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RESEARCH PAPER

Comparative bioactivation of the novel anti-tuberculosis agent PA-824 in *Mycobacteria* and a subcellular fraction of human liver

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BACKGROUND AND PURPOSE

PA-824 is a 2-nitroimidazooxazine prodrug currently in Phase II clinical trial for tuberculosis therapy. It is bioactivated by a deazaflavin (F_{420})-dependent nitroreductase (Ddn) isolated from *Mycobacterium tuberculosis* to form a des-nitro metabolite. This releases toxic reactive nitrogen species which may be responsible for its anti-mycobacterial activity. There are no published reports of mammalian enzymes bioactivating this prodrug. We have investigated the metabolism of PA-824 following incubation with a subcellular fraction of human liver, in comparison with purified Ddn, *M. tuberculosis* and *Mycobacterium smegmatis*.

EXPERIMENTAL APPROACH

PA-824 (250 μM) was incubated with the 9000× *g* supernatant (S9) of human liver homogenates, purified Ddn, *M. tuberculosis* and *M. smegmatis* for metabolite identification by liquid chromatography mass spectrometry analysis.

KEY RESULTS

PA-824 was metabolized to seven products by Ddn and *M. tuberculosis,* with the major metabolite being the des-nitro product. Six of these products, but not the des-nitro metabolite, were also detected in *M. smegmatis*. In contrast, only four of these metabolites were observed in human liver S9; M3, a reduction product previously proposed as an intermediate in the Ddn-catalyzed des-nitrification and radiolytic reduction of PA-824; two unidentified metabolites, M1 and M4, which were products of M3; and a haem-catalyzed product of imidazole ring hydration (M2).

CONCLUSIONS AND IMPLICATIONS

PA-824 was metabolized by des-nitrification in Ddn and *M. tuberculosis,* but this does not occur in human liver S9 and *M. smegmatis*. Thus, PA-824 was selectively bioactivated in *M. tuberculosis* and there was no evidence for 'cross-activation' by human enzymes.

Abbreviations

Ddn, deazaflavin (F_{420})-dependent nitroreductase; Des-nitro PA-824, (6*S*)-6-{[4-(trifluoromethoxy)benzyl]oxy}-6, 7-dihydro-5*H*-imidazo[2,1-*b*][1,3]oxazine); FGD1, F_{420}-dependent glucose-6-phosphate dehydrogenase; HPLC,

high-performance liquid chromatography; LC, liquid chromatography; LC/MS, liquid chromatography mass spectrometry; M5, (6*S*)-2-nitro-6,7-dihydro-5*H*-imidazo[2,1-*b*][1,3]oxazin-6-ol; PA-824, (6*S*)-2-nitro-6-{[4-(trifluoromethoxy)benzyl]oxy}-6,7-dihydro-5*H*-imidazo[2,1-*b*][1,3]oxazine; NO, nitric oxide; PA-824 amine, (6*S*)-6-{[4-(trifluoromethoxy)benzyl]oxy}-6,7-dihydro-5*H*-imidazo[2,1-*b*][1,3]oxazin-2-amine; RNS, reactive nitrogen species; Rt, retention time; SN 30725, (6*R*)-2-nitro-6,7-dihydro-5*H*-imidazo[2,1-*b*][1,3]oxazin-6-yl [4-(trifluoromethoxy) phenyl] carbamate; SN 31280, (6*S*)-6-[(2,4-dibromobenzyl)oxy]-2-nitro-6,7-dihydro-5*H*-imidazo[2,1-*b*][1,3]oxazine; SN 30440, (2-nitro-6,7-dihydro-5*H*-imidazo[2,1-*b*][1,3]oxazin-7-yl)methanol; TB, tuberculosis

Introduction

Tuberculosis (TB) killed about 1.8 million people in 2008 (WHO, 2009) and the presence of multi- and extensive-drug resistant strains as well as HIV-TB co-infection have made this disease a global health priority. New drugs for the treatment of TB are being sought and the Global Alliance for TB drug development (TB Alliance) has recently begun Phase II clinical trials of PA-824 (Figure 1). This drug is effective against both drug-sensitive and multi-drug resistant *Mycobacterium tuberculosis*. In addition, it is both a potent bactericidal and a sterilizing agent in mouse models of the disease (Stover *et al.*, 2000). Hence, PA-824 holds great potential for shortening the duration of current therapy and also for the treatment of multi-drug resistant TB and TB-HIV co-infections.

PA-824 is a 2-nitroimidazooxazine prodrug that undergoes bioreductive activation in susceptible strains of *M. tuberculosis* to exert its anti-tubercular effect through a novel mechanism of action (Spigelman, 2007). Initial reports indicated that an F_{420}-dependent glucose-6-phosphate dehydrogenase (FGD1) was important in this activation (Stover *et al.*, 2000; Manjunatha *et al.*, 2006) and it is possible that the polar metabolites formed may then inhibit bacterial mycolic acid and protein synthesis by inhibiting oxidation of hydroxymycolic acid to ketomycolate (Stover *et al.*, 2000). However, it has become clear more recently that PA-824 is activated by a deazaflavin (F_{420})-dependent nitroreductase (Ddn) in *M. tuberculosis* (Singh *et al.*, 2008). During the process of bioactivation, a des-nitro metabolite (6*S*)-6-{[4-(trifluoromethoxy)benzyl]oxy}-6,7-dihydro-5*H*-imidazo[2,1-*b*][1,3]oxazine (Figure 1), is formed and reactive nitrogen species (RNS), including nitric oxide (NO), are released. These RNS are responsible for the anti-mycobacterial activity (Singh *et al.*, 2008). The bioactivation of PA-824 and the resulting formation of NO are thought to be specific to microorganisms which utilize cofactor F_{420} (Singh *et al.*, 2008). The safety, tolerability and pharmacokinetics of PA-824 has been reported recently in healthy volunteers (Ginsberg *et al.*, 2009a,b) and pulmonary TB patients (Diacon *et al.*,

