susceptible strain of *M. tuberculosis* (RG500) and three INH-resistant clinical isolates (RG102, RG103 and RG113). In general, derivatives 4, 14, 15 and 16 (MIC=1.92, 1.96, 1.96 and 1.86 μM, respectively) showed relevant activities against RG500 strain, while the derivative 13 (MIC=0.98 μM) was more active than INH (MIC=1.14 μM). However, these derivatives were inactive against RGH102, which displays a mutation in the coding region of *inhA*. These results suggest that the activities of these compounds depend on the inhibition of this enzyme. However, the possibility of other mechanisms of action cannot be excluded, since compounds 2, 4, 6, 7, 12-17, 19, 21 and 23 showed good activities against *katG*-resistant strain RGH103, being more than 10-fold more active than INH.

Introduction

Today, the widespread development of multidrug-resistant tuberculosis (MDR-TB) has become one of the biggest challenges for health professionals worldwide. According to the World Health Organization (WHO), in 2008, the highest level ever reported was found in a region of Northwestern Russia, where 28% of all TB cases were MDR-TB.[1] In the same year, it was estimated that 440,000 people developed MDR-TB, which correspond to 3.6% of all incident TB cases globally, and about 150,000 deaths were registered.[1]

MDR-TB is defined as *Mycobacterium tuberculosis* strains resistant to, at least, rifampicin (RIF) and isoniazid (INH). INH displays a critical role in the initial phase of tuberculosis (TB) therapy, when its bactericidal activity rapidly reduces the viable bacilli count in sputum.[2]

The most accepted mechanism of action for INH involves the inhibition of biosynthesis of mycolic acids, a class of compounds found in the cell wall of the *M. tuberculosis*. Initially, INH is activated *in vivo* by the catalase-peroxidase enzyme *katG*, yielding electrophilic and radical species. During this process, an isonicotinoyl radical is formed and reacts with the nicotinamide group of NAD (nicotinamide adenine dinucleotide) to yield an INH-NAD adduct. This adduct binds to and inhibits the enzyme *inhA*, a NADH-dependent enoyl acyl carrier protein reductase, involved in mycolic acid biosynthesis.[3] Mutations or deletions in the *katG* gene or in the *inhA* gene correspond to 70-80% of INH- resistance in clinical isolates of *M. tuberculosis*. Other genes involved in INH- resistance are the *ahpC* and *ndh*.[4]

Due to the high impact of resistant strains in TB treatment, there is an urgent need for new drugs. A strategy commonly used in drug research is to synthesize analogs of drugs in current use in an attempt to improve the biological activity or pharmacokinetic parameters. In this context, INH could be considered a good starting point for the discovery of new anti-TB drugs, due to its high potency and critical role in TB treatment.

Given this, our previous studies reported the synthesis and anti-mycobacterial evaluation against *M. tuberculosis* $H_{37}R_v$ strain (ATCC 27294, susceptible both to RIF and INH) of a series of *N*-acylhydrazones derived from INH.[5-18] The design concept used to prepare these compounds was molecular hybridization which explores the introduction of monosubstituted benzaldehydes moieties (Figure A) into isoniazid core (Figure B) to obtain *N*-acylhydrazones groups (Figure C). This modification is aimed at investigating the influence of some substituents at the phenyl ring (Figure A) on the *in vitro* biological activity presented by these compounds (Figure 1). It is well known that *N*-acylhydrazones are attractive target compounds for new drug development, because this scaffold is associated to many pharmacological properties, such as antidepressant, anticonvulsant, anti-inflammatory, antimicrobial[19] and antitubercular activities.[5-7,20,21] Moreover, the synthesis of hydrazones can be considered an important strategy to improve pharmacokinetic parameters of some substances, because its introduction promotes an increase of lipophilicity. This feature is extremely important for anti-TB drug discovery, since the mycobacterium cell wall is composed of a thick hydrophobic layer.

