Identification of novel diphenyl urea inhibitors of Mt-GuaB2 active against *Mycobacterium tuberculosis*

Veeraraghavan Usha,¹ Sudagar S. Gurucha,¹ Andrew L. Lovering,¹ Adrian J. Lloyd,² Athina Papaemmanouil,¹ Robert C. Reynolds³ and Gurdyal S. Besra¹

Correspondence

Gurdyal S. Besra
g.besra@bham.ac.uk

¹School of Biosciences, University of Birmingham, Edgbaston, Birmingham B15 2TT, UK

²Department of Biological Sciences, University of Warwick, Coventry, UK

³Drug Discovery Division, Southern Research Institute, Birmingham, AL 35255, USA

In contrast with most bacteria, which harbour a single inosine monophosphate dehydrogenase (IMPDH) gene, the genomic sequence of *Mycobacterium tuberculosis* H37Rv predicts three genes encoding IMPDH: *guaB1*, *guaB2* and *guaB3*. These three genes were cloned and expressed in *Escherichia coli* to evaluate functional IMPDH activity. Purified recombinant Mt-GuaB2, which uses inosine monophosphate as a substrate, was identified as the only active GuaB orthologue in *M. tuberculosis* and showed optimal activity at pH 8.5 and 37 °C. Mt-GuaB2 was inhibited significantly *in vitro* by a panel of diphenyl urea-based derivatives, which were also potent anti-mycobacterial agents against *M. tuberculosis* and *Mycobacterium smegmatis*, with MICs in the range of 0.2–0.5 µg ml⁻¹. When Mt-GuaB2 was overexpressed on a plasmid *in trans* in *M. smegmatis*, a diphenyl urea analogue showed a 16-fold increase in MIC. Interestingly, when Mt-GuaB orthologues (Mt-GuaB1 and 3) were also overexpressed on a plasmid *in trans* in *M. smegmatis*, they also conferred resistance, suggesting that although these Mt-GuaB orthologues were inactive *in vitro*, they presumably titrate the effect of the inhibitory properties of the active compounds *in vivo*.

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INTRODUCTION

Every year, 9 million people are newly diagnosed with tuberculosis (TB) and there are about two million TB deaths each year (Corbett *et al.*, 2003). Furthermore, increased incidences of multi-drug resistant (MDR)-TB and extensively drug resistant (XDR)-TB strains have caused an alarming rise in the number of TB cases (Kaufmann & Parida, 2007). Since effective new drugs are urgently needed for the treatment of MDR-TB, XDR-TB and HIV-associated TB cases, there is a need to identify and characterize potential new drug targets.

Inosine monophosphate (IMP) is synthesized via several steps from phosphoribosyl pyrophosphate. IMP is a common metabolic precursor for both adenine and guanine nucleotides. Guanosine monophosphate (GMP) is synthesized in two stages from IMP (Lambden &

Drabble, 1973). The first step of GMP synthesis involves the conversion of IMP to xanthosine monophosphate (XMP) and is performed by inosine monophosphate dehydrogenase (IMPDH, IMP-NAD⁺ oxidoreductase, E.C. 1.1.1.205) with concomitant conversion of NAD⁺ to NADH, as shown in Fig. 1(a) (Jackson *et al.*, 1975). XMP is then subsequently converted to GMP by a GMP synthetase (Gilbert *et al.*, 1979). IMPDH catalyses these two very different chemical transformations, firstly a dehydrogenase reaction to form NADH and the covalent enzyme intermediate E-XMP*, and secondly a hydrolysis reaction, which converts E-XMP* into XMP (Hedstrom, 2009).

IMPDH is a rate-limiting enzyme in guanine nucleotide metabolism and inhibition of IMPDH depletes cellular guanine nucleotides (Hedstrom, 2009; Pimkin *et al.*, 2009). The IMPDH reaction was first reported in 1957 in extracts of *Aerobacter aerogenes* (Magasanik *et al.*, 1957). To date, IMPDH enzymes have been characterized from a variety of sources, which include humans (Carr *et al.*, 1993; Hager *et al.*, 1995; Hedstrom, 2009), protozoan parasites (Dobie *et al.*, 2007) and bacteria (Ashbaugh & Wessels, 1995; Park *et al.*, 2004; Tiedeman & Smith, 1985; Zhang *et al.*, 2009). Many bacteria harbour a single gene

Abbreviations: DPU, diphenyl urea; GMP, guanosine monophosphate; IMP, inosine monophosphate; IMPDH, IMP dehydrogenase; MDR, multi-drug resistant; TB, tuberculosis; XDR, extensively drug resistant; XMP, xanthosine monophosphate.

