Potent Anti-Mycobacterial Activity of the Pyridoxal Isonicotinoyl Hydrazone Analogue, 2-Pyridylcarboxaldehyde Isonicotinoyl Hydrazone: A Lipophilic Transport Vehicle for Isonicotinic Acid Hydrazide

Samantha Ellis, Danuta S. Kalinowski, Lisa Leotta, Michael L.H. Huang, Peter Jelfs, Vitali Sintchenko, Des R. Richardson, James A. Triccas

Microbial Immunity and Pathogenesis Group, Department of Infectious Diseases and Immunology, University of Sydney, Sydney, NSW, Australia (S.E., L.L., J.A.T.)

Molecular Pharmacology and Pathology Program, Department of Pathology and Bosch Institute, University of Sydney, Sydney, NSW, Australia (D.S.K., M.L.H., D.R.R.)

Centre for Infectious Diseases and Microbiology, Institute of Clinical Pathology and Medical Research-Pathology West, Westmead Hospital, Sydney, NSW, Australia (P.J., V.S.)
Running title: Lipophilic transport vehicles against mycobacteria

Address for correspondence: Des R. Richardson, Molecular Pharmacology and Pathology Program, Department of Pathology and Bosch Institute, University of Sydney, Sydney, NSW, 2006. Phone: (612) 9036 6548; Fax: (612) 9351 3429; Email: d.richardson@med.usyd.edu.au; James A. Triccas, Department of Infectious Diseases and Immunology, Blackburn Building (D06), University of Sydney, NSW 2006, Australia. Phone: (612) 9036 6582; Fax: (612) 9351 4731; Email: jamie.triccas@sydney.edu.au.

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Abbreviations: Analysis of variance, ANOVA; Bacille Camlette Guérin, BCG; colony forming units, CFU; desferrioxamine, DFO; Dulbecco’s Modified Eagle Media, DMEM; extensively-drug resistant, XDR; isonicotinic acid hydrazide, INH; multi-drug resistant, MDR; minimal inhibitory concentration, MIC; 2-pyridylcarboxaldehyde p-aminobenzoyl hydrazine, PCAH; 2-pyridylcarboxaldehyde benzoyl hydrazine, PCBH; 2-pyridylcarboxaldehyde 3-bromobenzoyl hydrazine, PC3BBH; 2-pyridylcarboxaldehyde 4-bromobenzoyl hydrazine, PC4BBH; 2-pyridylcarboxaldehyde p-hydroxybenzoyl hydrazine, PCHH; 2-pyridylcarboxaldehyde isonicotinoyl hydrazine, PCIH; 2-pyridylcarboxaldehyde p-nitrobenzoyl hydrazine, PCNH; 2-pyridylcarboxaldehyde 2-thiophenecarboxyl hydrazine, PCTH; 2-pyridylcarboxaldehyde p-trifluoromethylbenzoyl hydrazine, PCTFH.
ABSTRACT

The rise in drug-resistant strains of Mycobacterium tuberculosis is a major threat to human health and highlights the need for new therapeutic strategies. In this study, we have assessed if high-affinity iron chelators of the pyridoxal isonicotinoyl hydrazone (PIH) class can restrict the growth of clinically significant mycobacteria. Screening a library of PIH derivatives revealed one compound, namely 2-pyridylcarboxaldehyde isonicotinoyl hydrazone (PCIH), exhibited nanomolar in vitro activity against Mycobacterium bovis Bacille Camlette Guérin (BCG) and virulent M. tuberculosis. Interestingly, PCIH is derived from the condensation of 2-pyridylcarboxaldehyde with the first-line anti-tubercular drug, isoniazid (i.e., isonicotinic acid hydrazide; INH). PCIH displayed minimal host-cell toxicity and was effective at inhibiting growth of M. tuberculosis within cultured macrophages and also in vivo in mice. Further, PCIH restricted mycobacterial growth at high bacterial loads in culture, a property not observed with INH, which shares the isonicotinoyl hydrazide moiety with PCIH. When tested against Mycobacterium avium, PCIH was more effective than INH at inhibiting bacterial growth in broth culture and in macrophages, and also reduced bacterial loads in vivo. Complexation of PCIH with iron decreased its effectiveness, suggesting that iron chelation may play some role in its anti-mycobacterial efficacy. However, this could not totally account for its potent efficacy and structure-activity relationship studies suggest PCIH acts as a lipophilic vehicle for the transport of its intact INH moiety into mammalian cells and the mycobacterium. These results demonstrate that iron-chelating agents such as PCIH may be of benefit in the treatment and control of mycobacterial infection.
INTRODUCTION

Tuberculosis is the leading cause of disease from a single bacterial pathogen worldwide, infecting over a third of the global population (W.H.O, 2012). Almost 9 million new cases arise annually, and this is despite increased awareness of tuberculosis and implementation of strategies by the World Health Organization to reduce disease burden (W.H.O, 2012). Of particular concern is the increased incidence of multi-drug resistant (MDR) strains over the past decade, coupled with the emergence of extensively-drug resistant (XDR) strains of *Mycobacterium tuberculosis* (W.H.O, 2012). Strategies to improve tuberculosis drug therapy include the modification of existing drugs or the development of novel antibiotics, and a number of promising candidates have been described (Wong et al., 2013). There has also been interest in the implementation of nutrient supplementation to control tuberculosis, either as a stand-alone therapy or as an adjunct treatment for use with current drug regimens (Greenstein et al., 2012; Sinclair et al., 2011).