Therefore, in the present work, the antimicrobial activities of twenty-three *N*-acylhydrazones were evaluated against *M. tuberculosis* $H_{37}R_v$ and a set of clinical INH-resistant isolates.

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Materials and Methods

Compounds 1-23 (Table 1) were prepared according to our previous publications.[5-8] The determination of antimycobacterial activity was performed using the REMA (Resazurin Microtitre Assay), as previously described.[22] Briefly, the bacterial suspensions were homogenized by vortex agitation, and the turbidity was adjusted in accordance with tube 1.0 of the scale of McFarland (3.2×10^6 colony-forming units/mL). The inoculum was prepared by diluting the bacterial suspension 1:20 in Middlebrook 7H9 medium (4.7 g Middlebrook 7H9 base; Difco,Becton Dickinson), enriched with 10% (v/v) oleic acid-dextrose-albumin-catalase (OADC). The assay was performed in 96-well microplates and incubated at 37°C for seven days. After this period, 30 μL resazurin was added to each well, and the samples were incubated for another two days at 37°C. In order to evaluate cell viability, the oxi-reduction of resazurin was observed on the basis of

the color change during cellular growth process. Each experiment was carried out three times.

Results and Discussion

Twenty-three *N*-acylhydrazone derivatives were evaluated against INH-susceptible *M. tuberculosis* and three INH-resistant clinical isolates (Table 1). In general, all compounds showed significant activities against *M. tuberculosis* RG500, a pan-susceptible strain. Among them, the activities shown by compounds 4, 14, 15 and 16 (MIC=1.92, 1.96, 1.96 and 1.86 μM, respectively) were similar to those performed by INH, whereas the compound 13 (MIC=0.98 μM) was slightly more active than INH (MIC=1.14 μM).

The antimycobacterial evaluation against the resistant strain RGH102 showed that all tested compounds were inactive. This strain presents a C(-35)T promoter mutation and a S94A mutation in the *inhA* gene, responsible for disturbing the hydrogen-bonding network that could result in a reduced binding between INH-NAD adduct and *inhA*. Consequently, results indicate that the activities of such derivatives depend on the inhibition of this enzyme.

However, interesting results were found from the evaluation of the compounds against RGH103 and RGH113 strains, which carry the same S315T mutation in *katG*, namely a gene encoding the catalase-peroxidase responsible for the activation of INH. Compounds 2, 4, 6, 7, 12-17, 19, 21 and 23 showed good activities against RGH103 strain, being over 10-fold more active than INH. However, only the compounds 2, 14 and 21 still showed this activity profile against RGH113. This result could probably be explained by a different mechanism of resistance presented by this strain, such as drug efflux, reduced permeability or mutation in other loci like *nat*, *oxyR-ahpC* 23

In general, the synthesis of hydrazone derivatives is considered a useful approach in the development of compounds with better pharmacokinetic properties. These compounds used to be considered prodrugs, which are hydrolyzed in the cellular media and release the active compound.[24] However, our results indicate that the introduction of hydrazone moiety leads to compounds that could inhibit mycobacterial growth regardless of a mutation in *katG*. There are no structural modifications at the isonicotinic moiety. Subsequently, upon activation by *katG*, all compounds would lead to the same INH-NAD adduct, and we could infer that hydrazone interferes with *katG* activation. Another possible reason for the difference between the activities presented by this series could be related to the lipophilicity of the said

