Binding and processing of \(\beta\)-lactam antibiotics by the transpeptidase Ldt\(_{Mt2}\) from *Mycobacterium tuberculosis*

Eva Maria Steiner, Gunter Schneider and Robert Schnell

Department of Medical Biochemistry and Biophysics, Karolinska Institutet, Stockholm, Sweden

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\(\beta\)-lactam antibiotic; covalent adduct; faropenem; \(L,D\)-transpeptidase; protein structure

Correspondence
R. Schnell, Division of Molecular Structural Biology, Department of Medical Biochemistry and Biophysics, Karolinska Institutet, Scheeles väg 2, S-17177 Stockholm, Sweden
Fax: +46 8 327626
Tel: +46 8 52487695
E-mail: robert.schnell@ki.se
Website: http://www.msb.mbb.ki.se/

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\(\beta\)-lactam antibiotics represent a novel direction in the chemotherapy of tuberculosis that brings the peptidoglycan layer of the complex mycobacterial cell wall in focus as a therapeutic target. Peptidoglycan stability in *Mycobacterium tuberculosis*, especially during infection, relies on the nonconventional peptide cross-links formed by \(L,D\)-transpeptidases. These enzymes are known to be inhibited by \(\beta\)-lactams, primarily carbapenems, leading to a stable covalent modification at the enzyme active site. A panel of 16 \(\beta\)-lactam antibiotics was characterized by inhibition kinetics, mass spectrometry, and x-ray crystallography to identify efficient compounds and study their action on the essential transpeptidase, Ldt\(_{Mt2}\). Members of the carbapenem class displayed fast binding kinetics, but faropenem, a penem type compound showed a three to four time higher rate in the adduct formation. In three cases, mass spectrometry indicated that carbapenems may undergo decarboxylation, while faropenem decomposition following the acylation step results in a small 87 Da \(\beta\)-OH-butryl adduct bound at the catalytic cysteine residue. The crystal structure of Ldt\(_{Mt2}\) at 1.54 \(\AA\) resolution with this fragment bound revealed that the protein adopts a closed conformation that shields the thioester bond from the solvent, which is in line with the high stability of this dead-end complex observed also in biochemical assays.

Database
Structural data are available in Protein Data Bank under the accession numbers 5LB1 and 5LBG.

Introduction

In spite of considerable efforts of modern medicine, tuberculosis (TB) continues to be one of the leading causes of mortality worldwide [1]. *Mycobacterium tuberculosis*, the causative agent of TB, enters the lower airways, primarily infects macrophages, and establishes a long-term survival in the postendocytic vacuoles of these phagocytic cells [2]. The actions of a competent immune system lead to the formation of the

Abbreviations
AMO, amoxicillin; AMP, ampicillin; APA, 6-aminopenicillanicacid; BIA, biapenem; CA, carbapenem; CapLC, capillary liquid chromatography; CAR, carbenicillin; CEPH, cephalosporin C; Dap, diaminopimelate, 2,6-diaminopimelic acid; D-iGlu, d-isoglutamic acid; DORI, doripenem; DTNB, Ellmann’s reagent, Dithio-nitrobenzoate; EDTA, ethylenediaminetetraacetic acid; ERTA, ertapenem; ESI-MS, electrospray ionization mass spectrometry; ESI-Q-TOF, electrospray ionization quadrupole time-of-flight mass spectrometry; FARO, faropenem; FAROdal, faropenem daloxate; Ig, immunoglobulin; IMI, imipenem; Ldt\(_{Mt2}\), L,D-transpeptidase-2 in *M. tuberculosis*; MDR-TB, multidrug-resistant tuberculosis; MERO, meropenem; MES, 2(N-morpholinol)ethanesulfonic acid; meso-Dap, Meso-isomer of 2,6-diaminopimelic acid; MS, mass spectrometry; NAGA, N-acetylglucosamine; NAMA, N-acetylmuramic acid; PA, penam; PBP, penicillin-binding protein; PDB, Protein Data Bank; PE, penem; PEG MME, polyethylene glycol monomethyl ether; PEG, polyethylene glycol; PEN, penicillin G; PIP, piperacillin; PIV, pivmecillinam; r.m.s.d, root-mean-square deviation; SSM, secondary structure matching; TB, tuberculosis; TCEP, tris(2-carboxyethyl)phosphine; TEBI, tebipenem; TNB, thio-nitrobenzoate; XDR-TB, extensively drug-resistant tuberculosis.
granuloma in which noninfected macrophages, T cells, B cells, and fibroblasts surround the infected cells sealing off the infection [3]. The long-term survival of dormant bacteria in the host and the concomitant emerging multidrug-resistant (MDR-TB) strains make chemotherapy long, complicated, and less effective [4]. Novel validated targets and effective compounds with established mechanism of action are needed to improve TB treatment and to face the emerging antibiotic resistance.

