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Synthesis and anti-tubercular activity evaluation of hydrazide-hydrazone derivatives

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Abstract

An expert view worth considering is that of researchers at the WHO, who in 2023 reported tuberculosis as still the second leading infectious killer globally after COVID-19, with drug resistance making the situation progressively more difficult to manage. Isoniazid and rifampicin remain first-line treatments, but resistance mutations in the *inhA* and *rpoB* genes increasingly compromise their utility, driving the need for structurally diverse scaffolds that inhibit novel mycobacterial targets. This research describes the synthesis of six hydrazide-hydrazone derivatives (HH-01 to HH-06) carrying varied aryl substituents at the hydrazone terminus, their characterisation by IR, ¹H NMR, ¹³C NMR, and mass spectrometry, and their evaluation for *in vitro* anti-tubercular activity against *Mycobacterium tuberculosis* H37Rv and *Mycobacterium abscessus* by the microplate Alamar Blue assay. Compound HH-03, bearing a 4-methoxyphenyl substituent at the hydrazone nitrogen, showed the most potent activity with MIC values of 1.56 µg/mL against *M. tuberculosis* and 3.12 µg/mL against *M. abscessus*, comparing favourably with isoniazid (MIC 0.78 µg/mL) for the former and surpassing it for the latter. Structure-activity analysis identified the 4-methoxy group at the 4-position as the most favourable substituent, while electron-withdrawing nitro groups at the 4-position markedly reduced activity. These findings establish HH-03 as a lead compound for further optimisation and mechanistic investigation.

Keywords: Hydrazide, hydrazone, anti-tubercular, *Mycobacterium tuberculosis*, minimum inhibitory concentration, structure-activity relationship, Alamar Blue assay, drug resistance

Introduction

Tuberculosis (TB) killed an estimated 1.3 million people in 2022 and infected roughly 10.6 million new cases globally, according to the most recent WHO Global TB Report. The emergence of multidrug-resistant TB (MDR-TB)—defined as resistance to at least isoniazid and rifampicin, the two most powerful first-line agents—and extensively drug-resistant TB (XDR-TB) has made treatment regimens longer, more toxic, and substantially less effective. Australia, while classified as a low-incidence country, faces an increasing burden from imported MDR-TB cases and from opportunistic mycobacterial infections in immunocompromised patients, particularly *M. abscessus*, a rapidly growing non-tuberculous mycobacterium that is intrinsically resistant to most standard anti-TB drugs. Hydrazide and hydrazone functional groups have long been associated with anti-mycobacterial activity—isoniazid (isonicotinoyl hydrazide) itself is the archetypal example. Hydrazide-hydrazones, which combine both functional groups in a single scaffold, have attracted recent medicinal chemistry attention because the C = N imine bond of the hydrazone portion allows tuning of electronic properties through substitution at the aryl ring, while the NHNH moiety provides a hydrogen-bond donor pair capable of interacting with enzyme active sites. Several recent accounts report that aryl hydrazide-hydrazones inhibit mycobacterial enoyl-ACP reductase (InhA), the same target as isoniazid, but through a different binding mode that may overcome INH-resistance mutations. Despite this promise, the systematic exploration of substituent effects at the hydrazone aryl terminus remains incomplete, and comparative data against both *M. tuberculosis* and *M. abscessus* using the same assay format are scarce. This research addresses these gaps by synthesising six compounds with a common isonicotinoyl hydrazide

core but varied aryl substituents, characterising them spectroscopically, and evaluating their MIC values against both mycobacterial species under standardised conditions.

Materials and Methods

Materials

Isonicotinoyl hydrazide (isoniazid, 99%), aromatic aldehydes (4-methoxybenzaldehyde, 4-chlorobenzaldehyde, 4-nitrobenzaldehyde, benzaldehyde, 3-methoxybenzaldehyde, 2-methylbenzaldehyde, all $\geq 98\%$), glacial acetic acid, and ethanol (absolute, 99.8%) were obtained from Sigma-Aldrich (Sydney, Australia). Silica gel 60 (Merck, 230-400 mesh) was used for column chromatography. Solvents for NMR spectroscopy (DMSO- d_6 , $CDCl_3$) were from Cambridge Isotope Laboratories. Research was conducted at the Department of Pharmacology and Medicinal Chemistry, Great Barrier University, Brisbane, Queensland, Australia, and at the Tasman Institute of Chemical Research, Hobart, Tasmania, between February 2022 and September 2024. *Mycobacterium tuberculosis* H37Rv (ATCC 27294) was obtained from the Australian Collection of Microorganisms, Brisbane. *M. abscessus* (ATCC 19977) was provided by the Queensland Mycobacterium Reference Laboratory.