Iron is an obligate co-factor for over forty enzymes encoded within the mycobacterial genome (Cronje and Bornman, 2005; Yellaboina et al., 2006). Previous studies have shown that *M. tuberculosis* bacterial load is increased in iron-supplemented murine models relative to their to control counterparts (Lounis et al., 2001). In HIV-infected patients, the incidence of mycobacterial co-infection was more prominent in patients with a high iron grade (de Monye et al., 1999). The importance of iron for mycobacterial growth suggests a potential role for chelation therapy as a treatment option. Indeed, the use of iron chelation as a potential therapy for infectious diseases has been well documented, including the *in vitro* use of chelators to inhibit viral replication and reverse transcriptase activity in HIV treatment (Debebe et al., 2007; Traore and Meyer, 2007). Furthermore, in malarial infection, chelation therapy increased parasite clearance rate (Pradines et al., 2002; Walcourt et al., 2004). Iron chelation has been shown to inhibit the growth of *M. tuberculosis* in culture, although the compounds used displayed limited effect against bacilli internalized by
macrophages (Cronje et al., 2005). In vitro studies have also demonstrated a moderate inhibitory effect on Mycobacterium avium growth within macrophages and mice (Gomes et al., 2001). Both these latter studies acknowledged that more potent iron chelators may prove beneficial in the treatment of mycobacterial infection.

There has been significant progress in the development of new generations of iron chelators, both for use in iron overload disease and for their anti-proliferative activity in the inhibition of tumor growth (Richardson et al., 2009; Yu et al., 2009; Yu et al., 2006). Compounds that have been developed for such purposes include tridentate ligands such as those of the pyridoxal isonicotinoyl hydrazone (PIH) class that possess very high affinity for iron and much lower affinity for other essential metals e.g., Zn(II), Mg(II), Ca(II) (Richardson and Ponka, 1998a). The pharmacological advantages of PIH and its analogues include: (1) it can be simply prepared by a 1-step synthesis; (2) the compounds possesses high membrane permeability; (3) it can be orally administered (Richardson and Ponka, 1998a); and (4) it is neutral at biological pH (pH 7.4; (Richardson et al., 1990), allowing for penetration through cell membranes to reach intracellular iron stores (Richardson and Ponka, 1998b). Notably, structure-activity relationship studies have led to PIH analogues that have properties useful for the treatment of iron overload disease or cancer (Richardson et al., 1995; Sookvanichsilp et al., 1991). Some of these ligands have shown marked iron chelation activity in vitro and in vivo (Baker et al., 1992; Link et al., 2003), while other synthesized chelators demonstrate potent anti-proliferative effects with minimal toxicity in vivo (Kovacevic et al., 2011; Lovejoy et al., 2012; Whitnall et al., 2006).

PIH is the result of a simple condensation between pyridoxal and the potent anti-mycobacterial agent, isoniazid (i.e., isonicotinic acid hydrazide; INH), in which the latter structure is conserved (Table 1) (Hermes-Lima et al., 2000; Kang et al., 2006). Considering the structural similarity
between PIH and INH, in addition to the ability of PIH to bind the essential nutrient iron (Richardson and Ponka, 1998b), the current study examined the ability of a range of PIH analogues (Table 1) to inhibit the growth of pathogenic mycobacterial strains. This study identifies the PIH analogue, 2-pyridylcarboxaldehyde isonicotinoyl hydrazone (PCIH), as a potent inhibitor of mycobacterial growth. Our results indicate the pronounced activity of PCIH is mediated by a novel mechanism, as structure-activity relationship studies suggest PCIH acts as a lipophilic vehicle for the transport of the intact INH moiety into cells and the mycobacterium.
MATERIALS AND METHODS

Bacterial growth conditions

All mycobacterial strains (M. avium 104, M. bovis BCG Pasteur and M. tuberculosis H37Rv) were grown in complete Middlebrook 7H9 media (Bacto, Australia) containing albumin, dextrose and catalase (ADC), 20% Tween 80 and 50% glycerol (Sigma-Aldrich, Australia). Cultures were incubated on rollers at 37°C in a dry incubator. To generate luminescent M. bovis BCG and M. avium strain 104 (Saunders et al., 2002), bacteria were rendered electro-competent by standard methods (Lee et al., 2002) and transformed with 2 µL of pSMT1 (Snewin et al., 1999) by electroporation (25 uF, 2.5 kv, 200 Ω). Transformants were selected on Middlebrook 7H11 plates supplemented with 25 µg/mL hygromycin (Sigma-Aldrich). BCG::pSMT1 and M. avium::pSMT1 were cultured in complete Middlebrook 7H9 media supplemented with 25 µg/mL of hygromycin.

M. tuberculosis Beijing genotype INH-sensitive and mono-resistant (katG mutation) strains were sourced from the NSW Mycobacterium Reference Laboratory, Centre for Infectious Diseases and Microbiology Laboratory Services Strain Collection, Westmead Hospital, Sydney. The resistant strain displayed an INH MIC of >0.4 µg/mL. The INH and PCIH sensitivity of strains was assessed using the BACTEC MGIT™ 960 system (Becton Dickinson), according to the manufacturer’s recommendations. Briefly, a 0.5 McFarland suspension of M. tuberculosis was diluted (1:5) in sterile saline and 0.5 mL of this dilution was added to tubes containing PCIH or INH in critical concentrations (0.1, 0.4 or 2.5 µg/mL). A 1% growth control tube was inoculated with 0.5 mL of diluted suspension and was further diluted (1:100). The results for test samples were assessed once all tubes had been incubated longer than 4 days and the 1% growth control tube achieved a growth unit (GU) of 400. A GU of ≤100 in the drug-containing tube indicated the strain was susceptible. A GU of greater that 100 was interpreted as resistant.
Iron chelators

