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Short Report

Synthesis, Inhibition of *Mycobacterium tuberculosis* Enoyl-acyl Carrier Protein Reductase and Antimycobacterial Activity of Novel Pentacyanoferrate(II)-isonicotinoylhydrazones

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Tuberculosis remains among the top causes of death triggered by a single pathogen. Herein, a greener synthetic approach for isonicotinoylhydrazones is described using ultrasound energy. These compounds were used as starting materials for synthesizing pentacyanoferrate(II)-isonicotinoylhydrazones, which inhibited the reaction catalyzed by *Mycobacterium tuberculosis* 2-*trans*-enoyl-ACP(CoA) reductase (*Mt*InhA) in a time-dependent manner. The most active coordination complex showed an increase of more than ten-fold in the *Mt*InhA inhibition rate constant compared with lead pentacyano(isoniazid)ferrate(II) (IQG607). Additionally, the new series of metal-based compounds demonstrated antitubercular activity against a drug-susceptible *Mycobacterium tuberculosis* (Mtb) strain and was devoid of toxicity to mammalian cells (IC_{50} > 20 μmol L^{-1}, half maximal inhibitory concentration). Finally, one of the synthesized compounds showed intracellular activity similar to isoniazid in a macrophage model of Mtb infection, indicating that this chemical class may furnish novel structures to embark on the preclinical phase of anti-tuberculosis drug development.

Keywords: metallodrugs, tuberculosis, isoniazid, coordination complex

Introduction

Tuberculosis (TB) is an infectious disease caused mainly by *Mycobacterium tuberculosis* (Mtb). As reported by the World Health Organization (WHO), 9.6 million new cases of the disease and 1.5 million deaths were estimated worldwide in 2014.[1] A complicating factor in controlling this epidemic has been the emergence and spread of multidrug and extensively resistant Mtb strains (MDR and XDR, respectively).[1] Patients with susceptible TB are treated with isoniazid (INH), rifampicin, ethambutol and pyrazinamide, which form the basis of first-line drug therapy. However, the treatment of drug-resistant TB requires second-line drugs, which are more expensive, present a higher level of side effects and have limited effectiveness.[1] Therefore, there is an urgent need for innovative treatment options, which should be more effective and tolerable, for drug-susceptible and drug-resistant TB, including latent and dormant bacilli forms.[2]

Mtb is particularly susceptible to first-line INH drug; however, resistant strains have been reported since its early introduction for TB therapy.[3] INH is a pro-drug that must be activated by the mycobacterial katG-encoded catalase-peroxidase enzyme (katG), in the presence of manganese ions, NAD(H) and oxygen.[4-6] The katG-activated INH forms a covalent adduct with an NAD(H) cofactor, the isonicotinic acyl-NAD(H), which has been shown to be a slow, tight-binding competitive inhibitor of wild-type (WT) *Mycobacterium tuberculosis* 2-*trans*-enoyl-ACP(CoA)

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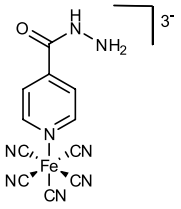
reductase (*Mt*InhA).[7,8] This enzyme, encoded by the InhA gene, belongs to the type II fatty acid synthase system (FAS-II) and catalyzes the NADH-dependent reduction of long-chain 2-*trans*-enoyl-ACP(CoA) substrates. The catalyzed conversion is the last reductive step of the elongation of mycolic acids, which have been considered the hallmark constituents of mycobacterial cell wall.[9] Inhibition of this enzyme by the isonicotinic acyl-NAD(H) adduct leads to the interruption of mycolic acid biosynthesis conducing to mycobacterial cell death.[10,11] It is noteworthy that the main molecular mechanisms of resistance to INH have been related to gene mutations in katG and InhA or its promoter region. The prevention of the activation of INH by the gene mutation S315T in katG has been found in up to 94% of the INH-resistant clinical isolates associated with high-level resistance.[12,13] Importantly, as mutations of the katG gene are the most common mechanism of resistance to INH, compounds that inhibit InhA without katG activation tend to be active against the majority of INH-resistant strains.[14-16]

Within this context, our research groups have described the pentacyano(isoniazid)ferrate(II) complex (IQG607), which is a slow-onset inhibitor of WT *Mt*InhA enzyme activity, with a true overall dissociation constant of 70 nmol L^{-1} (Figure 1).[17,18] Additionally, this compound is a slow-onset inhibitor of INH-resistant I21V, S94A and I47T InhA mutants, with overall dissociation constants similar to WT *Mt*InhA.[19] IQG607's mechanism of action has been hypothesized as an intramolecular electron transfer from the metallic center by a mechanism of self-activation, which favors a carbonyl-centered radical without katG participation.[20] It is important to mention that the broad uses of metals and the importance of their complexes in the medical area has increased over the years.[21] Metal-based complexes have showed advantages over small organic compounds such as improved aqueous solubility, controlled release, optimized pharmacokinetic parameters, specific tumor delivery and long-term anticancer efficiency prompting us to keep trying to develop a metal-containing anti-TB drug.[21]

Herein, some IQG607 analogs were synthesized and evaluated as possible inhibitors of *Mt*InhA-catalyzed reactions. The enhancement of the electron density on the hydrazide group could improve the stability of the proposed radical and, hopefully, lead to better *Mt*InhA-inhibition properties. First, the synthesis of isonicotinoylhydrazone precursors was performed through an ultrasound-assisted method. Afterward, isonicotinoylhydrazones were used for synthesizing novel pentacyanoferrate(II)-isonicotinoylhydrazones, which were evaluated as inhibitors of the catalytic activity of *Mt*InhA and the growth of the *M. tuberculosis* H37Rv strain. Finally, the intracellular activity of the compounds in a macrophage model of Mtb infection was also described.



