Peptide Deformylase Inhibitors as Potent Antimycobacterial Agents[†]

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Peptide deformylase (PDF) catalyzes the hydrolytic removal of the N-terminal formyl group from nascent proteins. This is an essential step in bacterial protein synthesis, making PDF an attractive target for anti-bacterial drug development. Essentiality of the *def* gene, encoding PDF from *Mycobacterium tuberculosis*, was demonstrated through genetic knockout experiments with *Mycobacterium bovis* BCG. PDF from *M. tuberculosis* strain H37Rv was cloned, expressed, and purified as an N-terminal histidine-tagged recombinant protein in *Escherichia coli*. A novel class of PDF inhibitors (PDF-I), the *N*-alkyl urea hydroxamic acids, were synthesized and evaluated for their activities against the *M. tuberculosis* PDF enzyme as well as their antimycobacterial effects. Several compounds from the new class had 50% inhibitory concentration (IC₅₀) values of <100 nM. Some of the PDF-I displayed antibacterial activity against *M. tuberculosis*, including MDR strains with MIC₉₀ values of <1 μM. Pharmacokinetic studies of potential leads showed that the compounds were orally bio-available. Spontaneous resistance towards these inhibitors arose at a frequency of $\leq 5 \times 10^{-7}$ in *M. bovis* BCG. DNA sequence analysis of several spontaneous PDF-I-resistant mutants revealed that half of the mutants had acquired point mutations in their formyl methyltransferase gene (*fnt*), which formylated Met-tRNA. The results from this study validate *M. tuberculosis* PDF as a drug target and suggest that this class of compounds have the potential to be developed as novel antimycobacterial agents.

Mycobacterium tuberculosis, a pathogen causing 2 million deaths and 8 million new cases of tuberculosis (TB) each year, is a leading health threat. This is further fueled by the human immunodeficiency virus epidemic and the appearance of multidrug-resistant TB (17, 18, 46). Infections involving multidrug-resistant TB are harder to treat and have limited the efficacy of TB control programs (29). This has necessitated the search for new antituberculosis drugs with a novel mode of action.

In prokaryotes and in certain organelles, like plastids and mitochondria of eukaryotes, nascent proteins carry an N-formylated methionine (40). After translational initiation, peptide deformylase (PDF) (EC 3.5.1.27) removes the *N*-formyl group from the nascent proteins (42). Deformylated proteins undergo further N-terminal processing to give mature proteins (61). PDF is an attractive candidate for the discovery of antibacterial agents, as the essentiality of the gene (*def*) encoding PDF has been shown for *Escherichia coli* and *Streptococcus pneumoniae* (8, 36). The enzyme has been characterized as a highly unstable metallopeptidase that uses Fe²⁺ as the catalytic metal (51). Oxidation of Fe²⁺ inactivates the enzyme, and purification of

active bacterial PDF has been a challenge (51). So far, Ni²⁺, Co²⁺, and Mn²⁺ have been described as cations that can stably replace Fe²⁺ while retaining high enzymatic activity (25, 49).

Naturally occurring antibiotics, like actinonin, inhibit the activity of the PDF enzyme (2). Based on structural (7, 39, 41) and mechanistic design, as well as high-throughput screening, several classes of PDF inhibitors (PDF-I) have been studied previously (64). Thus far, only compounds having hydroxamic acid-chelating or *N*-formyl hydroxylamine-chelating groups show potent enzyme inhibition, good antibacterial activity, and in vivo efficacy, including oral activity (9, 12, 27).

In this work, we validate *def* (gene encoding PDF) as a drug target for TB by proving its essentiality in *Mycobacterium bovis* BCG. PDF from *M. tuberculosis* strain H37Rv was cloned and expressed heterologously in *E. coli*. Recombinant His-tagged *M. tuberculosis* PDF enzyme was purified, and biochemical characterization was carried out. Activities of a novel class of PDF-I, the *N*-alkyl urea reverse hydroxamates (27), were determined by both *M. tuberculosis* PDF enzyme and cell-based assays for antimycobacterial activity. A potent lead, PDF-611 (LBK-611) (15), was identified as a novel antimycobacterial agent.

MATERIALS AND METHODS

Enzymes, chemicals, and antibiotics. Chemicals used were of the highest grade commercially available. Chemicals and antibiotics were purchased from Sigma (St. Louis, Mo.), unless otherwise indicated. Restriction enzymes and modifying enzymes were purchased from New England Biolabs, Inc. (Beverly, Mass.), and were used according to the manufacturer's recommendations.

Bacterial strains, plasmids, and media. Bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* strains were cultured in Luria-Bertani (LB)

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TABLE 1. Strains and plasmids used in this work