Two supplementary tables of primer sequences are available with the online version of this paper.

Fig. 1. (a) Reaction scheme of IMPDH. (b) Purification of recombinant *M. tuberculosis* GuaB orthologues expressed in *E. coli*. Purified Mt-GuaB1, Mt-GuaB2 and Mt-GuaB3 fractions from Ni²⁺-chelate affinity chromatography were pooled and analysed by 12 % SDS-PAGE followed by Coomassie brilliant blue G-250 staining. M, Molecular mass markers.

for IMPDH, which is the sole provider of XMP for the cell and is thus vital for survival.

The *Mycobacterium tuberculosis* H37Rv genome contains three genes predicted to encode IMPDH enzymes, designated *guaB1* (Rv1843c), *guaB2* (Rv3411c) and *guaB3* (Rv3410c) (Cole *et al.*, 1998). It is interesting to note that of the three orthologues of IMPDH, *guaB3* and *guaB2* are in an operon, and *guaB1* is elsewhere in the genome. The Mt-GuaB orthologues have approximately 41 % sequence identity at the amino acid level with human IMPDH (Shu & Nair, 2008). A genome-wide transposon mutagenesis study has suggested that Mt-GuaB2 is the only essential orthologue for growth of *M. tuberculosis* H37Rv (Sasseti *et al.*, 2001, 2003). In this study, we have biochemically characterized the Mt-GuaB orthologues, and have shown that Mt-GuaB2 is a drug target which is inhibited by a series of diphenyl urea-based compounds with potent *M. tuberculosis* activity.

METHODS

Materials. All restriction enzymes were purchased from New England Biolabs. Oligonucleotide primers were synthesized by MWG operon. All chemicals were obtained from Sigma. Diphenyl urea (DPU) analogues, 7834535 (DPU-1), 7285642 (DPU-2) and 7275166 (DPU-3) were purchased from ChemBridge.

Cloning of Mt-*guaB* orthologues in *Escherichia coli* and *Mycobacterium smegmatis*. The *M. tuberculosis* *guaB* genes were amplified by conventional PCR (see Supplementary Table S1 for

pET28b cloning primers and Supplementary Table S2 for pVV16 cloning primers, both available with the online version of this paper) and *M. tuberculosis* H37Rv genomic DNA as a template. The purified PCR products of *guaB2* and *guaB3* were digested with *Nde*I and *Hind*III, and ligated into similarly digested expression vector pET28b (Novagen). The *guaB1* PCR product was digested with *Eco*RI and *Hind*III and ligated into the pUC19 vector cut similarly and later subcloned into pET28b. The resulting recombinant plasmids were transformed into *E. coli* TOP-10 cells and subsequently recovered using QIAprep spin miniprep kit (Qiagen). For the pVV16 cloning of Mt-*guaB* orthologues, the PCR products were digested with *Nde*I and *Hind*III and cloned into pVV16 vector which had been digested with the same restriction enzymes, transformed into *E. coli* TOP-10 cells and plated on LB containing 25 µg kanamycin ml⁻¹ and 50 µg hygromycin ml⁻¹. The presence of an insert in positive clones was determined by digesting the plasmid with *Nde*I and *Hind*III or *Eco*RI and *Hind*III and separation on a 1 % agarose gel. The correct nature of the insert was confirmed by sequencing.

Functional expression of *M. tuberculosis* IMPDH orthologues cloned in *E. coli*.

Plasmids from positive clones were transformed into *E. coli* C41 (DE3) cells. A single colony was grown overnight at 37 °C using Luria-Bertani broth (LB; Difco) supplemented with 25 µg kanamycin ml⁻¹. A 1 % inoculum of the overnight culture was inoculated into fresh LB medium containing 25 µg kanamycin ml⁻¹ and incubated at 37 °C until the culture reached OD₆₀₀ 0.6–0.7. The culture was then cooled to 16 °C for 15 min before addition of 1 mM IPTG and incubation was continued for a further 20 h at 16 °C. The culture was harvested by centrifugation at 3500 g for 10 min at 4 °C and the resulting cell pellet was stored at –80 °C.

