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In vitro advanced antimycobacterial screening of isoniazid-related hydrazones, hydrazides and cyanoboranes: Part 14

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Abstract—As a part of an ongoing search for new isoniazid-related isonicotinoylhydrazones (ISNEs), 2′-monosubstituted isonicotinohydrazides and cyanoboranes, some analogues belonging to these three series of compounds were further evaluated in an in vitro advanced antimycobacterial screening. The results here reported allowed us to extend their structure–activity relationships. A general correlation emerged between their lipophilicity and effectiveness against intracellular *M. tuberculosis*. On the whole, the most interesting result of this research was that some hydrazides and ISNEs proved to be more effective antimycobacterial agents than parental isoniazid in a TB-infected macrophage model.

In the last two decades, the AIDS epidemic and the deadly synergy between *Mycobacterium tuberculosis* and HIV have favoured the recrudescence of tuberculosis (TB) throughout the world.[1–3] The worldwide appearance of drug-resistant strains of *M. tuberculosis*,[4–9] along with social problems such as homelessness, increased poverty and immigration,[6] significantly contributed to this resurgence in both developing and industrialised countries. In addition, a scarce compliance with the current anti-TB treatment regimens, which are rather complex and lengthy, may favour the diffusion of tubercular bacilli. Combinations of two or more anti-TB drugs are used, generally for 6–12 months, in order to prevent the frequent onset of relapses due to resistant mycobacteria.[9–12] However, these therapies are often not very effective in immunodepressed patients.

Consequently, although efficacious anti-TB drugs are available, TB is still a serious global threat to public health and continuing search is imperative for new antimycobacterial agents and therapeutical regimens.

In the last few years, we have synthesised and assayed a large number of lipophilic analogues of isoniazid (INH), a first-line anti-TB drug, and we have found that several of them have good in vitro antimycobacterial properties.[13–20] According to our design, the increased lipophilicity of these derivatives should facilitate diffusion through biomembranes, thus enhancing antimycobacterial effectiveness and, possibly, extending the activity spectrum to non-tubercular mycobacteria (NTM); these latter are often responsible for opportunistic infections in AIDS and hospitalised patients and are sensible only to very few drugs.

In particular, fluorinated isonicotinoylhydrazones (ISNEs), 2′-monosubstituted isonicotinohydrazides and, to a lesser extent, their cyanoborane adducts proved to be among the most promising INH derivatives.[14] Interestingly, we found that, at concentrations much lower than the corresponding MICs in culture media, most of such compounds were effective in killing *M. tuberculosis* growing within macrophages and, moreover, some isonicotinohydrazides were shown to be more active than parental INH in this macrophage assay.[14] These findings were considered to be of interest, because *M. tuberculosis* is an intracellular parasite living and multiplying inside macrophages and the goal of an antimycobacterial therapy is to eradicate this pathogen both in the extracellular and intracellular environment.

In this context, we now report on the in vitro antimycobacterial activities of some other ISNEs and related hydrazides and cyanoboranes (**1**–**10**, Fig. 1); the results of the advanced screening here discussed allowed us to

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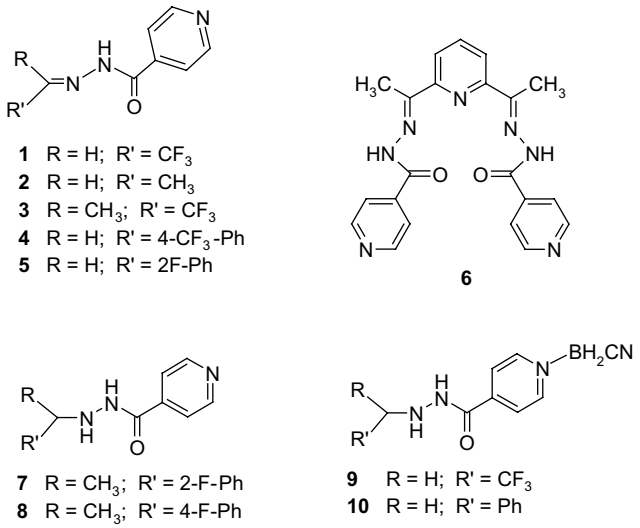

Figure 1.

acquire further information about the structure–activity relationships of these three classes of INH derivatives.