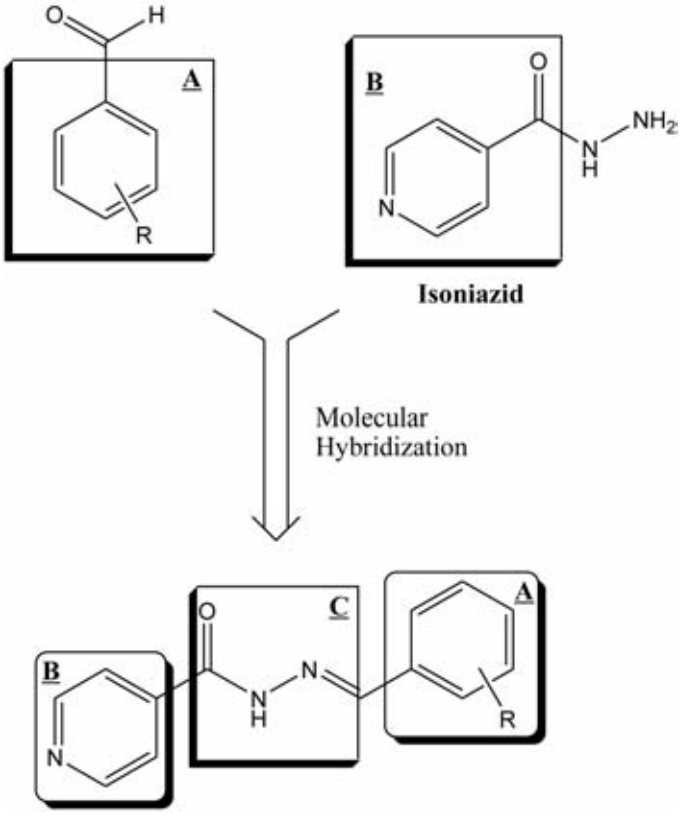

Figure 1. Design concept of *N*-acylhidrazones isoniazid derivatives.

Table 1. *In vitro* activity and *c*LogP measurements of compounds 1-23 against susceptible and INH-resistant *M. tuberculosis* strains.

		MIC (μM)				
Entry	R	RG500*	RGH102°	RGH103#	RGH113§	*c*LogP^
1	2-F	2.05	Res$	Res	Res	1.93
2	3-F	4.11	Res	8.22	8.22	1.95
3	4-F	4.11	Res	Res	Res	1.97
4	2-Cl	1.92	Res	7.70	Res	2.44
5	3-Cl	7.70	Res	Res	Res	2.46
6	4-Cl	3.85	Res	7.70	Res	2.49
7	2-Br	3.29	Res	6.58	Res	2.57
8	3-Br	3.29	Res	Res	Res	2.60
9	4-Br	3.29	Res	Res	Res	2.62
10	2-OH	4.14	Res	Res	Res	1.75
11	3-OH	2.07	Res	Res	Res	1.31
12	4-OH	2.07	Res	8.29	Res	1.33
13	2-OMe	0.98	Res	7.83	Res	1.82
14	3-OMe	1.96	Res	7.83	7.83	1.84
15	4-OMe	1.96	Res	7.83	Res	1.87
16	2-OEt	1.86	Res	7.43	Res	2.20
17	3-OEt	3.71	Res	7.43	Res	2.22
18	2-NO_2	Res	Res	Res	Res	1.72
19	3-NO_2	7.40	Res	7.40	Res	1.74
20	4-NO_2	7.40	Res	Res	Res	1.77
21	3-CN	3.99	Res	7.99	7.99	1.54
22	4-CN	3.99	Res	Res	Res	1.56
23	H	4.44	Res	8.88	Res	1.81
Isoniazid	----------	1.14	Res	Res	Res	-0.97

*RG500 (pan susceptible); °RGH102 (*inhA* S94A and *inhA* C(-35)T, #RGH103 (*katG* S315T), §RGH113 (*katG* S315T); ^*c*LogP: calculated at www.molinspiration.com; $Res.: Resistant (MIC >72.9 μM).

derivatives. The more lipophilic the compounds, the easier they can pass through the mycobacterial cell wall and show an improved activity. However, this can be ruled out since no linear correlation with the cLog was observed (Table 1).

In this context, two reasonable grounds for the observed activities against RGH103 and RGH113 strains would be the following: i) the substances could inhibit antimycobacterial growth, with different levels of activation by *katG* or the substances could inhibit antimycobacterial growth, without previous activation by *katG*, or ii) considering that S315T mutant strains have a reduced ability to metabolize INH, but do not lose their catalase-peroxidase activity,[25] the hydrazone derivatives could be better metabolized than INH.