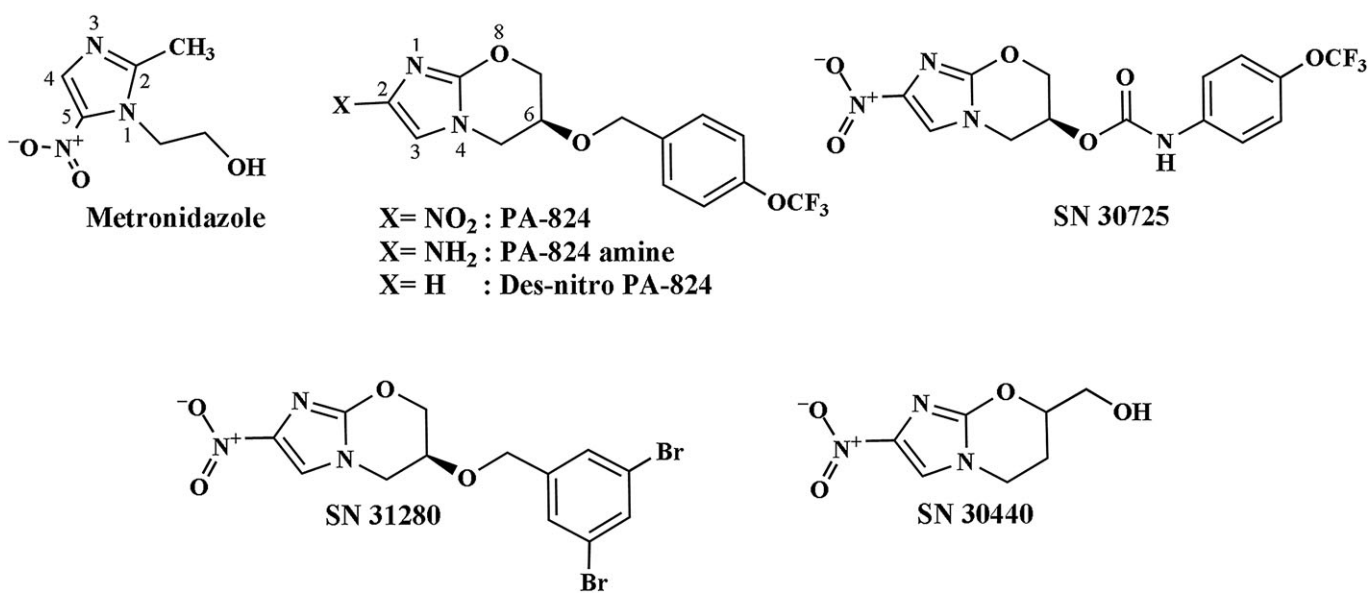

Figure 1

Chemical structures of metronidazole, PA-824 and derivatives (PA-824 amine and des-nitro PA-824), SN 30725 and internal standards (SN 31280 and SN 30440).

2010); however, little is known about the ability of mammalian enzymes to bioactivate PA-824.

Electrochemical studies suggested that the nitro group of PA-824 is a site for reduction (Yanez *et al.*, 2001); however, the nitro radical anion requires more energy for its formation and is less stable than is the case for metronidazole, a 5-nitroimidazole (Figure 1) (Bollo *et al.*, 2004). Moreover, radiolytic reduction of PA-824 demonstrated that reduction occurs at the imidazole ring in preference to the nitro group and a number of products are observed (Anderson *et al.*, 2008). The metabolic products of PA-824 observed in *M. tuberculosis* also include a number of intermediates which result from an initial hydride transfer to the imidazole ring prior to formation of the des-nitro metabolite (Singh *et al.*, 2008). It is not known if PA-824 also undergoes imidazole ring reduction by human liver enzymes or if des-nitrification occurs.

We have investigated the metabolic profile of PA-824 following incubation with a subcellular fraction (the 9000× *g* supernatant; S9) of human liver and compared this with the profile generated in the presence of purified Ddn, *M. tuberculosis* and *Mycobacterium smegmatis*.

Methods

Ddn cloning, expression and purification

The open reading frame (ORF) encoding Ddn (Rv3547) was PCR amplified from *M. tuberculosis* H37Rv genomic DNA using Platinum® Pfx polymerase (Invitrogen, Auckland, New Zealand). The first round of Gateway® amplification used the gene-specific primers (Forward-GGCAGCGGCGCGATGC CGAAATCACCGCCG, Reverse-GAAAGCTGGGTGT CAGGGTTCGCAAACCACGATCGG) with a temperature gradient (50–70°C). A second Gateway® amplification was performed using the first amplification round as a template with generic primers (Forward- GGGGACAAGTTTGTACAAAAAAGCAGG CTTCGAAAACCTGTATTTTCAGGGCAGCGGCGCG ATG, Reverse- GGGGACCACTTTGTACAAGAAAGC TGGGTG). The PCR product was purified using a QIAquick gel extraction kit (Qiagen, Auckland, New Zealand) to set up the BP reaction (Invitrogen) and the plasmid was then transformed into *Escherichia coli*. Positive clones were then selected using restriction digestion with *Bsr*GI. The Ddn ORF was transferred into a range of Gateway® destination vectors (pDEST17, pDEST15, pDEST566 and pDESTsmg) to produce N-terminal His-, glutathione-*S*-transferase (GST)- and maltose binding protein (MBP)-tagged proteins. Positive clones were selected on lysogeny broth (LB) agar plates containing ampicillin (100 μg·mL^{-1}) and restriction digestion with *Bsr*GI was used to select the correct size inserts. pDESTsmg positive clones were selected on low salt LB agar plates (pH 8.0) containing hygromycin B (50 μg·mL^{-1}) (Goldstone *et al.*, 2008).

E. coli BL21pRP cells were used to express His-(pDEST17), GST-(pDEST15) and MBP-Ddn (pDEST566) constructs. Protein expression was performed using autoinduction media at 18°C (Studier, 2005). A single transformed colony was selected to inoculate an MDG starter culture. The starter culture was grown overnight at 37°C and used to inoculate ZYM-5052 expression cultures which were grown in 2 L baffled flasks in a shaking incubator for ~4 h at 37°C and then for ~20 h at 18°C. The His-tagged Ddn was also expressed in *M. smegmatis* (mc^{2}4517) using autoinduction media (Bashiri *et al.*, 2007) and cultured at 37°C for ~4 days.

Cells expressing the different Ddn constructs were harvested by centrifugation and lysed by cell disruption (18 psi) followed by centrifugation (20 000 g for 30 min). The His-Ddn supernatant was subjected to immobilized metal affinity chromatography and the bound protein was eluted using an imidazole gradient. The GST- and MBP-Ddn fusion supernatant proteins were incubated with glutathione–sepharose beads and amylose resin, respectively, at 4°C. The proteins were then batch purified by elution using 20 mM glutathione and maltose, respectively, in lysis buffer. The protein solutions were then concentrated and filtered using a 10/300 S200 gel column equilibrated in lysis buffer. FGD1 and the cofactor F_{420} were expressed and purified following previously published methods (Bashiri *et al.*, 2007; 2008).

Metabolism of PA-824 in vitro by enzyme preparations

Purified Ddn (0.6 μM) and the co-enzyme FGD1 (0.1 μM) were pre-incubated with the cofactor F_{420} (20 μM) and glucose-6-phosphate (100 μM) for 5 min in a final volume of 0.5 mL of phosphate buffer (pH 7.4, 67 mM) at 37°C. The reaction was initiated by the addition of PA-824 (250 μM) and incubations (n = 3) were carried out for 3 h.