Strain or plasmid	Relevant characteristic(s)	Source or reference
Strains		
<i>E. coli</i>		
XL1-Blue	<i>recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac</i> [F' <i>proAB lacI^qΔM15 Tn10</i> (Tet ^r)]; cloning strain	Stratagene
M15(pREP4)	Nal ^s Str ^s Rif ^s Thi ⁻ Lac ⁻ Ara ⁺ Gal ⁺ Mtl ⁻ F ⁻ RecA ⁺ Uvr ⁺ Lon ⁺ ; expression strain for P _{T5} -regulated gene expression	QIAGEN
<i>M. bovis</i> BCG	Pasteur strain ATCC 35734	ATCC
XO22	Single-crossover strain; Suc ^s Hyg ^r	This work
XOdef	Single-crossover strain XO22 carrying complementation plasmid pMV262-def	This work
Plasmids		
pCR-XL-TOPO	Amp ^r ; TA-cloning vector for longer PCR products	Invitrogen
pCR2.1-TOPO	Amp ^r ; TA-cloning vector	Invitrogen
pQE30	Amp ^r ; low-copy-number plasmid with inducible T5 promoter for production of N-terminal His ₆ tag translational fusion	QIAGEN
pCR2.1-PDF	<i>M. tuberculosis</i> <i>def</i> gene cloned into pCR2.1-TOPO vector	This work
pQE30-PDF	<i>M. tuberculosis</i> <i>def</i> gene cloned into BamHI site of pQE30	This work
pYUB657	Amp ^r , Hyg ^r ; <i>sacB</i> counterselectable marker, <i>E. coli</i> - <i>Mycobacterium</i> shuttle vector	47
pYUB657-dimer	DNA fragment containing an in-frame <i>def</i> deletion cloned into pYUB657 suicide vector	This work
pMV262	<i>E. coli</i> - <i>Mycobacterium</i> shuttle plasmid, <i>hsp60</i> promoter; Kan ^r ColE1 OriM	62
pMV262-def	pMV262 containing the <i>def</i> gene, expressed as a translational fusion with the first six amino acids of <i>hsp60</i>	This work

broth (Becton Dickinson, Franklin Lakes, NJ) and LB agar plates supplemented with either 100 µg/ml of ampicillin or 50 µg/ml of kanamycin for cloning and maintenance. Terrific broth (12 g tryptone, 24 g yeast extract, 4 ml glycerol, 2.3 g potassium phosphate [monobasic], 9.4 g potassium phosphate [dibasic], pH 7.5, water to make 1 liter) was used as the growth medium for the expression of PDF from *E. coli*.

M. bovis BCG was cultured in Middlebrook 7H9 (catalog no. 271310; Difco) broth supplemented with 10% (vol/vol) albumin-dextrose saline (0.81% NaCl, 5% bovine serum albumin fraction V [Roche, Mannheim, Germany], and 2% glucose), 0.2% glycerol, and 0.05% Tween 80 or on Middlebrook 7H10 (catalog no. 262710; Difco) agar plates supplemented with 10% (vol/vol) oleic acid-albumin-dextrose-catalase enrichment (catalog no. 212240; BBL), 0.5% glycerol, and 0.05% Tween 80. When required, hygromycin B (Roche) was used at the concentration of 50 µg/ml. Sucrose was used in the 7H10 medium at a 4% concentration.

Buffers. Lysis buffer was composed of 50 mM HEPES (pH 7.5), 5 mM NiSO₄, 5 mM MgSO₄, 10 µg/ml catalase, 0.25 mM Tris(2-carboxyethyl)phosphine hydrochloride, 10 µg/ml DNase I, 1 ml protease cocktail inhibitor, 1 mg/ml lysozyme, and 1% Triton X-100. Buffer I was composed of 50 mM NaH₂PO₄, 300 mM NaCl, and 10 mM imidazole, pH 8.0. Buffer II was composed of 50 mM NaH₂PO₄, 300 mM NaCl, and 20 mM imidazole, pH 8.0. Buffer III was composed of 50 mM NaH₂PO₄, 300 mM NaCl, and 250 mM imidazole, pH 8.0. Gel filtration buffer was composed of 50 mM NaH₂PO₄, 1 M NaCl, and 5% glycerol.

Construction of the BCG *def* suicide plasmid and the *def* complementation plasmid. Gene regions flanking the *def* gene were PCR amplified and ligated together to obtain a DNA fragment in which the *def* gene (636 bp) was excluded in frame (Fig. 1A). Primers BglF (5'-TGGTAAATAGAGATCTGTGTGCGA CGTCATAGCCGAGTT-3') and NdeR (5'-TGTCTTTTCATATGTAATGGT CTCGTGGCCGGGGC-3') amplify an 1,150-bp fragment upstream of *def* (left fragment). Primers NdeFF (5'-GAAGTGTACATATGGGGCTGAGGAGCG GGGCAAT-3') and BglIRR (5'-TTTAATTTTATAGTCTGGCGGGCTTTGGC ATAGCG-3') amplify an 1,173-bp fragment downstream of *def* (right fragment). Primers BglF/BglIRR and NdeFF/NdeFF carry BglII and NdeI restriction sites, respectively, which enabled the left and right fragments to be ligated and cloned into pCR-XL-TOPO (Table 1). The dimer fragment was then cloned into the suicide vector pYUB657 (47), generating the *def* suicide plasmid pYUB657-dimer (Table 1). The *def* complementation vector was constructed by cloning the *def* gene into the BamHI site of pMV262 (Table 1), generating pMV262-def. This vector was introduced into a *def* single-crossover strain to allow chromosomal *def* to be deleted.

Genomic DNA isolation and Southern blot analysis. Genomic DNA from *M. bovis* BCG was isolated using the protocol described in a DNeasy tissue kit

(QIAGEN, Valencia, CA). For Southern blot analysis, DNA samples were digested with the appropriate restriction enzyme and fragments separated on a 1% agarose gel and transferred to Hybond-N⁺ nylon membrane (Amersham). Southern hybridization was carried out using enhanced chemiluminescence direct nucleic acid labeling and detection systems (Amersham).