The complex mycobacterial cell envelope contains a plethora of mycobacterium-specific components involved in pathogenesis. Several proteins needed for cell wall biosynthesis, maintenance, and repair are established targets for antibiotics used today [5,6]. The lipid-rich external layer consisting of mycolic acids and other glycolipids forms a permeability barrier. It is linked to arabinogalactan, a branching polysaccharide that is, in turn, covalently attached to the peptidoglycan layer. Like in most bacteria, the peptidoglycan layer provides the foundation of the cell wall being responsible for the mechanical and osmotic stability of the cell [7]. The *M. tuberculosis* peptidoglycan contains unique features in the glycan chains and in the peptide stem cross-links [8]. The sequence of the tetrapeptide stems is the conventional (L-Ala-D-Glu-meso-Dap-d-Ala; Dap: diaminopimelate), but the amidation of carboxylates (in D-Glu and meso-Dap) and the cross-linking pattern makes it unique. While most bacteria rely on Dap-Ala (so called 3–4 cross-links), the *M. tuberculosis* peptidoglycan contains a high density (up to 80%) of Dap-Dap cross-links (Scheme 1), specifically in the intra-macrophage state [9,10]. These 3–3 cross-links are produced by the L,D-transpeptidases [11] that are not related to the D,D-transpeptidases. L,D-transpeptidases are targeted by β-lactam-type antibiotics, for instance carbapenems [12–19]. The carbapenem-type antibiotic meropenem in combination with the β-lactamase inhibitor clavulanate showed a remarkable bactericidal effect on dormant mycobacteria and drug-resistant strains in the mouse TB model [6,20,21]. Encouraging results have been obtained by treatment of extensively drug-resistant tuberculosis (XDR-TB) patients with clavulanate-carbapenem combinations [22–24]. Moreover, a penem type β-lactam, faropenem, proved to be remarkably successful even in the absence of clavulanate in killing *M. tuberculosis* in cultures *in vitro* and inside macrophages [25]. These recent achievements suggest that inhibition of peptidoglycan biosynthesis by β-lactam antibiotics hold a potential for future TB therapy.

In the *M. tuberculosis* H37Rv genome, five genes are found that encode L,D-transpeptidases. Gene knock-out studies indicate that they are required for cell wall stability with the concomitant effects on alteration of cell size and increased susceptibility to antibiotics such as amoxicillin and imipenem [26–28]. LdtMt2 (Rv2518c) catalyzes the formation of the predominant Dap–Dap cross-links within the peptidoglycan meshwork in *M. tuberculosis* and exhibits the highest expression level among the L,D-transpeptidases. The ldtMt2 gene was shown to be essential for infectivity in the mouse model of TB [26] consistent with the profound effects of the ldtMt2 knock-out phenotype [26–28].

L,D-transpeptidase-2 in *M. tuberculosis* consists of a catalytic domain with the characteristic ErfK/YbiS/YhnG fold and two consecutive domains both belonging to the bacterial immunoglobulin fold family [12–15]. The binding of β-lactam compounds imipenem, ampicillin, and meropenem as covalent adducts at the catalytic cysteine residue was established through mass spectrometry and crystal structure analysis [12–15,29].