PIH and its analogues were synthesized by established procedures by a simple 1-step synthesis between commercially available aromatic aldehydes and hydrazides (Sigma-Aldrich) (Kalinowski et al., 2008). The following PIH analogues were examined in the current study: 2-pyridylcarboxaldehyde isonicotinoyl hydrazone (PCIH); 2-pyridylcarboxaldehyde benzoyl hydrazone (PCBH); 2-pyridylcarboxaldehyde 3-bromobenzoyl hydrazone (PC3BBH); 2-pyridylcarboxaldehyde 4-bromobenzoyl hydrazone (PC4BBH); 2-pyridylcarboxaldehyde p-hydroxybenzoyl hydrazone (PCHH); 2-pyridylcarboxaldehyde p-aminobenzoyl hydrazone (PCAH); 2-pyridylcarboxaldehyde 2-thiophenecarboxyl hydrazine (PCTH); 2-pyridylcarboxaldehyde p-nitrobenzoyl hydrazone (PCNH); and 2-pyridylcarboxaldehyde p-trifluoromethylbenzoyl hydrazone (PCTFH). Log $P_{\text{calc}}$ values of the ligands were the average log $P$ values calculated in ChemDraw v4.5 using Crippen’s fragmentation (Ghose and Crippen, 1987), Viswanadhan’s fragmentation (Viswanadhan et al., 1989), and Broto’s methods (Broto, 1984).

Resazurin assay of growth inhibition

To determine the minimal inhibitory concentration (MIC) of test compounds and the effect of iron complexation on their inhibitory activity, the chelator alone or chelator complexed with iron in a 2:1 chelator:iron ratio using FeSO$_4$ were serially diluted in 10 µL of purified H$_2$O in triplicate (0.01-10 µM). The bacterial suspension (90 µL) at varying OD$_{600nm}$ (0.001, 0.01, 0.1 or 1.0) was added to the wells and incubated for 5 or 7 days for *M. avium* or BCG/*M. tuberculosis*, respectively. Resazurin (10 µL; 0.05% w/v; Sigma-Aldrich, Australia) was then added, incubated for 4 h at 37°C, and fluorescence measured at 590 nm using a FLUOstar Omega microplate reader (BMG Labtech, Germany) (Dixit et al., 2012). After subtraction of background fluorescence from all wells, the percentage mycobacterial survival was determined by comparing the fluorescence of wells containing PCIH or INH compared to control wells not treated with compound.
Compound intracellular efficacy and toxicity

THP-1 cells (TIB-202R), a human monocyte cell line (American Type Culture Collection, USA), were grown in complete Dulbecco’s Modified Eagle Media (DMEM; LifeTechnologies, Australia) including 10% fetal bovine serum (FBS), 200 µM L-glutamine (LifeTechnologies, Australia), and 1 mM HEPES buffer solution (LifeTechnologies, Australia). Cells (1 x 10^5) in media containing 50 ng/mL phorbol 12-myristate 13-acetate (PMA) were added to 96-well plates and were then incubated for 48 h at 37°C to allow adherence and differentiation. Cells were then infected with 10^6 BCG pSMT1 or M. avium pSMT1 prepared in DMEM for 4 h at 37°C. After 4 h, cells were washed 3 times with PBS/2% FCS and complete DMEM added after the final wash. Compounds (0.3-10 µM) were then added to plates and incubated for up to 7 days at 37°C in 5% CO₂. Decanal (1%; Sigma-Aldrich, Australia) substrate was added to each well and the luminescence measured after 7 s of incubation. To establish the effect of certain compounds over time, experiments were conducted over a 7-day period and readings were taken at various time points. Survival was calculated as percentage luminescence compared to the untreated control.

For determination of intracellular bacterial load, cells were infected with OD₆₀₀ₙₐₐ 0.01 M. tuberculosis H37Rv for 4 h at 37°C, compounds added (1 or 10 µM) and then incubated for 7 days at 37°C in 5% CO₂. Cells were then lysed after this period and bacterial number determined after plating the suspension onto 7H11 Middelbrook agar, using standard procedures (Pinto et al., 2004).

To examine the toxicity of selected compounds, 1 x 10^5 THP-1 cells/well were added to a 96-well plate and left for 48 h at 37°C to adhere. Compounds (0.3-10 µM) were added to the wells in 3-fold dilutions and incubated for 7 days at 37°C. Then 0.05% w/v resazurin (4 h) was added and the fluorescence measured. Cell viability was calculated as percentage fluorescence in comparison to untreated cells.
Murine studies

C57BL/6 mice were purchased from the Animal Resources Centre (Perth, Australia) and all experiments were approved by the University of Sydney Animal Ethics Committee. Mice were infected either intra-nasally with $1 \times 10^5$ CFU *M. avium* or aerosol infected with *M. tuberculosis* H37Rv, as previously described (Pinto et al., 2004). At four weeks post-infection, INH (10 mg/kg or 100 mg/kg) or PCIH (10 mg/kg or 100 mg/kg) or vehicle only (50 % DMSO in PBS) were administered daily via the intra-peritoneal route for 21 days (*M. avium*, BCG) or 28 days (*M. tuberculosis*). Bacterial loads in the lung and spleen were determined by plating of tissue homogenates on 7H11 Middelbrook agar via standard methods (Pinto et al., 2004).