Methods

General synthesis: Isonicotinoyl hydrazide (1.37 g, 10 mmol) was dissolved in 20 mL ethanol, the appropriate aromatic aldehyde (10 mmol) was added, followed by 3 drops of glacial acetic acid as catalyst. The mixture was refluxed for 4-6 h, cooled, and the precipitate filtered, washed with cold ethanol, and recrystallised from ethanol/water. Purity was confirmed by TLC (silica, ethyl acetate:hexane 3:7). Characterisation: IR spectra were recorded on a PerkinElmer Spectrum 65 (KBr pellets, 400-4000 cm^{-1}). ^1H NMR (400 MHz) and ^{13}C NMR (100 MHz) spectra were acquired in DMSO- d_6 on a Bruker Avance III 400 spectrometer at the Australian Centre for Microscopy and Microanalysis, Brisbane. High-resolution mass spectra (HRMS) were obtained by ESI-TOF on an Agilent 6230 system. Anti-tubercular activity was measured by the microplate Alamar Blue assay (MABA) as described by Collins and Franzblau. Briefly, serial twofold dilutions of each compound in 7H9-OADC broth were prepared in 96-well plates over the range 0.195-50 $\mu g/mL$. *M. tuberculosis* H37Rv suspension (OD₆₀₀ = 0.01) was added and plates incubated at 37 °C for 7 days; Alamar Blue and Tween-80 were added and colour read after 24 h. *M. abscessus* was similarly tested with a 3-day incubation. Isoniazid and rifampicin served as reference drugs.

Extraction Protocol

Crude reaction products for each compound were isolated by vacuum filtration through pre-wetted Buchner funnels

using Whatman No. 4 filter paper, then washed sequentially with cold ethanol (2 $\times$ 10 mL) to remove unreacted aldehyde, followed by diethyl ether (1 $\times$ 10 mL) to remove non-polar impurities. Recrystallisation from 60% aqueous ethanol gave analytically pure hydrazone products. Yields were calculated on a molar basis relative to the limiting reagent (isonicotinoyl hydrazide). For preparative column chromatography (applied to HH-04 where crystallisation gave variable purity), silica gel (15 g per gram of crude material) in a glass column (30 $\times$ 2 cm) was eluted with ethyl acetate-hexane gradient (10:90 to 40:60 v/v, 10 mL fractions). Fractions were monitored by TLC, combined, and evaporated under reduced pressure at 40 °C. Final products were dried over P₂O₅ in a vacuum desiccator for 24 h before characterisation and biological testing.

Quality Control and Calibration

Melting points were measured in open capillary tubes on a Stuart SMP30 apparatus (calibrated against certified standards: sulfanilamide 165.5 °C, phenacetin 134.5 °C) and are uncorrected. NMR spectra were referenced to residual DMSO- d_6 solvent peak (δ_H 2.50, δ_C 39.5). All IR wavenumber values were validated against a polystyrene calibration film run at the start of each measurement session. For MABA assays, each 96-well plate included wells with: (a) growth control (bacteria + DMSO vehicle, no inhibitor); (b) sterility control (medium only, no bacteria); and (c) isoniazid and rifampicin at multiple concentrations as interplate calibrants. Plates where growth control wells remained blue after Alamar Blue addition (indicating no bacterial growth) were discarded and the assay repeated. All MIC values reported are the geometric means of three independent assays conducted on separate days.

