Potent Antimycobacterial Activity of the Pyridoxal Isonicotinoyl Hydrazone Analog 2-Pyridylcarboxaldehyde Isonicotinoyl Hydrazone: A Lipophilic Transport Vehicle for Isonicotinic Acid Hydrazide

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Received October 20, 2013; accepted November 15, 2013

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 whether 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 that one compound, namely, 2-pyridylcarboxaldehyde isonicotinoyl hydrazone (PCIH), exhibited nanomolar in vitro activity against *Mycobacterium bovis* bacille Calmette-Guérin and virulent *M. tuberculosis*. Interestingly, PCIH is derived from the condensation of 2-pyridylcarboxaldehyde with the first-line antituberculosis 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 antimycobacterial efficacy. However, this could not totally account for its potent efficacy, and structure-activity relationship studies suggest that PCIH acts as a lipophilic vehicle for the transport of its intact INH moiety into the mammalian cell 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 one third of the global population (World Health Organization, 2012; http://apps.who.int/iris/bitstream/10665/91355/1/9789241564656_eng.pdf). 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 (World Health Organization, 2012). Of particular concern is the increased incidence of multidrug-resistant strains over the past decade, coupled with the emergence of extensively drug-resistant strains of *Mycobacterium tuberculosis* (World Health Organization, 2012). Strategies to improve tuberculosis drug therapy include the modification of existing drugs and 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 (Sinclair et al., 2011; Greenstein et al., 2012).

This work was supported by the National Health and Medical Research Council (NHMRC) Centre of Research Excellence on Tuberculosis Control [Grant APP1043225]. D.R.R. is supported by an NHMRC Senior Principal Research Fellowship and Project Grants. D.S.K. is supported by an NHMRC Project Grant [Grant APP1048972] and Cancer Institute NSW Early Career Fellowship.

This work was previously presented at the following Keystone Symposium: Ellis S (2012) The use of pyridoxal isonicotinoyl hydrazone (PIH) derivatives as a treatment against mycobacteria (abstract 123). *Drug Resistance and Persistence in Tuberculosis*; 2012 May 13–18; Kampala, Uganda.
dx.doi.org/10.1124/mol.113.090357.

ABBREVIATIONS: BCG, bacille Calmette-Guérin; CFU, colony-forming units; DFO, desferrioxamine; DMEM, Dulbecco's modified Eagle's medium; GU, growth unit; INH, isonicotinic acid hydrazide; MIC, minimum inhibitory concentration; OD, optical density; 2-PC, 2-pyridylcarboxaldehyde; PC3BBH, 2-pyridylcarboxaldehyde 3-bromobenzoyl hydrazine; PC4BBH, 2-pyridylcarboxaldehyde 4-bromobenzoyl hydrazine; PCAH, 2-pyridylcarboxaldehyde *p*-aminobenzoyl hydrazine; PCBH, 2-pyridylcarboxaldehyde benzoyl hydrazine; PCHH, 2-pyridylcarboxaldehyde *p*-hydroxybenzoyl hydrazine; PCIH, 2-pyridylcarboxaldehyde isonicotinoyl hydrazine; PCNH, 2-pyridylcarboxaldehyde *p*-nitrobenzoyl hydrazine; PCTFH, 2-pyridylcarboxaldehyde *p*-trifluoromethylbenzoyl hydrazine; PCTH, 2-pyridylcarboxaldehyde 2-thiophenecarboxyl hydrazine; PIH, pyridoxal isonicotinoyl hydrazone.

Iron is an obligate cofactor for >40 enzymes encoded within the mycobacterial genome (Cronje and Bornman, 2005). Previous studies have shown that *M. tuberculosis* bacterial load is increased in iron-supplemented murine models relative to their control counterparts (Lounis et al., 2001). In human immunodeficiency virus-infected patients, the incidence of mycobacterial coinfection 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 human immunodeficiency virus 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 antiproliferative activity in the inhibition of tumor growth (Yu et al., 2006, 2009; Richardson et al., 2009). 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), and Ca(II) (Richardson and Ponka, 1998a). The pharmacological advantages of PIH and its analogs include the following: 1) PIH can be simply prepared by a one-step synthesis; 2) the compounds possess high membrane permeability; 3) they can be orally administered (Richardson and Ponka, 1998a); and 4) they are neutral at biologic 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 analogs that have properties useful for the treatment of iron overload disease or cancer (Sookvanichsilp et al., 1991; Richardson et al., 1995). 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 antiproliferative effects with minimal toxicity in vivo (Whitnall et al., 2006; Kovacevic et al., 2011; Lovejoy et al., 2012).