Statistics

The significance of differences for linear and log-transformed assays was evaluated by one-way analysis of variance (ANOVA) with pair-wise comparison of multi-grouped data sets achieved using the Bonferroni post hoc test.
RESULTS

In vitro inhibition of mycobacterial growth by tridentate iron chelators

Initially, a screen was conducted on 10 ligands derived from (and including) the parent compound, PIH (Table 1), to determine if they could effectively reduce mycobacterial growth in culture. The 9 PIH analogues shown in Table 1 all demonstrate marked structural similarity, being members of the PCIH series of chelators (Becker and Richardson, 1999; Kalinowski et al., 2008). Each of the latter have a conserved tridentate iron-binding site (aromatic nitrogen, imine nitrogen and carbonyl oxygen) with substitutions distal to the coordination sphere (Becker and Richardson, 1999; Kalinowski et al., 2008). This group of analogues constitute a series of compounds that all are effective ligands with varying lipophilicity, as reflected by their calculated partition coefficient (log $P_{\text{calc}}$; Table 1). Examination of this series enables an assessment of the structure-activity relationships in terms of the role of lipophilicity and chelation efficacy in the anti-bacterial efficacy observed. Notably, INH was included as a positive control due to its potent anti-mycobacterial activity (Vilcheze and Jacobs, 2007) and similarity to the isonicotinoyl hydrazone moiety of PIH (Richardson and Ponka, 1998b).

BCG was exposed to a single chelator dose of 10 µM for 7 days and survival was determined in comparison to vehicle-treated bacterial cells (control). Significantly, INH, PIH and PCIH were able to markedly and significantly ($p<0.01$) restrict the growth of BCG at 10 µM, with the two chelators leading to effective (PIH) and almost complete growth inhibition (PCIH; Table 1). Calculation of the MIC$_{90}$ for PCIH (0.39 µM) and INH (0.39 µM) indicated a similar level of inhibitory activity of the two compounds (Fig. 1A). The analogue, PC4BBH, showed inhibitory activity that was comparable to PIH, but less than INH or PCIH (Table 1). Despite their very similar structures, the other 7 compounds screened showed limited efficacy, with PCBH, PCHH, PCAH, and PCNH causing less than a 20% reduction in BCG growth at 10 µM in comparison to the control (Table 1).
Remarkably, the slight structural change between PCIH and PCBH due to the alteration of the isonicotinoyl nitrogen to a C-H group, led to an almost total loss of anti-mycobacterial activity (Table 1). This observation indicated the critical importance of the isonicotinoyl nitrogen for efficacy. This dramatic alteration was unanticipated, as the replacement of the N with the phenyl C-H increased lipophilicity (log $P_{\text{calc}}$ increases from 0.96 to 2.21; Table 1), which could potentially enhance membrane permeability relative to PCIH. Indeed, previous studies using a human cell line have demonstrated the increased permeability and iron chelation efficacy of PCBH over PCIH (Becker and Richardson, 1999).

Increasing the lipophilicity of PCBH by the synthesis of PCTH, PC3BBH, PC4BBH, and PCTFH (log $P_{\text{calc}}$: 2.49, 3.05, 3.05 and 3.14, respectively) significantly ($p<0.01$) improved the anti-mycobacterial efficacy relative to PCBH (Table 1). However, despite the greater lipophilicity of these analogues than PCBH and especially PCIH, the anti-mycobacterial activity was considerably less than PCIH (Table 1). Moreover, there was little correlation between the log $P_{\text{calc}}$ of these analogues and efficacy at inhibiting bacterial growth ($r^2 = 0.46$; data not shown), indicating that lipophilicity did not appear to be the primary criterion for efficacy.

Considering the high activity of PCIH against BCG, this agent was also tested against $M. avium$ to determine if it displays broad anti-mycobacterial activity (Fig. 1B). Interestingly, PCIH also effectively restricted the growth of $M. avium$ in culture, being significantly ($p<0.01$) more effective than INH at concentrations less than 1 µM (namely, 0.31 and 0.63 µM), although the MIC$_{90}$ of both compounds was similar (PCIH: 1.25 µM; INH: 2.5 µM; Fig. 1B). Therefore, PCIH showed potent inhibitory activity against both BCG and $M. avium$. 
We next determined if PCIH was able to inhibit growth at a variety of bacterial concentrations, as this is a factor that may influence drug efficacy in a clinical setting. PCIH showed no significant difference in effectiveness against BCG relative to INH at the lower starting OD$_{600\text{nm}}$ of 0.001, 0.01 and 0.1 (Fig. 2A-C). However, notably, at an OD$_{600\text{nm}}$ of 1.0, PCIH was able to significantly ($p<0.001$) inhibit BCG growth compared to INH at 1 µM and 10 µM (Fig. 2D). Testing the effect of PCIH against varying bacterial loads of *M. avium* (Fig. 2E-H), resulted in a significantly ($p<0.01$) greater reduction in bacterial survival compared to INH at lower bacterial loads (OD$_{600\text{nm}}$ 0.001; Fig. 2E) and higher concentrations of the agents when bacterial load was increased (OD$_{600\text{nm}}$ 0.01-0.1; Fig. 2F,G). However, none of the compounds showed growth inhibition at an OD$_{600\text{nm}}$ of 1.0 (Fig. 2H), indicating a higher intrinsic resistance to both PCIH and INH by *M. avium*. Taken together, these results indicate that PCIH can restrict the growth of both BCG and *M. avium* over varying bacterial concentrations, an important advantage over the currently used drug, INH.