Expression and purification of *M. tuberculosis* PDF. The *M. tuberculosis* H37Rv genomic DNA sequence (GenBank accession number NC_000962) was used to design primers 5'-ATGGATCCATGGCAGTCGTACCCATCCG-3' and 5'-ATGGATCCGTGACCGAACGGGTCGGG-3', which had BamHI sites (underlined) incorporated in them. The native TAA stop codon from the *M. tuberculosis* *def* gene was not included to utilize the vector-encoded stop codon, resulting in the fusion protein MRGSHHHHHGS-*M. tuberculosis* PDF-GSAC ELGTPGRPAAKLN, with residues in italics originating from the vector. PCR was carried out using a high-fidelity Platinum PCR Supermix (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. PCR products were initially cloned into the pCR2.1-TOPO cloning vector (Invitrogen), generating plasmid pCR2.1-PDF; from this, the *def* gene was subcloned into BamHI-linearized *E. coli* expression vector pQE30 (QIAGEN), generating plasmid pQE30-PDF (Table 1). Both pCR2.1-PDF and pQE30-PDF were sequenced. DNA sequences were analyzed using the software Vector NTI, version 7.0 (InforMax, Frederick, MD). Amino acid sequence alignments were prepared using CLUSTALW, version 1.83 (11). All DNA manipulations were performed under standard conditions as described previously (55).

E. coli M15(pREP4) cells carrying pQE30-PDF were grown in terrific broth containing 50 µg/ml of kanamycin and 100 µg/ml of ampicillin at 37°C until the optical density at 600 nm reached ~0.5. The culture was induced with 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and incubated at 37°C for an additional 4 h. Cells (from 1 liter) were harvested, and the cell pellet was washed twice with sterile phosphate-buffered saline and suspended in 20 ml lysis buffer. The cells were lysed by incubation on ice for 30 min, followed by sonication. Cell debris was removed by centrifugation at 35,000 × g at 4°C for 15 min. The proteins in the supernatant were precipitated by adjustment to 60% saturation with ammonium sulfate. The pellet was dissolved in 10 ml of buffer I and added to Ni-nitrilotriacetic acid (NTA) slurry (QIAGEN) for batch purification by metal affinity chromatography. The resulting mixture was incubated for 1 h with gentle shaking at 4°C. The slurry was washed twice with buffer II, and the bound PDF was eluted with buffer III. Active fractions obtained by Ni-NTA batch purification were further purified by gel filtration using a HiLoad Superdex 26/60 prep grade column (Amersham) that had been equilibrated with gel filtration buffer. Elution was done with the same buffer at a flow rate of 1 ml/min. The active fractions were pooled and concentrated using a Vivaspin 20 centrifugal

FIG. 1. (A) Arrangement of *def* and neighboring genes in the *M. tuberculosis* genome. PCR fragments generated for the construction of the *def* Δ suicide vector are indicated by the solid arrows. See Materials and Methods for details. The genetic map is not drawn to scale. (B) Southern analysis of *def* Δ mutants. *def* Δ mutants (lanes 2 to 4) could be obtained only when the single-crossover strain was complemented, in *trans*, with *def* carried on plasmid pMV262. Lane 1, wild-type BCG; lane 5, single-crossover strain; lane 6, single-crossover strain X0def, harboring the *def* gene on plasmid pMV262-def; lane 7, plasmid pMV262-def; lane M, 1-kb DNA marker. Genomic DNA was digested with HindIII and probed with *def*. Wild-type BCG yields a 10.3-kb fragment, while the single-crossover strain yields an 8.3-kb fragment. Neither of these fragments was seen in complemented *def* Δ mutants, indicating that the chromosomal copy of the *def* gene had been deleted.

concentrator (Sartorius, Hanover, Germany). Glycerol was added to a final concentration of 33% (vol/vol), and the enzyme was stored frozen at -80°C .

PDF enzyme assay. The PDF enzyme assay format was based on a published method (12), and the assay was carried out in a final volume of 50 μl in black, 96-well, half-area plates (Corning Costar, Etobicoke, Ontario, Canada). The reaction mixture contained 50 mM HEPES (pH 7.5) and ~ 30 ng of *E. coli*-expressed *M. tuberculosis* PDF per well. The reaction was initiated by adding *N*-formyl-methionine-alanine-serine (fMAS) substrate to a final concentration of 2 mM (Bachem, Germany). The reaction was done at room temperature for 2.5 min and terminated by adding 25 μl of fluorescamine (0.02 mg/ml in dioxane). The microplate was further incubated for 5 min and read for fluorescence by a Tecan Safire plate reader using an excitation wavelength of 380 nm and an emission wavelength of 470 nm.

Biochemical characterization of *M. tuberculosis* PDF. Biochemical characterization of *M. tuberculosis* PDF was performed using pure and batch-purified recombinant enzyme. The effect of substrate concentrations on *M. tuberculosis* PDF activity was studied using two *N*-formylated peptide substrates, *N*-formyl-methionine-alanine (fMA) and fMAS (Bachem), with concentrations ranging from 0 to 10 mM. The effects of the divalent metal ions Ca^{2+} , Mg^{2+} , Mn^{2+} , Co^{2+} , Cu^{2+} , Ni^{2+} , and Zn^{2+} on PDF activity were assessed by the addition of different ions at a final concentration of 1 mM to the assay buffer.

For inhibition studies of *M. tuberculosis* PDF, a 30-min preincubation of the inhibitor with the enzyme was included prior to initiating the reaction. The concentrations of the inhibitors varied from 0 to 10 μM . Fifty percent inhibitory concentration (IC_{50}) values were obtained by fitting the data to a sigmoid dose-response equation using GraphPad Prism software, version 3.0 (GraphPad Software, Inc., San Diego, CA).