Samples of human liver were obtained with full informed consent and ethical approval (NZ Northern X Regional Ethics Committee, 98/040) from three patients (40, 70 and 74 years; two males, one female; one smoker) following resection for metastases from colorectal carcinoma. There was no prior use of medication noted. The 9000× *g* supernatant (S9) was prepared from human liver homogenate (25%) as described previously (Zhou *et al.*, 2000) and the preparations were pooled. Hepatic S9 (4 mg·mL^{-1}) was pre-incubated (3 min) with cofactors (NADPH and NADH, 1 mM) in a final volume

of 0.5 mL of phosphate buffer (pH 7.4, 67 mM) at 37°C. The reaction was initiated by the addition of PA-824 (250 μM) and incubations (n = 3) were carried out for 3 h.

Metabolism of PA-824 by M. tuberculosis and M. smegmatis

M. tuberculosis (H37Ra) and *M. smegmatis* (mc^2 4517) were grown in Middle Brook 7H9 broth supplemented with OADC (10%), Tween 80 (0.05%) and glycerol (0.5%). Early log phase cultures (A_{650} nm 0.2; 40 mL) were harvested by centrifugation (3500× g for 15 min) and resuspended in fresh media (4 mL). Following a 3 min pre-incubation, the reaction was initiated by the addition of PA-824 (250 μM) in a final incubation volume of 0.5 mL and incubations (n = 4) were carried out for 3 h at 37°C.

Extraction procedures

Reactions were terminated by the addition of ice-cold acetonitrile (1 mL) containing 1 M hydrochloric acid (10% v/v). SN 31280 (6*S*)-6-[(3,5-dibromobenzyl)oxy]-2-nitro-6,7-dihydro-5*H*-imidazo[2,1-*b*][1,3]oxazine (5 nmol) was added as an internal standard for semi-quantitative analysis. The proteins were precipitated (–80°C, 12 h) and then centrifuged (21 000× g, 20 min). The supernatant was concentrated under vacuum to 100 μL and analysed by liquid chromatography mass spectrometry (LC/MS).

The polar metabolites of PA-824 were extracted from the supernatant using normal phase solid phase extraction. Firstly, the supernatant was concentrated to a dried residue under vacuum and the residue was resuspended in 0.5 mL ethyl acetate/methanol (80:20 v/v). This was then loaded onto a silica column (Biotage, Hengoed, UK) preconditioned with the resuspension solvent (2 × 1 mL) and analytes eluted with 1 mL ethyl acetate/methanol (80:20 v/v). The extract was dried and resuspended in 1% DMSO in water (100 μL) prior to analysis by high-performance liquid chromatography (HPLC). SN 30440 (2-nitro-6,7-dihydro-5*H*-imidazo[2,1-*b*][1,3]oxazin-7-yl)methanol (5 nmol) was used as an internal standard. Calibration curves (1.0–10.0 μM) were prepared using (6*S*)-2-nitro-6,7-dihydro-5*H*-imidazo[2,1-*b*][1,3]oxazin-6-ol.

HPLC and LC/MS analysis

An Agilent 1100 HPLC system (Agilent Technologies, Santa Clara, CA, USA) interfaced with a diode array detector (Hewlett-Packard, Santa Clara, CA, USA) was used and separation was achieved on a 4.6 × 250 mm Allsphere C18, 5 μm column (Alltech, Auckland, New Zealand). The mobile phase comprised: (A) 10 mM ammonium formate (pH 3.5); and (B) 80% v/v acetonitrile in water at 0.5 mL·min^{-1}. The gradient conditions were 5% B increasing to 50% B over 0–10 min; 50–60% over 10–12 min followed by 60% B at 12–40 min then decreasing back to 5% B at 40–45 min. For analysis of polar metabolites, the conditions were 15% B at 0–14 min; 15–90% B over 14–16 min followed by 90% B at 16–35 min; and 15% B at 37 min. The eluent was monitored at 254 and 330 nm, with a reference wavelength of 550 nm.

Online mass spectra were obtained using the said system, interfaced with a single quadrupole mass spectrometer (Agilent 1100 LC/MSD) using positive and negative mode electrospray ionization (ESI) with nitrogen as the drying gas (12 L·min^{-1}) for the routine analysis of samples. The following parameters were used: fragmentor voltage 125 V, nebulizer pressure 35 psi, gas temperature 350°C, capillary voltage 3000 V and the mass/charge (m/z) ratio was scanned from 100 to 800.

Fragmentation analysis (MS^n) was performed using liquid chromatography-ion trap mass spectrometry, with capillary LC for structural elucidation. The system consisted of an Agilent 1100 system with a capillary pump, diode array detector and ion trap mass spectrometer with Agilent LC/MSD trap software. Separation was achieved on a 0.5 × 150 mm Zorbax C18, 5 μm column (Agilent Technologies). The mobile phase comprised: (A) 10 mM ammonium formate (pH 3.5); and (B) 80% (v/v) acetonitrile in water at 15 μL·min^{-1}. Initial conditions were 5% B increasing to 50% B at 0–17 min followed by 60% B at 22–30 min, then 40% B at 32–40 min, then returning to 5% B between 40 and 45 min. The metabolites were identified using ESI source with nitrogen as the drying gas (4.4 L·min^{-1}), set at negative mode with auto MS(n). The following parameters were used: nebulizer pressure 12 psi, gas temperature 325°C, capillary voltage 4500 V and the mass/charge (m/z) ratio was scanned from 100 to 800.

Statistical analysis

Statistical analysis was performed by Student's *t*-test using SigmaStat v3.5 (Systat Software Inc, San Jose, CA, USA). The test was run at the 95% confidence interval and P values <0.05 were considered significant.

Materials

PA-824 (6*S*)-2-nitro-6-{[4-(trifluoromethoxy)benzyl]oxy}-6,7-dihydro-5*H*-imidazo[2,1-*b*][1,3]oxazine; SN 31280 (6*S*)-6-[(3,5-dibromobenzyl)oxy]-2-nitro-6,7-dihydro-5*H*-imidazo[2,1-*b*][1,3]oxazine; SN 30440 (2-nitro-6,7-dihydro-5*H*-imidazo[2,1-*b*][1,3]oxazin-7-yl)methanol; des-nitro PA-824 (6*S*)-6-{[4-(trifluoromethoxy)benzyl]oxy}-6,7-dihydro-5*H*-imidazo[2,1-*b*][1,3]oxazine); PA-824 amine (6*S*)-6-{[4-(tri