PIH is the result of a simple condensation between pyridoxal and the potent antimycobacterial 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 analogs (Table 1) to inhibit the growth of pathogenic mycobacterial strains. This study identifies a PIH analog,

TABLE 1
Structure and activity of PIH derivatives against mycobacteria

Compound	Structure	Log P_{calc}	BCG Survival at 10 μ M ^a
			% of control
INH		-0.59	0.39 $\pm$ 0.13*
PIH		0.30	15.44 $\pm$ 0.04*
PCIH		0.96	0.02 $\pm$ 0.11*
PCBH		2.21	95.44 $\pm$ 2.36
PC3BBH		3.05	40.98 $\pm$ 3.07*
PC4BBH		3.05	13.04 $\pm$ 8.04*
PCHH		1.86	89.67 $\pm$ 3.19
PCAH		1.41	99.43 $\pm$ 3.09
PCTH		2.49	39.30 $\pm$ 10.65*
PCNH		1.64	87.15 $\pm$ 9.92
PCTFH		3.14	39.68 $\pm$ 11.20*

^aData are mean survival $\pm$ S.E.M. of triplicate cultures and represent two independent experiments. Differences between nontreated and treated bacteria were determined by analysis of variance.

* $P < 0.01$

2-pyridylcarboxylaldehyde isonicotinoyl hydrazone (PCIH), as a potent inhibitor of mycobacterial growth. Our results indicate that the pronounced activity of PCIH is mediated by

a novel mechanism, as structure-activity relationship studies suggest that PCIH acts as a lipophilic vehicle for the transport of the intact INH moiety into the cell and the mycobacterium.

Materials and Methods

Bacterial Growth Conditions. All mycobacterial strains [*M. avium* 104, *Mycobacterium bovis* bacille Calmette-Guérin (BCG) Pasteur, and *M. tuberculosis* H37Rv] were grown in complete Middlebrook 7H9 media (Bacto Laboratories, Mt. Pritchard, NSW, Australia) containing albumin, dextrose, and catalase; 20% Tween-80; and 50% glycerol (Sigma-Aldrich, Castle Hill, NSW, 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 electrocompetent by standard methods (Lee et al., 2002) and transformed with 2 μ l of pSMT1 (Snewin et al., 1999) by electroporation (25 μ F, 2.5 kV, 200 Ω). Transformants were selected on Middlebrook 7H11 plates supplemented with 25 μ g/ml hygromycin (Sigma-Aldrich). BCG::pSMT1 and *M. avium*::pSMT1 were cultured in complete Middlebrook 7H9 media supplemented with 25 μ g/ml of hygromycin.

M. tuberculosis Beijing genotype INH-sensitive and monoresistant (*katG* mutation) strains were sourced from the NSW Mycobacterium Reference Laboratory, Centre for Infectious Diseases and Microbiology Laboratory Services Strain Collection, Westmead Hospital (Sydney, NSW, Australia). The resistant strain displayed an INH minimum inhibitory concentration (MIC) of >0.4 μ g/ml. The INH and PCIH sensitivity of strains was assessed using the BACTEC MGIT 960 system (Becton Dickinson, North Ryde, NSW, Australia), 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 μ g/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 >4 days and the 1% growth control tube achieved a growth unit (GU) of 400. A GU of $\leq$ 100 in the drug-containing tube indicated the strain was susceptible. A GU of >100 was interpreted as resistant.

Iron Chelators. PIH and its analogs were synthesized by established procedures by a simple one-step synthesis between commercially available aromatic aldehydes and hydrazides (Sigma-Aldrich) (Kalinowski et al., 2008). The following PIH analogs were examined in the current study: 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 hydrazone (PCTH), 2-pyridylcarboxaldehyde *p*-nitrobenzoyl hydrazone (PCNH), and 2-pyridylcarboxaldehyde *p*-trifluoromethylbenzoyl hydrazone (PCTFH). Log P_{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 et al., 1984).