It is worthy of note that these compounds were not cytotoxic to host cells.[5-8] Therefore, it is conceivable that *N*-acylhydrazones derived from INH could exhibit fewer side effects than this drug itself, because the highly reactive hydrazine group within INH is chemically protected in the *N*-acylhydrazones derivatives.

In conclusion, we described the antimycobacterial evaluation of a series of twenty-three *N*-acylhydrazones derived from INH. In general, these derivatives showed relevant activities against RG500 strain, while the derivative 13 (MIC=0.98 μM) was more active than INH (MIC=1.14 μM). However, these derivatives were inactive against RGH102, which displays a mutation in the coding region of *inhA*. These results suggest that the activities of these compounds depend on the inhibition of this enzyme. Nevertheless, the possibility that other mechanisms of action are involved cannot be excluded, since several derivatives were active against the *katG*-resistant strains RGH103 and RGH113, which carry the same S315T mutation. This finding may be considered important as it refers to the mutation that is the most frequently detected in clinical isolates, accounting for up to 60% of the INH-resistance worldwide.

Research on structure-activity relationship (QSAR) and other computational studies are ongoing in our laboratory, aimed at understanding the possible mechanisms of action presented by this series of compounds.

References

1. WHO. Global tuberculosis control: WHO report 2010. Available from: http://www.who.int/tb/publications/global_report/2010/en/index.html
2. Gillespie SH. Evolution of drug resistance in *Mycobacterium tuberculosis*: Clinical and molecular perspective. Antimicrob Agents Ch 2002;46:267-74.
3. de Souza MVN, Ferreira MD, Pinheiro AC, et al. Synthesis and biological aspects of mycolic acids: An important target against *Mycobacterium tuberculosis*. Thescientificworldjournal 2008;8:720-51.
4. Victor TC, Johnson R, Streicher EM, et al. Drug resistance in *Mycobacterium tuberculosis*. Curr Issues Mol Biol 2006;8:97-111.
5. Junior IN, Lourenco MCS, das Gracas M, et al. Synthesis and anti-mycobacterial activity of N '-[(E)-(disubstituted-phenyl) methylidene]isonicotino-hydrazide derivatives. Lett Drug Des Discov 2005;2:563-6.
6. de Souza MVN, Lourenco MCD, Ferreira MD, et al. Synthesis and anti-mycobacterial activity of (E)-N '-(monosubstituted-benzylidene)isonicotinohydrazide derivatives. Eur J Med Chem 2008;43:1344-7.
7. Ferreira MD, Cardoso LND, Goncalves RSB, et al. Synthesis and antitubercular evaluation of N'-[(E)-(hydroxy, methoxy and ethoxy-substituted-phenyl) methylidene]isonicotinohydrazide derivatives. Lett Drug Des Discov 2008;5:137-40.
8. de Souza MVN, Ferreira MD, Goncalves RSB, et al. Synthesis and Antitubercular Activity of Heteroaromatic Isonicotinoyl and 7-Chloro-4-Quinolinyl Hydrazone Derivatives. Thescientificworldjo 2010;10: 1347-55.
9. Glidewell C, Wardell SMSV, de Souza MVN, et al. Supramolecular structures in *N*-isonicotinoyl arylaldehydehydrazones: multiple hydroge*N*-bonding modes in series of geometric isomers. Acta Crystallogr B 2007;63: 879-95.
10. Glidewell C, de Souza MVN, Wardell SMSV, et al. 4-Cyanobenzaldehyde isonicotinoylhydrazone monohydrate: a three dimensional hydroge*N*-bonded framework structure. Acta Crystallogr C 2007;63:O166-O8.