ISNEs **1**–**6**, isonicotinohydrazides **7**, **8** and cyanoboranes **9**, **10** were synthesised according to previously reported procedures[20–22] and characterised by means of IR, ^{1}H and ^{13}C NMR.[23] Their lipophilicity was measured by means of a reversed-phase TLC system[24] and expressed as Rm[25] (Table 1). Chromatographic Rm values are well-known lipophilicity indexes, calculated as $\log(1/R_f - 1)$; the higher the lipophilicity of a compound, the higher the Rm value.

The in vitro antimycobacterial evaluation was performed according to the Tuberculosis Antimicrobial Acquisition and Coordinating Facility (TAACF) antituberculosis drug discovery program,[6] which consists of different subsequent antimycobacterial assays.

In the TAACF primary screening all compounds **1**–**10** had exhibited good in vitro activity against *M. tuberculosis H37Rv*, with MIC values ranging between 0.025 and 1.6 μg/mL,[14,17] and *M. tuberculosis* Erdman, with MICs between 0.025 and 12.5 μg/mL.[14] These data were included in Table 1 in order to complete the discussion. In addition, compounds **1**–**10** had low in vitro cytotoxicity in VERO cells: in fact, their selectivity indexes (SI = IC_{50} Vero cells/MICs) were generally higher than 78 up to >2500 (Table 1).

Therefore, compounds **1**–**10** were selected for further advanced antimycobacterial assays.

In particular, they were tested for killing *M. tuberculosis* Erdman (ATCC 35801) in monolayers of mouse bone marrow macrophages, as previously described,[26] at four fold concentrations equivalent to 0.25, 1, 4 and 16 times the MIC. EC_{90} and EC_{99} are the lowest concentrations, respectively, effecting 90% and 99% reductions in colony forming units (CFU) at seven days compared to drug-free controls. This assay is considered a highly predictive method for evaluating the ability of new antimycobacterial agents to enter macrophages and kill intracellular bacilli. In fact, many factors can be considered that influence the activity of compounds within the infected cells (e.g., transport mechanisms, chemical modification in the macrophage cytoplasm, potential cytotoxic side effects). This test may thus be more useful for eventual therapeutical use in patients than broth culture assays.[26–28]

All tested compounds **1**–**10** were effective in killing *M. tuberculosis* Erdman within macrophages.

In particular ISNEs **1**–**6** displayed EC_{90} values ranging between 0.03 and 0.09 μg/mL and EC_{99} values ranging between 0.08 and 0.41 μg/mL (Table 1); their EC_{90}s were similar to that of INH, whereas their EC_{99}s (with the

Table 1. Rm values and in vitro antimycobacterial activities of compounds **1**–**10** against *M. tuberculosis H37Rv* and *M. tuberculosis* Erdman in broth culture and in infected macrophages

Compd	Rm[a]	MIC[b] H37Rv	MIC[b] Erdman	SI[c]	EC_{90}[d]	EC_{99}[e]	EC_{90}/MIC Erdman[f]	MBC[b] H37Rv
1	−0.265	0.05[g]	n.d.	302	0.05	0.25	n.d.	n.d.
2	−0.849	0.025[h]	0.025	>2500	0.03	0.08	1.2	0.05
3	−0.198	0.05[h]	0.1	1160	0.046	0.41	0.46	0.2
4	−0.049	0.05	0.1	>1250	0.04	0.09	0.4	0.2
5	−0.225	0.05[g]	0.05[g]	168[g]	0.05	0.14	1.00	n.d.
6	n.d.	⩽0.2	0.39	>320.5	0.09	0.29	0.23	12.5
7	−0.094	1.6[g]	3.13[g]	78.12[g]	2.54	6.06	0.81	n.d.
8	−0.082	1.6[g]	1.56[g]	>78[g]	1.84	5.25	1.18	n.d.
9	−0.220	0.78[g]	12.5	122	1.14	10.58	0.091	3.13
10	−0.089	0.80[g]	12.5	n.d.	0.116[g]	1.49[g]	0.0093	1.56
INH	−0.861	0.025–0.05	n.d.	>20000	0.03	0.42	n.d.	0.1