Antimicrobial agents and drug susceptibility testing. Streptomycin (STR), isoniazid (INH), rifampin (RIF), linezolid (LZD), and erythromycin (ERY) were obtained from Sigma (St. Louis, Mo.). Stock solutions of STR, LZD, and ERY were prepared at 10 mM concentration in deionized water, while INH, RIF, and PDF-I were dissolved in 90% dimethyl sulfoxide. Drug susceptibility testing was based on a previously published method (20). Logarithmic-phase *M. bovis* BCG cultures were diluted in complete 7H9 broth to obtain an optical density at 600 nm of ~ 0.04 , which corresponded to approximately 10^6 CFU/ml, before being added to the test plate. The plates were incubated at 37°C for 4 days. On the fourth day, 50 μl of a freshly prepared 1:1 mixture of Alamar Blue (Serotec Ltd., Oxford, United Kingdom) and 10% Tween 80 was added to each

well. The plates were reincubated overnight at 37°C . STR was used as a reference drug in each plate. The MICs were obtained by quantifying the fluorescence of Alamar Blue, using excitation and emission wavelengths of 530 nm and 590 nm, respectively. Values below 150,000 relative fluorescence units were considered to reflect no growth.

Frequency of spontaneous resistant mutants. Log-phase BCG cultures at 10^9 CFU/ml were plated onto 7H10 plates containing drug at $10\times$ MIC and onto drug-free media. Plates were incubated at 37°C for approximately 6 weeks or until colonies appeared. The frequency of spontaneous resistance was calculated as the ratio of the number of colonies that grew on drug-supplemented plates to that of drug-free plates.

Mutational analyses of *def* and *fnt* genes. The *def* and *fnt* genes of PDF-I-resistant BCG mutants were analyzed for sequence variation. The *def* gene region was amplified using primers Updef (5'-GGTCCCGTCTTGGTCTGC A-3') and Dndef (5'-CGGCGATGATGCCGCCG-3'), generating an 838-bp amplicon. The primers Upfnt (5'-TAGCGTGCCTTGCCTACCA-3') and Dnfnt (5'-CAGCGCGGCAACACCAG-3') would amplify a 1,178-bp amplicon. High-fidelity PCR was carried out using Proof Start DNA polymerase (QIAGEN). The PCR products were purified from agarose gels using a QIAquick gel extraction kit (QIAGEN). The purified PCR products were sequenced at Research Biolabs Technologies (Singapore). Jalview software was used for visualizing the alignment (11), and another software was used for bioinformatics analysis and secondary-structure prediction of the mutants (13).

In vivo pharmacokinetic studies. CD-1 female outbred mice (BioArc, India) were used for pharmacokinetic studies of selected PDF inhibitors. For intravenous (i.v.) and oral (p.o.) administration, the compounds were formulated in 4% and 10% polyethylene glycol 300, respectively. After 1 week of acclimation, mice received a dose of 1 mg/kg of body weight i.v. via tail vein injection or a dose of 5 mg/kg p.o. by gavage. Groups of four mice were euthanized by CO_2 inhalation at different intervals from 5 min to 4 h following dosing. Blood samples were collected by cardiac puncture into heparinized tubes. Blood was centrifuged, and plasma was recovered and stored frozen at -80°C . Plasma samples were extracted with 1 ml ethyl acetate, analyzed, and quantified for drug content by liquid chromatography coupled with triple quadrupole mass spectrometry (X-Terra, C_{18} , 4.6 by 50 mm, 5 μm), with carbamazepine as the internal standard. For quantitative calibration, standard curves were established using spiked compounds in plasma. The limit of detection was 10 ng/ml. A noncompartmental model (WINNONLIN, version 4.1) was used to calculate pharmacokinetic pa-

FIG. 2. Expression of *M. tuberculosis* PDF in *E. coli*. (A) SDS-PAGE analysis of PDF expressed in *E. coli* M15(pREP4) carrying pQE30-PDF. Lane 1, total cell protein uninduced; lane 2, total cell protein induced; lane 3, soluble fraction; lane 4, insoluble fraction; lane 5, Ni-NTA batch-purified *M. tuberculosis* PDF; lane 6, *M. tuberculosis* PDF purified by gel filtration; lane M, low-range molecular mass marker (Amersham). Arrows indicate the position of recombinant His-tagged PDF. (B) Effects of various substrate (fMA and fMAS) concentrations on recombinant *M. tuberculosis* PDF activity. RFU, relative fluorescence units.

rameters. The p.o. bioavailability was calculated as the ratio of the area under the curve (AUC) for p.o. administration to the AUC for i.v. administration corrected for the dose ($F = \text{AUC}_{\text{p.o.}} \times \text{dose}_{\text{i.v.}} / \text{AUC}_{\text{i.v.}} \times \text{dose}_{\text{p.o.}}$).

RESULTS

Essentiality of *def*, the gene encoding peptide deformylase in *M. bovis* BCG. In order to determine the essentiality of peptide deformylase in *M. bovis* BCG, a two-step allelic-exchange methodology (47) was employed to generate *def* deletion mutants. A suicide plasmid, pYUB657-dimer (Table 1), was electroporated into BCG, and a single-crossover strain, XO22, confirmed by Southern hybridization (Table 1; Fig. 1B), was obtained. This strain had a sucrose sensitivity and hygromycin resistance ($\text{Suc}^s \text{Hyg}^r$) phenotype conferred through the integration of the suicide vector into the chromosome. After the second recombination event, the selection markers were eliminated and 75 revertant ($\text{Suc}^r \text{Hyg}^s$) colonies were screened by PCR. All were positive for chromosomal *def*. The 28 colonies that were *hyg* negative by PCR were analyzed by Southern blotting by probing with *def*. Hybridization results showed that all of the recombinants retained the wild-type *def* gene (3.8-kb band), while the single-crossover strain from which the secondary recombinants were derived generated a larger, 4.6-kb fragment (data not shown), suggesting that the *def* gene is essential and cannot be deleted.