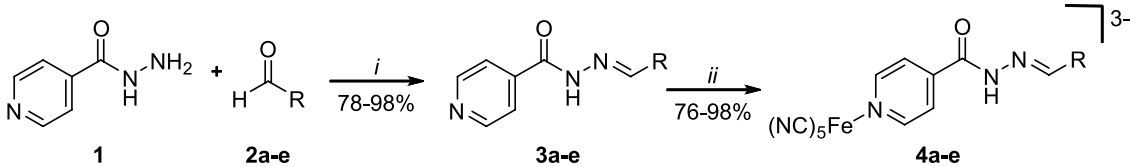
IQG607

Figure 1. Chemical structure of pentacyano(isoniazid)ferrate(II) (IQG607).

Results and Discussion

First, isonicotinoylhydrazones **3a**-**e** were synthesized by condensation reactions between isoniazid (**1**) and aldehydes **2a**-**e** using an ultrasound-mediated synthetic protocol (Scheme 1). In general, sonochemistry can provide important advantages over conventional thermal heating procedures, including generation of the products under milder and faster conditions with improved yields and purities.[22,23] Moreover, ultrasound-assisted techniques have been used in medicinal chemistry programs to accelerate the synthesis of drug-like compounds in accordance with green chemistry concepts. In particular, isonicotinoylhydrazones have been synthesized employing ultrasound and conventional thermal heating protocols.[24,25] In these procedures, the products were obtained with 87-99% yields after sonication of reaction mixtures by 40-80 min using an ultrasonic bath.[24,25] By contrast, conventional thermal heating furnished the isonicotinoylhydrazones with 73-97% yields after reflux for 6-8 h.[24,25]

The synthesis of hydrazones **3a**-**e** was accomplished in the presence of 10 mol% of acetic acid (AcOH) after sonication for 15 min, leading to the products in 78-98% yields (Scheme 1). Although water was also evaluated as a solvent in the sonochemical method, better yields were obtained with ethanol (see Supplementary Information (SI) section). Comparing both solvents, the yields were, on average, 30% higher using 2.5, 5.0 or 10.0 mol% of catalyst in ethanol (Scheme S1 in the SI section). The compounds **3a**-**e** were obtained in elevated purity and, thus, utilized in the subsequent reaction step without requiring any further purification. Comparing with already reported ultrasound metodology, the disparity in reaction times may be related to differences in power and form of energy transfer between the used equipment. Pentacyanoferrate(II)-isonicotinoylhydrazones **4a**-**e** were prepared by a similar procedure used before for the



1 **2a-e** **3a-e** **4a-e**

Compounds **2**, **3**, **4**	R
a	Ph
b	Me-4-C_6H_4
c	MeO-4-C_6H_4
d	F-4-C_6H_4
e	Fur-2-yl

Scheme 1. Conditions: (*i*) AcOH (10 mol%),))), EtOH, 15 min; (*ii*) $[Fe(CN)_5(NH_3)]^{3-}$, H_2O, 25 °C, 10 h.

lead compound IQG607.[17,26] Aminpentacyanoferrate(II) $[Fe(CN)_5(NH_3)]^{3-}$ was synthesized from sodium nitroprusside dissolved in ammonium solution followed by NH_3 gas bubbling into the reaction mixture, in accordance with a previously described protocol.[26] The complexes **4a**-**e** were obtained from the reaction between hydrazones **3a**-**e** and aminpentacyanoferrate(II) in water, at room temperature for 10 h, with yields of 76-98% (Scheme 1). The *E*-stereochemistry of imine-double bond was assigned for both series of compounds based on the magnitude of the imine-proton chemical shifts.[27]

The pentacyanoferrate(II)-isonicotinoylhydrazones **4a**-**e** were evaluated in line with previous studies describing the inhibitory activity of IQG607 on the reaction catalyzed by *Mt*InhA.[17] It is noteworthy that coupling of the pentacyanoferrate(II) group alters the physicochemical properties of compounds, such as aqueous solubility, polarizability and reactivity.[20] These chemical features are directly linked with the satisfactory pharmacokinetic profile, intermolecular binding and toxicological behavior of drug candidates. It is important to mention that compounds **3a**-**e** did not inhibit the *Mt*InhA activity (data not shown). By contrast, incubation of the enzyme with compounds **4a**-**e** in the presence and in the absence of NADH resulted in time-dependent inactivation. Notably, this has become a desirable feature for chemical compounds in drug discovery research because, unlike classical enzymatic inhibitors, accumulation of the substrate for the enzyme cannot reverse time-dependent inhibition processes.[28] The values of the observed first-order rate constants (k_{obs}) indicated that these compounds required no activation by mycobacterial katG as they were able to directly inhibit *Mt*InhA (Table 1). For purposes of comparison, the k_{obs} value for IQG607 was also determined. For the lead compound, k_{obs} was 0.89 min^{-1}, which is different from a previously described value[17] because of the different final concentration of the inhibitor. Among the synthesized series, **4a** and **4e** presented the lowest inhibition activities, with k_{obs} values of 0.38 and 0.53 min^{-1}, respectively. By contrast, compounds **4b** and **4c** were more effective than IQG607, with k_{obs} of 2.00 and 1.66 min^{-1}, respectively. These values represent increments of 2.2 and 1.8-fold in the *Mt*InhA inhibition rate constant compared with IQG607. Interestingly, compound **4d** was the most effective, presenting a k_{obs} value of 9.4 min^{-1} with $t_{1/2}$ of 0.07 min in the absence of NADH (Table 1). Compared with the lead complex, IQG607, **4d** increased more than ten-fold the *Mt*InhA inhibition rate constant. These findings reveal that the presence of a 4-fluoro substituent appears to favorably alter the inhibitory features of the synthesized complexes. Notably, compounds with bulkier moieties in the region of the aromatic ring, such as **4b** (R = Me) and **4c** (R = OMe), were more effective than non-substituted **4a** or furan-2-yl substituted **4e**. Moreover, the inhibitory activity of IQG607 was similar to that of the compounds with smaller molecular volumes. Indeed, previous works have reported the importance of bulky and hydrophobic moieties for *Mt*InhA inhibition.[29] However, further studies are needed to clarify the structure-activity relationship for this class of anti-TB drug candidates.