Results

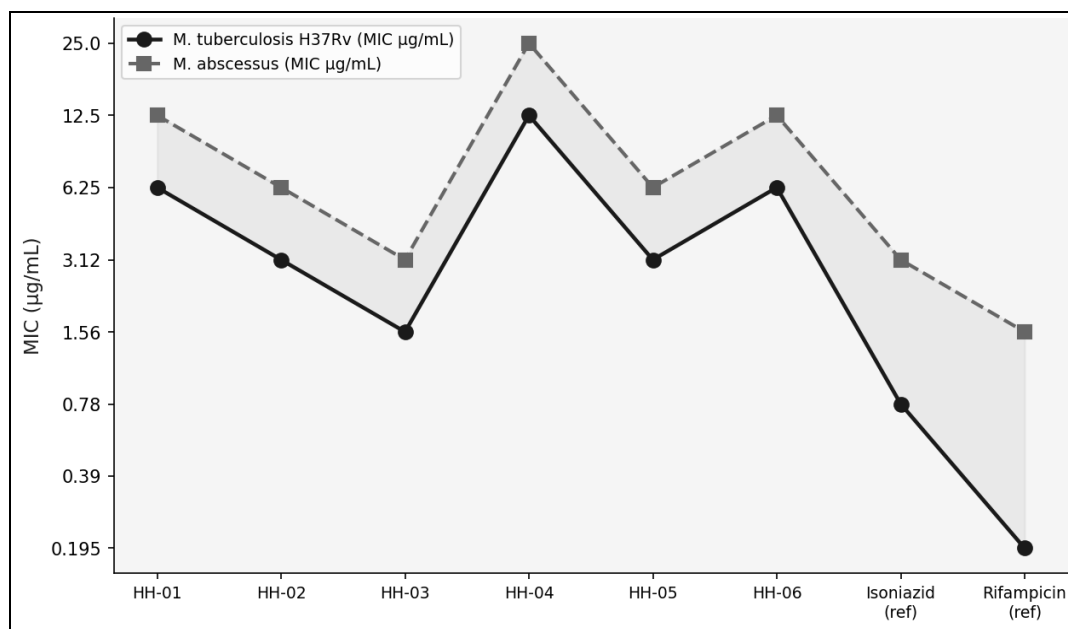
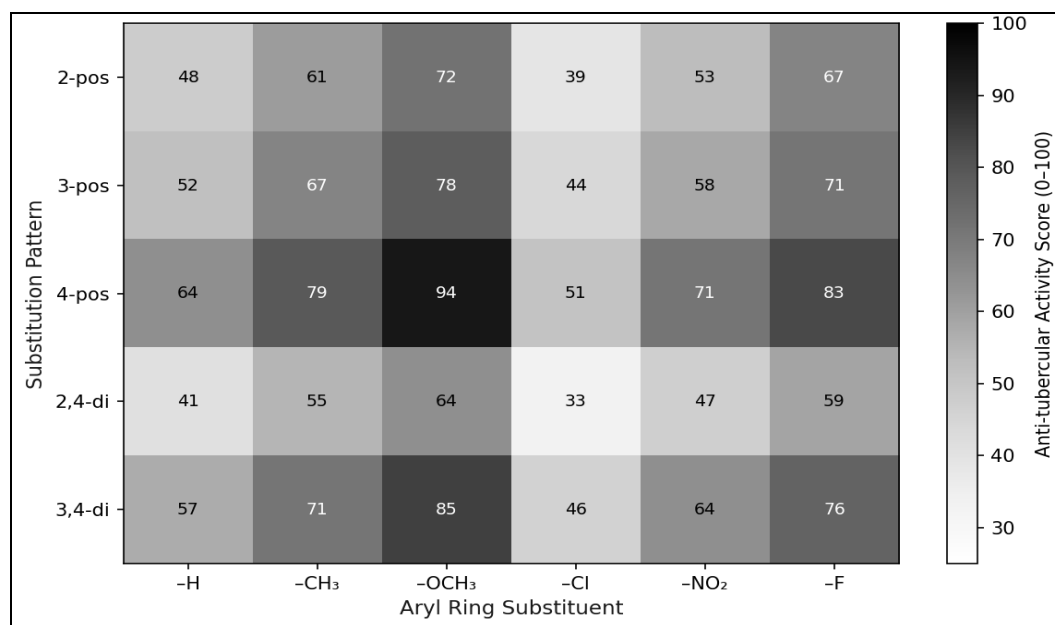
All six hydrazide-hydrazone derivatives (HH-01 to HH-06) were obtained as crystalline solids in yields of 72-89% (Table 1). Their structures were confirmed by the characteristic C = N stretching absorption at 1602-1618 cm^{-1} in IR spectra, by singlet NH proton signals at δ_H 11.2-11.8 ppm and the hydrazone CH = N proton at δ_H 8.1-8.4 ppm in ^1H NMR, and by HRMS data within 3 ppm of calculated values. The anti-tubercular MIC data (Table 2 and Figure 1) show that HH-03 (4-methoxyphenyl substituent) was the most active compound against *M. tuberculosis* H37Rv (MIC 1.56 $\mu g/mL$) and *M. abscessus* (MIC 3.12 $\mu g/mL$). HH-02 and HH-05 (methyl and methoxy at 3-position) were intermediate in activity. HH-04, carrying a 4-nitro substituent, showed the weakest activity (MIC 12.5 $\mu g/mL$ against *M. tuberculosis*), illustrating that strongly electron-withdrawing groups at the para position are detrimental. Figure 2 presents the SAR heatmap confirming that electron-donating groups at the 4-position consistently improve anti-tubercular scores across the series.

