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Synthesis and bioevaluation of some new isoniazid derivatives

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ABSTRACT

The aim of the study was to synthesize some new compounds with potential anti-tuberculosis activity, containing isoniazid and α,β-unsaturated thiocinnamamide-like thioamides as precursors. The obtained derivatives were evaluated regarding their biological activity (antioxidant and antibacterial), as well as their influence on the eukaryotic cell cycle. The results suggested that the newly obtained derivatives of isoniazid exhibited different biological activities, depending on their structure; thus, the most active compound in terms of anti-oxidant and anti-*Mycobacterium tuberculosis* effects proved to be the isonicotinic acid *N*′-(1-amino-1-mercapto-3-phenyl-propen-1-yl)-hydrazide. This compound also increased the expression of NAT1 and NAT2 genes, which are implicated in the metabolism of the isoniazid, demonstrating that it could be rapidly metabolized, and thus well tolerated. The largest spectrum of antibacterial activity (excluding *M. tuberculosis*) was noticed for the isonicotinic acid *N*′-[1-amino-1-mercapto-3-(*p*-chloro-phenyl)-propen-1-yl]-hydrazide, which was also the most cytotoxic, especially at high concentrations, although not significantly affecting the cellular cycle phases. The obtained results showed that the new derivatives could represent potential candidates for the treatment of *M. tuberculosis* infections, but further research is needed in order to improve their pharmacological properties, by increasing their antimicrobial activity and reducing the risk of side-effects.

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1. Introduction

Tuberculosis is an infectious disease with a large spectrum of manifestations caused by *Mycobacterium tuberculosis*.[1] Today, tuberculosis is among the top five causes of global mortality.[2] According to the World Health Organization, one third of people are infected with *M. tuberculosis*.[3] In 2011 there were 8.7 million new tuberculosis cases, including 1.1 million cases among people with HIV, and 1.4 million people died from tuberculosis, including half a million women and 430,000 people with HIV.[4]

At present, the treatment of tuberculosis is based on two groups of drugs. The first-line drugs (isoniazid (INH), rifampin, pyrazinamide (PZA), ethambutol (EMB) and streptomycin) have a greater efficacy and acceptable toxicity, while the second-line ones (kanamycin, amikacin, capreomycin, cycloserine (CS), ethionamide (ETH), para-aminosalicylic acid (PAS), and fluoroquinolones (FQ)) are usually characterized by a lower efficacy or a greater toxicity.[1,5,6]

However, new anti-tubercular drugs with new mechanisms of action have not been developed in the last years.[7] The resurgence of tuberculosis and the emergence of drug resistant *M. tuberculosis* isolates, in the recent years, have renewed the interest in the development of new effective anti-tubercular drugs[6,8] (e.g., derivatives of thiadiazole,[2,8] pyrazine,[9] ethionamide[10]).

INH is the most frequently prescribed antibiotic in the treatment of tuberculosis.[11] Although it is being used for more than 60 years for tuberculosis treatment,[12] its mechanisms of action are questionable.[13] INH is a prodrug that is activated in vivo by KatG (mycobacterial catalase–oxidase).[13,14] INH-derived reactive species inhibit the synthesis of cell wall lipids and of nucleic acids[13] and also interfere with bacterial respiratory metabolism.[15] INH derivatives have proved good anti-mycobacterial activity,[6,16–20] some of them being more active than the prodrug they derived from.[11,16,20] So, INH derivatives might be useful in increasing the effectiveness of standard drug regiments in the therapy of *M. tuberculosis* infections and may serve as promising compounds for future anti-mycobacterial drug development.[6,16,17]

The drug toxicity is another problem in tuberculosis treatment. INH is metabolized by acetylation and dehydrazination and the N-acetylhydrazine metabolite is believed to be responsible for

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the hepatotoxic effects seen in treated patients. In eukaryotic cells, N-acetyltransferases (NAT) are involved in the biotransformation of aromatic amines and hydrazines by transferring the acetyl group from acetyl coenzyme A to the free amino group of the parent compound, the N-acetylation of the parent amines being considered a detoxification step.[21,22] NATs are cytosolic enzymes found in many tissues, with distinct tissue distribution and substrate specificity. In humans, two forms with high level of homology are known, that is, NAT1 and NAT2. While NAT1 has a ubiquitous tissue distribution and its expression has been demonstrated to be also related to cancers, NAT2 activity has been described in liver, colon and intestinal epithelium.[22] The rate of acetylation is genetically determined and has not been shown to significantly alter the effectiveness of INH. Slow acetylation may lead to the accumulation of high drug concentrations, with an increased risk of toxicity. Although the implication of NAT gene polymorphism in drug metabolism was intensively studied, to date, the modulation of NATs expression by different drugs was less evaluated.

The aim of this article is to present a series of newly synthesized INH derivatives and the preliminary evaluation of their biological activity.