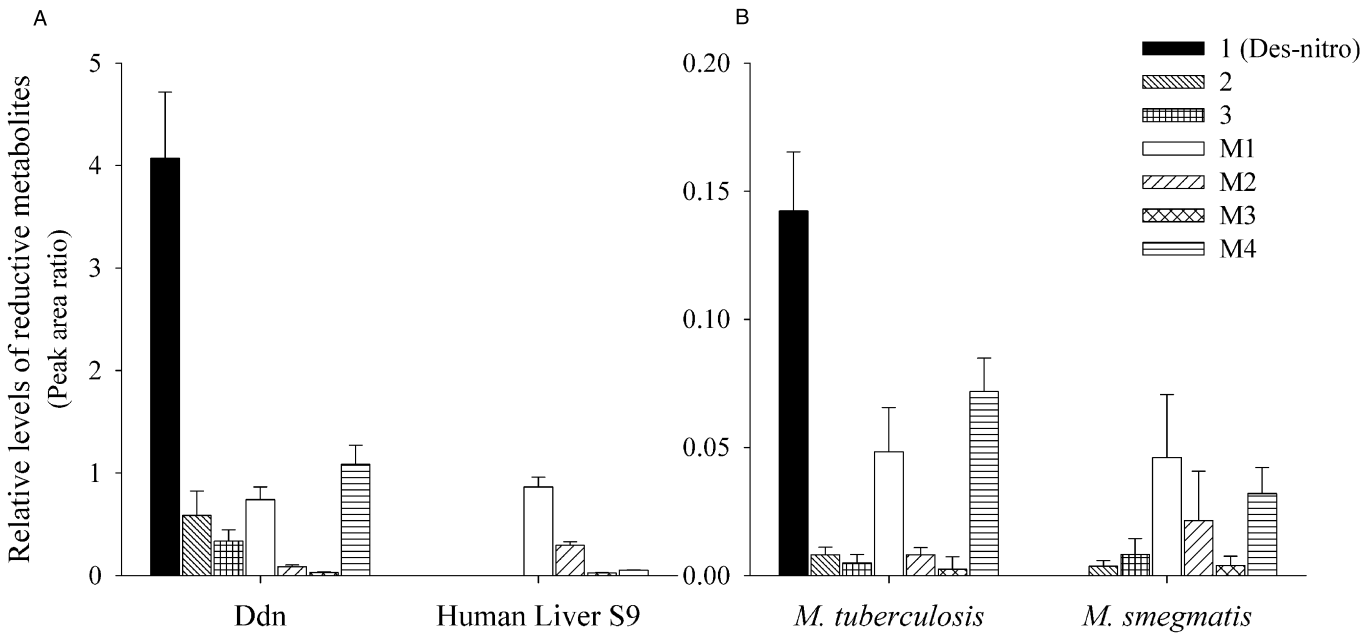

Figure 2

Relative levels of reductive metabolites formed following incubation of PA-824 with (A) *in vitro* enzyme preparations of deazaflavin-dependent nitroreductase (Ddn) and human liver S9 as well as (B) whole cells of *Mycobacterium tuberculosis* and *Mycobacterium smegmatis*. *In vitro* preparations were supplemented with appropriate cofactors/coenzymes (Ddn: F_{420} and FGD1; human liver S9: NADPH and NADH). All the values are mean ± SD peak area ratio (relative to internal standard, SN 31280) of $n = 3$ or 4 incubations.

fluoromethoxy)benzyl]oxy}-6,7-dihydro-5*H*-imidazo[2,1-*b*][1,3]oxazin-2-amine; M5 (6*S*)-2-nitro-6,7-dihydro-5*H*-imidazo[2,1-*b*][1,3]oxazin-6-ol; and SN 30725 (6*R*)-2-nitro-6,7-dihydro-5*H*-imidazo[2,1-*b*][1,3]oxazin-6-yl [4-(trifluoromethoxy) phenyl] carbamate were synthesized following published procedures (Baker *et al.*, 2000; Singh *et al.*, 2008; Thompson *et al.*, 2009) (Figure 1). The two-electron reduction product of PA-824 was prepared by radiolytic reduction of PA-824 using a previously published method (Anderson *et al.*, 2008). NADPH and NADH were obtained from Applichem GmbH (Darmstadt, Germany). Glutathione, reduced form, was purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany); ferrous sulphate ($FeSO_4.7H_2O$) was from May and Baker Ltd (Dagenham, UK); hydrochloric acid was from Biolab (Aust) Ltd (Clayton, Australia); and ammonium formate was from Acros Organics (Fair Lawn, NJ, USA). Acetonitrile was purchased from Scharlau Chemie S.A. (Barcelona, Spain). All other reagents and solutions used were of analytical grade.

Table 1

The retention times and masses of the metabolites of PA-824

Metabolite	Retention time (min)	Mass/charge ratio (m/z) $[M+H]^+$	$[M-H]^-$
1 (des-nitro)	23.1	315.0	–
2	19.2	331.0	–
3	18.9	291.0	–
M1	17.2	–	363.0
M2	17.6	–	376.0
M3	17.7	362.0	360.0
M4	19.0	–	361.0

PA-824 (250 μM) was incubated with purified Ddn, *Mycobacterium tuberculosis, Mycobacterium smegmatis* or pooled human liver S9 for 3 h. While seven metabolites were observed with Ddn or *M. tuberculosis*, only six were observed with *M. smegmatis* due to lack of formation of the des-nitro metabolite ('1'). Only four of these metabolites (M1–M4) were observed with human liver S9.

Results

Functional activity of purified Ddn in the presence of F_{420} and FGD1 was confirmed because 25% of PA-824 (retention time 24.9 min, $[M+H]^+$ 360.0 m/z) was consumed after 3 h incubation and seven products were detected by ESI LC/MS analysis (Figure 2). The major metabolite, '1' (Table 1) was identified as the des-nitro product of PA-824 by co-elution with the authentic standard (6*S*)-6-{[4-(trifluoromethoxy)benzyl]oxy}-6,7-dihydro-

5*H*-imidazo[2,1-*b*][1,3]oxazine (Figure 1). Following incubation of PA-824 with human liver S9 for 3 h, 19% of PA-824 was consumed but only four metabolites were detected. Importantly, in contrast to Ddn, the des-nitro metabolite '1' was not a product of human liver metabolism (Figure 2). This metabolite was also not observed following a shorter incubation (0.5 h) of PA-824 with human liver S9.

Two metabolites detected in Ddn incubations, '2' and '3' (Table 1) were consistent with products reported previously following the Ddn-catalyzed metabolism of PA-824 (Singh *et al.*, 2008). These metabolites ('2' and '3'), were not observed in human liver S9 incubations. Four unknown products M1, M2, M3 and M4 (Table 1) were detected in both Ddn and human liver S9 incubations. These products were also observed following shorter incubations (0.5 h) with human liver S9.

Metabolite M3 had an identical mass and co-eluted with the product of two electron radiolytic reduction of PA-824. M3 has also been proposed as a Ddn-catalyzed intermediate (A) in the formation of the des-nitro product ('1') (Singh *et al.*, 2008). An incubation of the product of two electron radiolytic reduction of PA-824, which comprised M3 and small amounts of PA-824, with Ddn, resulted in the formation of M1, M4 and the des-nitro product, '1'. In contrast, incubation of the same material with human liver S9 resulted in the formation of M1 and M4, but no des-nitro ('1') was detected.