A copy of *def* cloned into pMV262 (pMV262-*def*, Kan^r [Table 1]) was introduced into the single-crossover strain XO22, resulting in transformant XOdef (Table 1). Double crossovers were isolated from XOdef by culturing the strain in the absence of hygromycin, thus allowing the integrated vector sequence to be lost. All 28 $\text{Suc}^r \text{Hyg}^s \text{Km}^r$ colonies which were selected had lost the integrated suicide vector. Of these, 25 contained the wild-type *def* gene in the chromosome (data not shown). Southern hybridization of the remaining three isolates

confirmed that they contained a *def* deletion in the chromosome (Fig. 1B). When these mutants were probed with *def*, the chromosomal copy of *def* could not be detected (Fig. 1B).

Expression and purification of *M. tuberculosis* PDF. As shown in Fig. 2A, recombinant *M. tuberculosis* PDF was expressed to high levels in *E. coli*. However, the expressed protein was largely in the insoluble form (Fig. 2A, lane 4). Insolubility of recombinant *M. tuberculosis* PDF has been reported earlier (57). Despite the low solubility of *M. tuberculosis* PDF, purification from *E. coli* extracts was performed under native conditions. The enzyme was purified to homogeneity by a combination of ammonium sulfate fractionation, Ni-NTA, and gel filtration chromatography. Typical yields were 1.2 mg/liter. The resulting purified protein showed a single band on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels (Fig. 2A, lane 6) and showed enzymatic activity on *N*-formylmethionyl peptides.

SDS-PAGE analysis (Fig. 2A, lane 6) showed that the purified protein had a molecular mass of ~30 kDa. This was slightly larger than the predicted molecular mass of 23.96 kDa (inclusive of the His_6 tag and an additional 17 amino acids at the C terminus which was introduced from the vector). Such anomalous migration of recombinant *M. tuberculosis* PDF was observed earlier (57). Analytical gel filtration on a HiLoad Superdex 26/60 prep grade column gave an apparent molecular mass of 24.5 kDa, close to that deduced from its amino acid sequence (23.96 kDa). This suggested that the peptide deformylase enzyme behaved as a monomeric species under the native conditions used for purification. Identity of the 30-kDa protein was further confirmed by Western blotting using anti-PDF antibody and anti-His antibody (data not shown).

There was a significant loss of protein and enzyme activity from fractions obtained by gel filtration which were not suit-

TABLE 2. Effects of PDF-I on *M. tuberculosis* PDF and *H. sapiens* PDF enzyme activities (IC_{50} s) and antibacterial activities (MIC_{90} s) in *M. bovis* BCG

Compound	Structure	IC_{50} (nM)		MIC_{90} (μ M) in <i>M. bovis</i> BCG ^a	Reference(s)
		<i>M. tuberculosis</i> PDF ^a	<i>H. sapiens</i> PDF		
Actinonin		50.5 $\pm$ 7.7	43	>25	12, 32
PDF-709		90.0 $\pm$ 4.2	147 ^a	6.25	27
PDF-611 (LBK-611)		69.5 $\pm$ 0.7	115 ^a	0.78	15
BB-3497		23.9 $\pm$ 7.5	8	3.125	12, 45

^a This work.

able for large-scale screens. Instead, as reported earlier with *E. coli* PDF (10), batch-purified *M. tuberculosis* PDF (Fig. 2A, lane 5) was used for the screening of the large library. After the background (induced *E. coli* M15 cell extracts transformed with the pQE30 vector, treated in a similar way) was subtracted, the enzyme activity in the batch was very specific and could be completely inhibited by PDF-I. The hits were reconfirmed using the purified enzyme.

Biochemical characterization of *E. coli*-expressed *M. tuberculosis* PDF. Biochemical characterization was done with both pure and batch-purified enzyme, and results were found to be similar. During optimization of substrate concentrations of fMA and fMAS, the latter was found to be a more optimal substrate for *M. tuberculosis* PDF (Fig. 2B). A prominent feature of the kinetics observed for both substrates tested was that the enzyme showed no saturation kinetics up to ~10 mM substrate, and concentrations above 12.5 mM fMAS were inhibitory for *M. tuberculosis* PDF. This finding was similar to that from an earlier report (57) (Fig. 2B). Subsequently, 2 mM fMAS was used for evaluating other parameters of the enzyme assays. Velocity versus substrate concentration plots typically produced a straight line, indicating that the K_m values for these substrates are >5 mM (data not shown).

The enzyme displayed a pH optimum of 7.5, retaining less than 20% of its optimal activity at extreme pH, for example, at pH 5.5 or pH 10 (data not shown). Ionic strength had a significant detrimental effect on *M. tuberculosis* PDF activity at concentrations greater than 0.1 M KCl (data not shown). This result agreed with earlier results with *M. tuberculosis* PDF (57). Co^{2+} , Ni^{2+} , and Zn^{2+} were partially inhibitory, while Cu^{2+} ions completely abolished PDF activity (data not shown). Ear-

lier reports had indicated that Co^{2+} , Ni^{2+} , and Zn^{2+} had no effect on *M. tuberculosis* PDF enzyme activity (57).

Enzyme inhibition studies. Several inhibitors from an available PDF-I library (27) were tested in an *M. tuberculosis* PDF enzyme assay (Table 2). The most active compound, PDF-611, or LBK-611 (15), had an IC_{50} of 69.5 nM, while a similar compound, PDF-709, had an IC_{50} of 90 nM. Two other known antibacterial PDF-I, actinonin (10) and BB-3497 (12, 14), showed potent inhibition of the *M. tuberculosis* PDF enzyme, with IC_{50} s of 50.5 and 23.9 nM, respectively.