The *in vitro* inactivation of *Mt*InhA by the compounds **4a**-**e** did not require the presence of the NADH cofactor, such as reported for IQG607.[17] This can be observed in the higher value of k_{obs} when 10 μmol L^{-1} of NADH was pre-incubated with the compounds and *Mt*InhA (Table 1). Moreover, this finding suggests that the evaluated complexes could interact with *Mt*InhA at the NAD(H) binding site. Together, these results indicate that the mode of action of the metal-based compounds synthesized in this work is similar to that of IQG607, which exhibits a slow-binding inhibition profile and appears to act as a competitive inhibitor for both enzyme substrates, NADH and 2-*trans*-dodecenoyl-CoA (DD-CoA).[17,19]

In particular, the aqueous solubility of the pentacyanoferrate(II)-containing compounds was

Table 1. Observed first-order rate constant for the pentacyanoferrate(II)-isonicotinoylhydrazones **4a**-**e**

Compound	k_{obs} / min^{-1}		$t_{1/2}$ / min	
	0 μmol L^{-1} NADH	10 μmol L^{-1} NADH	0 μmol L^{-1} NADH	10 μmol L^{-1} NADH
4a	0.38 ± 0.03	0.024 ± 0.002	1.83 ± 0.17	28.8 ± 2.8
4b	2.00 ± 0.16	0.318 ± 0.02	0.35 ± 0.02	2.17 ± 0.16
4c	1.66 ± 0.15	0.11 ± 0.01	0.42 ± 0.03	6.07 ± 0.31
4d	9.44 ± 1.55	0.53 ± 0.04	0.07 ± 0.01	1.29 ± 0.1
4e	0.53 ± 0.05	0.048 ± 0.002	1.31 ± 0.13	14.40 ± 0.72
IQG607	0.89 ± 0.07	0.22 ± 0.01	0.77 ± 0.06	3.12 ± 0.16

k_{obs}: first-order rate constants; $t_{1/2}$: half-life of the first-order rate constant for enzyme inhibition.

increased compared with the isonicotinoylhydrazones. The presence of pentacyanoferrate group reduced the ClogP values, on average, 0.67 logarithmic units (Table 2). Incidentally, the complexes were solubilized in water in all experiments whereas the isonicotinoylhydrazones required dimethyl sulfoxide (DMSO) as co-solvent. The isonicotinoylhydrazones **3a**-**e** showed minimal inhibitory concentration (MIC) values, varying in the range of 5.9 to 6.9 μmol L^{-1} (Table 2). Compared with isoniazid dissolved in 2.5% DMSO, the compounds were approximately 2.7 to 3.1-fold less active against the *M. tuberculosis* H37Rv strain than the first-line drug. By contrast, hydrazones **3a**-**e** presented similar activity against the bacilli compared with the lead compound IQG607, which exhibited an MIC of 7.7 μmol L^{-1}. The pentacyanoferrate(II)-isonicotinoylhydrazones **4a**-**c** and **4e** presented MICs in the range of 6.0 to 12.4 μmol L^{-1}. Interestingly, the presence of a metallic center completely altered the potency order elicited by the hydrazone precursors. Among the pentacyanoferrate(II) series, compound **4a** displayed the highest activity, with an MIC of 6.0 μmol L^{-1}. It is noteworthy that the association of a metallic center attached at the pyridine ring produced a compound with an improved inhibitory capacity compared with its precursor isonicotinoylhydrazone **3a** (which had an MIC of 6.9 μmol L^{-1}). In addition, **4a** was more potent than the lead compound IQG607, based on their MIC values (6.0 *vs.* 7.7 μmol L^{-1}, respectively). On the other hand, the 4-fluoro-substituted complex **4d** did not show antimycobacterial activity at the maximum tested concentration of 24 μmol L^{-1}. It is important to keep in mind that this metal-based complex exhibited the most effective inhibition of *Mt*InhA activity among the evaluated structures. This fact reinforces the known difficulty of uniting potent target inhibition with chemical properties that allow for cellular potency in anti-TB drug discovery. Moreover, as no correlation was observed between *Mt*InhA inhibition and MIC values, further studies need to be provided to ascertain whether or not *Mt*InhA is the molecular target responsible for the antimycobacterial activity presented by the compounds.

Considering their MIC values, compounds **4a**-**c** and **4e** were selected for further investigation of possible *in vitro* cytotoxic effects on mammalian cells using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) protocol. HaCat (human keratinocyte) and RAW 264.7 (murine macrophage) cells were used in these experiments. Cellular viability was evaluated after exposing the cell lineages to the metal-based compounds, and the results were expressed as percentages of cell viability (Table 3). The *in vitro* incubation of the compounds, at concentrations of 10 and 20 μmol L^{-1}, did not significantly

Table 2. ClogP values and activity of the synthesized isonicotinoylhydrazones **3a**-**e** and pentacyanoferrate(II)-isonicotinoylhydrazones **4a**-**e** against the *M. tuberculosis* H37Rv strain

Compound	ClogP[a]	MIC / (μmol L^{-1})	Compound	ClogP[a]	MIC / (μmol L^{-1})
3a	1.62	6.9	**4a**	0.92	6.0
3b	2.12	6.4	**4b**	1.42	23.5
3c	1.69	5.9	**4c**	1.14	11.3
3d	1.76	6.3	**4d**	1.06	> 24
3e	0.79	6.7	**4e**	0.09	12.4
IQG607	–1.41	7.7	INH	–0.67	1.1[b]/2.2[c]

[a]ClogP calculated by ChemBioDraw Ultra version 13.0.0.3015; [b]INH (isoniazid) dissolved in DMSO; [c]INH dissolved in water.

