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Research Paper

Identification of Serine 119 as an Effective Inhibitor Binding Site of *M. tuberculosis* Ubiquitin-like Protein Ligase PafA Using Purified Proteins and *M. smegmatis*

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ABSTRACT

Owing to the spread of multidrug resistance (MDR) and extensive drug resistance (XDR), there is a pressing need to identify potential targets for the development of more-effective anti-*M. tuberculosis* (*Mtb*) drugs. PafA, as the sole Prokaryotic Ubiquitin-like Protein ligase in the Pup-proteasome System (PPS) of *Mtb*, is an attractive drug target. Here, we show that the activity of purified *Mtb* PafA is significantly inhibited upon the association of AEBSF (4-(2-aminoethyl) benzenesulfonyl fluoride) to PafA residue Serine 119 (S119). Mutation of S119 to amino acids that resemble AEBSF has similar inhibitory effects on the activity of purified *Mtb* PafA. Structural analysis reveals that although S119 is distant from the PafA catalytic site, it is located at a critical position in the groove where PafA binds the C-terminal region of Pup. Phenotypic studies demonstrate that S119 plays critical roles in the function of *Mtb* PafA when tested in *M. smegmatis*. Our study suggests that targeting S119 is a promising direction for developing an inhibitor of *M. tuberculosis* PafA.

1. Introduction

Tuberculosis (TB), caused by *M. tuberculosis* (*Mtb*), is one of the most common causes of death worldwide (WHO, 2016). At present, this global health problem is exacerbated by the emergence and spread of *M. tuberculosis* strains that are resistant to antibiotics including multidrug resistant (MDR) and extensively drug resistant (XDR) strains. According to the latest statistics, only 52% of patients with MDR-TB and 28% with XDR-TB can be treated effectively (WHO, 2016). In the past 50 years, only two new drugs, bedaquiline (Goel, 2014) and delamanid (Hoagland et al., 2016), have been successfully developed to address MDR-TB (Zumla et al., 2013, Mdluli et al., 2015). To obtain more effective treatment options for MDR-TB, there is an urgent need to develop new drugs with different mechanisms of action.

Ubiquitin-dependent protein degradation in eukaryotes plays a central role in many cellular functions, such as post-translational quality control, cell proliferation, differentiation and development (Grabbe et al., 2011, Yau and Rape, 2016). Ubiquitin is covalently attached to specific lysine residues of target proteins through a complicated multi-step ligation reaction and eventually delivers doomed proteins for proteasomal degradation (Hershko et al., 2000). Similar to this process in eukaryotic cells, proteins are targeted to the proteasome via a prokaryotic ubiquitin-like protein modifier termed Pup in *Mtb* (Pearce et al., 2008, Striebel et al., 2009). The inactive form of Pup has a C-terminal glutamine: conversion of this residue to glutamate (Pup^E) by the enzyme Dop (Striebel et al., 2009) activates Pup for ligation. Activated Pup is then attached to target proteins by PafA, the sole ligase in

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the Pup-proteasome System (PPS) (Pearce et al., 2006, Pearce et al., 2008, Striebel et al., 2009, Sutter et al., 2010, Guth et al., 2011). Pupylated proteins are then directed into the proteasome via recognition of Pup by *Mycobacterium* proteasomal ATPase (Mpa) (Sutter et al., 2009, Wang et al., 2009, Striebel et al., 2010, Wang et al., 2010). Analogous to deubiquitination, depupylation also occurs in *Mtb* and is catalyzed by Dop (Burns et al., 2010, Imkamp et al., 2010b) and PafA (Zhang et al., 2017). Previous studies showed that the Pup-proteasome System (PPS) of *Mtb* is required for resistance to nitric oxide and is essential for *Mtb* to cause lethality in mice (Darwin et al., 2003, Darwin et al., 2005, Lamichhane et al., 2006, Gandotra et al., 2007, Samanovic et al., 2015). To our knowledge, the PPS is only present in the Nitrospira and Actinobacteria (Imkamp et al., 2015) and is not present in most other bacteria, including gut microbiota. These unusual properties of the *Mtb* PPS make it an attractive target for drug development. Previous strategies for inhibiting the *Mtb* PPS focused on the 20S proteasome (Lin et al., 2009, Cheng and Pieters, 2010, Lin et al., 2010, Clements et al., 2013, Lin et al., 2013, Zheng et al., 2014, Totaro et al., 2017), however, owing to the high degree of mechanistic and structural conservation of mammalian and mycobacterial proteasomes, inherent toxicity is inevitable (Cheng and Pieters, 2010). On the other hand, PafA shares no homology with ubiquitin ligases in eukaryotes (Festa et al., 2007, Burns et al., 2009, Bode and Darwin, 2014), suggesting that there may be no or few side effects for drugs that target PafA. Unfortunately, to date, effective inhibitors of PafA have not been identified.

Here, we show that the serine protease inhibitor, AEBSF (4-(2-aminoethyl) benzenesulfonyl fluoride), is a potent inhibitor of purified *Mtb* PafA. We further show that this compound binds to PafA via S119. Biochemical analysis demonstrated that substitution of S119 with aromatic amino acid residues, imitating the binding of AEBSF, almost completely abolishes the pupylase and depupylase activity of *Mtb* PafA. Further structural analysis showed that this inhibition of PafA activity is a consequence of defective Pup binding to PafA even though this residue is far from the PafA catalytic site. Finally, phenotypic studies demonstrated that S119 is critical for the function of *Mtb* PafA and essential for *Msm* △*PafA* survival under nitrogen limitation and in macrophages. Overall, our work shows that S119 is situated within a critical, small-molecule accessible region of PafA whose modification inhibits PafA activity and is thus a highly promising target for the development of inhibitors of *M. tuberculosis* PafA.