In order to assess the issue of selectivity, the compounds were also tested against purified *Homo sapiens* PDF and human matrilysin (MMP-7). Matrilysin belongs to the matrix metalloproteinase (MMP) family of zinc-containing enzymes related to PDF (44). PDF-611 (LBK-611) and PDF-709 inhibited *H. sapiens* PDF with IC_{50} s of 115 nM and 147 nM, respectively (Table 2). BB-3497 had an IC_{50} of 8 nM with *H. sapiens* PDF (45) and actinonin an IC_{50} of 43 nM (32). However, no compounds displayed significant inhibition of MMP-7 with concentrations up to 10 μ M or inhibited the growth of the K562 cell line with concentrations up to 10 μ M, except actinonin, which had an IC_{50} of 8.5 μ M (data not shown).

Antimycobacterial activities of PDF-611 (LBK-611) and PDF-709. PDF-611 (LBK-611) was the most potent in terms of antimycobacterial activity, with a MIC_{90} value of 0.78 μ M (0.25 μ g/ml) (Table 2). Both PDF-709 and BB-3497 displayed good antimycobacterial activities against growing *M. bovis* BCG cultures (MIC_{90} s of 6.25 μ M and 3.125 μ M, respectively); however, actinonin showed a poor antimycobacterial effect, with a MIC_{90} of >25 μ M (>16 μ g/ml). Based on the results obtained

TABLE 3. MICs of PDF-611 (LBK-611) against various *M. tuberculosis* isolates

Strain ^a	MIC (μ g/ml) ^b		
	PDF-611	BB-3497	Gatifloxacin
H37Rv (ATCC 27294)	0.125–0.5	0.125–0.5	0.03–0.06
Beijing MDR	0.125	0.125–0.25	0.03–0.06
W-Beijing MDR	0.125	0.125	0.25
Beijing F29 MDR	0.03–0.25	0.03–0.5	0.015
Inh ^r SDR	0.06–0.125	0.125	0.015
Str ^r SDR	0.5	1.0	0.03
Rif ^r SDR	0.25	1.2	0.015
Pza ^r SDR	0.5	1.0	0.03
Clinical MDR	0.06–1.0	0.06–2	<0.008–0.06

^a MDR, multidrug resistance; SDR, single-drug resistance; Pza, pyrazinamide. The clinical MDR isolates are 10 clinical isolates which were described as MDR; however, a detailed susceptibility pattern for TB drugs is not available.

^b All MIC determinations were done twice. Single MIC values represent identical values in both experiments, while multiple values indicate different results in two experiments. For clinical MDR, the multiple values indicate the range of MIC values for 10 different clinical isolates.

from *M. tuberculosis* PDF inhibition and antimycobacterial studies, the compounds PDF-709 and PDF-611 (LBK-611) were selected for further analyses.

The bactericidal effects of PDF-611 (LBK-611) and PDF-709 on *M. bovis* BCG over 4 days were determined through time-kill studies (data not shown). At both 1 $\times$ and 10 $\times$ MIC for each compound, there was <1 log unit decrease in viable counts, demonstrating a bacteriostatic effect for the time period tested. However, on increasing incubation time with the inhibitor, higher bacterial killing was observed at the same concentrations (data not shown), indicating that the PDF-I are slow acting. PDF-611 (LBK-611) was also shown to be effective against several clinical isolates of *M. tuberculosis*. The compound demonstrated low MICs (<1 μ g/ml) for several multidrug-resistant clinical TB isolates (Table 3).

Spontaneous mutation rates and analysis of PDF-I-resistant *M. bovis* BCG isolates. The frequency of spontaneous resistance development was evaluated using a concentration of 10 $\times$ MIC. PDF-611- and PDF-709-resistant mutants arose at frequencies of $\leq 5 \times 10^{-7}$ in *M. bovis* BCG. These mutation frequencies were lower than the development of resistance to INH, which occurred at a frequency of 7×10^{-5} , but higher than that for RIF, which was $\sim 1 \times 10^{-8}$ (data not shown).

The development of PDF-I resistance in other bacteria can progress through at least two main mechanisms, mutations in the *def* gene or mutations in the formyl methyltransferase gene (*fnt*), which catalyzes the formylation of methionylated initiator tRNA (12, 21, 34, 35). Twenty-nine spontaneous PDF-I-resistant *M. bovis* BCG mutants were characterized. The complete *def* and *fnt* genes from these isolates were sequenced. None of the isolates had mutations in the *def* gene. Of the 29 isolates, 16 were found to have missense mutations in the *fnt* gene (Table 4). In the remaining 13 isolates, no changes were detected in the *def* and *fnt* genes. It was noted that independently isolated mutants had the same amino acid mutation (fmt-32a, fmt-32b, and fmt-32c had the T32N mutation; fmt-222a and fmt-222b had the E222K mutation; and fmt-117a and fmt-117b had the G117C mutation). Nonconserved amino acid changes were observed for half of the mutants. About half of

TABLE 4. Bioinformatics analysis of *fnt* mutants

Mutant	Mutation in FMT	Predicted structural location ^a	Predicted to be within 8 Å of fMet-tRNA binding site ^a
fmt-100	P100L	Loop	No
fmt-222a	E222K	Helix	No
fmt-53	A53V	Helix	No
fmt-32a	T32N	Loop	Yes
fmt-32b	T32N	Loop	Yes
fmt-230	P230S	Loop	Yes
fmt-105	V105F	Sheet	Yes
fmt-32c	T32N	Loop	Yes
fmt-192	R192P	Loop	No
fmt-276	T276A	Loop	No
fmt-104a	W104L	Loop	No
fmt-104b	W104L	Loop	No
fmt-117a	G117C	Sheet	Yes
fmt-124	A124G	Helix	Yes
fmt-222b	E222K	Helix	No
fmt-117b	G117C	Sheet	Yes

^a Deduced from a model of *M. tuberculosis* FMT, based on the *E. coli* FMT crystal structure (59).

the formyl methyltransferase (FMT) mutants had mutations that mapped to regions around the active site, deduced from a homology model of *M. tuberculosis* FMT based on *E. coli* FMT crystal structure (Table 4; also see Fig. S3 in the supplemental material).