M2 was characterized using ion trap LC/MS (MS^n) analysis. M2 had a molecular ion of $[M\text{-}H]^-$ 434.2 m/z, which is consistent with an adduct (+58.1) of the molecular ion $[M\text{-}H]^-$ 376.1 m/z, which was previously observed by single quadrupole LC/MS. The adduct was only observed in negative mode and subsequent fragmentation of the adduct resulted in an MS^2 at 376.1 m/z and a major fragment ion MS^3 of 183.9 m/z. The mass of M2 was confirmed by the detection of a molecular ion peak at $[M+H]^+$ 378.7 m/z in positive mode. Three additional fragments were identified as the NH_4^+, Na^+ and K^+ adducts of M2. Subsequent fragmentations resulted in MS^2 and MS^3 of 175.0 and 176.0 m/z respectively. The molecular fragmentation pattern is consistent with the structure shown in Figure 3.

The metabolite M2 was also formed in the presence of denatured human liver S9 and at $t = 0$, prior to the initiation of the incubation. To determine whether this is an iron-catalyzed reaction, the ability of ferrous ions (Fe^{2+}) to facilitate the formation of M2 was determined. Ferrous sulphate (1 M) was incubated with PA-824 (250 μM) at 37°C in the presence or absence of glutathione (1 mM). Extensive formation of metabolite M2 was observed (Figure 4A). This reaction required the presence of both Fe^{2+} and glutathione. To confirm this mechanism, PA-824 (250 μM) was further incubated with denatured S9 (4 mg·mL^{-1}) in the presence of varying concentrations of an iron chelator, desferoxamine. Significantly lower levels ($P < 0.05$) of M2 were observed following co-incubation with desferoxamine (7.5 mM) compared with the control sample (no desferoxamine) (Figure 4B). Hence, the formation of M2 may continue to occur after the termination of the incubation by a non-enzymatic haem (Fe^{2+})-catalyzed reaction (Figure 4C).

The comparison of the incubation chromatogram with that of authentic PA-824 amine (6*S*)-6-{[4-(trifluoromethoxy)benzyl]oxy}-6,7-dihydro-5*H*-imidazo[2,1-*b*][1,3]oxazin-2-amine (Figure 1), a potential product of reduction of the nitro group, indicated that six electron reduction of PA-824 was not catalyzed by either human liver S9 or Ddn, as no PA-824 amine was observed.

Following a 3 h incubation of PA-824 with *M. tuberculosis*, seven metabolites (1–3, M1–M4) were detected; these were identical to those observed following Ddn incubation (Figure 2). In contrast with *M. smegmatis*, only six of these metabolites were observed, with no formation of the des-nitro compound ('1'). However, in contrast to human liver, *M. smegmatis* did form metabolites '2' and '3', which were observed in both *M. tuberculosis* and Ddn incubations.

An interrogation of the LC/MS chromatogram indicated that human liver S9 could catalyze the formation of a polar metabolite of PA-824. Detection of this early eluting metabolite was optimized using normal phase extraction. This metabolite, M5, had a molecular ion of $[M+H]^+$ 186.0 m/z and the putative structure, (6*S*)-2-nitro-6,7-dihydro-5*H*-imidazo[2,1-*b*][1,3]oxazin-6-ol), was confirmed by co-elution with a synthetic standard. To confirm that oxidation of the benzyl linker chain resulted in the formation of M5, an analogue with a carbamate substitution in the benzyl linker chain was synthesized (SN 30725; (6*R*)-2-nitro-6,7-dihydro-5*H*-imidazo[2,1-*b*][1,3]oxazin-6-yl [4-(trifluoromethoxy) phenyl] carbamate; Figure 1). M5 was not observed after incubation of this analogue with human liver S9 (data not shown). Quantification of M5 indicated that low concentrations (<1 μM) were observed following the incubation of PA-824 (250μM) with increasing amounts of human liver S9 (0–12 mg·mL^{-1}). As the formation of this metabolite was very low (0.24 % of the parent compound), it appears to be a relatively minor route of PA-824 metabolism in human liver S9.

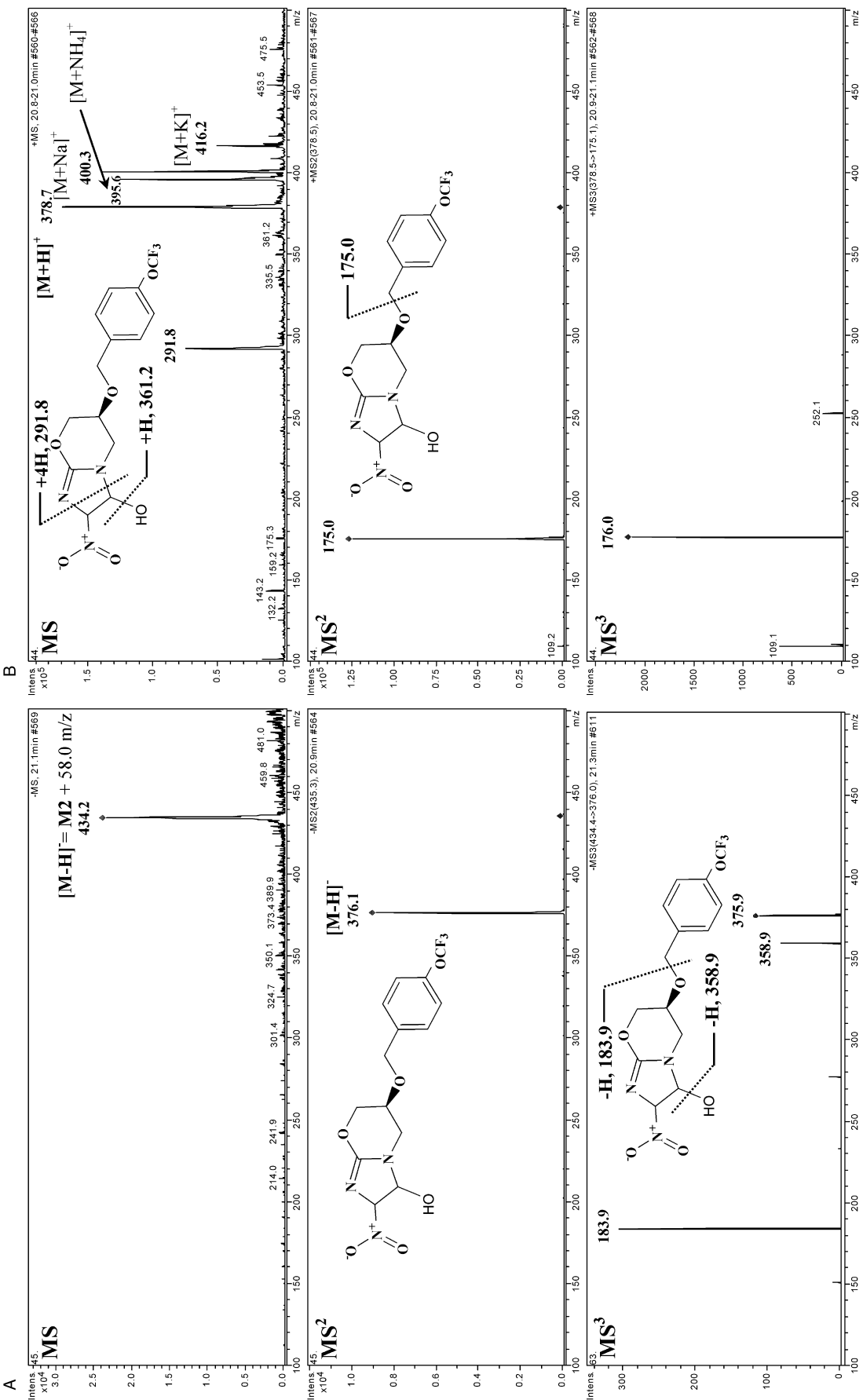

Figure 3
Elucidation of metabolite M2 using MS^n analysis in (A) negative and (B) positive ion mode.