**Iron-dependent activity of PCIH**

Despite PCIH showing effectiveness at restricting mycobacterial growth, it was unknown whether PCIH was functioning primarily by iron chelation or by direct anti-mycobacterial activity due to its isonicotinoyl hydrazone sub-structure (Table 1). Notably, PCIH has been demonstrated to be an effective tridentate iron chelator (Bernhardt et al., 2001), showing marked activity at mobilizing iron from mammalian cells (Becker and Richardson, 1999; Richardson et al., 2001). To test the role of iron chelation in its activity, PCIH was pre-complexed in a 2:1 ratio with iron, to saturate its iron-binding site, and thus, preventing further iron chelation from cells. BCG was then treated with either the free ligand or the iron complex and the MIC$_{90}$ was calculated. The iron complex of PCIH resulted in a 2-fold change of the MIC$_{90}$ in these experiments (0.31 µM to 0.63 µM) relative to PCIH alone (data not shown), suggesting the ability of PCIH to complex iron had some effect on its capacity to restrict BCG growth. When screened against *M. avium*, formation of the PCIH-iron
complex affected its anti-mycobacterial activity, resulting in an approximate 8-fold increase in MIC$_{90}$ (1.25 μM to >10 μM; data not shown). This observation indicated that iron chelation may play a limited role in the activity of PCIH against mycobacteria. Previous studies have demonstrated that PCIH and its iron complex have similar partition coefficients (i.e., log $P$: 1.98 cf. 1.89, respectively; Bernhardt et al., 2007) and this suggests they possess comparable ability to permeate the cell membrane. Thus, it is unlikely that the small change in the partition coefficient of PCIH relative to its iron complex would contribute to the increase in the MIC$_{90}$ observed upon complexation. This finding supports the suggestion that iron chelation, rather than changes in membrane permeability, may play some role in the anti-mycobacterial activity of PCIH.

**Restriction of bacterial growth by PCIH within host cells**

Considering the efficacy of PCIH in mycobacterial culture, we determined if PCIH could restrict mycobacterial growth within host THP-1 cells. Both INH and PCIH resulted in dose-dependent inhibition of BCG growth by day 7 post-infection of THP-1 cells, as determined by examination of luminescence emitted by luciferase-expressing BCG within THP-1 cells (Fig. 3A). PCIH was more effective than INH at inhibiting BCG growth at 0.3, 1 and 10 μM, although this did not reach statistical significance (Fig. 3A). Both INH and PCIH displayed a similar level of effectiveness when tested at 3 and 5 days post-infection (data not shown).

When tested against *M. avium*-infected THP-1 cells, PCIH exhibited a similar trend of anti-mycobacterial activity to INH at high drug concentrations (Fig. 3B). However, PCIH was significantly ($p<0.05$) more effective than INH at a concentration of 0.3 μM (Fig. 3B). The inhibitory effect of PCIH was independent of any toxic effect on THP-1 cells, as the compound had no significant effect on THP-1 viability, irrespective of the inhibitor concentration tested (Fig. 3C).
This result was in good correlation with the low anti-proliferative activity of PCIH observed in previous studies using human cells *in vitro* (Becker and Richardson, 1999).

**PCIH effectively restricts mycobacterial growth *in vivo***

Having determined that PCIH effectively restricted bacterial growth *in vitro*, we examined if the compound also displayed activity *in vivo*. Mice were infected with *M. avium* and treated daily with 100 mg/kg PCIH or INH for 21 days. Although PCIH did appear to decrease the bacterial load in the lung, neither INH or PCIH resulted in a significant decrease in *M. avium* load in this organ compared to the vehicle-only treated animals (Fig. 4). However, in the spleen, PCIH treatment led to an approximate 1.5-log reduction in *M. avium* colony forming units (CFU) compared to untreated mice (*p*<0.05) (Fig. 4). This was similar to the reduction observed with INH treatment. Therefore, PCIH retained its activity in an *in vivo* model of mycobacterial infection.

**PCIH displays anti-tubercular activity**

As PCIH was able to restrict the growth of both BCG and *M. avium*, we determined if it also displayed activity against *M. tuberculosis*. Notably, PCIH showed the same trend of growth inhibition compared to INH when tested against *M. tuberculosis* grown in culture media (Fig. 5A) with an MIC<sub>90</sub> of 0.31 μM and 0.63 μM for INH and PCIH, respectively. Moreover, PCIH was able to significantly (*p*<0.05) reduce the number of bacteria recovered from *M. tuberculosis*-infected THP-1 cells compared to untreated cells by more than 3-log units (Fig. 5B). When PCIH was assessed for potency in *M. tuberculosis*-infected mice, a reduction of CFU was observed in the lung and a significant (*p*<0.05) decrease was found in the spleen compared to control mice (Fig. 5C-D). Further, treatment with PCIH caused a similar level of reduction of bacterial load as that found for
INH (Fig. 5C-D). Collectively, these data indicate that PCIH displays broad activity against mycobacterial pathogens.

To further explore the mechanism of action of PCIH, we examined activity against INH-sensitive and -resistant clinical strains of \textit{M. tuberculosis} at a defined set of concentrations (0.1, 0.4 and 2.5 \(\mu\text{g/mL}\)). We selected a \textit{M. tuberculosis katG} mutant strain for this study, due to the critical role of KatG in INH activation and the dominance of \textit{katG} mutations in \textit{M. tuberculosis} strains displaying an INH-resistant phenotype (Vilcheze and Jacobs, 2007). We observed that both PCIH and INH may target the same pathway in \textit{M. tuberculosis} and require activation by KatG to exert their activity, as the \textit{M. tuberculosis katG} mutant strain was resistant to INH and PCIH at all concentrations tested (data not shown). In contrast, wild-type \textit{M. tuberculosis} was fully susceptible to both compounds. These findings indicate that PCIH may, in part, target a similar pathway as INH to exert its anti-mycobacterial activity.
DISCUSSION

Of the 9 PIH analogues tested in this investigation (Table 1), PCIH was the most effective at inhibiting mycobacterial growth both in vitro and in vivo. In fact, this study showed that several PIH analogues demonstrate substantial activity against mycobacteria. In the initial screen (Table 1), a chelator concentration of 10 µM was chosen as this concentration is pharmacologically achievable in vivo. Considering this, the related chelator, salicylaldehyde isonicotinoyl hydrazone, reached a concentration of 100 µM in the plasma of rabbits upon i.v. administration at 10 mg/kg (Kovarikova et al., 2005).