For further studies of mutant strains, fmt-192, fmt-276, fmt-104a, and fmt-104b were characterized for growth and antimicrobial susceptibility, as all of them were generated against PDF-709. Two isolates, fmt-192 and fmt-104a, had slower doubling times (32 h and 35 h, respectively) than wild-type *M. bovis* BCG (23 h), while fmt-276 and fmt-104b had doubling times (22 h and 24 h, respectively) similar to that of wild-type *M. bovis* BCG (23 h). In MIC testing, all four FMT mutants were found to have at least a 16-fold increase in MIC (>100 μ M) against both PDF-611 (LBK-611) and PDF-709. However, the mutants retained susceptibility to other TB drugs, like RIF and STR, and unrelated drugs, like LZD and ERY. Target specificity was further confirmed by expression of extra chromosomal copies of the *M. tuberculosis* *def* gene in BCG, resulting in a 16-fold increase in the MIC for PDF-611 (LBK-611) (data not shown). For comparative purposes, a mutant without a mutation in the *def* or the *fnt* gene was included in the study. This mutant had a slightly slower doubling time (26 h) than wild-type *M. bovis* BCG, with a susceptibility profile similar to that of the *fnt* mutants. All *fnt* mutants regained susceptibility to PDF-I when a wild-type *fnt* gene was supplied in *trans*, confirming that the resistance arose through a mutation in *fnt* (data not shown).

Pharmacokinetic studies with mice. Clearance of PDF-709 and PDF-611 (LBK-611) in mouse serum was measured after the compounds were administered i.v. and p.o. The concentrations in serum at different times were used to calculate the corresponding pharmacokinetic parameters presented in Table 5. PDF-611 (LBK-611) had a lower half-life (0.5 h) than PDF-709 (1.6 h) after i.v. administration. However, PDF-611 (LBK-611) had a higher half-life (3.7 h) than PDF-709 (2.7 h) after p.o. administration. Both compounds had similar bioavailabilities ($\sim 40\%$).

TABLE 5. Pharmacokinetic parameters^a of PDF-611 (LBK-611) and PDF-709 in mice

Compound	Type of administration	Dose (mg/kg)	$C_{\max}$ (ng/ml)	$T_{\max}$ (h)	AUC_{inf} (ng · h/ml)	$t_{1/2}$ (h)	CL (ml/min/kg)	V_{ss} (liter/kg)	Bioavailability (%)
PDF-611(LBK-611)	i.v.	1.000	603.6	0.083	253.5	0.500	65.8	3.0	45.0
	p.o.	5.000	194.3	0.250	566.0	3.70			
PDF-709	i.v.	1.000	902.0	0.083	145.6	1.600	114.5	3.9	40.0
	p.o.	5.000	186.0	0.500	293.0	2.700			

^a $C_{\max}$, maximum concentration of drug in serum; $T_{\max}$, time to maximum concentration of drug in serum; AUC_{inf} , area under the curve extrapolated to ∞ ; $t_{1/2}$, half-life; CL, clearance; V_{ss} , volume of distribution at steady state.

DISCUSSION

M. tuberculosis PDF was earlier proposed as a potential drug target (14) since BB-3497, a PDF inhibitor, had potent in vitro activity against *M. tuberculosis*. The gene encoding *M. tuberculosis* PDF had also been shown to be one of the genes required for optimal growth by *M. tuberculosis* through transposon site hybridization (56). In this work, the failure to obtain *def* deletion mutants in *M. bovis* BCG strongly suggested that the deletion of *def* was lethal. After the second recombination event, the chromosomal wild-type *def* gene could be deleted only in the presence of an extra chromosomal complementing copy of *def*. Taken together, these results show that the *def* gene is essential in *M. bovis* BCG.

It has been observed previously with *E. coli* PDF that the metal ions and reaction conditions (substrate and type of assay) can dramatically change the activity and properties of the enzyme (10, 38, 49, 50). During biochemical characterization of *M. tuberculosis* PDF, we observed some differences from an earlier published work (57, 58). These differences could possibly be attributed to the difference in assay protocol and expression/purification conditions followed in this study compared to the earlier published work. In the earlier work, the investigators had not purified the enzyme in the presence of excess Ni^{+2} and enzyme activity was determined using fMA as the substrate in a trinitrobenzenesulfonic acid assay (57). Moreover, the assay temperatures were also different (room temperature in this study versus 30°C in the previous study).

Structure-activity relationship studies of actinonin and other *N*-alkyl urea hydroxamates (9, 27) indicate that efficacious PDF-I share common structural features, the first being a metal-chelating group, usually a hydroxamate/reverse hydroxamate, and the second being an *n*-alkyl butyl residue at the P1' position that mimics the methionine side chain (4). The PDF-I tested in this work were reverse hydroxamates, carrying an *n*-butyl residue at the P1' position (Table 2). This class of compounds inhibit *M. tuberculosis* PDF with IC_{50} s of <100 nM and are bacteriostatic against *M. bovis* BCG, as was observed previously for other PDF-I against both *Staphylococcus aureus* and *E. coli* (3, 12, 35). The frequency of resistance to this class of inhibitors in *M. bovis* BCG is similar to what was previously reported for *S. aureus* (12, 35) and *E. coli* (3, 12).