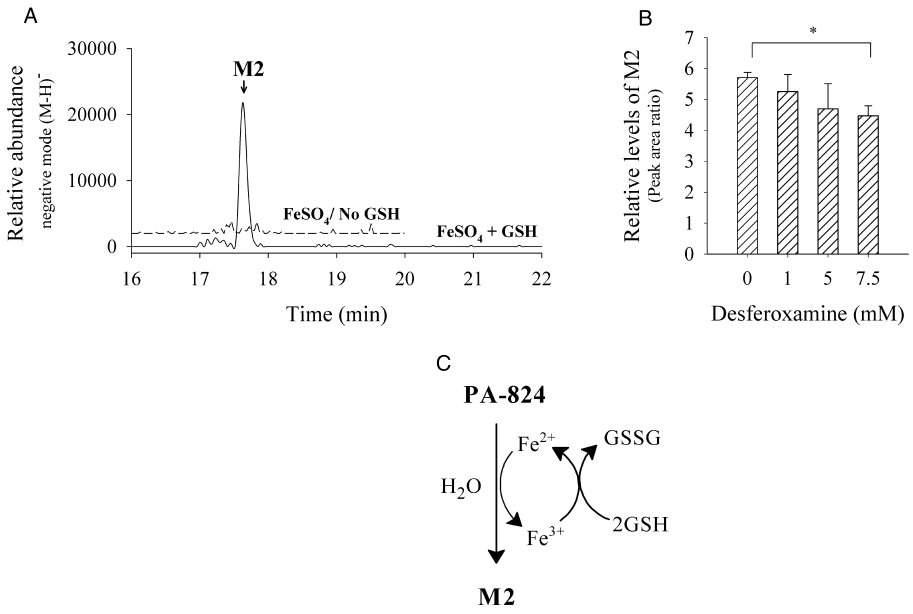

Figure 4

Formation of the metabolite M2 following 30 min incubation of PA-824 (250 μM) with ferrous sulphate (1 M) and glutathione (1 mM). (A) Extracted ion chromatogram of M2 in negative mode. (B) Relative levels of M2 in the presence of desferoxamine. (C) Proposed mechanism of M2 formation. *$P < 0.05$.

Discussion and conclusions

Des-nitrification of PA-824 by Ddn and *M. tuberculosis* has been reported previously with the concomitant formation of RNS responsible for the anti-mycobacterial activity of PA-824 (Singh *et al.*, 2008). However, this is the first time that the reductive metabolism of PA-824 by human liver S9 or *M. smegmatis* has been reported. We have confirmed that both Ddn and *M. tuberculosis* catalyze des-nitrification of PA-824 (Figure 5). In contrast, neither human liver S9 nor *M. smegmatis* could catalyze this reaction. However, we observed a number of additional metabolic products in both *Mycobacteria* and human liver S9. Indeed, thin layer chromatography of [^{14}C]-PA-824 has previously demonstrated the presence of multiple metabolites following incubation with either Ddn or *M. tuberculosis* (Singh *et al.*, 2008). As in this earlier report, Ddn and *M. tuberculosis* formed products which had a molecular mass consistent with metabolites '2' and '3'. Moreover, both these metabolites were detected in *M. smegmatis*, but not in human liver S9. The formation of metabolite '2' is also a des-nitrification reaction and is expected to result in the formation of RNS (Singh *et al.*, 2008). Hence, although *M. smegmatis* does not form the des-nitro metabolite ('1'), it may still produce RNS from PA-824. However, the formation of '2' appears to be minor compared with the formation of '1' in *M. tuberculosis*, confirming the previous observation (Singh *et al.*, 2008). The correlation between des-nitro metabolite formation and anaerobic killing of *M. tuberculosis* has been demonstrated in this earlier report. Hence, the anti-tubercular activity of PA-824 may be mediated predominantly through the formation of the des-nitro metabolite. Indeed, previous reports indicate that PA-824 has poor activity against *M. smegmatis* (Stover *et al.*, 2000).

An additional four metabolites (M1–M4) were observed following *in vitro* metabolism of PA-824 in *M. tuberculosis*. The presence of these metabolites is consistent with the multiple thin layer chromatography spots observed previously (Singh *et al.*, 2008). One of these metabolites, M3, was described as a stable intermediate in the formation of des-nitro PA-824 ('1') (Singh *et al.*, 2008) and a product of radiolytic reduction of PA-824 (Anderson *et al.*, 2008). Two-electron reduction is required for the formation of M3, via transfer of a hydride to the C-3 position of the imidazole ring and further protonation of the resulting nitronic acid (Singh *et al.*, 2008). The formation of des-nitro PA-824 from M3 is assumed to be due to the elimination of nitrous acid (Singh *et al.*, 2008). As neither human liver S9 nor *M. smegmatis* supported the formation of the des-nitro compound ('1'), this second step is also likely to be enzymically catalyzed in *M. tuberculosis*. Ddn, but not human liver S9, could catalyze the formation of des-nitro product from M3, whereas,

