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Synthesis and in vitro antimycobacterial activity of 3-substituted 5-hydroxy-5-trifluoro[chloro]methyl-4,5-dihydro-1 *H*-1-(isonicotinoyl) pyrazoles

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Abstract

A series of 3-substituted 5-hydroxy-5-trifluoro[chloro]methyl-1*H*-1-isonicotinoyl-4,5-dihydropyrazoles (**2a–i**) were synthesised by the cyclocondensation reaction of 4-methoxy-1,1,1-trifluoro[chloro]-4-(substituted)-alk-3-en-2-ones (**1a–i**) and isoniazid (INH). Their in vitro antimicrobial activity was tested against INH-susceptible *Mycobacterium tuberculosis* H37Rv, INH-resistant clinical *M. tuberculosis* isolates and non-tuberculous mycobacteria. Amongst the synthesised compounds, 5-hydroxy-5-trifluoromethyl-4,5-dihydro-1*H*-1-(isonicotinoyl)-pyrazole (**2a**) and 5-hydroxy-3-(4-methylphenyl)-5-trifluoromethyl-4,5-dihydro-1*H*-1-(isonicotinoyl) pyrazole (**2d**) were found to be the two most active agents against susceptible *M. tuberculosis* and several INH-resistant strains. The compound 3-(2-furyl)-5-hydroxy-5-trifluoromethyl-4,5-dihydro-1*H*-1-(isonicotinoyl)pyrazole (**2f**) was active against all the INH-resistant strains regardless of the genetic background at concentrations two- to four-fold its minimum inhibitory concentration against *M. tuberculosis* H37Rv. These compounds were inhibitors of mycolic acid biosynthesis, in agreement with the utilisation of the INH scaffold for their design. Interestingly, the most active compound against *M. tuberculosis*, 5-hydroxy-5-trifluoromethyl-4,5-dihydro-1*H*-1-(isonicotinoyl)-pyrazole (**2a**), was even more potent than INH against non-tuberculous mycobacteria.
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Keywords: Pyrazoles; Pyrazolines; *Mycobacterium tuberculosis*; Antimycobacterial activity

1. Introduction

Annually, nine million new cases of tuberculosis (TB) occur worldwide causing two million deaths [1]. In addition to human immunodeficiency virus (HIV) co-infection and the decline of socioeconomic conditions in several places of the world, the increase in the number of cases of TB with multidrug-resistant *Mycobacterium tuberculosis* (MDR-TB) has increased the difficulty in controlling this disease. Each year, 424 000 people develop MDR-TB, a form of TB that does not respond to standard treatment [2,3].

In the absence of an effective vaccine, treatment is the main tool for controlling the dissemination of TB. However, characteristics of the tubercle bacillus, such as slow growth, an impermeable envelope, ability to enter a latent stage and its intracellular location, restrict the number of available drugs for treatment. Moreover, the length of treatment (usually 6 months) makes it difficult for patients to comply with treatment [4].

In addition to TB, there are other mycobacterial species that are opportunistic pathogens and the number of cases of infections due to these non-tuberculous mycobacteria (NTM)

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is on the rise. Considering that most of the usual antimycobacterial drugs have little or no activity against NTM, it is important to have new drugs to treat these infections [5]. Isoniazid (INH), together with rifampicin and pyrazinamide, constitutes the backbone of a good outcome in the treatment of TB. INH has a simple structure, containing a pyridine ring and a hydrazide group, and both molecules are essential for its high activity against *M. tuberculosis* [6–9].

An important aspect to highlight is that INH is a pro-drug whose antimycobacterial action depends on its activation by a mycobacterial catalase–peroxidase enzyme (KatG) to generate a range of reactive radicals that attack multiple targets in *M. tuberculosis* [10–12].