In this work, we present a ranking of 16 β-lactam compounds as potential LdtMt2 inhibitors based on mass spectrometry and kinetic assays. Our study revealed that faropenem (penem class) reacts at a more than three to four time higher rate than the tested carbapenems including meropenem, doripenem, ertapenem, or piperacillin. Mass spectrometry and structural analysis of the protein–ligand complexes by X-ray crystallography were used to reveal the binding mode and the events following the acylation reaction. Three carbapenem compounds showed partial decomposition, consistent with a decarboxylation event following the acylation step. Binding of faropenem on the other hand resulted in rapid formation of a 87-Da fragment remaining as a stable covalent adduct. The 1.54 Å resolution X-ray structure of LdtMt2 with the faropenem-derived adduct bound at the catalytic cysteine residue was determined. The results presented point out faropenem as the LdtMt2 inhibitor superior to carbapenems according to binding kinetics, provide further insights to the events following inhibitor binding, and to the structural basis of the high stability of the inhibitor-bound complex.

Replacement of the catalytic dyad residue His336 by alanine resulted in a drop of the acylation rate by several orders of magnitude demonstrating involvement of this residue in the inhibition mechanism.

**Results**

**Acylation of the active site Cys354 in LdtMt2 by β-lactam compounds**

The covalent binding of β-lactam antibiotics (Fig. 1) at the LdtMt2 active site starts with the nucleophilic attack of the catalytic Cys354 on the carbonyl carbon of the
β-lactam ring leading to the acyl-enzyme adduct, analogous to the action of these inhibitors on D,D-transpeptidases. The amino acid sequence of LdtMt2 contains two cysteine residues; Cys35 following the predicted export signal sequence is a probable lipid attachment site, and Cys354 in the catalytic domain. In the two-domain protein construct comprising one of the Ig-domains and the catalytic domain (residues 149–408, B-C module) used in this study, the only cysteine residue is the catalytic Cys354 [12,14]. This opens the possibility to follow the kinetics of β-lactam-driven acylation (e.g., covalent modification of the active site cysteine Cys354) by the thiol-specific reagent dithio-nitrobenzoate (DTNB) spectrophotometrically at 412 nm (Fig. 2A). The standard curve recorded for LdtMt2 showed good agreement with a standard curve recorded with L-cysteine confirming that the spectrophotometric signal is generated specifically at the single cysteine residue of the protein. Under the conditions used in our assay the thio-nitrobenzoate (TNB)-derivatization of the protein active site finishes in seconds, that is 10–30 times faster than the fastest acylation reaction by the β-lactams studied here. Due to this difference in rate and the large excess of DTNB over the β-lactams, the amount of free cysteine can be reliably monitored without interference by the much slower acylation via β-lactams. Mass spectra and spectroscopy of the DTNB-derivatized enzyme in the presence of β-lactams faropenem or 6-aminopenicillanic acid shows that the TNB-adduct is stable, not released from the protein and does not react with the β-lactams (Fig. 2B,C).

The exposure of LdtMt2 to a panel of different β-lactams [seven penam, two penem, six carbapenem, and one cephem type, Fig. 1] at 1 mM concentration for 7 min followed by DTNB-derivatization was used to rank the compounds with regard to their comparative reactivity in acylation kinetics at pH 8 and at pH 6. The carbapenem class [tebipenem, meropenem, ertapenem, doripenem, biapenem], faropenem, and faropenem daloxate (penem class) and three penams (6-aminopenicillanic acid, piperacillin, penicillin-G) showed a high degree of Cys354 modification (Fig. 2D).

Electron spray mass spectrometric analysis was used to confirm the stable covalent complexes formed between the LdtMt2 active site and the tested 16 β-lactam compounds. Allowing 30 min of reaction time and an excess of β-lactams, the covalent complexes could be identified by the dominant mass values found in the spectrum (Table 1, Fig. 3). The typical error, estimated from the differences between the expected and observed mass of the native protein (28 483.7 Da calculated from the sequence) is 3–4 Da.