Table 3. Cytotoxicity effects and intracellular activity of the pentacyanoferrate(II)-isonicotinoylhydrazones **4a-c** and **4e** against the virulent *M. tuberculosis* H37Rv strain in macrophages

Compound	Cell viability ± SEM[a] / %				Intracellular activity[b]
	HaCat		RAW 264.7		Mean $\log_{10}$ CFU *per* well ± SEM
	10 μmol L^{-1}	20 μmol L^{-1}	10 μmol L^{-1}	20 μmol L^{-1}	
4a	95 ± 9	95 ± 7	100 ± 10	95 ± 6	4.96 ± 0.16
4b	90 ± 6	94 ± 79	97 ± 1	92 ± 4	4.94 ± 0.15
4c	98 ± 2	98 ± 9	86 ± 5	95 ± 5	4.78 ± 0.04[d]
4e	94 ± 5	96 ± 5	93 ± 4	99 ± 2	4.86 ± 0.05[c]
INH	–	–	–	–	4.63 ± 0.12[d]
UNT	–	–	–	–	5.34 ± 0.06

[a]Untreated control wells were considered as 100% of cell viability. [b]Data are expressed as the means ± standard error (SEM) of triplicates for each compound tested at 10 μmol L^{-1}; [c]$p < 0.05$; [d]$p < 0.01$ compared with the untreated group (Dunnett post-test). HaCat: human keratinocyte cells; RAW 264.7: murine macrophage cells; INH: isoniazid; UNT: untreated.

affect the cell viability of either of the cell lines tested, indicating an apparent low toxicity of the compounds to mammalian cells and selectivity for Mtb at evaluated concentrations.

The favorable features elicited by the compounds prompted us to investigate their intracellular activity in a macrophage model of Mtb infection. The INH (10 μmol L^{-1})-treated group showed a decrease of 0.71 $\log_{10}$ ($p < 0.01$) in the CFU counts (colony forming units) compared with the untreated control (Table 3). Unfortunately, exposing infected macrophages to the compounds **4a** and **4b** did not show a statistically significant reduction in the CFU counts. It is important to mention that compound **4a** presented the best MIC value of this series, indicating a possible difficulty for the molecule in crossing cellular and mycobacterial barriers to inhibit Mtb growth inside macrophages. By contrast, treatment with the compounds **4c** and **4e** resulted in significant reductions in the CFU counts compared with the untreated control (Table 3). Notably, compound **4c** exhibited intracellular activity similar to INH, with no statistical difference compared with the INH CFU counts. This finding indicates that compound **4c**, even with higher MIC value than INH, was able to inhibit intracellular mycobacteria growth with similar potency to that elicited by first-line drug. Together with increment of 1.8-fold in *Mt*InhA inhibition rate constant compared with the lead IQG607, the coordination complex **4c** becomes a promising starting point of a new class of metallo-based drug candidates for the tuberculosis treatment.

Conclusions

Herein, we have proposed an improved method for synthesizing isonicotinoylhydrazones using ultrasound energy. This environmentally friendly synthetic protocol produced the compounds with satisfactory to elevated yields, shorter reaction times and high purity compared with conventional thermal heating protocols. Furthermore, pentacyanoferrate(II)-isonicotinoylhydrazones were also synthesized in satisfactory to high yields (76-98%) and with good purity. The coordination complexes were able to inhibit *Mt*InhA in a time-dependent manner at comparable or greater velocities than the lead compound IQG607. Additionally, the compounds showed activity against drug-sensitive *M. tuberculosis* H37Rv with no apparent toxicity for mammalian cells. Finally, some pentacyanoferrate(II)-isonicotinoylhydrazones presented activity in a macrophage-infected model, and one of them exhibited similar activity to that of the first-line drug isoniazid. Altogether, these results indicate that this class of compounds may furnish candidates for future progression from basic research to pre-clinical pharmacological and, if feasible, toxicological studies aiming at new anti-TB agents. Studies to try to understand the structural requirements for *Mt*InhA inhibition and cellular potency are in progress and these results will be communicated in the future.

Experimental

Synthesis and structure: apparatus and analysis

All commercially available solvents and reagents were obtained from commercial suppliers and used without further purification. The sonochemical method for the synthesis of isonicotinoylhydrazones was carried out with a standard probe (25 mm) connected to a 1500 Watt Sonics Vibra-Cell ultrasonic processor (Newtown, Connecticut, USA) equipped with integrated temperature control. The device operates at 20 kHz, and the amplitude was set to