11. Glidewell C, Wardell SMSV, Wardell JL, et al. 3-Nitrobenzaldehyde isonicotinoylhydrazone monohydrate redetermined at 120 K: sheets built from O-H center dot center dot center dot O, O-H center dot center dot center dot N, *N*-H center dot center dot center dot O and C-H center dot center dot center dot O hydrogen bonds. Acta Crystallogr C 2007;63:O42-O4.
12. Glidewell C, Peralta MA, de Souza MNV, et al. 2,3-Dimethoxybenzaldehyde isonicotinoylhydrazone chloroform monosolvate, and the mono- and dihydrates of 3,4,5-trimethoxybenzaldehyde isonicotinoylhydrazone: hydroge*N*-bonded supramolecular structures in one, two and three dimensions. Acta Crystallogr C 2007;63:O68-O72.
13. Glidewell C, de Souza MVN, Wardell SMSV, et al. *N*-(5-Chloro-2-nitrobenzoyl)-N '-isonicotinoyl-hydrazine: a three-dimensional framework containing four types of hydrogen bond. Acta Crystallogr E 2007;63: O230-O2.
14. Glidewell C, Wardell SMSV, de Souza MVN, et al. 5-Chloro-2-nitrobenzaldehyde isonicotinoyl-hydrazone: a three-dimensional framework built from *N*-H center dot center dot center dot N and C-H center dot center dot center dot O hydrogen bonds. Acta Crystallogr E 2006;62:O3361-O3.
15. Silva FP, Ellena J, Ferreira MD, et al. Experimental and theoretical structure characterization of two isoniazid derivatives: 2,4-difluoro-N '-isonicotinoylbenzohydrazide and 2,4-dichloro-N '-isonicotinoylbenzohydrazide hydrochloride. J Mol Struct 2006;788:63-71.
16. Wardell SMSV, de Souza MVN, Wardell JL, Low JN, Glidewell C. Pyrrole-2-carbaldehyde isonicotinoylhydrazone monohydrate redetermined at 120 K. Acta Crystallogr C. 2006 Jan;62:O47-O9.
17. Wardell SMSV, de Souza MVN, Ferreira MD, et al. pi-Stacked hydroge*N*-bonded chains of rings in 2,4-difluorobenzaldehyde isonicotinoylhydrazone and hydro-ge*N*-bonded sheets in 2,3-dichlorobenzaldehyde isonicotinoylhydrazone. Acta Crystallogr C 2005; 61:O617-O20.
18. Wardell SMSV, de Souza MVN, Wardell JL, et al. Four nitrobenzaldehyde isonicotinoylhydrazones at 120 K: four different supramolecular structures in two and three dimensions. Acta Crystallogr C 2005; 61:O683-O9.
19. Rollas S, Kucukguzel SG. Biological activities of hydrazone derivatives. Molecules 2007;12:1910-39.
20. Carvalho SA, da Silva EF, de Souza MV, et al. Synthesis and antimycobacterial evaluation of new trans-cinnamic acid hydra-zide derivatives. Bioorg Med Chem Lett 2008;18:538-41.
21. Souza MVN, Lourenco MCS, Pinheiro AC, et al. Evaluation of anti-tubercular activity of nicotinic and isoniazid analogues. Arkivoc 2007:181-91.
22. Palomino JC, Martin A, Camacho M, et al. Resazurin microtiter assay plate: Simple and inexpensive method for detection of drug resistance in *Mycobacterium tuberculosis*. Antimicrob Agents Ch 2002;46: 2720-2.
23. da Silva PEA, Ramos DF, Bonacorso HG, et al. Synthesis and *in vitro* antimycobacterial activity of 3-substituted 5-hydroxy-5-trifluoro[chloro]methyl-4,5-dihydro-1H-1-(isonicotinoyl) pyrazoles. Int J Antimicrob Ag 2008;32:139-44.
24. Scior T, Garces-Eisele SJ. Isoniazid is not a lead compound for its pyridyl ring derivatives, isonicotinoyl amides, hydrazides, and hydrazones: a critical review. Curr Med Chem. 2006;13(18):2205-19.
25. Ramaswamy S, Musser JM. Molecular genetic basis of antimicrobial agent resistance in *Mycobacterium tuberculosis*: 1998 update. Tuber Lung Dis 1998;79:3-29.