The main target of INH is the pathway synthesising mycolic acids, essential components of the mycobacterial cell wall [13]. After several years of research, it is now widely accepted that INH inhibits InhA (enoyl-acyl carrier protein reductase) and part of the FASII system responsible for the synthesis of mycolic acids [14–16].

Resistance against INH is associated mostly with mutations or deletions in *katG* that block the activation step of the drug. In addition to mutations in this gene, other mutations associated with INH resistance have been identified in the promoter and coding region of *inhA* responsible for encoding the target for INH action. Further mutations in other genes have been reported to be associated with INH resistance, but occur less frequently and their association with INH resistance is less clear [16–20].

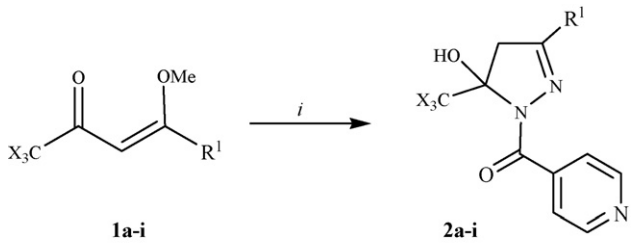
Development of new drugs against TB derived from already known molecules that have been in use for several years and have been proven safe and efficient is an attractive strategy from an economic, pharmaceutical and clinical viewpoint.

Since INH is a very important drug in the therapeutic arsenal for TB treatment, efforts are being made toward the development of new INH derivatives with greater activity, lower toxicity and fewer side effects [4,6,21–29].

Recently, the INH molecule was incorporated on a pyrazoline nucleus, showing activity against strains of *M. tuberculosis* both susceptible and resistant to INH. Interestingly, other compounds with a halogen-substituted phenyl group showed even greater activity [30]. In another study, a hydrophobic derivative of INH, 1-isonicotinyl-2-nonanoyl hydrazine, showed enhanced antimycobacterial activity against *M. tuberculosis* H37Rv. Therefore, it is possible that attaching chemical groups that aid the penetration of INH would make *M. tuberculosis* strains more susceptible to this drug [31]. Although many methods for the synthesis of 1*H*-pyrazoles and their derivatives have been published, attempts to perform the synthesis of simple 4,5-dihydro-1*H*-pyrazoles (2-pyrazolines) have not yet been successful [32,33]. By conventional procedures, pyrazoles have been obtained by directly reacting β-diketones with hydrazine. However, in most cases 5-hydroxy-4,5-dihydro-1*H*-pyrazoles have been obtained when the N-1 atom is substituted with a strong electron-withdrawing group that stabilises the –OH group and possibly hinders the elimination of the water molecule and the subsequent aromatisation of the pyrazoline ring. In particular, the synthetic potential of β-alkoxyvinyl trihalomethyl ketones to obtain a series of novel trihalomethylated 4,5-dihydro-1*H*-pyrazoles (2-pyrazolines) has been explored and reported by our group [34].

The 1-isonicotinoyl-3-alkyl(aryl/heteroaryl)-5-trihalomethyl-5-hydroxy-4,5-dihydro-1*H*-pyrazoles **2a–d** [35], **2e**, **2f**, **2h** and **2i** [36] and **2g** [37] were prepared from the reaction of 4-alkyl(aryl/heteroaryl)-1,1,1-trihalo-4-alkoxy-3-alken-2-ones with isonicotinic acid hydrazide, according to our previous publications (Fig. 1).

In this study, the activity of nine 3-substituted 5-hydroxy-5-trifluoro[chloro]methyl-1*H*-1- isonicotinoyl- 4,5-dihydropyrazoles was evaluated against *M. tuberculosis* H37Rv, clinical INH-resistant isolates and a set of clinical NTM isolates.