20%. In all experiments, the temperature was raised to 77-81 °C (ethanol) or 88-94 °C (water) after sonication for 5-8 min and was maintained at this level until the end of the reaction times. Melting points were measured using a Microquímica MQAPF-302 apparatus. ^{1}H nuclear magnetic resonance (NMR) spectra were acquired on an Anasazi EFT-60 or Varian 400 spectrometer (^{1}H at 60.13 or 400.13 MHz, respectively). ^{13}C NMR spectra were acquired on a Varian 400 spectrometer (^{13}C at 100.6 MHz) (Federal University of Rio Grande do Sul, UFRGS/Brazil). DMSO-d_6 or D_2O were used as the solvent in 5 mm sample tubes. Splitting patterns were designated as follows: s, singlet; d, doublet; m, multiplet. High-resolution mass spectra (HRMS) were obtained for all compounds on an LTQ Orbitrap Discovery mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). This hybrid system combines an LTQ XL linear ion-trap mass spectrometer and an Orbitrap mass analyzer. The experiments were performed via direct infusion of the sample in MeOH-H_2O (1:1) with 0.1% formic acid (flow rate 10 μL min^{-1}) in positive-ion mode using electrospray ionization (ESI). Elemental composition calculations were executed using the specific tool included in the Qual Browser module of the Xcalibur (Thermo Fisher Scientific, release 2.0.7) software. Cyclic voltammetry measurements were obtained with a 757 VA Computrace (Metrohm, Switzerland). Voltammograms were acquired using a glassy carbon electrode as the working electrode and a platinum auxiliary electrode in 0.1 mol L^{-1} KCl solution at 25 °C. Fourier transform infrared (FTIR) spectra were recorded using a universal attenuated total reflectance (UATR) attachment on a PerkinElmer Spectrum 100 spectrometer in the wavenumber range of 650-4000 cm^{-1} at a resolution of 4 cm^{-1}. The CHN elemental analyses were performed on a PerkinElmer 2400 CHN elemental analyzer (São Paulo University, USP/Brazil).

General procedure for synthesis of isonicotinoylhydrazones **3a**-**e**

Isoniazid (**1**) (0.144 g, 1.05 mmol) and the appropriate aldehyde **2a**-**e** (1.0 mmol) were mixed in ethanol (20 mL) in the presence of 10 mol% of acetic acid as catalyst in a 50 mL beaker. The reaction mixture was sonicated for 15 min using an ultrasonic probe. After cooling to room temperature, the solvent was evaporated under reduced pressure. The obtained solids were washed with water (4 × 10 mL) under vigorous stirring followed by centrifugation. Afterward, the products were dried under reduced pressure and isolated in satisfactory purity without need of subsequent purification steps.

(*E*)-*N*'-Benzylideneisonicotinohydrazide (**3a**)

Yield: 95%; mp 190-191 °C (194-196 °C);[24] IR (UATR) ν / cm^{-1} 3,198 (NH), 3,027 (CH), 1,682 (C=O), 1,562 (C=N), 1,282, 1,149, 766, 684; ^{1}H NMR (60.1 MHz, DMSO-d_6) δ 7.48-7.91 (m, 7H, Ar-H, Py-H*), 8.52 (s, 1H, vinylic-H), 8.81 (d, 2H, *J* 5.9 Hz, Py-H), 12.08 (s, 1H, NH); ^{13}C NMR (100.6 MHz, DMSO-d_6) δ 121.5, 128.8, 130.3, 134.0, 140.5, 149.1, 150.3, 161.6; HRMS (FTMS + pESI) *m/z*, calcd. for $C_{13}H_{11}N_3O$ [M + H]$^+$: 226.0975, found: 226.0957; *Py-H: pyridine hydrogens.

(*E*)-*N*'-(4-Methylbenzylidene)isonicotinohydrazide (**3b**)

Yield: 85%; mp 188-190 °C (190-192 °C);[30] IR (UATR) ν / cm^{-1} 1,651 (C=O), 1,550 (C=N), 1,301, 814, 686; ^{1}H NMR (60.1 MHz, DMSO-d_6) δ 2.35 (s, 3H, CH_3), 7.29 (d, 2H, *J* 7.5 Hz, Ar-H), 7.68 (d, 2H, *J* 7.8 Hz, Ar-H), 7.85 (d, 2H, *J* 6.0 Hz, Py-H*), 8.47 (s, 1H, vinylic-H), 8.81 (d, 2H, *J* 5.6 Hz, Py-H), 12.02 (s, 1H, NH); ^{13}C NMR (100.6 MHz, DMSO-d_6) δ 21.0, 121.5, 127.2, 129.4, 131.3, 140.2, 140.5, 149.1, 150.3, 161.5; HRMS (FTMS + pESI) *m/z*, calcd. for $C_{14}H_{13}N_3O$ [M + H]$^+$: 240.1131, found: 240.1136; *Py-H: pyridine hydrogens.

(*E*)-*N*'-(4-Methoxybenzylidene)isonicotinohydrazide (**3c**)

Yield: 98%; mp 166-168 °C (170-173 °C);[24] IR (UATR) ν / cm^{-1} 3,202 (NH), 3,047 (CH), 1,654 (C=O), 1,596 (C=N), 1,512, 1,256, 830, 688; ^{1}H NMR (60.1 MHz, DMSO-d_6) δ 3.83 (s, 3H, OMe), 7.04 (d, 2H, *J* 8.8 Hz, Ar-H), 7.65-7.89 (m, 4H, Ar-H, Py-H*), 8.44 (s, 1H, vinylic-H), 8.80 (d, 2H, *J* 6.1 Hz, Py-H), 11.95 (s, 1H, NH); ^{13}C NMR (100.6 MHz, DMSO-d_6) δ 55.3, 114.3, 121.5, 126.5, 128.9, 140.6, 148.9, 150.3, 161.1, 161.4; HRMS (FTMS + pESI) *m/z*, calcd. for $C_{14}H_{13}N_3O_2$ [M + H]$^+$: 256.1081, found: 256.1076; *Py-H: pyridine hydrogens.

(*E*)-*N*'-(4-Fluorobenzylidene)isonicotinohydrazide (**3d**)

Yield: 78%; mp 193-195 °C (216-220 °C);[31] IR (UATR) ν / cm^{-1} 3,185 (NH), 2,955 (CH), 1,654 (C=O), 1,551 (C=N), 1,508, 1,231, 835, 687; ^{1}H NMR (60.1 MHz, DMSO-d_6) δ 7.17-7.46 (m, 2H, Ar-H), 7.72-7.89 (m, 4H, Ar-H, Py-H*), 8.49 (s, 1H, vinylic-H), 8.81 (d, 2H, *J* 5.6 Hz, Py-H), 12.09 (s, 1H, NH); ^{13}C NMR (100.6 MHz, DMSO-d_6) δ 115.8 (d, *J* 21 Hz), 121.4, 129.3 (d, *J* 8.7 Hz), 130.6 (d, *J* 2.5 Hz), 140.4, 147.9, 150.2, 162.7 (d, *J* 230 Hz), 164.4; HRMS (FTMS + pESI) *m/z*, calcd. for $C_{13}H_{10}FN_3O$ [M + H]$^+$: 244.0881, found: 244.0876; *Py-H: pyridine hydrogens.