The protein–TNB adduct and protein–β-lactam complexes with tebipenem, biapenem, carbenicillin, and piperacillin were detected as the major peaks in the electrospray ionization mass spectrometry (ESI-MS) spectra with the expected m/z values (Table 1, Fig. 3). In earlier studies, ampicillin and imipenem were found to bind as unmodified adducts [12]. The other carbapenems, meropenem, ertapenem, and doripenem resulted in the expected adduct masses indicating covalent binding of these compounds, however, additional peaks corresponding to 40–47 Da lower masses also appeared in these three cases (Table 1, Fig. 3E). This difference from the calculated mass of the unmodified protein ligand adducts may indicate a degradation or modification after the initial binding event. Considering the 3–4 Da error of the mass spectrum these mass differences are in accordance with the decarboxylation (–44 Da) of the β-lactam ring.
detected in previous studies as a spontaneous event following the cleavage by β-lactamase enzymes [30].

In the cases of 6-aminopenicillanic acid and faropenem (penam and penem class, respectively), the ESI-MS results indicated unexpected adduct formation. Faropenem binding to LdtMt2 resulted in a dominant mass peak with a bound 87-Da fragment bound (Table 1, Fig. 3G) indicating a fast degradation following the initial acylation event. The faropenem analog, faropenem daloxate, resulted in the same 87-Da fragment bound to LdtMt2 (Fig. 3H). The addition of 6-aminopenicillanic acid gave a mass peak with twice

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**Fig. 1.** The β-lactam compounds used in this study. Structures are categorized in groups as penem, penam, and carbapenem and cephem. Names and the abbreviations (e.g., faropenem: FARO) are indicated.
the mass of the bound antibiotic (Table 1, Fig. 3F), suggesting a second target site or a possible coupling reaction (transpeptidation) taking place at the enzyme active site. Blocking the active site cysteine-354 by DTNB derivatization prior to exposure to 6-aminopenicillanic acid returned the TNB-derivatized species.
(28,678 Da) detected by mass spectrometry. Hence, a second target site can be excluded, pointing at the transpeptidation reaction producing the 6-aminopenicillanic acid dimer at the enzyme active site (Table 1).

Table 1. Evaluation of covalent adduct formation of LdtMt2 with β-lactam antibiotics by mass spectrometry. β-lactam compounds are selected to represent penem, carbapenem, and penam families.

| Beta-lactam | Ligand MW (Da) | Mass detected (Da) | Mass bound (Da) | Mass deviation (Da) | Conclusion |
|-------------|----------------|-------------------|----------------|--------------------|------------|
| Faropenem (PE) | 285.1 | 28,571.1 | 87.4 | −197.7 | Fragment bound |
| Faropenem daloxate (PE) | 397.4 | 28,570.9 | 87.3 | −310.1 | Fragment bound |
| Ertapenem (CA) | 475.5 | 28,955.5 | 471.8 | −3.7 | Full mass bound |
| | 28,912.0 | 428.4 | −47.2 | Decarboxylated |
| Meropenem (CA) | 383.5 | 28,821.9 | 338.3 | −45.2 | Decarboxylated |
| | 28,866.1 | 382.5 | −1 | Full mass bound |
| Doripenem (CA) | 420.5 | 28,863.6 | 379.9 | −40.6 | Decarboxylated |
| | 28,907.4 | 423.7 | 3.2 | Full mass bound |
| Teipenem (CA) | 497.6 | 28,978.3 | 494.6 | −3 | Full mass bound |
| Piperacillin (PA) | 517.6 | 29,004.7 | 521 | 3.5 | Full mass bound |
| 6-aminopenicillanic acid (PA) | 216.3 | 28,919.6 | 435.9 | 3.3 | 6-aminopenicillanic acid dimer bound |
| 6-aminopenicillanic acid dimer | 432.6 | | | | |

No covalent adduct was observed with penicillin-G, amoxicillin, and pivmecillinam, the spectra clearly gave the mass of the unmodified BC module of LdtMt2. Finally, the reaction mixture with cephalosporin gave a non-evaluable spectrum.