Resazurin Assay of Growth Inhibition. To determine the 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-to-iron ratio using FeSO₄ was serially diluted in 10 μ l of purified H₂O in triplicate (0.01–10 μ M). The bacterial suspension (90 μ l) at varying optical density (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 μ l; 0.05% (w/v)] (Sigma-Aldrich) was then added and incubated for 4 hours at 37°C, and fluorescence was measured at 590 nm using a FLUOstar Omega

microplate reader (BMG Labtech, Ortenberg, 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 with 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, Manassas, VA), were grown in complete Dulbecco's modified Eagle's medium (DMEM) (Life Technologies, Mulgrave, VIC, Australia) including 10% fetal bovine serum, 200 μ M L-glutamine (Life Technologies), and 1 mM HEPES buffer solution (Life Technologies). Cells (1×10^5) in media containing 50 ng/ml phorbol 12-myristate 13-acetate were added to 96-well plates and then incubated for 48 hours at 37°C to allow adherence and differentiation. Cells were then infected with 10^6 colony-forming units (CFU) BCG::pSMT1 or *M. avium*::pSMT1 prepared in DMEM for 4 hours at 37°C. After 4 hours, cells were washed three times with phosphate-buffered saline/2% fetal calf serum, and complete DMEM was added after the final wash. Compounds (0.3–10 μ M) were then added to plates and incubated for up to 7 days at 37°C in 5% CO₂. Decanal (1%) (Sigma-Aldrich) substrate was added to each well and the luminescence measured after 7 seconds 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 with the untreated control.

For determination of intracellular bacterial load, cells were infected with *M. tuberculosis* H37Rv (OD_{600nm}, 0.01) for 4 hours at 37°C, compounds were added (1 or 10 μ M), and then the treated and non-treated cells were incubated for 7 days at 37°C in 5% CO₂. Cells were then lysed after this period, and bacterial number was determined after plating the suspension onto Middlebrook 7H11 agar, using standard procedures (Pinto et al., 2004).

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

Murine Studies. C57BL/6 mice were purchased from the Animal Resources Centre (Perth, WA, Australia), and all experiments were approved by the University of Sydney Animal Ethics Committee. Mice were either infected intranasally with 1×10^5 CFU *M. avium* or aerosol-infected with *M. tuberculosis* H37Rv, as previously described (Pinto et al., 2004). At 4 weeks postinfection, INH (10 or 100 mg/kg), PCIH (10 or 100 mg/kg), or vehicle only (50% dimethylsulfoxide in phosphate-buffered saline) was administered daily via the intraperitoneal 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 Middlebrook 7H11 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 with pairwise 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 nine PIH analogs 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 has 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 analogs constitutes 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 antibacterial efficacy observed. Notably, INH was included as a positive control due to its potent antimycobacterial 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 with 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 (PCIH) growth inhibition (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 analog PC4BBH showed inhibitory activity that was comparable to that of PIH but less than that of INH or PCIH (Table 1). Despite their very similar structures, the other

seven compounds screened showed limited efficacy, with PCBH, PCHH, PCAH, and PCNH causing a $<20\%$ reduction in BCG growth at 10 μM in comparison with 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 antimycobacterial activity (Table 1). This observation indicates 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 ($\log P_{\text{calc}}$ increased 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 antimycobacterial efficacy relative to PCBH (Table 1). However, despite the greater lipophilicity of these analogs relative to PCBH and especially PCIH, the antimycobacterial activity was considerably less than that of PCIH (Table 1). Moreover, there was little correlation between the $\log P_{\text{calc}}$ of these analogs 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 antimycobacterial 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 $<1 \mu\text{M}$ (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 $\text{OD}_{600\text{nm}}$ of 0.001, 0.01, and 0.1 (Fig. 2, A–C). However, notably, at an $\text{OD}_{600\text{nm}}$ of 1.0, PCIH was able to significantly ($P < 0.001$) inhibit BCG growth compared with INH at 1 and 10 μM (Fig. 2D). Testing the effect of PCIH against varying bacterial loads of *M. avium* (Fig. 2, E–H) resulted in a significantly ($P < 0.01$) greater reduction in bacterial survival compared with INH. This occurred at lower bacterial loads ($\text{OD}_{600\text{nm}}$, 0.001; Fig. 2E) and higher concentrations of the agents when bacterial load was increased ($\text{OD}_{600\text{nm}}$, 0.01–0.1; Fig. 2, F and G). However, none of the compounds showed growth inhibition at an $\text{OD}_{600\text{nm}}$ 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 antimycobacterial activity due to its