(*E*)-*N*'-(Thiophen-2-ylmethylene)isonicotinohydrazide (**3e**)

Yield: 92%; mp 170-172 °C (165-167 °C);[25] IR (UATR) ν / cm^{-1} 3,268 (NH), 3,121 (CH), 1,646 (C=O), 1,618, 1,533 (C=N), 1,351, 1,291, 839, 764, 681; ^{1}H NMR (60.1 MHz,

DMSO-d_6) δ 6.67 (Br, 1H, Fur-H*), 7.00 (d, 1H, *J* 3.3 Hz, Fur-H), 7.78-7.88 (m, 3H, Fur-H, Py-H*), 8.38 (s, 1H, vinylic-H), 8.80 (d, 2H, *J* 5.8 Hz, Py-H), 12.02 (s, 1H, NH); ^{13}C NMR (100.6 MHz, DMSO-d_6) δ 112.2, 114.2, 120.9, 121.4, 138.6, 145.5, 150.1, 150.3, 163.8; HRMS (FTMS + pESI) *m/z*, calcd. for $C_{11}H_9N_3O_2$ $[M + H]^+$: 216.0768, found: 216.0773; *Fur-H: fur-2-yl hydrogens; *Py-H: pyridine hydrogens.

General procedure for synthesis of compounds **4a-e**

The coordination complexes **4** were prepared by a two-step synthetic procedure with minor modifications from an earlier reported method described for the isoniazid ligand (IQG607).[17,23] All synthetic processes were carried out at controlled temperature and protected from light. In a 250 mL flask, 0.298 g (1 mmol) of sodium nitroprusside was dissolved in 10 mL of ammonium solution (25%) with stirring. NH_3 gas was bubbled into the reaction mixture for 4 h on an ice bath at temperatures ranging from –1 to –2 °C. After the reaction was complete, a yellow solid was formed, characteristic of aminpentacyanoferrate(II) as an intermediate compound.[23] Lastly, the solid formed was filtered off, washed with absolute ethanol, and dried under reduced pressure. Without any further purification, 0.326 g (1 mmol) of aminpentacyanoferrate(II) ($Na_3[Fe(CN)_5(NH_3)]\cdot 3H_2O$) was dissolved in 8 mL of ultrapure water under argon flow. To the resulting solution, the respective isonicotinoylhydrazone (3 mmol) was added and the mixture was stirred for 10 h at 25 °C. Subsequently, the reaction mixture was filtered and the resulting solution was added dropwise to another vessel containing 100 mL of cold NaI solution in ethanol (1:10, m/v). Because of the efficiency of the precipitation process, the flask was stored at 4 °C for at least 16 h. Finally, the solid product was separated by centrifugation, washed with cold ethanol (2 × 25 mL) and dried under reduced pressure.

Pentacyano[(*E*)-benzylideneisonicotinohydrazide]ferrate(II) (**4a**)

Yield: 94%; λ (H_2O) / nm 456; IR (UATR) ν / cm^{-1} 2,038 (CN), 1,653 (C=O), 1,546 (C=N), 1,295, 758, 690; ^{1}H NMR (400.1 MHz, D_2O) δ 7.24-7.36 (m, 5H, Ar-H), 7.59 (d, 2H, *J* 8 Hz, Py-H*), 8.15 (s, 1H, vinylic-H), 8.99 (d, 2H, *J* 4 Hz, Py-H); ^{13}C NMR (100.6 MHz, D_2O) δ 120.3, 127.7, 129.0, 131.7, 132.5, 152.4, 157.3, 164.3, 174.9, 179.3; $E_{1/2}$: 390 mV (*vs.* Ag|AgCl); anal. calcd. for $C_{18}H_{11}FeN_8Na_3O + 3H_2O$: C, 40.47; H, 3.21; N, 20.98, found: C, 40.33; H, 3.09; N, 20.56%; *Py-H: pyridine hydrogens.

Pentacyano[(*E*)-(4-methylbenzylidene)isonicotinohydrazide]ferrate(II) (**4b**)

Yield: 95%; λ (H_2O) / nm 451; IR (UATR) ν / cm^{-1} 2,032 (CN), 1,626 (C=O), 1,528 (C=N), 1,265, 711, 670; ^{1}H NMR (400.1 MHz, D_2O) δ 2.17 (s, 3H, Me), 7.15 (d, 2H, *J* 8 Hz, Ar-H), 7.32 (d, 2H, *J* 8 Hz, Py-H*), 7.56 (d, 2H, *J* 8 Hz, Ar-H), 8.15 (s, 1H, vinylic-H), 9.02 (d, 2H, *J* 8 Hz, Py-H); ^{13}C NMR (100.6 MHz, D_2O) δ 20.7, 120.4, 127.9, 129.7, 130.0, 142.5, 152.7, 156.8, 163.1, 175.0, 179.5; $E_{1/2}$: 337 mV (*vs.* Ag|AgCl); anal. calcd. for $C_{19}H_{13}FeN_8Na_3O + 3H_2O$: C, 41.63; H, 3.49; N, 20.44, found: C, 41.26; H, 3.27; N, 20.19%; *Py-H: pyridine hydrogens.