2. Results and discussion

2.1. Synthesis and structural characterization

Starting from INH we have synthesized five new derivatives, containing moieties of thiocinnamamide-like thioamides. All the compounds were synthesized by a simple coupling reaction between INH and thioamides **1**–**5** (thioamides obtained previously by Pappalardo method[23,24] Fig. 1A). Several methods, using different solvents, were used, like dimethyl sulfoxide, tetrahydrofuran, ethanol, but the best results were obtained using a mixture of ethanol and water, and adding a drop of hydrochloric acid. Starting materials were added in equimolar quantity, refluxed for 1–2 h, and then left for 8 days at room temperature. Product formation (mixture *Z*/*E*) was monitored by thin layer chromatography on silica gel. The R_f values obtained are presented in Table 1. The most polar compounds have been found to be **7**, **8** and **9**, followed by compounds **10** and **6**. Table 1 compiles also the yields of the synthesis and the melting points of new derivatives.

The chemical structure of the newly synthesized compounds was confirmed by ^{1}H and ^{13}C NMR spectral analysis and by elemental analysis. The ^{1}H NMR spectra showed two singlets at 10.06–10.10 ppm and 9.52–9.56 ppm, which represent the two hydrazide protons (NH–NH) that are deuterable. The ^{13}C NMR spectra also showed the C(SH)(NH_2) signals at 76.25–76.59 ppm. The complete interpretation of ^{1}H and ^{13}C NMR spectra and the elemental analyses of new compounds are presented in the experimental part.

2.2. Biological evaluation

2.2.1. Antioxidant activity of INH derivatives

One of the most important properties of the biological active substances is their antioxidant activity, which reflects their capacity to protect the organism from the damage caused by free radicals. In literature data, several methods are used to measure the

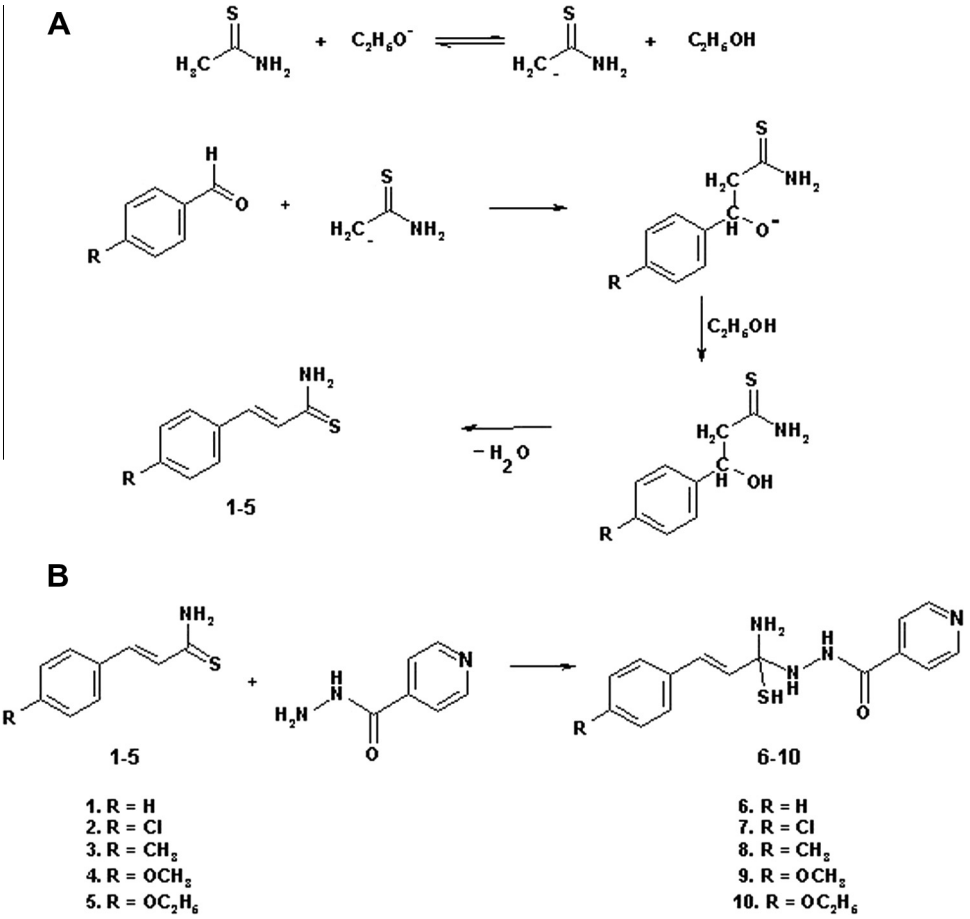

Figure 1. Synthesis of thioamides **1**–**5** (A) and of the new isoniazid derivatives **6**–**10** (B).