Purification of recombinant Mt-GuaB orthologues. The *E. coli* cell pellets were resuspended in buffer A [50 mM Tris/HCl pH 8.0, 500 mM NaCl, 10 mM imidazole, 10 % glycerol, 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM benzamidinium and lysozyme (1 mg ml⁻¹)] and stored for 30 min on ice. The cells were disrupted by sonication using eight pulses of 30 s with 30 s cooling intervals between pulses. The unbroken cells and cell debris were removed by centrifugation at 27 000 g for 45 min at 4 °C. The clear supernatant was applied to a 1 ml chelating Sepharose HisTrap column charged with Ni²⁺ that had been equilibrated with buffer B (buffer A minus lysozyme and protease inhibitors). The column was extensively washed with buffer B before elution with 150 and 200 mM imidazole in the same buffer. Fractions containing recombinant protein were pooled and dialysed against 50 mM Tris/HCl pH 8.0, 150 mM NaCl, 10 % glycerol, 1 mM EDTA and 1 mM DTT and concentrated using a Centricon concentrator (Amicon). The concentrated enzyme was stored at –80 °C. Protein concentration was determined using the bicinchoninic acid (BCA) protein assay kit (Pierce). The purified Mt-GuaB orthologues were analysed by 12 % SDS-PAGE and stained with Coomassie brilliant blue R250.

MIC of DPU compounds against Mt-GuaB orthologues over-expressed in *M. smegmatis* and wild-type *M. tuberculosis* H37Rv.

The susceptibility of wild-type *M. smegmatis*, *M. smegmatis* harbouring either the control pVV16 vector or pVV16-Mt-*guaB1*, pVV16-Mt-*guaB2* and pVV16-Mt-*guaB3* plasmids was determined in the presence of different concentrations of DPU-2 and DPU-3. The Mt-GuaB over-expressed and control strains were grown in tryptic soy broth (TSB) containing 0.05 % Tween 80 at 37 °C until OD₆₀₀ 0.7. Serial tenfold dilutions (10⁻¹–10⁻⁸) of each culture were spotted (5 µl) on TSB agar plates supplemented with 25 µg kanamycin ml⁻¹ and 50 µg hygromycin ml⁻¹ and serial twofold dilutions of the respective DPU compound ranging from 0.125 to 8 µg ml⁻¹. Plates were incubated at 37 °C for 3–4 days. The MIC was defined as the minimal concentration that inhibited 99 % of bacilli growth. Lysates of pVV16 vector control and Mt-GuaB overexpressed strains were prepared and the expression was confirmed by Western blotting and immunological detection with anti-

His tag antibodies (Qiagen) according to the manufacturer's protocol. The MIC of DPU compounds for wild-type *M. tuberculosis* H37Rv was determined essentially as described by measurement of AlamarBlue fluorescence (Ananthan *et al.*, 2009; Maddry *et al.*, 2009). The DPU compounds were screened initially at 10 µg ml⁻¹ and later at a dose-response with serial twofold dilutions covering a range from 0.1 to 50 µg ml⁻¹ (Ananthan *et al.*, 2009; Maddry *et al.*, 2009).

Enzyme assays. IMPDH activity was assayed at 37 °C in a heat-controlled UV spectrophotometer (Jenway) by following the production of NADH ($\epsilon_{340}=6.22 \text{ mM}^{-1} \text{ cm}^{-1}$) at 340 nm in a 1 cm path length cuvette. After pre-incubation of the reaction mixture for 10 min at 37 °C to achieve thermal equilibration, the reaction was started by the addition of purified enzyme and incubated at 37 °C for 20 min. The IMPDH activity was assayed essentially as described by Pal *et al.* (2002) with minor modifications. The 1 ml reaction mixture consisted of 50 mM Tris/HCl pH 8.5, 150 mM KCl, 1.25 mM IMP, 3 mM NAD⁺, 1 mM DTT, 1 mM EDTA and a final enzyme concentration of 4 µg. Control experiments contained all the assay components except the enzyme or substrate (minus IMP or NAD⁺). Assays were performed in duplicate. One unit of enzyme activity was defined as the amount of enzyme required to transform 1 µmole substrate min⁻¹ at 37 °C. The specific activity was expressed as µmole NADH produced min⁻¹ (mg protein)⁻¹.