Pentacyano[(*E*)-(4-methoxybenzylidene)isonicotinohydrazide]ferrate(II) (**4c**)

Yield: 76%; λ (H_2O) / nm 450; IR (UATR) ν / cm^{-1} 2,031 (CN), 1,606 (C=O), 1,513 (C=N), 1,262, 833, 702; ^{1}H NMR (400.1 MHz, D_2O) δ 3.55 (s, 3H, OMe), 6.81 (d, 2H, *J* 8 Hz, Ar-H), 7.32 (d, 2H, *J* 4 Hz, Py-H*), 7.58 (d, 2H, *J* 8 Hz, Ar-H), 7.99 (s, 1H, vinylic-H), 9.06 (d, 2H, *J* 4 Hz, Py-H); ^{13}C NMR (100.6 MHz, D_2O) δ 55.7, 114.4, 120.3, 125.7, 129.8, 138.1, 146.1, 157.4, 161.3, 164.6, 175.1, 179.4; $E_{1/2}$: 381 mV (*vs.* Ag|AgCl); anal. calcd. for $C_{19}H_{13}FeN_8Na_3O_2 + 3H_2O$: C, 40.45; H, 3.39; N, 19.86, found: C, 40.19; H, 3.16; N, 19.49%; *Py-H: pyridine hydrogens.

Pentacyano[(*E*)-(4-fluorobenzylidene)isonicotinohydrazide]ferrate(II) (**4d**)

Yield: 98%; λ (H_2O) / nm 457; IR (UATR) ν / cm^{-1} 2,027 (CN), 1,626 (C=O), 1,527 (C=N), 1,263, 711; ^{1}H NMR (400.1 MHz, D_2O) δ 7.11-7.15 (m, 2H, Ar-H), 7.40 (d, 2H, *J* 4 Hz, Py-H*), 7.72-7.75 (m, 2H, Ar-H), 8.25 (s, 1H, vinylic-H), 9.02 (d, 2H, *J* 8 Hz, Py-H); ^{13}C NMR (100.6 MHz, D_2O) δ 116.2, 120.5, 129.5, 130.4, 138.4, 151.8, 157.7, 162.9, 164.0, 175.1, 179.7; $E_{1/2}$: 377 mV (*vs.* Ag|AgCl); anal. calcd. for $C_{18}H_{10}FFeN_8Na_3O + 3H_2O$: C, 39.15; H, 2.92; N, 20.29, found: C, 38.91; H, 2.74; N, 20.01%; *Py-H: pyridine hydrogens.

Pentacyano[(*E*)-(thiophen-2-ylmethylene)isonicotinohydrazide]ferrate(II) (**4e**)

Yield: 78%; λ (H_2O) / nm 452; IR (UATR) ν / cm^{-1} 2,034 (CN), 1,622 (C=O), 1,542 (C=N), 1,296, 757, 686; ^{1}H NMR (400.1 MHz, D_2O) δ 6.44 (s, 1H, Fur-H*), 6.77 (s, 1H, Fur-H), 7.31 (d, 2H, *J* 4 Hz, Py-H*), 7.50 (s, 1H, Fur-H), 8.04 (s, 1H, vinylic-H), 8.99 (d, 2H, *J* Hz, Py-H); ^{13}C NMR (100.6 MHz, D_2O) δ 112.5, 117.3, 120.3, 138.2, 140.9, 146.3, 147.8, 157.3, 164.4, 175.0, 179.5; $E_{1/2}$: 426 mV (*vs.* Ag|AgCl); anal. calcd. for $C_{16}H_9FeN_8Na_3O_2 + 3H_2O$:

C, 36.66; H, 2.88; N, 21.38, found: C, 36.31; H, 2.64; N, 21.05%; *Fur-H: fur-2-yl hydrogens; *Py-H: pyridine hydrogens.

Biological assays

Time-dependent inhibition assays

Expression and purification of the recombinant WT InhA from *M. tuberculosis* were performed as previously described.[9,32] The protein concentration was spectrophotometrically determined by diluting 10 µL of purified enzyme to 500 µL in 100 mmol L^{-1} Pipes (pH 7.0) at 25 °C, assuming an extinction coefficient of 29,600 M^{-1} cm^{-1} at 282 nm. The substrate DD-CoA was synthesized and purified as described elsewhere.[9,33] Time-dependent inactivation reactions were performed using a UV-2550 UV-Vis spectrophotometer (Shimadzu, Japan) by monitoring the oxidation of NADH to NAD^+ at 340 nm (ε_{NADH} = 6220 M^{-1} cm^{-1}) due to the reduction of the DD-CoA substrate for a period of 60 s at 25 °C. The enzymatic assay was performed essentially as previously described.[17] In brief, 3 µmol L^{-1} of InhA was incubated with 500 µmol L^{-1} isonicotinoylhydrazone-pentacyanoferrate(II) compounds in the presence or absence of 10 µmol L^{-1} of NADH cofactor in 100 mmol L^{-1} Na_2HPO_4 pH 7.5 buffer. Aliquots from the mixture were taken at different times, and were added to a cuvette containing 100 µmol L^{-1} of DD-CoA and 200 µmol L^{-1} of NADH with a final volume of 500 µL in order to measure the remaining enzymatic activity after the incubation. It is noteworthy that stability of compounds in solution was evaluated by voltammetric methods for at least three days without any apparent modification.

The following equations were employed to determine k_{obs} and $t_{1/2}$ of the inhibitors, respectively:

$$v = v_0 e^{-k_{obs} t} \quad (1)$$

$$t_{1/2} = \frac{\ln 2}{k_{obs}} \quad (2)$$

where v is the remaining InhA enzyme activity at time t, v_0 is the enzyme activity at time zero, k_{obs} is the observed first-order rate constant for InhA inhibition, t is time in seconds, and $t_{1/2}$ is the half-life of the first-order rate constant for enzyme inhibition.