Table 1
Results of the physic-chemical characterization of the obtained compounds **6**–**10**

Compd	Yield (%)	Mp (°C)	R_f [a]	TAC (%)
INH	–	171–173	0.29	90.00
1	44	144–145	0.65	35.71
2	43	192–193	0.80	15.71
3	47	195–197	0.86	25.71
4	52	173–174	0.79	60.00
5	41	175–176	0.70	37.85
6	74	120–123	0.58	88.23
7	75	198.6–201	0.65	25.73
8	80	186–188	0.63	12.50
9	65	169–170	0.61	13.97
10	60	161–164	0.59	25.73

[a] AcOEt:CH_2Cl_2:CH_3OH = 5:3:2.

total antioxidant activity (TAC). In the present study we have used the DPPH method, based on the properties of a stable free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH).[25] TAC values (%) were calculated using the Eq. 1:

$$\text{TAC}\ (\%) = \text{Abs}_{\text{ini}} - \text{Abs}_{30\ \text{min}}/\text{Abs}_{\text{ini}} \times 100 \quad (1)$$

where Abs_{ini} = the initial absorption of DPPH radical (measured at 517 nm), $\text{Abs}_{30\ \text{min}}$ = the absorbance recorded at the same wavelength after 30 min.

A comparative study of the antioxidant capacity of both precursors, thioamides and INH, as well as of the products of the coupling reaction showed that the newly synthesized compounds **6** and **7** exhibited a better antioxidant activity than their precursors, that is, the compounds **1** and **2**, respectively (Table 1). As it could be noticed, INH presented the highest antioxidant activity (90%), followed by the compounds **6** (88.23%) and **4** (60%). All the other tested derivatives exhibited a weaker antioxidant activity with percentages of inhibition lower than 40%.

2.2.2. Antimicrobial activity

In order to discover new agents potentially useful in the treatment of tuberculosis, α,β-unsaturated thioamides combinations of INH were synthesized and tested for their in vitro anti-tubercular activity. The tested compounds were assayed in serial binary dilutions ranging from 25 to 0.012 μg/mL. The compound **6** exhibited the best anti-*Mycobacterium* activity, with a Minimal Inhibition Concentration (MIC) of 0.391 μg/mL, followed by the compounds **8** and **10** (MIC = 0.781 μg/mL). The compound **7** exhibited the lowest inhibitory activity (MIC = 6.25 μg/mL). This result is surprising, knowing that the chlorine substituents are generally expected to improve the antimicrobial activity.[19,26]

Although all tested compounds displayed an anti-tubercular activity lower than that of INH (MIC = 0.098 μg/mL) (Table 2), their activity is comparable to other INH derivatives reported in the literature,[27–31] Excepting the compound **7**, all other synthesized compounds displayed MICs below the 6.25 μg/mL value, postulated by the Global Program for the Discovery of New Anti-Tuberculosis Drugs as the upper threshold for the evaluation of new anti-*M. tuberculosis* agents.[27]

Table 2
In vitro activity of INH derivatives against *M. tuberculosis*

Compd	MIC (μg/mL)
INH	0.098
6	0.391
7	6.25
8	0.781
9	1.563
10	0.781

In order to establish the spectrum of the antibacterial activity of the novel INH derivatives, they were also tested on other non-tuberculosis bacterial strains isolated from clinical specimens, that is: Gram-positive (*Streptococcus hominis*, *Staphylococcus aureus*, *Enterococcus faecalis*) and Gram-negative (*Escherichia coli*, *Klebsiella pneumoniae*, *Proteus mirabilis*, *Citrobacter koseri*, *Morganella morganni*, *Acinetobacter baumanii*, *Pseudomonas aeruginosa*) using an adapted diffusion technique. Interestingly, the most active compound proved to be the compound **7**, substituted with a chlorine atom in *para*-position (active against all tested bacterial strains), followed by the compounds **6** and **9**. All new synthesized derivatives proved to be more active on the tested strains than INH and the thioamides they derived from, excepting compound **10**, that was less active than the compound **5** (Table 3).

2.2.3. Cytotoxicity and effects on cell cycle

All tested compounds were further tested for their cytotoxicity and the apoptotic effect on HCT-8 cell line. The discrimination between intact and apoptotic cells was performed by flow cytometry using FITC-labeled annexin-V and propidium iodide. The most toxic compounds proved to be the thioamides **1** and **2** (at the tested concentration of 50 μg/mL) and the new derivative **7** (at the tested concentration of 25 μg/mL) (see Supplementary data for more information details).