1, 2	a	b	c	d	e	f	g	h	i
R^1	H	Me	Ph	4-MePh	2-thienyl	2-furyl	4-MePh	2-thienyl	2-furyl
X	F	F	F	F	F	F	Cl	Cl	Cl

Reagents and conditions: (*i*) **2a:** $NH_2NHC(O)C_5H_4N$, MeOH, r.t., 48 h; **2b-d:** $NH_2NHC(O)C_5H_4N$, MeOH, 60 - 65 °C, 16 h; **2e-i:** $NH_2NHC(O)C_5H_4N$, MeOH, 20 - 25 °C, 24 h;

Scheme 1. Preparation of the 1-isonicotinoyl-3-alkyl(aryl/heteroaryl)-5-trihalomethyl-5-hydroxy-4,5-dihydro-1*H*-pyrazoles **2a–i** from the reaction of 4-alkyl(aryl/heteroaryl)-1,1,1-trihalo-4-alkoxy-3-alken-2-ones with isonicotinic acid hydrazide.

2. Material and methods

2.1. Chemistry

The 1-isonicotinoyl-3-alkyl(aryl/heteroaryl)-5-trihalomethyl-5-hydroxy-4,5-dihydro-1*H*-pyrazoles (**2a–i**) were prepared from the reaction of 4-alkyl(aryl/heteroaryl)-1,1,1-trihalo-4-alkoxy-3-alken-2-ones with isonicotinic acid hydrazide, according to our previous publications [35–37] (Scheme 1). Compounds **2a–d** were prepared according to reference [35], compounds **2e**, **2f**, **2h** and **2i** were prepared according to reference [36] and compound **2g** was prepared according to reference [37,38]. Compounds **2a–i** were fully characterised by spectroscopic methods and gave satisfactory analytical and spectral data.

2.2. Minimum inhibitory concentration (MIC) determination

The antimicrobial activity of the INH derivatives was evaluated by determination of the MIC against INH-susceptible *M. tuberculosis* H37Rv (ATCC 27294) (MIC = 0.2 μg/mL or 1.45 μM) and several genetically characterised INH-resistant clinical isolates with MICs > 10 μg/mL (>72.9 μM) displaying the following genotypes: RGH101, *inhA* C(–15)T; RGH102, *kasA* (T6T), *inhA* (orf) S94A, *inhA* C(–35)T; RGH103, *kat*G S315T; and RGH104, *kas*A G269S. The isolates were maintained in Ogawa–Kudoh medium for ca. 14 days. The bacterial suspensions were prepared in sterile water containing 3 mm glass beads. The suspensions were homogenised by vortex agitation and the turbidity was adjusted in agreement with tube one of the scale of McFarland (3.2×10^6 colony-forming units/mL). The inoculum was prepared by diluting the bacterial suspension 1:25 in Middlebrook 7H9 OADC medium (4.7 g Middlebrook 7H9 base; Difco, Becton Dickinson) enriched with 10% (v/v) oleic acid–dextrose–albumin–catalase (BBL).

The resazurin method [39] was used for the determination of the MIC. Briefly, the assay is performed in 96-well microplates using resazurin as an indicator of cellular viability in 7H9 OADC medium with the INH and pyrazole derivatives dissolved in dimethyl sulfoxide (DMSO). MIC determination was carried out by two-fold serial dilutions of the drugs (range 100.0–0.2 μg/mL) dispensed into each well of a 96-well microtitre plate.

The NTM tested were clinical isolates of the species *Mycobacterium avium*, *Mycobacterium kansasii* and *Mycobacterium fortuitum*. Microplates inoculated with *M. kansasii* and *M. avium* were incubated for 5 days, whilst plates inoculated with *M. fortuitum* were incubated for 48 h before addition of resazurin. All determinations were performed in triplicate.