K_m (*app*) determination experiments for Mt-GuaB2 in the forward reaction were carried out by varying the concentration of one substrate while the concentration of the second substrate was maintained at a constant saturating level. The apparent K_m values for IMP and NAD⁺ were assessed from the initial rate measurements in duplicate by nonlinear curve fitting using the Graphpad prism software. For Mt-GuaB1, Mt-GuaB2 and Mt-GuaB3, the concentration of IMP was varied between 0.01 and 3 mM, NAD⁺ was varied between 0.03 and 4.5 mM with a final enzyme concentration of 4 µg. The pH optimum for the purified recombinant Mt-GuaB2 was determined by performing the assay at various pH values with increments of 0.5 pH units. The thermal stability of the protein was determined by pre-incubating Mt-GuaB2 at a variety of temperatures for 10 min and then the reaction mixture was added to start the assay. To determine the effect of temperature on enzyme activity, the reactions were incubated at different temperatures for 20 min at pH 8.5. The effect of monovalent cations (KCl, RbCl, CsCl and NH₄Cl) on Mt-GuaB2 activity was also examined at a concentration of 150 mM. The enzymic activity of Mt-GuaB2 was measured in the presence of three DPU compounds (DPU-1, DPU-2 and DPU-3). The inhibition constant, K_i , of the DPU inhibitors were measured at saturating amounts of NAD⁺ (3 mM; K_m 610.5 µM), with varying concentrations of IMP (64, 128 and 256 µM; K_m 128.1 µM) in the absence and presence of increasing concentrations of DPU-1 (0, 100, 300, 500, 650 and 800 µM), DPU-2 (0, 300, 1000, 1300, 1600 and 2000 µM) or DPU-3 (0, 10, 30, 100, 300 and 1000 µM). The reactions were initiated by the addition of NAD⁺ and the absorbance was measured at 340 nm using the assay as described above. The initial velocity (*V*) without inhibitor and at each inhibitor concentration was plotted on the *y*-axis versus IMP concentration on the *x*-axis. K_i (for noncompetitive inhibition) and αK_i (for uncompetitive inhibition) values were determined from non-linear regression analysis using the Graph pad prism program by fitting data to the equation for noncompetitive and uncompetitive inhibition, respectively.

RESULTS

Expression and purification of recombinant Mt-GuaB orthologues cloned in *E. coli*

To compare the three GuaB orthologues in *M. tuberculosis* H37Rv, the genes encoding these proteins were cloned and

expressed in the heterologous host *E. coli* as hexa-histidine fusion proteins using the pET28b vector under the control of a T7 promoter. *E. coli* C41 (DE3) cells were transformed with the pET28b-guaB constructs. To prevent inclusion body formation, it was necessary to induce expression of recombinant protein production at 16 °C for 20 h. Under these conditions, sufficient soluble protein was produced and subsequently purified using HisTrap Ni²⁺ chelating Sepharose affinity chromatography (Fig. 1b). The Mt-GuaB orthologues were purified with a satisfactory yield of approximately 1.7 mg (Mt-GuaB1), 3.3 mg (Mt-GuaB2) and 1.8 mg (Mt-GuaB3) per litre of culture. To confirm that the cloned genes were indeed IMPDHs, the activities of Mt-GuaB1, Mt-GuaB2 and Mt-GuaB3 were assayed spectrophotometrically.

Biochemical properties of recombinant Mt-GuaB orthologues

Analysis of the purified recombinant Mt-GuaB1, Mt-GuaB2 and Mt-GuaB3 revealed that only Mt-GuaB2 (Rv3411c) encoded a functional IMPDH (see below). The purified enzymes from the other two orthologues, Mt-GuaB1 and Mt-GuaB3, lacked IMPDH activity (data not shown).