It has been shown that M. avium viability and growth is highly dependent on iron (Dhople et al., 1996). This observation could indicate that a relatively small reduction in iron levels may have a greater effect on the growth of M. avium compared to BCG, and indeed, such a result was observed in this study. PCIH is known to effectively chelate iron levels within cells (Becker and Richardson, 1999; Richardson et al., 2001), while its precursor INH is not an effective ligand as it lacks the tridentate ligating site (Ponka et al., 1979). Hence, considering the important role of Fe in a variety of metabolic processes, depletion of iron by PCIH would have a profound impact on mycobacterial growth.

Notably, PCIH has greater membrane permeability when compared to the “gold standard” chelator in clinical use, desferrioxamine (DFO) (Becker and Richardson, 1999; Richardson et al., 2001). This is due to several factors, including that PCIH is: (1) more lipophilic than DFO; (2) it is predominantly neutral at physiological pH; (3) it is less than half the molecular weight of DFO; and (4) it mainly forms neutral iron complexes that can diffuse readily from cells (Becker and Richardson, 1999; Bernhardt et al., 2001; Richardson et al., 2001). Thus, high membrane permeability would permit greater access to intracellular bacteria that reside within phagolysosomes.
(Ehrt and Schnappinger, 2009) (Fig. 6), thereby reducing the effective dose required. Since PCIH is known to rapidly permeate cells and organelles to bind iron (Becker and Richardson, 1999; Richardson et al., 2001), it may be a viable option as an anti-mycobacterial agent.

The enhanced intracellular activity of PCIH may also be due to its greater lipophilicity compared to the parent molecule, PIH, due to the absence of the alcohol side chains present in the former ligand (Table 1) (Becker and Richardson, 1999). This factor means PCIH may more readily enter host cells and mycobacteria to impart its anti-mycobacterial action. However, an argument merely relying on the greater lipophilicity of PCIH as the sole determinant of its efficacy does not explain the total lack of activity observed with PCBH (Table 1). Notably, PCBH only differs very slightly in structure to PCIH and due to the slightly greater lipophilicity of PCBH relative to PCIH (Table 1), it demonstrates greater activity at mobilizing cellular iron from tumor cells in vitro (Becker and Richardson, 1999). Moreover, increasing the lipophilicity of PCBH by preparing PC3BBH, PC4BBH, PCTH and PCTFH, led to improved efficacy over PCBH, but did not lead to an anti-mycobacterial agent as effective as PCIH (Table 1). Hence, other factors, in addition to lipophilicity, played a role in the anti-bacterial activity observed.

In this study, the greatest anti-mycobacterial activity was observed with PIH and PCIH, both of which have the INH moiety conserved within the ligand. Taking into account the pronounced loss of activity observed with PCBH (Table 1), it is notable that relative to INH, benzoic acid hydrazide (which is the analogous hydrazide used to synthesize PCBH) displays little anti-mycobacterial activity (Quemard et al., 1991). Thus, hydrolysis of PCBH intracellularly to its components, 2-pyridylcarboxaldehyde (2-PC) and benzoic acid hydrazide, would not be expected to lead to an effective anti-tuberculosis agent. Considering this, it can be speculated that the efficacy of PCIH relative to INH could be related to the ligand acting as a lipophilic vehicle for INH transport into...
mammalian cells and the mycobacterium, rather than via iron chelation. In this case, hydrolysis of PCIH in the cell or bacterium may lead to its components, namely 2-PC and the potent INH moiety (Fig. 6). The later agent would lead to bactericidal activity via its well-described action on mycobacterial wall synthesis (Fig. 6). Indeed, we determined that INH and PCIH may share a similar mode of action as they are both require KatG-mediated activation for activity against *M. tuberculosis*. Hence, PCIH may act as a lipophilic transport vehicle that facilitates INH entrance into the cell and bacterium, after which INH is liberated. Subsequently, through well described classical mechanisms INH is activated leading to inhibition of mycolic acid synthesis (Vilcheze and Jacobs, 2007). Furthermore, acid-catalyzed hydrolysis of PIH analogues is well known (Richardson et al., 1989), and it is notable that mycobacteria-containing phagolysosome are acidic (Geisow et al., 1981), which could facilitate this process (Fig. 6). Collectively, the formation of a “Trojan Horse” (*i.e.*, PCIH) that is lipophilic and enables the effective penetration of the active constituent, INH.

Interestingly, regarding this latter mechanism and particularly the role of PIH or PCIH as an active agent in vivo, it is known that INH treatment of patients results in a depletion of vitamin B₆ (pyridoxal or pyridoxal phosphate) and this is probably due to the fact that INH condenses with the latter to directly generate PIH in vivo (Whitehouse et al., 1983). Hence, it cannot be excluded whether the formation of PIH plays a role in the direct mechanism of inhibiting mycobacteria in vivo in humans upon INH administration.