Spontaneous PDF-I-resistant isolates in *M. bovis* BCG demonstrated elevated MICs specifically towards the PDF-I and not towards other drug classes. In our work, the resistance arose through missense mutations in the *fnt* gene in 16 out of 29 mutants, while the rest had no mutation in either the *def* or the *fnt* gene. Several *fnt* mutants in this study were found to

have mutations in residues important for function (by use of the homology model with *E. coli* FMT crystal structure) (Table 4; also see Fig. S3 in the supplemental material) (23, 26, 59). Three independently isolated mutants (*fnt*-32a, *fnt*-32b, and *fnt*-32c [with the T32N mutation]) had a Ser-to-Ala mutation in a region involved in the recognition of the initiator tRNA (53, 54). Mutant *fnt*-53 (with the A53V mutation) lies just outside this region. Several mutants with mutations in the C-terminal region involved in the recognition of the tRNA by FMT were obtained (*fnt*-222a, *fnt*-222b, *fnt*-230, and *fnt*-276) (23). Three mutants, *fnt*-104a and *fnt*-104b (with the W104L mutation) and *fnt*-100 (with the P100L mutation), mapping close to conserved residues (Asn-106, His-108, and Asp-144) are predicted to participate in the catalytic steps (1, 28) (see Fig. S3 in the supplemental material). Without formylation assays of Met-tRNA^{fMet}, we are unsure about the extent to which different *fnt* mutations affect transformylase activity. Further studies are needed to understand the functional effect of the mutations.

Mutants that did not have mutations in the *def* or the *fnt* gene could be efflux pump mutants, as observed for *E. coli* and *Haemophilus influenzae* (12, 15). However, the actual mechanism of resistance for these mutants remains to be elucidated. Thus, in the case of mycobacteria, more than one mechanism is operative to confer resistance to PDF-I.

Recently, several eukaryotic PDF enzymes have been identified and characterized, e.g., from *Arabidopsis thaliana* (22), *Plasmodium falciparum* (5), and mitochondria of *H. sapiens* (22, 32, 33, 45, 60). The mRNA for *H. sapiens* PDF has been shown to be expressed at similar levels in all types of human tissues (22), with the enzyme being an active deformylase both in vitro and in vivo (32, 33, 60). Actinonin has been found to inhibit cell growth in various human tumor cell lines and in tumor models (32, 33, 63). In addition, two well-characterized bacterial PDF inhibitors, BB-3497 and actinonin (also used in this study), inhibit *H. sapiens* PDF at nanomolar concentrations similar to those at which its bacterial counterpart is inhibited (32, 45).

However, despite the inhibition of cell-free *H. sapiens* PDF and antiproliferative effects on tumor cell lines, a number of normal cell lines were found to be resistant to actinonin (33) and BB-3497 (45). Actinonin has been well tolerated in mice at doses of up to 400 mg/kg (24), and successors of BB-3497 (BB-83698) have been tested in humans at levels of up to 475 mg without any clinically significant adverse effects (52). Furthermore, recent biochemical studies with *H. sapiens* PDF showed that the enzyme had

significantly lower activity (k_{cat}/K_m) than the bacterial enzyme (*E. coli* PDF) (32, 33, 45, 52, 60).

In addition, the unambiguous localization of *H. sapiens* PDF in the mitochondria is important, as evidence suggests that the mitochondria of tumor cells may be more sensitive to mitochondrial insult (6, 48). There are examples of drugs selectively toxic to cancer cells working through the inhibition of mitochondrial respiration (19, 43). Mitochondrial membranes are less permeable to small molecules than most other membranes and act as an efficient supplementary filter for many drugs. For this reason, a number of antibiotics (tetracycline, macrolides, etc.) that inhibit mitochondrial targets (16, 30, 31, 37) are nevertheless currently used in human medicine. Thus, actinonin's inhibition of *H. sapiens* PDF, resulting in mitochondrial disruption, could have a similar tumor specificity (33). A major, striking difference between *H. sapiens* PDF (also mouse PDF) and bacterial PDF (*E. coli* PDF) is in the highly conserved EGCLS motif (motif III), where leucine is mutated into glutamic acid in mammalian PDF (Glu-173 in *H. sapiens* PDF), which results in the low activity of *H. sapiens* PDF (60). From the sequence similarity, *M. tuberculosis* PDF is 11% identical overall and has only 6% identity at the active site with *H. sapiens* PDF. These differences can be exploited to make compounds with decreased affinity for *H. sapiens* PDF without affecting their potency with respect to *M. tuberculosis* PDF. In this study, the PDF-I do not show selectivity towards *M. tuberculosis* PDF, but they were considered for further studies due to their tolerability in mice, in addition to the lack of effect on MMP-7 and the K562 cell line up to 10 μ M (~100-fold-higher concentration than *H. sapiens* PDF IC_{50}).

In order to ensure treatment compliance, antituberculosis therapy requires an oral dosing regimen of once a day or less. The oral bioavailabilities of PDF-611 (LBK-611) and PDF-709 were in the range of 40% in the mouse, indicating potential oral administration in humans. This was also reinforced by the relatively long half-lives of 3.7 h and 2.7 h, respectively, following oral administration. Assuming interspecies scaling within normal range, such a half-life in the mouse would support once-daily oral dosing in humans. These figures constitute an improvement over previous half-life reports for PDF inhibitors (27). Oral bioavailabilities of PDF-611 (LBK-611) and PDF-709 are also a clear improvement over those reported for other PDF inhibitors of the class *N*-alkyl urea hydroxamic acids (0.1 to 3.2%) (27) and that for BB-83698 (i.v. application only) (52). The total clearance levels were moderate for both compounds, i.e., 66 and 114 ml/min/kg for PDF-611 (LBK-611) and PDF-709, respectively, which lie in the range of mouse liver blood flow (90 ml/min/kg). Thus, this group of PDF inhibitors has the potential to be developed further as a new class of antituberculosis agent.

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