Occurrence of apoptosis for these three compounds was also observed in cell cycle analysis, indicated by the presence of one sub-G0/G1 (left) peak. At lower concentrations (25 μg/mL for compounds **1** and **2** and 12.5 μg/mL for compound **7**), these compounds also affected cell cycle. Compound **1** increased S and G2/M phases and decreased G0/G1 phase, effect that was not observed for its derivative, compound **6**, which only slightly affected the cell cycle. Compound **2** also determined the increase of S and G2/M phases and the decrease of G0/G1 phase, but its derivative, compound **7**, although more toxic, determined a small decrease of S phase and an increase of G2/M phase. Compound **3** increased G2/M phase and decreased G0/G1 and S phases, although its derivative, compound **8**, increased G0/G1 phase and decreased S and G2/M phases. Compound **9** did not significantly affect the cell cycle, while compound **4** increased G2/M and decreased S phases. Compound **5** slightly increased G0/G1 phase and decreased S phase, and compound **10** increased both G0/G1 and G2/M phases and decreased S phase (see also Supplementary data).

2.2.4. Influence of new INH derivatives on NATs expression

NAT protein is essential in the INH metabolization, being critical for the control of the magnitude of the side-effects exhibited by this drug. NAT protein is also essential for the survival of *Mycobacterium bovis* BCG inside macrophages,[32] being implicated in the degradation of cholesterol, which seems to be essential for intracellular mycobacterial survival. It is not yet demonstrated if its expression is correlated with other stress response genes in mycobacteria. So far, mycobacterial NAT genes have been identified in the slow growing mycobacteria including *M. tuberculosis* and also in the non-pathogenic model strain *Mycobacterium bovis* BCG.[33]

In the present study we have investigated the influence of new INH derivatives on the expression of NAT genes in the eukaryotic cells, as an additional marker for their cytotoxicity. The obtained results varied for the tested compounds. INH significantly decreased the expression of NAT1 and NAT2 genes (Fig. 2). A similar behavior with that of INH was noticed for the compounds **1**, **3**, **4** and **9**, in case of NAT1 and for the compounds **2**, **3**, **4**, **5** and **9** in case of NAT2 gene. The rest of the tested compounds induced the expression of NAT1 and NAT2 genes with different intensities. Concerning the compound **6**, which exhibited the best antimicrobial activity, it induced a slight increase of NAT1 and NAT2 genes, suggesting a better rate of metabolization and thus, a lower risk for

Table 3
The antimicrobial evaluation of the new compounds **6**–**10** and their precursors **1**–**5** expressed as bacterial growth inhibition (+), or not (−)

Compound	INH	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Micrococcaceae											
Streptococcus hominis 1813	−	+	+/−	+/−	+/−	+/−	+	+	+	+/−	+/−
Staphylococcus aureus	−	+	+	+/−	+/−	+/−	+	+	+	+/−	+/−
Enterobacteriaceae											
Enterobacter cloacae 1845	+	+	+/−	+/−	+/−	+	+	+	+	+	−
Citrobacter koseri 1742	−	+	+/−	+/−	+/−	+	+	+	+/−	+/−	+/−
Morganella morganni 2810	−	+/−	−	−	−	+	+/−	+	+/−	+/−	−
Proteus mirabilis 1751	−	+	−	−	−	+	+/−	+	−	−	+/−
Escherichia coli 1777	−	+	+/−	+/−	+	+/−	+/−	+	+/−	+/−	−
Klebsiella pneumoniae 1756	−	+	−	−	−	−	+/−	+	+/−	+/−	−
Pseudomonadaceae											
Pseudomonas aeruginosa ATCC 27853	−	+/−	−	−	−	+	+	+	−	+/−	−
Pseudomonas aeruginosa 165	−	+	+/−	−	−	+/−	+/−	+	+	+	+/−
Pseudomonas aeruginosa 1144	−	+/−	+/−	+	+	+	+	+	+	+	+
Pseudomonas putida 160	+/−	+	+	+	+	+	+	+	+	+	+
Enterococcaceae											
Enterococcus faecalis 2920	−	+/−	+/−	+/−	+/−	+	+/−	+	+/−	+/−	+/−
Moraxellaceae											
Acinetobacter baumanii 122	−	+	+/−	+/−	+/−	+/−	+/−	+	+/−	+/−	+/−
Acinetobacter baumanii 125	−	+/−	−	−	−	−	+/−	+	+/−	+/−	+/−
Acinetobacter baumanii 135	+	+	+	+	+	+	+	+	+	+	+
Acinetobacter baumanii 136	+/−	+	+	+	+	+	+	+	+	+	+
Acinetobacter baumanii 192	+	+/−	+/−	+/−	+/−	+	+	+	−	+/−	+/−
Acinetobacter baumanii 247	−	+/−	−	−	−	−	+/−	+	−	+/−	+/−

