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. 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* H37Rv 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, antimicrobial19 and antitubercular activities.5,17,20,21 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* H37Rv, and a set of clinical INH-resistant isolates.

Materials and Methods

Compounds 1-23 (Table 1) were prepared according to our previous publications.5,4 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<sup>8</sup> 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, 1.86 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. 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

| Entry | R | MIC (µM) | RG500 | RG102 | RGH103 | RGH113 | cLogP |
|-------|---|----------|-------|-------|--------|--------|-------|
| 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 | 7.70 | 2.44 |
| 5     | 3-Cl | 7.70 | Res | Res | Res | 2.46 |
| 6     | 4-Cl | 3.85 | Res | 7.70 | 7.70 | 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 | 7.83 | 1.82 |
| 14    | 3-Ome | 1.96 | Res | 7.83 | 7.83 | 1.84 |
| 15    | 4-Ome | 1.96 | Res | 7.83 | 7.83 | 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-NO2 | Res | Res | Res | Res | 1.72 |
| 19    | 3-NO2 | 7.40 | Res | 7.40 | Res | 1.74 |
| 20    | 4-NO2 | 7.40 | Res | Res | Res | 1.77 |
| 21    | 2-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 394A and inhB C(-35)T); RGH103 (katG S315T); RGH113 (katG S315T); cLogP 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 antitymocobacterial growth, with different levels of activation by katG or the substances could inhibit antitymocobacterial 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, the hydrazone derivatives could be better metabolized than INH.

It is worthy of note that these compounds were not cytoxic to host cells. 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 antitymocobacterial 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 M) was more active than INH (MIC=1.14 µM). However, these derivatives were inactive against RGH112, 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.

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