The Mt-GuaB2 enzyme exclusively catalysed the oxidative deamination (forward reaction) of IMP in the presence of NAD⁺. In the absence of enzyme or substrates (IMP or NAD⁺) no activity was detectable. The optimum protein concentration for Mt-GuaB2 activity was determined to be 4 µg ml⁻¹ which was in the most linear part of the curve (Fig. 2a). The optimal temperature for catalytic activity was 37 °C (Fig. 2b). To determine the pH activity profile of Mt-GuaB2, the enzyme activity was monitored at saturating substrate conditions at several different pH values (Fig. 2c). The pH optima of Mt-GuaB2 activity was determined to be approximately 8–8.5 (Fig. 2c). Mt-GuaB2 enzyme could be stored at –80 °C and pH 8.5 for several months with negligible loss of enzymic activity. The enzyme was thermostable and retained activity upon incubation at 60 °C for 10 min (Fig. 2d). Above 70 °C, there was a complete inhibition in activity due to thermal inactivation of the enzyme (Fig. 2d).

The presence of DTT and EDTA, typically used in the routine assays, had no stimulatory effect on Mt-GuaB2 activity. Monovalent cations, such as K⁺, activate IMPDH (Hedstrom, 2009; Kerr *et al.*, 2000; Zhou *et al.*, 1997) and in a similar fashion Mt-GuaB2 exhibited a requirement for KCl (Fig. 3a). In order to investigate the effect of other salts on Mt-GuaB2 activity, the reaction was carried out in the presence of NH₄Cl, RbCl and CsCl. The presence of RbCl or NH₄Cl at the same concentration was also stimulatory but to a lesser extent than that observed for KCl (Fig. 3b).

Determination of apparent kinetic parameters of Mt-GuaB2

The apparent kinetic parameters for IMPDH activity of Mt-GuaB2 were determined at the optimum pH of 8.5.

Fig. 2. Characterization of recombinant Mt-GuaB2 expressed in *E. coli*. (a) Protein dose-response. (b) Optimum assay temperature. (c) pH optimum. (d) Thermotolerance of Mt-GuaB2. After pre-incubation of the reaction mixture for 10 min at 37 °C the reaction was started by the addition of purified enzyme and incubated at 37 °C for 20 min. (d) Thermotolerance of Mt-GuaB2. Recombinant Mt-GuaB2 (4 μg) was pre-incubated at various temperatures for 10 min in the reaction buffer before starting the reaction by adding the substrate. The remaining activity was assayed at 37 °C. Data shown are mean $\pm$ SEM.

Plots of initial velocity versus either IMP or NAD^+ concentration showed Michaelis–Menten kinetics. The apparent Michaelis constant (K_m) values for the substrates IMP and NAD^+ were calculated to be $128.1 \pm 10.85 \mu\text{M}$ and $610.5 \pm 69.16 \mu\text{M}$, respectively. The K_m value for IMP was close to that observed with *Streptococcus pyogenes* IMPDH which had a K_m of 62 μM (Prosise *et al.*, 2002; Zhang *et al.*, 1999). Generally, bacterial IMPDHs have been reported to have higher K_m values for IMP and NAD^+ than non-bacterial enzymes (Kerr & Hedstrom, 1997; Umejiego *et al.*, 2004; Zhou *et al.*, 1997).

The results established that Mt-GuaB2 encodes a functional enzyme that possesses IMPDH activity. The enzyme did not catalyse the reverse reaction, i.e. the conversion of XMP to IMP. Product inhibition of the forward reaction by XMP and NADH is characteristic of several IMPDH enzymes studied to date (Verham *et al.*, 1987; Xiang *et al.*, 1996). The Mt-GuaB2 enzyme exhibited substrate inhibition *in vitro* at high concentrations of NADH and XMP. The activity was strongly inhibitory with more than 95 % inhibition at a concentration of 1 mM NADH. Higher

concentrations of NADH completely abolished Mt-GuaB2 activity. The *in vitro* activity was also 40 % inhibited by XMP at a concentration of 1 mM XMP.

Inhibition studies

Screening results for over 100 000 compounds from the ChemBridge collection of drug-like molecules has been made publicly accessible through PubChem (<http://pubchem.ncbi.nlm.nih.gov/>) and has been reported recently (Ananthan *et al.*, 2009). A cursory informatic analysis of the numerous hits was done which identified a number of potentially interesting compounds and their targets (Ananthan *et al.*, 2009). Among these, the DPU compounds are known inhibitors of kinases and IMPDH (Ananthan *et al.*, 2009). Ideally, the known, potent activity against *M. tuberculosis in vitro* would be an ideal starting point for drug design presuming a target can be identified. Typically, antibacterial drug design programs have been hindered when starting from enzyme-based inhibition and later building on antibacterial activity. Unfortunately, pure phenotypic screens often lead to inhibitors that require

Fig. 3. K^+ dependence (a) and monovalent cation dependence (b) of recombinant Mt-GuaB2 activity. The initial velocities of IMPDH activity were measured by varying the concentration of KCl between 18.75 and 150 mM. The activities of each monovalent cation were determined at a fixed concentration of 150 mM. The specific activity was expressed as $\mu\text{mol NADH produced min}^{-1} (\text{mg protein})^{-1}$.

extensive investigation prior to identification of an optimal and specific enzyme activity.