2.3. Analysis of mycolic acid and fatty acid biosynthesis

The effect of the synthesised compounds on mycolic and fatty acid biosynthesis was determined using mid log phase cultures of *M. tuberculosis* H37Rv grown on 7H9 ADST (albumin–dextrose–sodium chloride–Tween 80, 0.1% v/v). Treatment of 5 mL aliquots of this culture with each compound was performed at two concentrations, the MIC and 4 × MIC for 18 h, followed by addition of 1[^{14}C] acetic acid (1 μCi/mL of culture) and further incubation for another 18 h. Cleavage, derivatisation and extraction of fatty acids and mycolic acids such as methyl esters was carried out as described previously [40]. Mycolic acid methyl esters (MAMEs) and fatty acid methyl esters (FAMEs) were separated by thin layer chromatography (TLC) on silica gel G plates using six developments with petroleum ether and ethyl ether (95:5 v/v) as the solvent system. Autoradiography was performed by exposing the TLC plates to radiography film for 24 h at −80 °C before development.

3. Results and discussion

Nine pyrazole derivatives were evaluated against INH-susceptible *M. tuberculosis* and four INH-resistant clinical isolates. Five pyrazole derivatives (**2a**, **2b**, **2c**, **2d** and **2f**) showed activity against *M. tuberculosis* H37Rv with a MIC between 0.77 μM and 18.66 μM (MICs are given in μM for the sake of comparison with INH) (MIC = 1.45 μM; Table 1).

Analysis of the correlation between activity against *M. tuberculosis* H37Rv and the compound's chemical structure allows us to draw some conclusions. It is important to observe that trifluoromethyl-substituted pyrazoles were more active than the respective trichloromethyl-substituted pyrazoles. For example, compound **2d** (MIC = 2.2 μM) was ca. 114 times more active than its analogous compound **2g** (MIC ≥ 251.89 μM). The same trend was also observed for compounds **2f** (trifluoromethyl substituted; MIC = 9.6 μM) and its trichloromethylated pyrazole analogue **2i** (MIC > 268 μM). Antimicrobial activity was dependent on the R^1 substituent. The two most active compounds were **2a** (R^1 = H) and **2d** (R^1 = 4-Me-Ph), with very different substituents. However, antimicrobial activity decreased by many folds when R^1 = furyl (**2f**) and became inactive when R^1 = thienyl (**2e**). Furthermore, the substituents of the phenyl ring appear to have a significant effect on antimicrobial activity because the tolyl-substituted compound **2d** increases the activity by eight times with respect to phenyl-substituted compound **2c**. In addition, the results show that although they have a higher MIC than that observed for the susceptible strain, these molecules present activity against INH-resistant strains. The most active compounds against H37Rv (**2a** and **2d**) were also approximately six to eight times more active than INH against the RGH101 resistant strain that presents a C(−15)T promoter mutation in *inhA*, the second most common molecular basis of INH resistance in clinical isolates [41]. As expected, strain RGH102, which presents simultaneous mutations C(−35T) and S94A in *inhA,* showed a higher resistance level than RGH101. The main genetic

Table 1
In vitro antimycobacterial activity of pyrazole derivatives **2a–i** against five mycobacterium strains

Compound	Molecular formula	MIC (μM)				
		H37Rv	RGH101	RGH102	RGH103	RGH104
Isoniazid	$C_6H_7N_3O$	1.45	>72.9	>72.9	>72.9	>72.9
2a	$C_{10}H_8F_3N_3O_2$	0.77	12	24.13	48	24.13
2b	$C_{11}H_{10}F_3N_3O_2$	5.71	22.89	366.3	366.3	5.71
2c	$C_{16}H_{12}F_3N_3O_2$	18.66	74.63	>298.5	>298.5	18.66
2d	$C_{17}H_{14}F_3N_3O_2$	2.23	8.94	71.63	286.53	4.47
2e	$C_{14}H_{10}F_3N_3O_2S$	>293.26	>293.26	>293.26	>293.26	>293.26
2f	$C_{14}H_{10}F_3N_3O_3$	9.6	38.46	38.46	19.23	38.46
2g	$C_{17}H_{14}Cl_3N_3O_2$	≥251.89	≥251.89	>251.89	≥251.89	251.89
2h	$C_{14}H_{10}Cl_3N_3O_2S$	>257.07	>257.07	>257.07	≥257.07	>257.07
2i	$C_{14}H_{10}Cl_3N_3O_3$	>268.09	>268.09	>268.09	≥268.09	>268.09