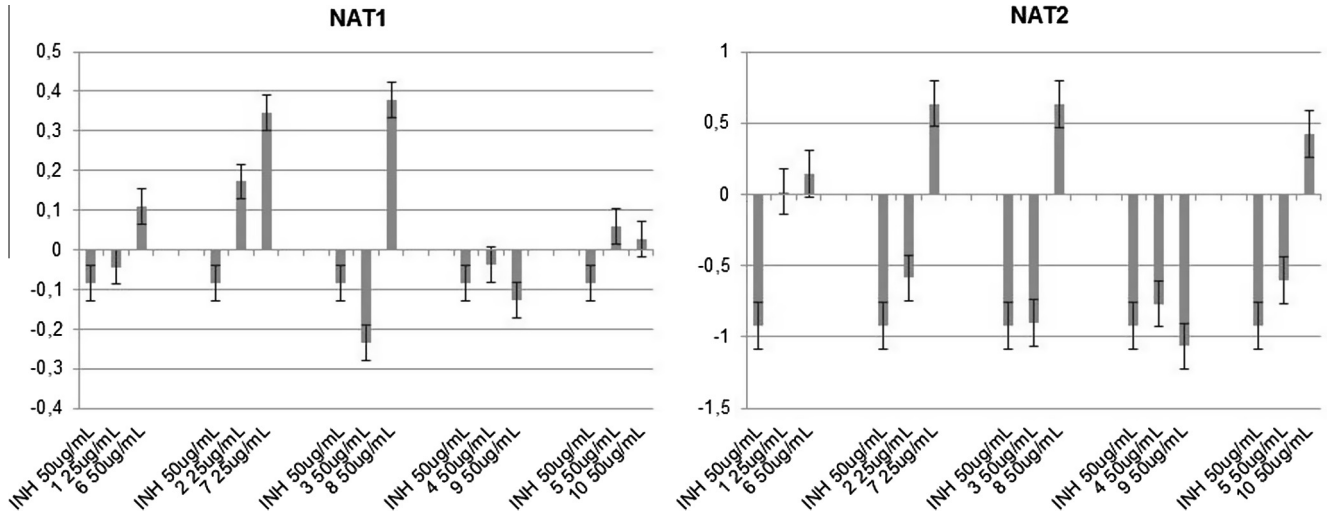

Figure 2. The influence of the new isoniazid derivatives **6**–**10** and their precursors **1**–**5** on the expression of NAT genes in the eukaryotic cells.

side effects that could be induced by the accumulation of the compound in high concentrations within the host. A similar behavior was also noticed for the compounds **8** and **10**, also exhibiting a good MIC on *Mycobacterium tuberculosis*. These results suggest that these compounds could be better tolerated than INH in the human host.

3. Conclusions

The synthesis of the new INH derivatives was performed with good yields and their structure was confirmed by physico-chemical analyses. In relation to the biological studies, the obtained results showed that the newly obtained derivatives of INH exhibit different biological activities, depending on their structure. The most active compound in terms of anti-oxidant and anti-*Mycobacterium tuberculosis* effects proved to be the compound **6**. This compound also increased the expression of NAT1 and NAT2 genes, which are implicated in the metabolism of the INH, demonstrating that it could be rapidly metabolized, and thus well tolerated within the human host. The largest spectrum of antibacterial activity was noticed for the compound **7** that was also the most cytotoxic one. The obtained results showed that the obtained compounds could represent potential candidates for the development of new anti-*M. tuberculosis* agents. However, more research is needed to improve their pharmacological properties, by increasing their antimicrobial activity and by reducing the risk of side-effects.

4. Materials and methods

4.1. Apparatus and chemicals

Starting materials (chemicals, TLC plates) and solvents were purchased from Sigma–Aldrich and Chimopar and used as received. ^{1}H and ^{13}C NMR spectra were recorded on a Varian Inova-300 spectrometer (at selected temperatures, in deuterated solvent DMSO-d_6, isotopic purity 99.9%); UV–vis spectra were

recorded on a UVD-3500 spectrometer, in methanol at ambient temperature. Melting points were determined with a Böethius apparatus and device Krüss. For biological tests the tested compounds were initially dissolved into DMSO to prepare a concentrated stock solution. Following additional dilutions were prepared in culture medium.

4.2. INH derivatives synthesis

Thioamides **1**–**5** were synthesized using the Pappalardo method.[23,24] Reaction mechanism is shown in Figure 1A.

The isonicotinoyl hydrazide derivatives were prepared by reaction between the appropriate thiocinnamamide-like thioamides **1**–**5** (1.0 equiv) with INH (1.0 equiv).

To 1 mmol (0.137 g) of INH dissolved in 3.5 mL water was added 1 mmol of appropriate thioamide dissolved in 5 mL ethanol and 10 μL of hydrochloric acid (37%). The mixture was refluxed for 1–2 h and then stirred for 8 days at room temperature. The products were separated on preparative TLC using silica gel plates or on chromatographic columns filled with silica gel and the mixture AcOEt:CH_2Cl_2:CH_3OH = 5:3:2 as eluent.