With the hypothesis in mind that these types of molecules (DPUs) inhibited IMPDH, we purchased a small set of the more active and selective samples that were available from ChemBridge. A total of 16 DPU analogues were initially screened at a concentration of 1 mM in the *in vitro* Mt-GuaB2 activity assay. Among the analogues screened, three compounds (DPU-1, DPU-2 and DPU-3) displayed >90 % inhibition at 1 mM. These three compounds were retested at different concentrations in order to determine K_i and alpha K_i values for each compound (Table 1). The alpha K_i values of DPU-1 and DPU-3 were 231.8 μ M and 181.2 μ M, respectively, and the K_i value of DPU-2 was 1203 μ M. DPU-1 and -3 showed uncompetitive inhibition with respect to IMP, and DPU-2 showed noncompetitive inhibition versus IMP (Fig. 4a–c). The Lineweaver–Burk plots (Fig. 4a–c, inset) were generated only to indicate the type of inhibition observed. The three inhibitors were also tested for their ability to inhibit whole cell growth of *M. tuberculosis* and *M. smegmatis*. All three inhibitors were found to possess anti-mycobacterial activity against *M. tuberculosis* H37Rv (Table 1) with MIC values ranging from 0.2 to 0.4 μ g ml⁻¹. DPU-2 and -3 inhibited *M. smegmatis* growth to a similar extent, with an MIC value of 0.5 μ g ml⁻¹ for each compound. DPU-1 did not inhibit *M. smegmatis* growth even at the highest concentration (40 μ g ml⁻¹) tested.

Drug susceptibility of Mt-GuaB orthologues overexpressed in *M. smegmatis*

To confirm whether Mt-GuaB and IMPDH activity is the target of the DPU compounds (Table 1), the *M. smegmatis* pVV16 overexpressing strains were screened for susceptibility to the DPU compounds (Table 2 and Fig. 5a). The overexpressed Mt-GuaB orthologues in *M. smegmatis*

showed increased resistance to DPU-2 and -3 compared with the pVV16 vector control (Table 2 and Fig. 5a). The MIC values of DPU-3 against pVV16-Mt-*guaB1*, -Mt-*guaB2* and -Mt-*guaB3* strains were 16-fold higher than that of the pVV16 vector control (Table 2). The data indicated that the target protein of the DPU compounds was indeed IMPDH. The MIC of *M. smegmatis* to compound DPU-2 was enhanced twofold, indicating that the overproduction of Mt-GuaB orthologues also conferred resistance to compound DPU-2 (Table 2). It is interesting that although Mt-GuaB1 and Mt-GuaB3 were functionally inactive, the above gene dosage experiment suggests that the DPU compounds presumably bind to Mt-GuaB1 and Mt-GuaB3 proteins, thus requiring a higher concentration for inhibition. The Western blot after immunological detection with anti-His tag antibody clearly confirmed the expression in the pVV16-Mt-GuaB1–3 constructs (Fig. 5b).

DISCUSSION

Purine biosynthetic enzymes, and IMPDH in particular, are considered to be attractive targets for development of novel anti-mycobacterial agents. To further our knowledge of purine metabolism we have investigated the IMPDH enzyme of *M. tuberculosis*. IMPDH, encoded by the *guaB* gene catalyses the reversible conversion of IMP to XMP. In *E. coli*, at least two different enzymic pathways lead to the synthesis of XMP (Neuhard & Nygaard, 1996; Pimkin *et al.*, 2009) which are well characterized (Gilbert *et al.*, 1979; Gilbert & Drabble, 1980; Kerr & Hedstrom, 1997; Kerr *et al.*, 2000; Tiedeman & Smith, 1985). However, the *M. tuberculosis* H37Rv purine *de novo* biosynthesis pathway enzymes have received little attention to date and are poorly studied.