n.d. = not determined.
[a] Chromatographic Rm values were determined by means of a reversed-phase TLC as $\log(1/R_f - 1)$.
[b] MIC and MBC values are expressed in μg/mL.
[c] SI = IC_{50} Vero cells/MIC *H37Rv*.
[d] Concentration (μg/mL) effecting 90% reduction in CFU, at seven days compared to drug-free controls.
[e] Concentration (μg/mL) effecting 99% reduction in CFU, at seven days compared to drug-free controls.
[f] Compounds with EC_{90}/MIC < 16 are considered active.
[g] Ref. 14.
[h] Ref. 17.

exception of ISNE **3**) were lower than that of the parent drug (Table 1). Acetaldehyde isonicotinoylhydrazone **2** was shown to be the most effective among them; it had similar activity levels both within the macrophages and in broth culture, whereas ISNEs **3**, **4** and **6** were 2, 2.5 and 4.3 times more active within the infected host cells than in culture medium, respectively (Table 1). The increase in antimycobacterial activity within infected macrophages exhibited by these latter ISNEs might be correlated to their lipophilicity which, as their Rm values revealed (Table 1), was higher than that of compound **2**.

In the macrophage model, 2′-arylethylisonicotinohydrazides **7**, **8** displayed EC_{90}s similar to their MICs in culture medium (Table 1). Their good activity, however, was lower than that of the corresponding 2′-arylmethylisonicotinohydrazides;[14] these data were in accordance with our previous results that suggested that the arylmethyl moiety on N-2′ of isonicotinohydrazides was generally more beneficial than the arylethyl one,[14] although this latter brought about slightly higher hydrophobicity levels.

Cyanoboranes **9**, **10** had good activity against *M. tuberculosis* Erdman growing within infected macrophages (EC_{99} = 1.14 and 0.116 μg/mL, respectively, Table 1) and, on the other hand, only moderate effectiveness against the same strain in culture medium (MICs = 12.5 μg/mL); thus, they proved to be about 10 and 100 times more effective against bacilli growing within macrophages than in broth culture, respectively. This significant increase in antimycobacterial activity might be related to their lipophilicity which, due to the presence of the cyanoborane moiety, was greater than that of the corresponding ISNEs and isonicotinohydrazides (Table 1).[29] In fact cyanoborane **10**, which possessed greater hydrophobicity than its congener **9** (Table 1), exhibited the most appreciable increase in potency within infected macrophages. Thus the coordination of pyridinic N to BH_2CN moiety appeared to be beneficial for the antimycobacterial activity in TB-infected cells, while in culture medium it brought about a decrease in activity with respect to ISNEs and hydrazides.[14]

Concurrently with the testing of compounds in macrophages, MICs were determined against some strains of singly-drug-resistant (SDR) *M. tuberculosis* using a broth microdilution assay, the Microplate Alamar Blue Assay (MABA).[30–32]

Tested compounds **2**, **3**, **4**, **6**, **9** and **10** displayed from excellent (ISNEs **2**–**4** with MICs ranging from 0.025 to 0.2 μg/mL, Table 2) to moderate (cyanoboranes **9**, **10** with MICs ranging from 6.25 to 12.5 μg/mL, Table 2) activity against ethambutol- and rifampin-resistant strains. They also exhibited low MBC values[33] against the rifampin-resistant strain, particularly ISNEs **2**–**4** (Table 2). As expected, similarly to the other analogues that we previously assayed,[14] they were ineffective against INH-, ethionamide- and thiacetazone-resistant strains. These results clearly suggested that such INH derivatives should act with the same molecular mechanism as the parental drug; in fact, cross resistance between INH and ethionamide, as well as INH and thiacetazone, is well known.[34–36]

In addition ISNEs **2**–**4** and cyanoboranes **9**, **10** displayed low MBCs, rather close to their MICs, against *M. tuberculosis H37Rv* (Table 1), proving to have a bactericidal effect against this strain.