2. Materials and Methods

2.1. Protein Cloning, Expression, and Purification

All genes were cloned from the *Mtb* reference strain H37Rv. PafA was cloned into pTrc99a as described previously (Striebel et al., 2009). All PafA variants were constructed using a QuikChange® Site-Directed Mutagenesis Kit (Agilent Technologies). C-terminal Flag-His6-tagged Mpa and PanB were cloned into pET28a. Sequences of primers used in this study are given in Table S1. All recombinant proteins were expressed in *E. coli* BL21 by growing recombinant *E. coli* BL21 cells in 1 L LB medium to an A_{600} of 0.6 at 37 °C. Protein expression was induced by the addition of 0.2 mM isopropyl-β-D-thiogalactoside (IPTG) before incubating cells overnight at 16 °C. Proteins were purified on Ni-NTA affinity columns and stored at −80 °C. Pup^E and N-terminal 5-Carboxyfluorescein-cys-Pup^E were synthesized by GL Biochem, Shanghai, China.

2.2. Inhibitor Profile of PafA Pupylase Activity

All chemicals or inhibitors were purchased from Sigma-Aldrich. PafA was incubated with different inhibitors at 25 °C for 0.5 h in pupylation buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 20 mM $MgCl_2$ and 10% (*v*/*v*) glycerol). Pupylation reactions included PanB-Flag (8 μM), Pup^E (10 μM) and PafA (0.5 μM) pre-incubated with inhibitors and were incubated at 25 °C for 6 h with 5 mM ATP in pupylation buffer. Samples were analyzed by SDS-PAGE, followed by Coomassie brilliant blue (CBB) staining and western blotting with an anti-Pup monoclonal antibody (Abmart) and an anti-Flag antibody (Sigma-Aldrich Cat# P2983, RRID: AB_439685).

2.3. Pupylation Assays

PanB pupylation assay reactions were carried out in pupylation buffer containing PanB-Flag (8 μM), Pup^E (10 μM) and PafA (0.5 μM) at 25 °C for 6 h, while Mpa pupylation assay reactions were carried out in pupylation buffer containing Mpa-Flag (6 μM), Pup^E (10 μM) and PafA (0.5 μM) at 25 °C for 2 h with 5 mM ATP. For pupylation assays using lysate as the substrate, reactions included *Msm* △*PafA* lysates (10 μg), Pup^E (10 μM) and PafA (0.5 μM) and were incubated at 25 °C for 20 min with 5 mM ATP in pupylation buffer. In AEBSF inhibitory assays, reactions were carried out as described above, except that PafA (0.5 μM) was pre-incubated in AEBSF for 30 min at 25 °C. Unbound AEBSF was removed by dialysis using a Spectra/Por(R) Dialysis Membrane (Sangon Biotech, Shanghai, China). Samples were analyzed by SDS-PAGE, followed by Coomassie brilliant blue staining and western blotting.

2.4. Identification of the AEBSF Binding Site on PafA by LC-MS/MS

PafA (0.5 μM) was incubated with AEBSF (0.25 mM or 0.5 mM) at 25 °C for 0.5 h in pupylation buffer. After SDS-PAGE and Coomassie staining, PafA bands were cut out and in-gel digested with trypsin. The tryptic peptide digests of the proteins were analyzed using an LC system (Nano Pump, Ultimate 3000, Dionex, Thermofisher) coupled with an ESI-Q-TOF mass spectrometer (MaXis, Impact, Bruker Daltonik, Germany). The peptide sequences were determined by searching MS/MS spectra against the Protein database using the Mascot (version 2.4, Matrix Science) software suite with a precursor ion mass tolerance of 20 ppm and fragment ion mass tolerance of 0.05 Da. Carbamidomethyl (C) was set as the fixed modification, and oxidation (M) was set as the variable modification. For modification of AEBSF, +183.0354 Da was set as a variable modification on serine, and a maximum of two missed cleavage of trypsin was chosen. MS data are available ("ProteomeXchange: PXD007045 and PXD006930").

2.5. Quantitative Pupylation Assays

Quantitative pupylation assays were performed according to a previously described procedure (Ofer et al., 2013), except that N-terminal 5-Carboxyfluorescein-cys-Pup^E was used instead of N-terminal 5-iodoacetamidofluorescein-cys-Pup^E. The fluorescence intensity of each sample was measured with a Synergy 2 microplate reader (Biotek Instruments) at 494 nm (excitation) and 522 nm (emission).

2.6. Determination of $PafA_{Mtb}$ Pupylase Activity in M. smegmatis

We cloned $PafA_{Mtb}$ and its variants with a C-terminal Flag-tag into the mycobacterial expression vector pMV261 or pSMT3 via *Bam*HI and *Hin*dIII restriction sites. These plasmids were then transformed into WT *M. smegmatis* (*Msm*) or *Msm* △*PafA*. *Msm* cells were grown at 37 °C in 100 ml 7H9 medium containing 30 μg/ml Kan or 50 μg/ml Hyg with 0.5% glycerol, 0.05% Tween-80 to an OD_{600} of 2.0. Cells were collected by centrifuging at 5000 rpm for 10 min and were then lysed in pupylation buffer with a high-pressure cracker (Union-Biotech, Shanghai, China). These lysates were analyzed by SDS-PAGE, followed by Coomassie brilliant blue staining and western blotting.

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