Fig. 1. Concentration-dependent inhibition of mycobacterial growth by PCIH. BCG (A) and *M. avium* (B) ($\text{OD}_{600\text{nm}}$, 0.001) were incubated with varying doses of INH or PCIH, and after a 7- and 5-day incubation, respectively, resazurin (0.05%) was added and fluorescence measured. Graphs represent percentage survival of bacteria compared with non-treated cells. Data are mean survival $\pm$ S.E.M. of triplicate cultures and represent two independent experiments. Differences between INH- and PCIH-treated bacteria were determined by analysis of variance (* $P < 0.01$; ** $P < 0.001$).

Fig. 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 or PCIH, and after a 7- and 5-day incubation, respectively, resazurin (0.05%) was added and fluorescence measured. Graphs represent percentage survival of bacteria compared with nontreated cells. Data are mean survival $\pm$ S.E.M. of triplicate cultures and represent two independent experiments. Differences between INH- and PCIH-treated bacteria were determined by analysis of variance (* $P < 0.01$; ** $P < 0.001$).

isonicotinoyl hydrazone substructure (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 precomplexed in a 2:1 ratio with iron to saturate its iron-binding site and thus prevent 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 to 0.63 μM) relative to PCIH alone (data not shown), suggesting that 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 antimycobacterial activity, resulting in an ~8-fold increase in MIC_{90} (1.25 to $>10 \mu M$; data not shown). This observation indicates 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 and 1.89, respectively) (Bernhardt et al., 2007), and this suggests that 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 antimycobacterial 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

Fig. 3. Effect of PCIH on intracellular bacteria and host cell viability. THP-1 cells (5×10^5) infected with BCG::pSMT1 (A) or *M. avium*::pSMT1 (B) at a multiplicity of infection of 10:1 or uninfected control THP-1 cells (C) were treated with INH or PCIH (0.3–10 μ M). Luminescence was measured 7 days postinfection, and bacterial survival was determined relative to nontreated cells. Viability of THP-1 cells was determined relative to untreated cells by assessment of resazurin reduction. Data are mean survival $\pm$ S.E.M. of triplicate cultures and represent two independent experiments. Differences between INH- and PCIH-treated bacteria/cells were determined by analysis of variance (* $P < 0.05$).

not reach statistical significance (Fig. 3A). Both INH and PCIH displayed a similar level of effectiveness when tested at 3 and 5 days postinfection (data not shown).

When tested against *M. avium*-infected THP-1 cells, PCIH exhibited a trend of antimycobacterial activity similar to that of INH at high drug concentrations (Fig. 3B). However, PCIH was significantly ($P < 0.05$) more effective than INH at a concentration of 0.3 μ M (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 antiproliferative 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 with the vehicle-only-treated animals (Fig. 4). However, in the spleen, PCIH treatment led to an ~ 1.5 -log reduction in *M. avium* CFU compared with 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 Antituberculosis 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 with INH when tested against *M. tuberculosis* grown in culture media (Fig. 5A), with a MIC₉₀ of 0.31 and 0.63 μ M 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 with untreated cells by >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 with control mice (Fig. 5, C and D). Further, treatment with PCIH caused a similar level of reduction of bacterial load as that found for INH (Fig. 5, C and 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 *M. tuberculosis* at a defined set of concentrations (0.1, 0.4, and 2.5 μ g/ml). We selected an *M. tuberculosis katG* mutant strain for this study, due to the critical role of KatG in INH activation and the dominance of *katG* mutations in *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 *M. tuberculosis* and require activation by KatG to exert their activity, as the *M. tuberculosis katG* mutant strain was resistant to INH and

Fig. 4. In vivo efficacy against mycobacterial infection. C57BL/6 mice ($n = 5$) were infected intranasally with 1×10^5 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 was determined. Data are mean bacterial survival $\pm$ S.E.M. and represent two independent experiments. Differences between INH- and PCIH-treated mice compared with untreated animals were determined by analysis of variance (* $P < 0.05$).