Each of the three Mt-*guaB* genes was successfully expressed in *E. coli* and *M. smegmatis*. In both *E. coli* and *M.*

Table 1. Structures of DPU analogues and their corresponding MIC values against *M. tuberculosis* and *M. smegmatis*, and the K_i and alpha K_i values obtained for the *in vitro* inhibition of Mt-GuaB2 purified from *E. coli*

Compound	Structure	MIC ₉₀ (μ g ml ⁻¹)		<i>In vitro</i> Mt-GuaB2 assay K_i (μ M)
		<i>M. tuberculosis</i>	<i>M. smegmatis</i>	
DPU-1		0.2	>40.0	alpha K_i 231.8
DPU-2		0.3	0.5	1203
DPU-3		0.4	0.5	alpha K_i 181.2

Fig. 4. The mechanism of inhibition of DPU-1 (a), DPU-2 (b) and DPU-3 (c) by IMP (64–256 μM). Mt-GuaB2 was assayed as described in Methods in the absence of inhibitor and with increasing concentrations of DPU-1, -2 and -3. Data were fitted in Graphpad prism to a non-linear regression function for uncompetitive (DPU-1 and -3) and noncompetitive (DPU-2) inhibition. The Lineweaver–Burk plots inset (top) of each plot were generated only to display the type of inhibition.

smegmatis, only the recombinant overexpressed Mt-GuaB2 exhibited IMPDH activity. Mt-GuaB1 and Mt-GuaB3 were obtained as soluble proteins but they lacked enzymic activity. The biochemical properties of the functional orthologue Mt-GuaB2 were extensively characterized.

Table 2. MIC of DPU-2 and -3 against Mt-GuaB pVV16-overexpressed *M. smegmatis* strains

<i>M. smegmatis</i> strain	MIC ₉₀ * ($\mu\text{g ml}^{-1}$)	
	DPU-3	DPU-2
mc ² 155	0.5	0.5
pVV16	0.5	0.5
pVV16:Mt-guaB1	8	1
pVV16:Mt-guaB2	8	1
pVV16:Mt-guaB3	8	1

*The MIC₉₀ value is expressed as the lowest concentration that inhibited visible growth of the organism on a TSB agar plate after incubation at 37 °C for 3–4 days.

Mt-GuaB2 had an absolute substrate specificity for IMP and NAD⁺. The K_m values calculated for NAD⁺ and IMP were within the range of K_m values determined for other known bacterial IMPDH enzymes (Kerr & Hedstrom, 1997; Prosise *et al.*, 2002; Zhang *et al.*, 1999). The specific features of the Mt-GuaB2 enzyme were its elevated stability towards higher temperatures, higher pH optimum and requirement of K⁺ for functional activity.

Our results led us to further investigate and attempt to explain the apparent non-functional nature of Mt-GuaB1 and Mt-GuaB3. Bioinformatic analysis of the three homologue sequences (and their comparison with that of human type II IMPDH) reveals potential reasons why Mt-GuaB2 is the only active orthologue amongst the three *M. tuberculosis* orthologues. The complex of human type II IMPDH with 6-chloropurine riboside 5'-monophosphate and NAD⁺ (PDB ID, 1NFB; Risal *et al.*, 2004) identifies several important active site residues, which can be mapped to Mt-GuaB1–3 in a multiple sequence alignment (Fig. 6). Most significantly, the Cys nucleophile that forms a covalent intermediate with the substrate is altered to Thr in Mt-GuaB3 (Thr226), and so would presumably be unable to initiate the first stage of catalysis. Furthermore,

Fig. 5. Growth of Mt-GuaB1, Mt-GuaB2 and Mt-GuaB3 overexpressing strains in the presence of 8 µg DPU-3 ml⁻¹ and 1 µg DPU-2 ml⁻¹. (a) pVV16 vector and the Mt-GuaB1–3 overexpression strains were transformed into *M. smegmatis* and grown in TSB containing 0.05 % Tween 80, until OD₆₀₀ 0.7. Numbers 1–8 indicate serial tenfold dilutions (10⁻¹–10⁻⁸) of each culture which were spotted on TSB plates supplemented with kanamycin, hygromycin and the DPU compound. (b) Western blot analysis of lysates of Mt-GuaB overexpressing *M. smegmatis* strains and pVV16 vector, to confirm expression.