Meropenem, doripenem, ertapenem (carbapenem class), piperacillin, 6-aminopenicillanic acid (penam class), faropenem (penem class), the compounds that fall in the more efficient category (Fig. 2D) were further characterized and the kinetic rates of the acylation reaction were determined at 50 μM β-lactam concentration (Fig. 4A,B). The comparison of the acylation rates by these six different β-lactams indicates that faropenem is 3–4 times faster than the tested carbapenems (ertapenem, meropenem, doripenem), 7 times faster than piperacillin, and 17 times faster than 6-aminopenicillanic acid (Fig. 4B). The rate of the faropenem-driven acylation reaction measured using the DTNB-based assay at pH 6 (0.020 s⁻¹), the expected pH of the mycobacterial cell wall, is approximately half of the value obtained at pH 8 (0.045 s⁻¹). Faropenem degradation was also monitored using a direct spectrophotometric assay at 299 nm in the presence of 50 μM enzyme and 50 μM β-lactam at pH 6 (Fig. 4C). The rate constant determined from this direct assay (0.029 s⁻¹) was in good agreement with the rate constant derived from the DTNB-based assay under the same conditions. After the reaction using this assay was completed no derivatization with DTNB could be observed, demonstrating that the cysteine residue had been modified by faropenem.

The intact catalytic site is required for the acylation by β-lactams

The active site of the L,D-transpeptidases contains a catalytic Cys-His dyad. The invariant cysteine (Cys354 in LdtMt2) is responsible for the nucleophilic attack to form the acyl-enzyme intermediate, while the histidine residue (His336 LdtMt2) activates the thiol group of cysteine and assists the acyl-transfer step to the acceptor substrate by deprotonation [13,16,31]. The replacement of the catalytic cysteine-354 to alanine resulted in an enzyme with no spectrophotometric signal for DTNB derivatization. Furthermore, no adduct formation with faropenem or 6-aminopenicillanic acid was detected by mass spectrometry confirming Cys354 as the sole binding site for β-lactams. There are two histidine residues at the active site in proximity of Cys354 that may have a role in not only catalysis but also in β-lactam-driven adduct formation. Therefore, the two mutant variants H336A (LdtMt2-H336A) and H352A (LdtMt2-H352A) of the LdtMt2 BC module construct were analyzed with respect to cysteine derivatization by the fast-binding β-lactam compound faropenem. In the case of the H336A mutant, no cysteine acylation could be detected in the 0–500-s time frame indicating a decrease in acylation activity by several orders of magnitude (Fig. 4D). Longer exposure (30 min) to the antibiotic led, however, to the formation of the same faropenem-derived adduct (85.2 Da adduct) as observed for the wild-type protein. Consistent with these observations, the formation of the...
double mass adduct of the 6-aminopenicillanic acid was not detected with the H336A mutant. The H352A mutation did not show a decrease in the rate of the acylation reaction recorded in the DTNB-based assay indicating that this residue is not involved in the activation of the cysteine nucleophile.

**Structures of covalent adducts**

As part of the assay and method validation, the DTNB-derivatized BC module of LdtMt2 was crystallized. The 1.55 Å crystal structure (Table 2) revealed the TNB-adduct bound to the catalytic cysteine residue, Cys354, in both chains of the asymmetric unit. The active site of LdtMt2 lays on the β-sheet platform providing the residues of the catalytic dyad (Cys354 and His336) and is covered by a β-hairpin (residues 300–323) as a lid. The formation of the covalent adduct induces changes in lid conformation leading to a variable, ligand-dependent accessibility of the active site (Fig. 5A). In the case of the TNB adduct, the lid moves away from the active site when compared to the ligand-free structure to accommodate the bound ligand. This movement, observed in both polypeptide...
chains in the asymmetric unit of this crystal form, corresponds to a displacement of Pro315 at the tip of the lid by 12.0 Å in comparison with the ligand free (closed) conformation (PDB: 4HUC; Fig. 5A) [12]. Since the tip of the lid (residues 309–316) is disordered in one of the chains (chain B), the structure observed in the complete chain (chain A) is used to describe the interactions between the TNB adduct and the protein.

The TNB adduct forms a covalent bond with the Cys354 via a mixed disulfide bridge in the cavity lined by Trp340, Val322, Thr307, Tyr308, His336, His352, Tyr318, and Thr320 (Fig. 5C,D). The TNB adduct interacts with LdtMt2 through direct hydrogen bonds. The carboxylate moiety forms hydrogen bonds with the hydroxyl group of Tyr318 (and three water molecules) and one of the nitro group oxygens interacts with the side chain of Thr320. The Accessible Surface Area (ASA) for the TNB-adduct is 71% of the total molecular surface of the ligand with 4.7 Å² ASA for the Cys354-linked sulfur atom of the ligand.