Purities of the synthesized compounds were checked by thin layer chromatography on silica gel.

4.2.1. Isonicotinic acid *N′*-(1-amino-1-mercapto-3-phenyl-propen-1-yl)-hydrazide (6)

Yield: 74%.

Elemental analysis: $C_{15}H_{16}N_4OS$, M = 300; calcd: C = 60.00, H = 5.33, N = 18.67, S = 10.67; found: C = 59.94, H = 5.36, N = 18.72, S = 10.56.

^{1}H NMR (DMSO-d_6, T = 303 K, δ ppm, J Hz): 10.08 (br s, 1H, deuterable); 9.53 (br s, 1H, deuterable); 9.25 (br s, 1H, deuterable); 8.71 (d, 2H, H-1, H-5, 6.1); 7.73 (d, 2H, H-2, H-4, 6.1); 7.65 (d, 1H, H-9, 15.6); 7.59 (dd, 2H, H-11, H-15, 1.3, 8.2); 7.41 (dd, 2H, H-12, H-14, 7.5, 8.2); 7.34 (tt, 1H, H-13, 1.3, 7.5); 7.01 (d, 1H, H-8, 15.6); 4.53 (br s, 2H, H-N, deuterable).

^{13}C NMR (DMSO-d_6, T = 303 K, δ ppm): 163.80 (C-6); 150.10 (C-1, C-5); 141.02 (C-9); 140.18 (C-3); 134.69 (C-10); 129.69 (C-8); 128.96 (C-11, C-15); 128.59 (C-13); 127.74 (C-12, C-14); 120.90 (C-2, C-4); 76.51 (C-7).

R_f (silicagel, AcOEt:CH_2Cl_2:CH_3OH = 5:3:2): 0.58.

4.2.2. Isonicotinic acid *N′*-[1-amino-1-mercapto-3-(*p*-chloro-phenyl)-propen-1-yl]-hydrazide (7)

Yield: 75%.

Elemental analysis: $C_{15}H_{15}ClN_4OS$, M = 334.5; calcd: C = 53.81, H = 4.48, Cl = 10.61, N = 16.74, S = 9.57; found: C = 53.77, H = 4.51, Cl = 10.67, N = 16.71, S = 9.59.

^{1}H NMR (DMSO-d_6, T = 303 K, δ ppm, J Hz): 10.11 (br s, 1H, deuterable); 9.54 (br s, 1H, deuterable); 9.25 (br s, 1H, deuterable); 8.73 (d, 2H, H-1, H-5, 5.9); 7.74 (d, 2H, H-2, H-4, 5.9); 7.63 (d, 1H, H-9, 15.4); 7.58 (d, 2H, H-11, H-15, 8.1); 7.42 (d, 2H, H-12, H-14, 8.1); 6.98 (d, 1H, H-8, 15.4); 4.54 (br s, 2H, H-N, deuterable).

^{13}C NMR (DMSO-d_6, T = 303 K, δ ppm): 163.96 (C-6); 150.17 (C-1, C-5); 140.58 (C-9); 140.25 (C-3); 134.42 (C-13); 134.02 (C-10); 129.52 (C-8); 128.65 (C-11, C-15); 127.56 (C-12, C-14); 120.98 (C-2, C-4); 76.59 (C-7).

R_f (silicagel, AcOEt:CH_2Cl_2:CH_3OH = 5:3:2): 0.65.

4.2.3. Isonicotinic acid *N′*-[1-amino-1-mercapto-3-(*p*-tolyl)-propen-1-yl]-hydrazide (8)

Yield: 80%.

Elemental analysis: $C_{16}H_{18}N_4OS$, M = 314; calcd: C = 61.15, H = 5.73, N = 17.83, S = 10.19; found: C = 61.28, H = 5.78, N = 17.86, S = 10.06.

^{1}H NMR (DMSO-d_6, T = 303 K, δ ppm, J Hz): 10.08 (br s, 1H, deuterable); 9.56 (br s, 1H, deuterable); 9.30 (br s, 1H, deuterable); 8.74 (d, 2H, H-1, H-5, 5.9); 7.71 (d, 2H, H-2, H-4, 5.9); 7.65 (d, 1H, H-9, 15.6); 7.54 (d, 2H, H-11, H-15, 8.3); 7.35 (d, 2H, H-12, H-14, 8.3); 7.04 (d, 1H, H-8, 15.6); 4.57 (br s, 2H, H-N, deuterable); 2.47 (s, 3H, H-16).

^{13}C NMR (DMSO-d_6, T = 303 K, δ ppm): 163.95 (C-6); 150.21 (C-1, C-5); 141.06 (C-9); 140.28 (C-3); 137.16 (C-13); 133.98 (C-10); 129.78 (C-8); 128.91 (C-11, C-15); 127.53 (C-12, C-14); 121.02 (C-2, C-4); 76.56 (C-7); 21.05 (C-16).