Mt-GuaB3 lacks two conserved residues that contact the nicotinamide headgroup of NAD⁺; human IMPDH residues Asp274 and Arg322 have Asp and Lys counterparts in Mt-GuaB1 and Mt-GuaB2, but align with Gln164 and Ile216 in Mt-GuaB3. A structural basis for the non-observable activity of Mt-GuaB1 is less obvious, but may come from a shift that alters the position of a conserved Tyr; human IMPDH Tyr411 contacts the edge of the nucleotide substrate and sits alongside the Cys nucleophile, but aligns with Ser384 in Mt-GuaB1. An experimental structure of Mt-GuaB1 would be required to confirm this, in particular determining that the neighbouring Tyr385 does not occupy this position. Another possibility for the lack of IMPDH activity in Mt-GuaB1 and Mt-GuaB3 may relate to the hydrolytic half-reaction, as only Mt-GuaB2 possesses an equivalent residue to Arg412 of human type II IMPDH postulated to activate a water molecule implicated in the hydrolysis of the covalent enzyme intermediate (Hedstrom, 2009). The metabolic function of Mt-GuaB1 and Mt-GuaB3 awaits further clarification; however, several ‘moonlighting’ roles for

IMPDH enzymes independent of classical catalytic activity have been proposed (Hedstrom, 2009).

We have shown that the DPU analogues exhibited potent anti-mycobacterial activity against *M. tuberculosis* and *M. smegmatis* growth. The mode of action of the DPU compounds was through *in vitro* and *in vivo* inhibition of Mt-GuaB2, and we established that Mt-GuaB orthologues were indeed the targets for the DPUs examined. However, the presence of other potential cellular targets may exist. Surprisingly, DPU-1, which was an effective Mt-GuaB2 inhibitor, did not inhibit *M. smegmatis* growth even at very high concentrations, although it inhibited *M. tuberculosis*. Antimycobacterial drugs, such as isoniazid and rifampicin have also been shown to have higher MIC values against *M. smegmatis* compared with *M. tuberculosis* (Chaturvedi *et al.*, 2007; Chung *et al.*, 1995; Reddy *et al.*, 2008). One possible explanation for this may be cell wall permeability, which is the major barrier and factor contributing to the intrinsic resistance of *M. smegmatis* to rifampicin (Hetherington *et al.*, 1995; Jarlier & Nikaido,

Fig. 6. Comparative sequence alignment of Mt-Gua1–3 and the human type II IMPDH. Possible amino acid differences in Mt-GuaB1 and Mt-GuaB3 responsible for a lack of IMPDH activity referred to in the text are blue. Partially conserved residues are in red, conserved residues are white on a red background. Numbering and secondary structure used above the alignment are taken from human type II IMPDH (PDB 1NFB).

1994; Piddock *et al.*, 2000). Resistance of *M. smegmatis* to isoniazid and rifampicin has also been attributed to drug efflux mechanisms (Chaturvedi *et al.*, 2007; Li *et al.*, 2004; Piddock *et al.*, 2000). Remarkably, these DPU inhibitors of Mt-GuaB2 have mechanistic and structural precedent in several compounds identified through high-throughput screening of *Cryptosporidium parvum* IMPDH (Macpherson

et al., 2010). Despite the protozoan nature of *C. parvum*, its IMPDH has long been thought to have been acquired via gene transfer from a bacterial source (Striepen *et al.*, 2002; Umejiego *et al.*, 2004). Observing the structure of *C. parvum* IMPDH with one of these inhibitors (inhibitor C64, PDB code 3KHJ) reveals a novel interaction with the enzyme, which partially stacks against the purine ring of IMP. A direct

comparison of DPU-2 with C64 yields a good match for structurally equivalent chemical groups; the chloroaniline, urea-linker and nitrotoluene groups of DPU-2 are presumably analogous to the bromoaniline, amide linker and thiazole groups of C64, respectively. This significant agreement in inhibitor structure further validates our conclusion that Mt-GuaB2 is the target of these compounds, and may also provide a potential basis for the optimization of DPU1–3. The mechanism of inhibition of DPU-3 was uncompetitive versus IMP and that of DPU-2 was noncompetitive with respect to IMP. In summary, we conclude that DPU-2 and DPU-3 exhibit anti-mycobacterial activity by specifically inhibiting Mt-GuaB2.

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