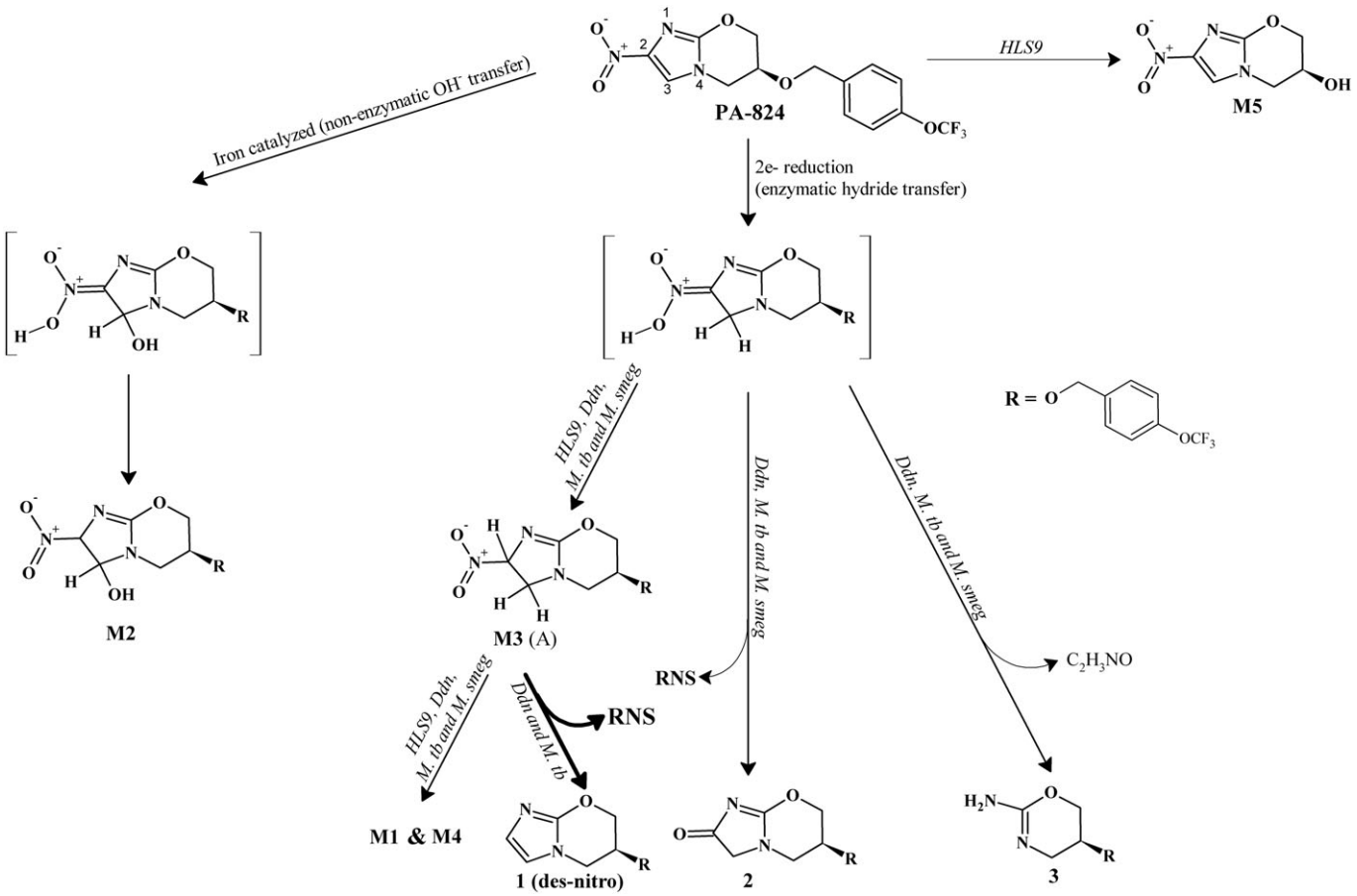

Figure 5

Proposed metabolic pathways of PA-824. '1' (des-nitro), '2' and '3' have been reported previously as products of Ddn-catalyzed reductive metabolism of PA-824 (Singh *et al.*, 2008). While M3, a precursor of the des-nitro product ('1'), was detected in human liver S9 (HLS9), Ddn, *Mycobacterium tuberculosis* (*M. tb*) and *Mycobacterium smegmatis* (*M. smeg*), the des-nitro product ('1') was only observed in Ddn and *M. tuberculosis*. Three metabolites (M1, M2 and M4) previously not reported in Ddn were also observed following incubation of PA-824 with human liver S9, Ddn, *M. tuberculosis* and *M. smegmatis*. A polar metabolite M5 was also observed in human liver S9. RNS, reactive nitrogen species.

incubation of M3 with human liver S9 resulted in the formation of two unidentified metabolites M1 and M4. These were also products of PA-824 metabolism in *M. tuberculosis*. M1 and M4 were three and one mass units greater than M3 (364.0 and 362.0 amu vs. 361.0 amu respectively). However, attempts to isolate these metabolites for further identification have not been successful and it is not known if these are products of further reduction of the imidazole ring.

The mechanism of reduction of PA-824 has been previously proposed as either an enzyme-catalyzed hydride transfer to the imidazole ring (Singh *et al.*, 2008), formation of a radical anion intermediate in the imidazole ring (Anderson *et al.*, 2008) or a nitro radical formation (Yanez *et al.*, 2001; Bollo *et al.*, 2004). Radiolytic reduction is reported to result in the formation of the six electron amino-product of PA-824 as well as the imidazole ring hydride and hydrate (Anderson *et al.*, 2008). No products of reduction of the nitro group were observed following mycobacterial and human liver enzymatic reduction of PA-824. Thus, enzyme-catalyzed reduction appears to occur preferentially at the C-3 of the imidazole ring rather than the nitro group.

The one electron reduction potential, $E(1)$, of compounds is considered to be a controlling factor for enzymic reduction. The $E(1)$ of PA-824 has been determined as -534 ± 7 mV, which is relatively low and was predicted to restrict the range of enzymes that may reduce this compound (Anderson *et al.*, 2008). However, we have clearly demonstrated that using NAD(P)H as cofactor, the reduction of PA-824 to a number of minor metabolites occurs in human liver S9. There have been no previous reports of the human metabolites of PA-824. The 9000× *g* supernatant (S9) of human liver homogenate, used in these studies with PA-824, contains a number of

reductive enzymes found in the cytosolic rather than microsomal fraction of the hepatocytes. Thus, the use of microsomal fractions, lacking cytosolic components, may underestimate the ability of human liver to reduce this class of nitroimidazole compounds. However, this has to be further confirmed. The enzyme(s) responsible for the metabolism of PA-824 has not been identified, but it may be pertinent that the redox potential of $NAD(P)^+$ is –320 mV, similar to that of F_{420} (–380 mV), the cofactor for the mycobacterial enzyme, Ddn.

PA-824 is a bicyclic 2-nitroimidazooxazine with a similar structure to the 4-nitroimidazoles. Although one-electron reduction of a 4-nitroimidazole compound by a partially purified oxygen sensitive hydrogenase 1 from *Clostridium pasteurianum* has been reported previously (Church *et al.*, 1990), we could not find any published reports of the reduction of 4-nitroimidazole compounds by human liver.

A gene with 72% identity to *M. tuberculosis* Ddn (Rv3547) is present in *M. smegmatis* (Manjunatha *et al.*, 2006) and although *M. smegmatis* is capable of metabolizing PA-824 to form six reductive metabolites that are identical to those observed in *M. tuberculosis*, this species does not form the des-nitro metabolite ('1'). *M. smegmatis* FGD, which facilitates the formation of reduced F_{420}, has a higher K_M for glucose-6-phosphate than the enzyme from *M. tuberculosis* (1.6 mM vs. 0.1 mM, respectively) (Bashiri *et al.*, 2008). The combination of potential differences in the tertiary structure of Ddn and/or lower formation of reduced F_{420} may explain the lack of production of the des-nitro metabolite ('1') in *M. smegmatis*, and hence, poor activity of PA-824 in this species.