MIC, minimum inhibitory concentration.

difference related to INH resistance between RGH101and RGH102 strains is the S94A mutation in the *inhA* gene. This mutation disturbs the hydrogen-bonding network, which could result in reduced binding of the INH–NAD adduct with InhA, the main target of INH. Thus, the combination of both mutations produces the expression of higher levels of a mutated InhA protein, which could be correlated with the high MIC values. Strain RGH103 carries a S315T mutation in *katG* that encodes a catalase–peroxidase responsible for the activation of INH. This is the most frequently detected mutation in clinical isolates accounting for up to 60% of the INH resistance in the world. Surprisingly, whilst this strain showed a high level of resistance to INH and compounds **2a** and **2d** (>50-fold, 62-fold and 128-fold, respectively, compared with the susceptible strain), it was killed by compound **2f** at concentrations two-fold higher than the MIC value obtained for the wild-type strain. This enables us to infer that the pyrazolinic constituent could be important for the antimicrobial action; however, the pyrazolidines alone did not demonstrate antimycobacterial activity (data not shown). Strain RGH104 displayed a 50-fold and 30-fold increase in the level of resistance to INH and compound **2a**, respectively, compared with the pan-susceptible strain. Interestingly, only a G269S mutation in *kasA* was identified, whilst no other mutations were found in *katG, inhA* or *ahpC*. It was demonstrated that this mutation could be found in INH-susceptible and INH-resistant strains, refuting its relationship with INH resistance. The clinical origin of this strain points towards the presence of yet unidentified mutations underlying the INH resistance phenotype, perhaps related to increased drug efflux or reduced permeability. Some other genes have been associated with resistance to INH such as *ndh*, *furA, nat* and *ahpC*, none of them targets for this drug. The complex scenario of the mechanism(s) of action and resistance to INH suggests that more, still undescribed, genes may play a role. Taking that into account, it is encouraging that compounds **2b**, **2c**, **2d** and **2f** showed activity against RGH104 with values of one-, two- and four-fold those determined for *M. tuberculosis* H37Rv. These quite unexpected results suggest that factors intrinsically related to the chemical structure of these compounds are critical for the antitubercular activity and warrant further work on these three compounds.

If we agree that all the molecules described here undergo activation by KatG, we could hypothesise that they would act like INH, inhibiting mycolic acid synthesis. Therefore, to evaluate this possibility the compounds were assayed at one- and four-fold their respective MIC values (only the results obtained at the highest concentration assayed are shown for simplicity). Our results showed that these compounds were active against *M. tuberculosis* in a manner comparable with that of INH, causing abrogation of mycolic acid synthesis without alteration of fatty acid synthesis (Fig. 1). However, we cannot rule out the possibility that secondary targets such as dihydrofolate reductase may exist, as has been reported for INH [12]. Moreover, the fact that compound **2f** is active against all the INH-resistant strains regardless of the genetic background at concentrations two- to four-fold its MIC against *M. tuberculosis* H37Rv strongly suggests that it might be having a different target or mechanism of action (Table 1).

Of practical consequence is our finding that compounds **2a**, **2b**, **2d** and **2e** are active (although to different extents) against NTM. Amongst this group of opportunistic pathogens that are responsible for an increasing number of infections, *M. avium* is the most prominent, being one of the leading causes of death in acquired immune deficiency syndrome (AIDS) patients. Other species such as *M. kansasii*, *Mycobacterium chelonae*, *M. fortuitum* and *M. abscessus* are steadily gaining importance as causes of infections in patients undergoing

Table 2
Activity of compounds **2a**, **2b**, **2d**, **2e** and isoniazid (INH) against non-tuberculous mycobacteria

Compound	MIC (μM)				
	2a	**2b**	**2d**	**2e**	INH[a]
M. avium	6.25	5.90	1.72	38.40	180
M. fortuitum	3.12	183	71.6	>306	180
M. kansasii	6.25	45.75	17.9	38.40	120

MIC, minimum inhibitory concentration.
[a] MIC values for INH are average values collected from the literature.