R_f (silicagel, AcOEt:CH_2Cl_2:CH_3OH = 5:3:2): 0.63.

4.2.4. Isonicotinic acid *N′*-[1-amino-1-mercapto-3-(*p*-methoxy-phenyl)-propen-1-yl]-hydrazide (9)

Yield: 65%.

Elemental analysis: $C_{16}H_{18}N_4O_2S$, M = 330; calcd: C = 58.18, H = 5.45, N = 16.97, S = 9.70; found: C = 58.24, H = 5.43, N = 16.95, S = 9.87.

^{1}H NMR (DMSO-d_6, T = 303 K, δ ppm, J Hz): 10.06 (br s, 1H, deuterable); 9.52 (br s, 1H, deuterable); 9.23 (br s, 1H, deuterable); 8.71 (d, 2H, H-1, H-5, 6.0); 7.72 (d, 2H, H-2, H-4, 6.0); 7.77 (d, 1H, H-9, 15.3); 7.51 (d, 2H, H-11, H-15, 8.6); 6.9 (d, 2H, H-12, H-14, 8.6); 6.77 (d, 1H, H-8, 15.3); 4.52 (br s, 2H, H-N, deuterable); 3.84 (s, 3H, H-16).

^{13}C NMR (DMSO-d_6, T = 303 K, δ ppm): 163.87 (C-6); 161.57 (C-13); 150.26 (C-1, C-5); 141.23 (C-9); 140.65 (C-3); 133.31 (C-10); 129.55 (C-8); 129.49 (C-11, C-15); 115.51 (C-12, C-14); 121.12 (C-2, C-4); 76.43 (C-7); 55.42 (C-16).

R_f (silicagel, AcOEt:CH_2Cl_2:CH_3OH = 5:3:2): 0.61.

4.2.5. Isonicotinic acid *N′*-[1-amino-1-mercapto-3-(*p*-ethoxy-phenyl)-propen-1-yl]-hydrazide (10)

Yield: 60%.

Elemental analysis: $C_{17}H_{20}N_4O_2S$, M = 344; calcd: C = 59.30, H = 5.81, N = 16.28, S = 9.30; found: C = 59.27, H = 5.78, N = 16.24, S = 9.36

^{1}H NMR (DMSO-d_6, T = 303 K, δ ppm, J Hz): 10.07 (br s, 1H, deuterable); 9.53 (br s, 1H, deuterable); 9.24 (br s, 1H, deuterable); 8.69 (d, 2H, H-1, H-5, 5.8); 7.67 (d, 2H, H-2, H-4, 5.8); 7.76 (d, 1H, H-9, 15.23); 7.50 (d, 2H, H-11, H-15, 8.59); 6.89 (d, 2H, H-12, H-14, 8.59); 6.76 (d, 1H, H-8, 15.23); 4.55 (br s, 2H, H-N, deuterable); 4.07 (q, 2H, H-16, 7.03), 1.43 (t, 3H, H-17, 7.03).

^{13}C NMR (DMSO-d_6, T = 303 K, δ ppm): 163.89 (C-6); 159.25 (C-13); 150.18 (C-1, C-5); 143.94 (C-9); 140.52 (C-3); 132.56 (C-10); 129.40 (C-8); 129.10 (C-11, C-15); 115.98 (C-12, C-14); 121.31 (C-2, C-4); 76.25 (C-7); 63.68 (C-16); 14.73 (C-17).

R_f (silicagel, AcOEt:CH_2Cl_2:CH_3OH = 5:3:2): 0.59.

4.3. Antioxidant activity of INH derivatives

A DPPH solution in methanol was prepared at a 2×10^{-4} M concentration. INH, thioamides **1**–**5** and new derivatives **6**–**10** were dissolved also in methanol at a 0.1 mg/mL concentration. DPPH solution (1.8 mL) was mixed with the solution of each compound (0.2 mL) and kept in dark for 30 min. The absorbance of solutions was measured at 517 nm. The blank mixture was obtained from 1.8 mL DPPH solution and 0.2 mL methanol. TAC values were calculated according to Eq. 1.

4.4. Antibacterial activity

In vitro antimicrobial tests were carried out by an adapted agar-disk diffusion technique using a bacterial suspension of 0.5 McFarland density obtained from 24 h cultures. The antimicrobial activity of the newly synthesized compounds was determined against the following strains: of *Escherichia Enterobacter cloacae*,

Citrobacter koseri, *Morganella morganii*, *Proteus mirabilis*, *Acinetobacter baumanii*, *Pseudomonas aeruginosa*, *Peudomonas putida*, *Streptococcus hominis*, *Enterococcus faecalis* and *Staphylococcus aureus*. The solutions of tested compounds (5 μL of 1 mg/mL solution) were spotted on the solid medium previously seeded with the microbial inocula. The inoculated plates were incubated for 24 h at 37 °C.