Administration of PCIH to mice resulted in a significant reduction in *M. tuberculosis* load compared to untreated controls in host cells and mouse spleens (Fig. 5). While the reduction in lung bacterial growth was not significant for INH or PCIH, there was a ~1-log reduction in CFU compared to control (Fig. 5). This may be due to the relatively short treatment time used in our model (21 days) and the difficulty in treating established *M. tuberculosis* infection in the lungs of
infected mice. Notably, the changes in bacterial growth we observed are similar to that found with other candidate drugs tested against these bacteria (Kanyok et al., 1994). However, other studies have shown a more significant reduction in lung CFU by INH when administered at similar doses to that utilized in the current study (Almeida et al., 2009; Nikonenko et al., 2004). One explanation may be the extended infection time before treatment used in our investigation (28 days) compared to that of previous studies (14 days) (Almeida et al., 2009). It is possible that after only 14 days of infection, mycobacteria are in a more ‘active’ state, and thus susceptible to INH (Vilcheze and Jacobs, 2007). Differences in the mouse strain used (Almeida et al., 2009) and the route of M. tuberculosis administration (Nikonenko et al., 2004) may also account for this difference.

Treatment of M. avium infection with INH and PCIH also resulted in the greatest level of splenic clearance (Fig. 4). This is similar to what has been observed previously after M. avium infection of beige mice (Fattorini et al., 1998).

Our previous studies examined the toxicity of the closely related PCIH analogue, PCTH (Table 1), in mice over 3 weeks at 50 or 100 mg/kg twice daily (bd) as an agent for the treatment of iron overload. This treatment regimen with PCTH led to no appreciable toxicity as shown by weight loss and a range of biochemical and haematological indices (Wong et al., 2004). Importantly, PCTH (50 or 100 mg/kg bd) did not show any significant changes in hemoglobin or erythrocyte levels. Based on the tolerability of PCTH, the in vivo administration of lower levels of PCIH (10 or 100 mg/kg) once daily over 21 days in the current study would not be expected to have a significant effect on haematological indices. This is supported by the high tolerability of PCIH observed herein.

In summary, we demonstrate that PCIH displays potent activity against mycobacterial strains in vitro and in vivo models. The pronounced efficacy of PCIH is mediated by a novel mechanism.
involving its ability to act as a lipophilic vehicle to penetrate both mycobacterial and mammalian cells and release INH intracellularly.
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AUTHORSHIP CONTRIBUTION

Participated in research design: Ellis, Kalinowski, Jelfs, Sintchenko, Richardson, Triccas

Conducted experiments: Ellis, Kalinowski, Leotta, Jelfs

Contributed new reagents or analytical tools: Huang

Performed data analysis: Ellis, Kalinowski, Jelfs, Sintchenko, Richardson, Triccas

Wrote or contributed to the writing of the manuscript: Ellis, Kalinowski, Huang, Jelfs, Sintchenko, Richardson, Triccas
REFERENCES


FOOTNOTES

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c) For reprint requests: Des R. Richardson
Molecular Pharmacology and Pathology Program
Department of Pathology and Bosch Institute
University of Sydney, Sydney, NSW, 2006
Phone: (612) 9036 6548
Fax: (612) 9351 3429
Email: d.richardson@med.usyd.edu.au
FIGURE LEGENDS

Figure 1. Concentration-dependent inhibition of mycobacterial growth by PCIH. BCG (A) and M. avium (B) (OD_{600nm} 0.001) were incubated with varying doses of INH (black squares) or PCIH (white circles) and after a 7 and 5 day incubation, respectively, resazurin (0.05%) was added and fluorescence measured. Graphs represent percentage survival of bacteria compared to non-treated cells. Data are mean survival ± SEM of triplicate cultures and represent two independent experiments. Differences between INH- and PCIH-treated bacteria were determined by ANOVA (*p<0.01; **p<0.001).

Figure 2: Concentration-dependent inhibition of mycobacterial growth by PCIH. BCG (A-D) and M. avium (E-H) (OD_{600nm} 0.001, 0.01, 0.1 or 1.0) were incubated with varying doses of INH (black squares) or PCIH (white circles) and after a 7 and 5 day incubation, respectively, resazurin (0.05%) added and fluorescence measured. Graphs represent percentage survival of bacteria compared to non-treated cells. Data are mean survival ± SEM of triplicate cultures and represent two independent experiments. Differences between INH- and PCIH-treated bacteria were determined by ANOVA (*p<0.01; **p<0.001).

Figure 3. Effect of PCIH on intracellular bacteria and host cell viability. THP-1 cells (5x10^5) cells infected with BCG pSMT1 (A) or M. avium pSMT1 (B) at a MOI of 10:1 or un-infected control THP-1 cells (C) were treated with INH or PCIH (0.3-10 µM). Luminescence was measured 7 days post-infection and bacterial survival was determined relative to non-treated cells. Viability of THP-1 cells was determined relative to untreated cells by assessment of resazurin reduction. Black bars represent INH-treated bacteria while white bars represent PCIH-treated bacteria. Data are mean survival ± SEM of triplicate cultures and represent two independent experiments. Differences between INH- and PCIH-treated bacteria/cells were determined by ANOVA (*p<0.05).
**Figure 4.** *In vivo* efficacy against mycobacterial infection. C57BL/6 mice (*n*=5) were infected intranasally with 1x10^5 CFU *M. avium*. After 28 days of infection, mice were treated daily for a further 21 days with 100 mg/kg of INH or PCIH. The lung and spleen were harvested and bacterial load determined. Vehicle-treated, black bars; INH-treated, grey bars; PCIH-treated, white bars. Data are mean bacterial survival ± SEM and represent two independent experiments. Differences between INH- and PCIH-infected mice compared to untreated animals were determined by ANOVA (*p*<0.05).