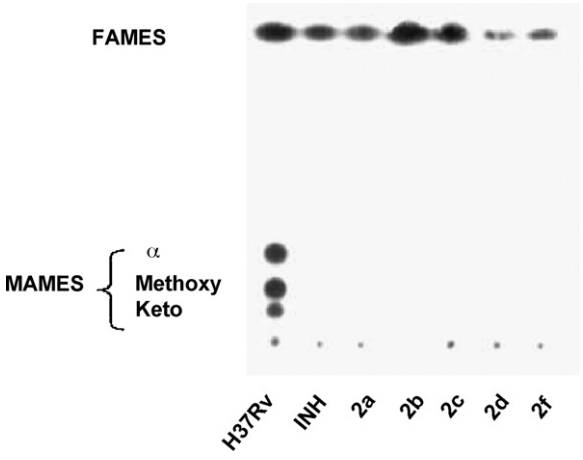


Fig. 1. Thin layer chromatography of [^{14}C] acetate-labelled cells of *Mycobacterium tuberculosis* H37Rv in the absence (lane 1) or presence (lanes 2–7) of isoniazid (INH) or compounds **2a**, **2b**, **2c**, **2d** and **2f**.

cosmetic or eye surgery. These mycobacteria are very difficult to eradicate using the current antimycobacterial drugs. They are quite resistant to INH (average MIC = 120 μM), this being a remarkable difference compared with *M. tuberculosis* (MIC = 1.45 μM) (Table 2). The results (Table 2) show that compound **2a** is a powerful agent against NTM, with MIC values in the range of 3.12–48 μM. Each compound showed activity against at least one NTM species, probably suggesting that there are subtle differences in the entry, activation or inhibition of the target in each species, a topic worth further investigation.

In summary, this study showed that at least two trifluoromethyl-substituted pyrazolidines, **2a** and **2d**, exhibited antimycobacterial activity in the same range as INH for the susceptible H37Rv strain and higher activity than INH against resistant strains such as RGH101, RGH102 and RGH103. More importantly, although compound **2f** is not the most active against *M. tuberculosis* H37Rv, it is the only compound having activity against all the tested INH-resistant strains, suggesting a different mechanism of action independent of KatG-mediated activation.

We believe that the INH moiety is not the only structure responsible for the antimycobacterial activity because pyrazolines with different substituents exhibited very different activities. For example, trifluoromethyl-substituted pyrazolines (**2a** and **2f**) were more active than their analogues, trichloromethyl-substituted pyrazolines (**2g** and **2i**). In addition, it was observed that the activity was also greatly influenced by the substituents R^1. Furthermore, it is conceivable that pyrazolidines derived from INH could exhibit lower toxicity and fewer side effects than INH itself because the highly reactive hydrazine group in the INH is chemically protected in the pyrazolidine derivatives. Our findings that at least one of the compounds described here exerted activity against clinical NTM isolates are very encouraging. Considering the difficulty of treating infections caused by these opportunistic pathogens, and the few drugs currently available for achieving this goal, we envision the possibility of using INH derivatives such as the ones reported here as additions to regular drug treatment. Finally, it is important to note that the diversity of the molecular basis to INH resistance determines different levels of resistance to INH analogues. The results reported herein should be useful in guiding future efforts to discover new compounds with increased antibiotic activity.

Acknowledgments

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Competing interests: None declared.

Ethical approval: Not required.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ijantimicag.2008.03.019.

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