4.5. Antimycobacterial activity

The antimycobacterial activities of the new synthesized compounds were assessed against *M. tuberculosis* clinical strain using the micro plate Alamar Blue assay (MABA) according to Franzblau et al.[34] Briefly, 200 μL of sterile deionized water was added to all outer-perimeter wells of sterile 96 well plates to minimize evaporation of the medium in the test wells during incubation. A serial dilution of the new synthesized compounds **6**–**10** and INH were made directly in 96 well on the plate in a volume of 50 μL of the Middlebrook 7H9 broth. The final drug concentrations tested ranged from 25 to 0.012 μg/mL.

An inoculum of *M. tuberculosis* (50 μL) was added in the wells. Plates were covered and sealed with parafilm and incubated at 37 °C for 8 days. After this time, 10 μL of a freshly prepared 1:1 mixture of Alamar Blue (0.1 mg/mL) reagent and 10% tween 80 was added to the plate and incubated at 37 °C for another 24 h. A blue color in the well was interpreted as no bacterial growth, and a pink color was scored as growth. The Minimal Inhibition Concentration (MIC) was defined as the lowest drug concentration, which prevented a color change from blue to pink.

4.6. Cell culture

The eukaryotic cell culture used in our assays was represented by HCT-8 (CCL-224) cells. The cells were maintained as an adherent culture in Dulbecco's Modified Essential Medium (DMEM) (Sigma, USA) supplemented with 10% heat-inactivated fetal bovine serum (Sigma, USA) at 37 °C, 5% CO_2, in a humid atmosphere.

4.7. Trypan blue exclusion assay

The cytotoxicity of new INH derivatives (**6**–**10**) was determined in HCT8 cells using Trypan Blue Exclusion method. A number of 3×10^5 HCT8 cells were seeded in 3.5-cm diameter wells and treated with 50 μg/ml INH derivatives. After 24 h, the cells were trypsinised, centrifuged 6 min at 1200 rpm and resuspended in 1 mL PBS. The cell suspension (50 μL) was incubated with 50 μL Trypan blue solution (0.4%). After 1 min, the mixture was placed in a haemocytometer and the viable (green)/non-viable (red) cells were counted. Each experiment was repeated three times.

4.8. Apoptosis detection

Apoptosis detection was made using Annexin V-FITC Apoptosis Detection Kit I (BD Bioscience Pharmingen, USA) according to manufacturer protocol.

A number of 3×10^5 HCT8 cells were seeded in 3.5-cm diameter wells and treated with 50 μg/mL INH derivatives (25 μg/mL for compound **7**) for 24 h. The total cells were resuspended in 100 μL of binding buffer and stained with 5 μL Annexin V-FITC and 5 μL propidium iodide for 10 min in dark. At least 10,000 events from each sample were acquired using a Beckman Coulter flow cytometer. The percentage of treatment affected cells was determined by subtracting the percentage of apoptotic/necrotic cells in the untreated population from percentage of apoptotic cells in the treated population.

4.9. Cell cycle analysis

The cells were harvested after treatment with 50 μg/mL of INH and compounds **1**–**10**, 25 μg/mL for compounds **1** and **2**, and 12.5 μg/mL for compound **7**, for 24 h, washed in a cold solution of PBS (pH 7.5), then fixed in cold 70% ethanol and stored at −20 °C overnight. The samples were then centrifuged, washed with PBS and then re-suspended in 100 μL PBS, treated with RNase A (1 mg/mL) and labeled with propidium iodide (100 μg/mL), incubated in the dark at room temperature for 30 min prior measurement. DNA content of cells was quantified on a Beckman Coulter EPICS XL flow cytometer and analyzed using FlowJo 8.8.6 software (Ashland, Oregon, USA).

4.10. Quantitative RT-PCR for NAT1 and 2 expressions

Total RNA was extracted with Trizol Reagent (Invitrogen, USA) according to the manufacturer' protocol from cells treated with INH and compounds **1**–**10** at non-toxic concentrations for 24 h. For each sample, 2 μg of total RNA was used for reverse transcription with High Capacity cDNA Reverse Transcription Kit with RNAse inhibitor (Applied Biosystem), and 50 ng cDNA from each sample was used in real time PCR reaction. Real Time PCR was performed on an ABI 7300 Real Time PCR System using pre-validated Taqman Gene Expression Assays kits (Applied Biosystems): NAT1 (Hs00265080_s1) and NAT2 (Hs01854954_s1). As endogenous control was used human beta actin. Each experiment was performed three times. Results were analyzed with RQ study software (Applied Biosystems). The $\Delta\Delta C_T$ method was used to compare the relative expression levels.

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Supplementary data

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