Mycobacterium tuberculosis inhibition assay

The measurement of MIC for each tested compound was performed in 96-well U-bottom polystyrene microplates. Isoniazid (control drug) and solutions of the compounds were prepared at concentrations of 1 mg mL^{-1} either in neat DMSO (**3a**-**k**) or in water (**4a**-**e** and IQG607). They were diluted in Middlebrook 7H9 medium containing 10% ADC (albumin, dextrose and catalase) to a concentration of 20 µg mL^{-1}. Serial two-fold dilutions of each drug in 100 µL of Middlebrook 7H9 medium containing 10% ADC were prepared directly in 96-well plates at concentration ranges of 10.0 to 0.02 µg mL^{-1}. Growth controls containing no antibiotic and sterility controls without inoculation were included. MIC was determined for the *M. tuberculosis* H37Rv strain, which was grown in Middlebrook 7H9 containing 10% OADC (oleic acid, albumin, dextrose and catalase) and 0.05% Tween 80. Cells were vortexed with sterile glass beads (4 mm) for 5 min to disrupt clamps and allowed to settle for 20 min. The supernatant was spectrophotometrically measured at an absorbance of 600 nm. The *M. tuberculosis* suspension was aliquoted and stored at –20 °C. Each suspension was appropriately diluted in Middlebrook 7H9 broth containing 10% ADC to achieve an optical density at 600 nm of 0.006, and 100 µL were added to each well of the plate except for the sterility controls. For the compounds solubilized in DMSO, a final concentration of 2.5% DMSO was maintained in each well. The plates were covered, sealed with parafilm, and incubated at 37 °C. After 7 days of incubation, 60 µL of 0.01% resazurin solution were added to each well, and incubated for an additional 48 h at 37 °C.[34] A change in color from blue to pink indicated the growth of bacteria, and the MIC value was defined as the lowest drug concentration that prevented the color change. Three tests were carried out independently, and the MIC values reported here were observed in at least two assays.

Cytotoxicity investigation

Cellular viability determination after incubation with the test compounds (**4a**-**c**, **4e**) was performed essentially as previously described.[35] RAW 264.7 and HaCat cells were grown in DMEM (Dulbecco's Modified Eagle medium) supplemented with 10% inactivated fetal bovine serum and 1% antibiotics (penicillin-streptomycin). The cells were maintained in culture bottles at 37 °C in a humidified atmosphere with 5% CO_2. Cells were seeded at 7×10^3 cells *per* well in a 96-well plate and incubated for 24 h to adhere. The medium was removed and replaced with 190 µL DMEM, and 10 µL of drug solutions, resulting in concentrations of 10 and 20 µmol L^{-1}. After incubation for 72 h for HaCat and 120 h for RAW 264.7 cells, MTT (1 mg mL^{-1}) was added and the resulting mixtures were incubated for 3 h at 37 °C under 5% of CO_2. The formazan crystals were dried at room temperature for at least 24 h and dissolved in DMSO. The absorbance was measured at 595 nm (Spectra Max M2e, Molecular Devices, USA).

The precipitated purple formazan crystals were directly proportional to the number of live cells with active mitochondria. The percentage of cell viability for the treated groups was reported considering the control wells (untreated) as 100% of cell viability:

$$\text{Cell viability (\%)} = \left(\frac{\text{absorbance of treated wells}}{\text{absorbance of control wells}} \right) \times 100$$

Data were expressed as mean of cell viability ± standard error of mean of three independent experiments performed in triplicate. The statistical analysis was performed by one-way analysis of variance, followed by the Dunnett post-test, using GraphPad Prism 5.0 (San Diego, CA, USA).

Intracellular activity investigation

To investigate the capacity of the compounds to kill intracellular mycobacteria, we tested the effects of **4a-c** and **4e** in a macrophage model of *M. tuberculosis* infection. The murine macrophage RAW 264.7 (obtained from Banco de Células do Rio de Janeiro (BCRJ), Brazil) was cultured in DMEM (Gibco) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% penicillin-streptomycin at 37 °C with 5% CO_2. Macrophage infection procedures were performed as previously described.[35] Briefly, macrophages were seeded in 24-well culture plates at a density of 105 cells *per* well in DMEM (supplemented with 10% FBS) and incubated for 24 h at 37 °C with 5% CO_2. The cells were then washed with sterile 0.9% saline solution to remove non-adherent cells. Infection of RAW 264.7 cells with *M. tuberculosis* H37Rv was performed at a multiplicity of infection of 1:1 (bacteria/macrophage) for 3 h at 37 °C with 5% CO_2. Infected RAW 264.7 cells were washed with sterile 0.9% saline solution to remove extracellular bacteria and replaced with 1 mL fresh DMEM (supplemented with 10% FBS).[35] Cells were then treated with the test compounds (at 10 μmol L^{-1}) and with the positive control drug isoniazid (10 μmol L^{-1}) in DMEM. After 5 days of incubation, each well was gently washed and the infected macrophages were then lysed with 0.025% SDS dissolved in sterile 0.9% NaCl solution. Lysates were serially diluted and plated on Middlebrook 7H10 Agar (Difco) supplemented with 10% OADC. Bacterial colony formation was registered after incubation of the plates for 15 days at 37 °C. To compare cell counts, the numbers were firstly converted into logarithms of CFU ($\log_{10}$ CFU). Data were evaluated by one-way analysis of variance (ANOVA), followed by the Dunnett post-test, using GraphPad Prism 5.0 (GraphPad, San Diego, CA, USA). Differences were considered significant at the 95% level of confidence. Each different drug was tested in triplicate, and the results are expressed as the log mean numbers of bacteria per well.

Supplementary Information

Supplementary information (optimization of ultrasound protocol and analytical data) is available free of charge at http://jbcs.sbq.org.br as PDF file.

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