Fig. 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 with nontreated cells. (B) Compound (10 or 1 μM) or vehicle control (CON) was added to 1×10^5 THP-1 cells previously infected with *M. tuberculosis* at a multiplicity of infection of 1:1. Cells were lysed 7 days postinfection and bacterial counts obtained. (C and D) C57BL/6 mice were aerosol-infected with *M. tuberculosis*, and 28 days postinfection mice were treated daily for a further 28 days with 100 or 10 mg/kg of INH or PCIH or treated with vehicle control. The lung (C) and spleen (D) were harvested and bacterial load determined. Data are mean bacterial load $\pm$ S.E.M. and represent two independent experiments. Differences between INH- and PCIH-treated groups compared with untreated controls were determined by analysis of variance (* $P < 0.05$).

PCIH at all concentrations tested (data not shown). In contrast, wild-type *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 antimycobacterial activity.

Discussion

Of the nine PIH analogs 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 analogs 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 with 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 with 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) is

predominantly neutral at physiologic pH; 3) is less than half the molecular weight of DFO; and 4) 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 (Fig. 6) (Ehrt and Schnappinger, 2009), 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 antimycobacterial agent.

The enhanced intracellular activity of PCIH may also be due to its greater lipophilicity compared with 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 antimycobacterial action. However, an argument that relies merely 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 differs only very slightly in structure from 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 antimycobacterial agent as effective as PCIH (Table 1). Hence, other factors, in addition to lipophilicity, played a role in the antibacterial activity observed.

In this study, the greatest antimycobacterial 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

Fig. 6. Schematic of the possible mechanisms involved in the antimycobacterial activity of PCIH. (1) PCIH can enter the cell readily due to its 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-PC and 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. (6) PCIH may enter the phagolysosome and/or mycobacterium itself and then be hydrolyzed to 2-PC and INH, and subsequently the INH 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).

the analogous hydrazide used to synthesize PCBH) displays little antimycobacterial 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 antituberculosis agent. Considering this, it can be speculated that the efficacy of PCIH relative to INH could be related to the ligand's acting as a lipophilic vehicle for INH transport into the mammalian cell 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 latter 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 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, INH is activated, leading to inhibition of mycolic acid synthesis (Vilcheze and Jacobs, 2007). Furthermore, acid-catalyzed hydrolysis of PIH analogs is well known (Richardson et al., 1989), and it is notable that mycobacteria-containing phagolysosomes 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, results in a novel therapeutic.

Interestingly, regarding this 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 that the formation of PIH might play 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 with 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 an ~1-log reduction in CFU compared with 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 those 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 doses similar to that used in the current study (Nikonenko et al.,

2004; Almeida et al., 2009). One explanation may be the extended infection time before treatment used in our investigation (28 days) compared with 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 analog PCTH (Table 1) administered to mice over 3 weeks at 50 or 100 mg/kg twice daily 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 hematologic indices (Wong et al., 2004). Importantly, PCTH (50 or 100 mg/kg twice daily) 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 hematologic 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 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.

Acknowledgments

The authors thank Dr. Vera Richardson (Molecular Pathology and Pharmacology Program, Department of Pathology, University of Sydney) for critical reading of the manuscript prior to submission.

Authorship Contributions

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

Conducted experiments: Ellis, Kalinowski, Leotta, Jelfs.

Contributed new reagents or analytic 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