We have also observed an additional metabolite (M2) in both mycobacteria and human liver S9. This putative hydrated product of PA-824, not previously described as an *M. tuberculosis* product, has been reported following the radiolytic reduction of PA-824 (Anderson *et al.*, 2008). The formation of M2 via one-electron reduction of the imidazole ring is unlikely, as such reduction will occur only under hypoxic conditions. We have demonstrated that M2 can be formed by the Fe^{2+} ion-catalyzed reduction of PA-824. The Fe^{2+} present within haem proteins has been reported previously to catalytically reduce compounds even under denaturing conditions (Carr *et al.*, 2006). The superoxide anion generated during the oxidation of Fe^{2+} may react with water molecules, resulting in the formation of hydroxyl ions. Thus, M2 may be the result of the transfer of hydroxyl ions to the C-3 position of the imidazole ring, via a mechanism similar to the enzymic hydride transfer.

In addition to reductive metabolism of PA-824, the formation of a polar metabolite, M5, was also observed following incubation with human liver S9. This product was identified as (6*S*)-2-nitro-6,7-dihydro-5*H*-imidazo[2,1-*b*][1,3]oxazin-6-ol (Figure 5), a product of benzyl linker side chain oxidation, although this appears to be a relatively minor pathway.

In conclusion, we have confirmed that PA-824 undergoes des-nitrification by *M. tuberculosis*. This may result in the selective release of NO within *M. tuberculosis* as neither *M. smegmatis* nor human liver S9 was capable of forming the des-nitro metabolite. Moreover, we have provided evidence to support the claim that 'cross-activation (of PA-824) by mammalian enzymes is highly unlikely' (Singh *et al.*, 2008).

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Conflicts of interest

None to declare

References

Anderson RF, Shinde SS, Maroz A, Boyd M, Palmer BD, Denny WA (2008). Intermediates in the reduction of the antituberculosis drug PA-824, (6S)-2-nitro-6-{[4-(trifluoromethoxy)benzyl]oxy}-6,7-dihydro-5H-imidazo[2, 1-b][1,3]oxazine, in aqueous solution. Org Biomol Chem 6: 1973–1980.

Baker WR, Shaopei C, Keeler EL (2000). Nitro-[2,1-b]imidazopyran compounds and antibacterial uses thereof. U.S. 6 087 358.

Bashiri G, Squire CJ, Baker EN, Moreland NJ (2007). Expression, purification and crystallization of native and selenomethionine labeled Mycobacterium tuberculosis FGD1 (Rv0407) using a Mycobacterium smegmatis expression system. Protein Expr Purif 54: 38–44.

Bashiri G, Squire CJ, Moreland NJ, Baker EN (2008). Crystal structures of F420-dependent glucose-6-phosphate dehydrogenase FGD1 involved in the activation of the anti-tuberculosis drug candidate PA-824 reveal the basis of coenzyme and substrate binding. J Biol Chem 283: 17531–17541.

Bollo S, Nunez-Vergara LJ, Squella JA (2004). Cyclic voltammetric determination of free radical species from nitroimidazopyran: a new antituberculosis agent. J Electroanal Chem 562: 9–14.

Carr JL, Tingle MD, McKeage MJ (2006). Satraplatin activation by haemoglobin, cytochrome C and liver microsomes in vitro. Cancer Chemother Pharmacol 57: 483–490.

Church DL, Rabin HR, Laishley EJ (1990). Reduction of 2-, 4- and 5-nitroimidazole drugs by hydrogenase 1 in Clostridium pasteurianum. J Antimicrob Chemother 25: 15–23.

Diacon AH, Dawson R, Hanekom M, Narunsky K, Maritz SJ, Venter A *et al*. (2010). Early bactericidal activity and pharmacokinetics of PA-824 in smear-positive tuberculosis patients. Antimicrob Agents Chemother 54: 3402–3407.

Ginsberg AM, Laurenzi MW, Rouse DJ, Whitney KD, Spigelman MK (2009a). Assessment of the effects of the nitroimidazo-oxazine PA-824 on renal function in healthy subjects. Antimicrob Agents Chemother 53: 3726–3733.

Ginsberg AM, Laurenzi MW, Rouse DJ, Whitney KD, Spigelman MK (2009b). Safety, tolerability, and pharmacokinetics of PA-824 in healthy subjects. Antimicrob Agents Chemother 53: 3720–3725.

Goldstone RM, Moreland NJ, Bashiri G, Baker EN, Shaun Lott J (2008). A new Gateway vector and expression protocol for fast and efficient recombinant protein expression in Mycobacterium smegmatis. Protein Expr Purif 57: 81–87.

Manjunatha UH, Boshoff H, Dowd CS, Zhang L, Albert TJ, Norton JE *et al*. (2006). Identification of a nitroimidazo-oxazine-specific protein involved in PA-824 resistance in Mycobacterium tuberculosis. Proc Natl Acad Sci USA 103: 431–436.

Singh R, Manjunatha U, Boshoff HI, Ha YH, Niyomrattanakit P, Ledwidge R *et al*. (2008). PA-824 kills nonreplicating Mycobacterium tuberculosis by intracellular NO release. Science 322: 1392–1395.

Spigelman MK (2007). New tuberculosis therapeutics: a growing pipeline. J Infect Dis 196 (Suppl. 1): S28–S34.

Stover CK, Warrener P, VanDevanter DR, Sherman DR, Arain TM, Langhorne MH *et al*. (2000). A small-molecule nitroimidazopyran drug candidate for the treatment of tuberculosis. Nature 405: 962–966.

Studier FW (2005). Protein production by auto-induction in high density shaking cultures. Protein Expr Purif 41: 207–234.

Thompson AM, Blaser A, Anderson RF, Shinde SS, Franzblau SG, Ma Z *et al*. (2009). Synthesis, reduction potentials, and antitubercular activity of ring A/B analogues of the bioreductive drug (6S)-2-nitro-6-{[4-(trifluoromethoxy)benzyl]oxy}-6,7-dihydro-5H-imidazo[2, 1-b][1,3]oxazine (PA-824). J Med Chem 52: 637–645.

WHO (2009). World Health Organization Report 2009: Global Tuberculosis Control Epidemiology, Strategy, Financing. WHO: Geneva, Switzerland.

Yanez C, Bollo S, Nunez-Vergara LJ, Squella JA (2001). Voltammetric determination of nitroimdazopyran candidate for the treatment of tuberculosis. Anal Lett 34: 2335–2348.

Zhou S, Paxton JW, Tingle MD, Kestell P (2000). Identification of the human liver cytochrome P450 isoenzyme responsible for the 6-methylhydroxylation of the novel anticancer drug 5,6-dimethylxanthenone-4-acetic acid. Drug Metab Dispos 28: 1449–1456.