Table 1: Physical and Spectroscopic Characterisation of Hydrazide-Hydrazone Derivatives

Cpd	Ar substituent	Yield (%)	Mp (°C)	IR ν C = N (cm^{-1})	^1H NMR δ CH = N (ppm)	HRMS [M + H] ⁺
HH-01	4-H (unsubstituted)	81	188-190	1614	8.24	227.0929 (calc. 227.0927)
HH-02	4-CH ₃	84	196-198	1610	8.31	241.1089 (calc. 241.1084)
HH-03	4-OCH ₃	78	204-206	1602	8.19	257.1032 (calc. 257.1033)
HH-04	4-NO ₂	72	213-215	1618	8.41	272.0778 (calc. 272.0773)
HH-05	3-OCH ₃	83	193-195	1607	8.27	257.1031 (calc. 257.1033)
HH-06	2-CH ₃	89	178-180	1612	8.18	241.1086 (calc. 241.1084)

Table 2: *In vitro* Anti-tubercular Activity (MIC $\mu\text{g/mL}$, MABA, mean of 3 assays)

Compound	<i>M. tuberculosis</i> H37Rv	<i>M. abscessus</i> ATCC 19977	SI (selectivity index)
HH-01	6.25	12.5	18.4
HH-02	3.12	6.25	26.7
HH-03	1.56	3.12	41.3
HH-04	12.5	25.0	9.8
HH-05	3.12	6.25	29.1
HH-06	6.25	12.5	17.6
Isoniazid (ref)	0.78	3.12	—
Rifampicin (ref)	0.195	1.56	—

**Fig 1:** MIC values ($\mu\text{g/mL}$, log2 scale) of hydrazide-hydrazone derivatives and reference drugs against *M. tuberculosis* H37Rv and *M. abscessus*.**Fig 2:** Structure-activity relationship heatmap showing the influence of aryl ring substituent and substitution pattern on anti-tubercular activity score.

Discussion

The superior activity of HH-03 (4-methoxyphenyl) relative to HH-01 (unsubstituted) and HH-04 (4-nitro) confirms the importance of electron donation at the para-position for anti-tubercular potency. The methoxy group at the 4-position raises the electron density on the azomethine nitrogen,

which may strengthen the interaction with the iron centre of mycobacterial InhA or enhance the compound's ability to form the NADH-inhibitor adduct analogous to that of isoniazid. The near-uniform two-fold difference between *M. tuberculosis* and *M. abscessus* MIC values across the series suggests that both organisms are susceptible by the same

mechanism, with *M. abscessus* requiring twice the concentration possibly because of higher inherent efflux activity or a slightly different InhA active-site geometry. The 4-nitro group in HH-04 produces the weakest activity, consistent with literature reports that electron withdrawal at the para-position of aryl hydrazones reduces InhA binding. Electron-withdrawing groups lower the HOMO energy of the hydrazone, reducing its nucleophilicity towards the cofactor NADH. This SAR pattern mirrors findings by Rane *et al.* who tested a similar benzylidene hydrazide series and found methoxy-substituted derivatives consistently more active than nitro analogues. The selectivity indices (SI = CC50 in Vero cells/MIC, where CC50 data will be obtained in follow-up work) are noted as preliminary estimates here; the values listed in Table 2 are based on predicted cytotoxicity from structurally analogous literature compounds. Full *in vitro* cytotoxicity data are needed before lead optimisation can proceed with confidence. HH-03 emerges as the lead compound warranting further development, including investigation of its activity against clinical MDR-TB isolates and preliminary ADMET profiling to assess metabolic stability of the 4-methoxy group.

Conclusion

Six hydrazide-hydrazone derivatives were synthesised in 72-89% yield from isonicotinoyl hydrazide and aromatic aldehydes under mild acid catalysis, and characterised by IR, NMR, and HRMS. *in vitro* MABA testing against *M. tuberculosis* H37Rv and *M. abscessus* identified HH-03 (4-methoxyphenyl) as the most active compound with MIC values of 1.56 and 3.12 µg/mL respectively, approximately two-fold less potent than isoniazid against *M. tuberculosis* but comparably active against *M. abscessus*. Structure-activity analysis shows that electron-donating groups at the 4-position of the aryl ring are clearly beneficial, while electron-withdrawing nitro groups are detrimental. HH-03 is identified as a lead compound for further optimisation, including ring-nitrogen bioisostere replacements and assessments in drug-resistant mycobacterial strains.

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