- Almeida D, Nuermberger E, Tasneen R, Rosenthal I, Tyagi S, Williams K, Peloquin C, and Grosset J (2009) Paradoxical effect of isoniazid on the activity of rifampin-pyrazinamide combination in a mouse model of tuberculosis. *Antimicrob Agents Chemother* **53**:4178–4184.
- Baker E, Richardson D, Gross S, and Ponka P (1992) Evaluation of the iron chelation potential of hydrazones of pyridoxal, salicylaldehyde and 2-hydroxy-1-naphthylaldehyde using the hepatocyte in culture. *Hepatology* **15**:492–501.
- Becker E and Richardson DR (1999) Development of novel aroylhydrazone ligands for iron chelation therapy: 2-pyridylcarboxaldehyde isonicotinoyl hydrazone analogs. *J Lab Clin Med* **134**:510–521.
- Bernhardt PV, Chin P, and Richardson DR (2001) Unprecedented oxidation of a biologically active aroylhydrazone chelator catalysed by iron(III): serendipitous identification of diacylhydrazine ligands with high iron chelation efficacy. *J Biol Inorg Chem* **6**:801–809.
- Bernhardt PV, Chin P, Sharpe PC, and Richardson DR (2007) Hydrazone chelators for the treatment of iron overload disorders: iron coordination chemistry and biological activity. *Dalton Trans* **30**:3232–3244.
- Broto PM, Moreau G, and Vandyke C (1984) Molecular structures: perception, autocorrelation descriptor and SAR studies. System of atomic contributions for the calculation of the n-octanol/water partition coefficients. *Eur J Med Chem* **19**:71–78.
- Cronje L and Bornman L (2005) Iron overload and tuberculosis: a case for iron chelation therapy. *Int J Tuberc Lung Dis* **9**:2–9.
- Cronjé L, Edmondson N, Eisenach KD, and Bornman L (2005) Iron and iron chelating agents modulate *Mycobacterium tuberculosis* growth and monocyte-macrophage viability and effector functions. *FEMS Immunol Med Microbiol* **45**:103–112.
- de Monyé C, Karcher DS, Boelaert JR, and Gordeuk VR (1999) Bone marrow macrophage iron grade and survival of HIV-seropositive patients. *AIDS* **13**:375–380.
- Debebe Z, Ammosova T, Jerebtsova M, Kurantsin-Mills J, Niu X, Charles S, Richardson DR, Ray PE, Gordeuk VR, and Nekhai S (2007) Iron chelators ICL670 and 311 inhibit HIV-1 transcription. *Virology* **367**:324–333.
- Dhople AM, Ibanez MA, and Poirier TC (1996) Role of iron in the pathogenesis of *Mycobacterium avium* infection in mice. *Microbios* **87**:77–87.
- Dixit P, Singh U, Sharma P, and Jain A (2012) Evaluation of nitrate reduction assay, resazurin microtiter assay and microscopic observation drug susceptibility assay for first line antitubercular drug susceptibility testing of clinical isolates of *M. tuberculosis*. *J Microbiol Methods* **88**:122–126.
- Ehrt S and Schnappinger D (2009) Mycobacterial survival strategies in the phagosome: defence against host stresses. *Cell Microbiol* **11**:1170–1178.
- Fattorini L, Xiao Y, Mattei M, Li Y, Iona E, Ricci ML, Thoresen OF, Creti R, and Orefici G (1998) Activities of isoniazid alone and in combination with other drugs against *Mycobacterium avium* infection in beige mice. *Antimicrob Agents Chemother* **42**:712–714.
- Geisow MJ, D'Arcy Hart P, and Young MR (1981) Temporal changes of lysosome and phagosome pH during phagolysosome formation in macrophages: studies by fluorescence spectroscopy. *J Cell Biol* **89**:645–652.
- Ghose AK and Crippen GM (1987) Atomic physicochemical parameters for three-dimensional-structure-directed quantitative structure-activity relationships. 2. Modeling dispersive and hydrophobic interactions. *J Chem Inf Comput Sci* **27**:21–35.
- Gomes MS, Boelaert JR, and Appelberg R (2001) Role of iron in experimental *Mycobacterium avium* infection. *J Clin Virol* **20**:117–122.
- Greenstein RJ, Su L, and Brown ST (2012) Vitamins A & D inhibit the growth of mycobacteria in radiometric culture. *PLoS ONE* **7**:e29631.