The TAACF antimycobacterial protocol also includes the screening of new agents against *M. avium* complex, an emerging NTM very dangerous for terminal AIDS patients in whom it frequently causes lethal infections.[6,37] In the preliminary screening, MICs were determined in the MABA against a strain of *Mycobacterium avium* (ATCC 25291) at the single concentration of 6.25 μg/mL. At this dose only ISNE **3** produced appreciable inhibition (93%), whereas INH displayed MIC > 32 μg/mL against the same strain.[31] Thus compound **3** was submitted to additional *M. avium* assays against five clinical isolates (labelled as 100, 101, 109, which are frequently encountered serotypes in AIDS patients, 108, 116) in Middlebrook 7H9 broth using the MABA and BACTEC 460 systems, at a range between 0.25 μg/mL and 32 μg/mL.[31,32] However, in this latter test MICs were equal or higher than 32 μg/mL.

Finally, the biological data here reported were consistent with our previous observations relevant to the antimycobacterial activities of ISNEs, 2′-monosubstituted isonicotinohydrazides and their cyanoborane adducts.[14] On the whole, the main results of this research can be summarised as follows: (a) in broth culture ISNEs and isonicotinohydrazides proved to be

Table 2. In vitro antimycobacterial activity of compounds **1**–**10** against SDR *M. tuberculosis* strains

Compd	MIC[a] INH-R	MIC[a] EMB-R	MIC[a] RIF-R	MIC[a] ETA-R	MIC[a] TAC-R	MBC[a] INH-R	MBC[a] RIF-R
2	>0.75	0.025	0.05	0.375	>0.75	> 0.75	0.05
3	>1.6	0.2	0.2	>1.6	>1.6	> 1.6	0.2
4	> 1.6	0.2	0.2	1.6	>1.6	> 1.6	0.4
6	>12.5	⩽0.2	⩽0.2	>12.5	>12.5	12.5	6.25
9	>25.0	12.5	12.5	>25.0	>25.0	> 25	6.25
10	>25.0	6.25	12.5	>25.0	>25.0	> 25	3.13
INH	>0.2	n.d.	n.d.	n.d.	n.d.	> 1.6	n.d.

INH-R = isoniazid-resistant. RIF-R = rifampin-resistant. EMB-R = ethambutol-resistant. ETA-R = ethionamide-resistant. TAC-R = thiacetazone resistant. n.d. = not determined.

[a] MIC and MBC values are expressed in μg/mL.

more effective antimycobacterial agents than cyanoboranes; (b) fluorine and trifluoromethyl groups were the most beneficial substituents on the benzene ring; the presence of the trifluoromethyl group linked to the iminic carbon of ISNEs or to the 2′-methylene group of hydrazides and cyanoboranes were also favourable; (c) the 2′-arylmethyl moiety had a more beneficial influence than the 2′-arylethyl one on the activity of isonicotinohydrazides and their cyanoborane adducts; (d) the in vitro cytotoxicity in Vero cells of cyanoboranes was higher than that of ISNEs and hydrazides. In fact we had previously found that they generally possessed antiproliferative properties, which appeared linked to the presence of the BH_2CN moiety;[29] (e) in the macrophage assay, most of the tested compounds displayed, at concentrations much lower than in culture media, excellent activity against *M. tuberculosis* growing within macrophages, which represent the relevant physiological environment for this pathogen. The increase in effectiveness within the infected cells generally appeared to be correlated to higher lipophilicity levels, observed in the fluorophenyl- and trifluoromethylphenyl substituted derivatives as well as in cyanoboranes. In this context, the most interesting goal achieved in this research was that some compounds belonging to the hydrazides and ISNEs series were shown to be more effective than parental INH against intracellular *M. tuberculosis*.

These promising results supported our initial work hypothesis that the increased lipophilicity of these INH derivatives should have a beneficial influence on their antimycobacterial activity, particularly enhancing the uptake and potency within cells.

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