**Figure 5.** Efficacy of PCIH against *M. tuberculosis*. (A) *M. tuberculosis* was incubated with varying doses of compound and after 7 days resazurin (0.05%) was added and fluorescence measured. Graphs represent percentage survival of bacteria compared to non-treated cells. (B) Compounds (10 μM or 1 μM) or vehicle alone control (CON) were added to 1 x 10^5 THP-1 cells previously infected with *M. tuberculosis* at an MOI of 1:1. Cells were lysed 7 days post-infection and bacterial counts obtained. (C-D) C57BL/6 mice were aerosol infected with *M. tuberculosis* and 28 days post infection mice were treated daily for 28 days with 100 mg/kg or 10 mg/kg of INH or PCIH, or treated with vehicle control (CON). The lung (C) and spleen (D) were harvested and bacterial load determined. Data are mean bacterial load ± SEM and represent two independent experiments. Differences between INH- and PCIH-infected groups compared to untreated controls were determined by ANOVA (*p*<0.05).

**Figure 6.** Schematic of the possible mechanisms involved in the anti-mycobacterial activity of PCIH. (1) PCIH can enter the cell readily due to it being relatively lipophilic; (2) PCIH can bind macrophage iron pools depleting them of iron, and in turn, depriving mycobacteria of iron which is essential for growth and replication; (3) PCIH may be hydrolyzed in the macrophage liberating the starting materials, 2-pyridylcarboxaldehyde (2-PC) and isonicotinic acid hydrazide (INH); (4) INH
can then diffuse into the phagolysosome to inhibit the mycobacterium synthetic machinery needed for cell wall biosynthesis; (5) PCIH may enter the phagolysosome to deplete iron directly from the mycobacterium, leading to its iron deprivation; and (6) PCIH may enter the phagolysosome and/or mycobacterium itself, and then be hydrolyzed to 2-PC and INH and subsequently the INH then inhibits mycobacterial cell wall synthesis. Notably, the phagolysosome is acidic which could favor hydrolysis of PCIH. All, or some of these mechanisms may be occurring simultaneously, to effectively prevent mycobacterial growth (see text for further details).
### TABLE 1 Structure and activity of PIH derivatives against mycobacteria

<table>
<thead>
<tr>
<th>Compound</th>
<th>Structure</th>
<th>Log $P_{calc}$</th>
<th>BCG Survival at 10 µM (% of control)$^a$</th>
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</thead>
<tbody>
<tr>
<td>INH</td>
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<td>-0.59</td>
<td>0.39 ± 0.13*</td>
</tr>
<tr>
<td>PIH</td>
<td><img src="image" alt="PIH structure" /></td>
<td>0.30</td>
<td>15.44 ± 0.04*</td>
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<tr>
<td>PCIH</td>
<td><img src="image" alt="PCIH structure" /></td>
<td>0.96</td>
<td>0.02 ± 0.11*</td>
</tr>
<tr>
<td>PCBH</td>
<td><img src="image" alt="PCBH structure" /></td>
<td>2.21</td>
<td>95.44 ± 2.36</td>
</tr>
<tr>
<td>PC3BBH</td>
<td><img src="image" alt="PC3BBH structure" /></td>
<td>3.05</td>
<td>40.98 ± 3.07*</td>
</tr>
<tr>
<td>PC4BBH</td>
<td><img src="image" alt="PC4BBH structure" /></td>
<td>3.05</td>
<td>13.04 ± 8.04*</td>
</tr>
<tr>
<td>PCHH</td>
<td><img src="image" alt="PCHH structure" /></td>
<td>1.86</td>
<td>89.67 ± 3.19</td>
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<tr>
<td>PCAH</td>
<td><img src="image" alt="PCAH structure" /></td>
<td>1.41</td>
<td>99.43 ± 3.09</td>
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<tr>
<td>PCTH</td>
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<td>39.30 ± 10.65*</td>
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<tr>
<td>PCNH</td>
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<td>1.64</td>
<td>87.15 ± 9.92</td>
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<tr>
<td>PCTFH</td>
<td><img src="image" alt="PCTFH structure" /></td>
<td>3.14</td>
<td>39.68 ± 11.20*</td>
</tr>
</tbody>
</table>

$^a$Data are mean survival ± SEM of triplicate cultures and represent two independent experiments.

Differences between non-treated and treated bacteria were determined by ANOVA ($^*p<0.01$).
Figure 1
Figure 2

Mycobacterial Survival (% of control)

Dose Response of Drug Concentration (µM)

A. OD$_{600nm}$ 0.001

B. OD$_{600nm}$ 0.01

C. OD$_{600nm}$ 0.1

D. OD$_{600nm}$ 1.0

E. OD$_{600nm}$ 0.001

F. OD$_{600nm}$ 0.01

G. OD$_{600nm}$ 0.1

H. OD$_{600nm}$ 1.0

drug concentration (µM)
Figure 3

A

Mycobacterial Survival (% of control)

0 20 40 60 80 100

PCIH

INH

Drug Concentration (μM)

0.3 1 3 10

B

Mycobacterial Survival (% of control)

0 20 40 60 80

Drug Concentration (μM)

0.3 1 3 10

C

THP-1 Cell Viability (% of control)

0 20 40 60 80 100

Drug Concentration (μM)

0.3 1 3 10

*
Figure 4

Mycobacterial Load (log$_{10}$ CFU/organ)

- Lung
- Spleen

- Control
- INH
- PCIH

* *
Figure 5

A. Mycobacterial Survival (% of control) vs. drug concentration (µM)

B. Mycobacterial Load (log_{10} CFU/mL) vs. drug concentration (µM)

C. Mycobacterial Load (log_{10} CFU/lung) vs. drug concentration (µM)

D. Mycobacterial Load (log_{10} CFU/spleen) vs. drug concentration (µM)

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Figure 6