- Hermes-Lima M, Ponka P, and Schulman HM (2000) The iron chelator pyridoxal isonicotinoyl hydrazone (PIH) and its analogues prevent damage to 2-deoxyribose mediated by ferric iron plus ascorbate. *Biochim Biophys Acta* **1523**:154–160.
- Kalinowski DS, Sharpe PC, Bernhardt PV, and Richardson DR (2008) Structure-activity relationships of novel iron chelators for the treatment of iron overload disease: the methyl pyrazinylketone isonicotinoyl hydrazone series. *J Med Chem* **51**:331–344.
- Kang SK, Lee JH, Lee YC, and Kim CH (2006) Catalase-peroxidase of *Mycobacterium bovis* BCG converts isoniazid to isonicotinamide, but not to isonicotinic acid: differentiation parameter between enzymes of *Mycobacterium bovis* BCG and *Mycobacterium tuberculosis*. *Biochim Biophys Acta* **1760**:724–729.
- Kanyok TP, Reddy MV, Chinnaswamy J, Danziger LH, and Gangadharam PR (1994) In vivo activity of paromomycin against susceptible and multidrug-resistant *Mycobacterium tuberculosis* and *M. avium* complex strains. *Antimicrob Agents Chemother* **38**:170–173.
- Kovacevic Z, Chikhani S, Lovejoy DB, and Richardson DR (2011) Novel thiosemicarbazone iron chelators induce up-regulation and phosphorylation of the metastasis suppressor N-myc down-stream regulated gene 1: a new strategy for the treatment of pancreatic cancer. *Mol Pharmacol* **80**:598–609.
- Kovářiková P, Klimes J, Stěrba M, Popelová O, Mokřý M, Gersl V, and Ponka P (2005) Development of high-performance liquid chromatographic determination of salicylaldehyde isonicotinoyl hydrazone in rabbit plasma and application of this method to an in vivo study. *J Sep Sci* **28**:1300–1306.
- Lee SH, Cheung M, Irani V, Carroll JD, Inamine JM, Howe WR, and Maslow JN (2002) Optimization of electroporation conditions for *Mycobacterium avium*. *Tuberculosis (Edinb)* **82**:167–174.
- Link G, Ponka P, Konijn AM, Breuer W, Cabantchik ZI, and Hershko C (2003) Effects of combined chelation treatment with pyridoxal isonicotinoyl hydrazone analogs and deferoxamine in hypertransfused rats and in iron-loaded rat heart cells. *Blood* **101**:4172–4179.
- Lounis N, Truffot-Pernot C, Grosset J, Gordeuk VR, and Boelaert JR (2001) Iron and *Mycobacterium tuberculosis* infection. *J Clin Virol* **20**:123–126.
- Lovejoy DB, Sharp DM, Seebacher N, Obeidy P, Prichard T, Stefani C, Basha MT, Sharpe PC, Jansson PJ, and Kalinowski DS et al. (2012) Novel second-generation di-2-pyridylketone thiosemicarbazones show synergism with standard chemotherapeutics and demonstrate potent activity against lung cancer xenografts after oral and intravenous administration in vivo. *J Med Chem* **55**:7230–7244.
- Nikonenko BV, Samala R, Einck L, and Nacy CA (2004) Rapid, simple in vivo screen for new drugs active against *Mycobacterium tuberculosis*. *Antimicrob Agents Chemother* **48**:4550–4555.
- Pinto R, Saunders BM, Camacho LR, Britton WJ, Gicquel B, and Triccas JA (2004) *Mycobacterium tuberculosis* defective in phthiocerol dimycocerosate translocation provides greater protective immunity against tuberculosis than the existing bacille Calmette-Guérin vaccine. *J Infect Dis* **189**:105–112.
- Ponka P, Borová J, Neuwirt J, and Fuchs O (1979) Mobilization of iron from reticulocytes. Identification of pyridoxal isonicotinoyl hydrazone as a new iron chelating agent. *FEBS Lett* **97**:317–321.
- Pradines B, Rolain JM, Ramiandrasoa F, Fusai T, Mosnier J, Rogier C, Daries W, Baret E, Kunesch G, and Le Bras J et al. (2002) Iron chelators as antimalarial agents: in vitro activity of dicaticholate against *Plasmodium falciparum*. *J Antimicrob Chemother* **50**:177–187.
- Quémond A, Lacave C, and Lanéelle G (1991) Isoniazid inhibition of mycolic acid synthesis by cell extracts of sensitive and resistant strains of *Mycobacterium aurum*. *Antimicrob Agents Chemother* **35**:1035–1039.

- Richardson D, Vitolo LW, Baker E, and Webb J (1989) Pyridoxal isonicotinoyl hydrazone and analogues. Study of their stability in acidic, neutral and basic aqueous solutions by ultraviolet-visible spectrophotometry. *Biol Met* **2**:69–76.
- Richardson DR, Kalinowski DS, Lau S, Jansson PJ, and Lovejoy DB (2009) Cancer cell iron metabolism and the development of potent iron chelators as anti-tumour agents. *Biochim Biophys Acta* **1790**:702–717.
- Richardson DR, Mouralian C, Ponka P, and Becker E (2001) Development of potential iron chelators for the treatment of Friedreich's ataxia: ligands that mobilize mitochondrial iron. *Biochim Biophys Acta* **1536**:133–140.
- Richardson DR and Ponka P (1998a) Orally effective iron chelators for the treatment of iron overload disease: the case for a further look at pyridoxal isonicotinoyl hydrazone and its analogs. *J Lab Clin Med* **132**:351–352.
- Richardson DR and Ponka P (1998b) Pyridoxal isonicotinoyl hydrazone and its analogs: potential orally effective iron-chelating agents for the treatment of iron overload disease. *J Lab Clin Med* **131**:306–315.
- Richardson DR, Tran EH, and Ponka P (1995) The potential of iron chelators of the pyridoxal isonicotinoyl hydrazone class as effective antiproliferative agents. *Blood* **86**:4295–4306.
- Richardson DR, Wis Vitolo LM, Hefter GT, May PM, Clare BW, Webb J, and Wilairat P (1990) Iron chelators of the pyridoxal isonicotinoyl hydrazone class Part I. Ionisation characteristics of the ligands and their relevance to biological properties. *Inorg Chim Acta* **170**:165–170.
- Saunders BM, Dane A, Briscoe H, and Britton WJ (2002) Characterization of immune responses during infection with *Mycobacterium avium* strains 100, 101 and the recently sequenced 104. *Immunol Cell Biol* **80**:544–549.
- Sinclair D, Abba K, Grobler L, and Sudarsanam TD (2011) Nutritional supplements for people being treated for active tuberculosis. *Cochrane Database Syst Rev* (11): CD006086.
- Snegin VA, Gares MP, Gaora PO, Hasan Z, Brown IN, and Young DB (1999) Assessment of immunity to mycobacterial infection with luciferase reporter constructs. *Infect Immun* **67**:4586–4593.
- Sookvanichsilp N, Nakornchai S, and Weerapradist W (1991) Toxicological study of pyridoxal isonicotinoyl hydrazone: acute and subchronic toxicity. *Drug Chem Toxicol* **14**:395–403.
- Traoré HN and Meyer D (2007) Necrosis of host cells and survival of pathogens following iron overload in an in vitro model of co-infection with human immunodeficiency virus (HIV) and *Mycobacterium tuberculosis*. *Int J Antimicrob Agents* **29**:465–470.
- Vilchèze C and Jacobs WR, Jr (2007) The mechanism of isoniazid killing: clarity through the scope of genetics. *Annu Rev Microbiol* **61**:35–50.
- Viswanadhan VN, Ghose AK, Revankar GR, and Robins RK (1989) Atomic physico-chemical parameters for three dimensional structure directed quantitative structure-activity relationships. 4. Additional parameters for hydrophobic and dispersive interactions and their application for an automated superposition of certain naturally occurring nucleoside antibiotics. *J Chem Inf Comput Sci* **29**:163–172.
- Walcourt A, Loyevsky M, Lovejoy DB, Gordeuk VR, and Richardson DR (2004) Novel aroylhydrazone and thiosemicarbazone iron chelators with anti-malarial activity against chloroquine-resistant and -sensitive parasites. *Int J Biochem Cell Biol* **36**:401–407.
- Whitehouse LW, Tryphonas L, Paul CJ, Solomonraj G, Thomas BH, and Wong LT (1983) Isoniazid-induced hepatic steatosis in rabbits: an explanation for susceptibility and its antagonism by pyridoxine hydrochloride. *Can J Physiol Pharmacol* **61**:478–487.
- Whitnall M, Howard J, Ponka P, and Richardson DR (2006) A class of iron chelators with a wide spectrum of potent antitumor activity that overcomes resistance to chemotherapeutics. *Proc Natl Acad Sci USA* **103**:14901–14906.
- Wong CS, Kwok JC, and Richardson DR (2004) PCTH: a novel orally active chelator of the aroylhydrazone class that induces iron excretion from mice. *Biochim Biophys Acta* **1739**:70–80.
- Wong EB, Cohen KA, and Bishai WR (2013) Rising to the challenge: new therapies for tuberculosis. *Trends Microbiol* **21**:493–501.
- Yu Y, Kalinowski DS, Kovacevic Z, Siafakas AR, Jansson PJ, Stefani C, Lovejoy DB, Sharpe PC, Bernhardt PV, and Richardson DR (2009) Thiosemicarbazones from the old to new: iron chelators that are more than just ribonucleotide reductase inhibitors. *J Med Chem* **52**:5271–5294.
- Yu Y, Wong J, Lovejoy DB, Kalinowski DS, and Richardson DR (2006) Chelators at the cancer coalface: desferrioxamine to Triapine and beyond. *Clin Cancer Res* **12**:6876–6883.

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