Fig. 4. Kinetic analysis of β-lactam binding at the LdtMt2 active site. (A) Acylation kinetics of meropenem, doripenem, ertapenem from the carbapenem class, piperacillin, 6-aminopenicillanic acid from the penem class, and faropenem from penem class were characterized to determine kinetic constants (kobs) of the reaction at 50 μM β-lactam concentration. The absorbance values were used to fit single turnover kinetic model for covalent complex formation between the antibiotics and LdtMt2. (B) The rate constants of the β-lactam-driven acylation reactions (kobs) at 50 μM compound concentration are shown for the six selected compounds (names indicated on horizontal axis). The columns represent the average of three measurements with the error bars showing the standard deviation. (C) The faropenem-driven acylation reaction by LdtMt2 monitored at 299 nm spectrophotometrically. (D) The kinetics of the FARO-driven acylation reaction following the derivatization of the active site Cys354 in the H336A mutant in comparison to the wild-type LdtMt2. Absorbance values are plotted versus time.
LdtMt2 crystals soaked with faropenem resulted in a crystal structure at 1.54 Å resolution (Table 2) with additional electron density linked to the catalytic Cys354 in one of the two polypeptide chains of the asymmetric unit. The size and shape corresponds well to a small fragment derived from faropenem observed as an adduct in our in ESI-MS analysis (87 Da, Fig. 3) and was modeled as a $\beta$-OH-butyryl moiety (calculated mass 87 Da). The $\beta$-hairpin lid (residues 300–323) adopts a closed conformation with this adduct bound similarly to the ligand-free structure (Fig. 5A; PDB: 4HUC) [12]. The largest main chain deviation between the LdtMt2 structure and LdtMt2-faropenem could be observed at the position of Tyr308 and Pro315 with a maximum shift in Cα positions of 1.7 and 1.5 Å. The faropenem-derived $\beta$-OH-butyryl adduct is bound at the active site cavity formed by the residues Trp340, Val333, Met303, Ser331, Tyr308, His336, His352, and Tyr318 (Fig. 5E,F). Besides, the covalent bond to the sulfur of Cys354 forms a hydrogen bond between the carbonyl oxygen and the backbone amine of Cys354 with a 2.7 Å distance. The adduct is wedged between Gly332 and the side chains of Tyr318, Val333, and His352. The faropenem-derived adduct displays 72% accessible surface area, but the carbon atom of the carbonyl group engaged in the covalent bond with Cys354 is completely buried (Fig. 5E) and inaccessible (0.1 Å² solvent accessible surface area).

**Faropenem-degradation products by small-molecule mass spectrometry**

Since the small 87 Da fragment detected as a Cys354-bound covalent adduct via mass spectrometry was observed in the crystal structure (Fig. 6A,B), we attempted to detect the remaining released fragment derived from faropenem by mass spectrometry. A total of 70 μM enzyme and 70 μM faropenem was incubated

**Table 2. X-ray diffraction data collection and refinement statistics.**

| Ligand                  | LdtMt2-TNB adduct | LdtMt2-$\beta$-OH-butyryl adduct |
|------------------------|-------------------|----------------------------------|
| PDB code               | 5LB1              | 5LBG                             |
| Beamline               | ESRF ID23-2       | ESRF ID29                        |
| Space group            | P1                | P2                              |
| Unit cell              |                   |                                  |
| a, b, c (Å)            | 53.37, 53.91, 57.17 | 45.06, 91.36, 67.55,             |
| α, β, γ (°)            | 68.8, 75.0, 88.6  | 90, 103.6, 90                    |
| Resolution (Å)         | 51.4–1.55 (1.57–1.55) | 43.80–1.54 (1.57–1.54)           |
| No. of unique reflections | 79 462 (3720)  | 77 780 (3846)                    |
| l/σ(l)                 | 9.7 (1.7)         | 14.6 (2.1)                       |
| Redundancy             | 2.0 (2.0)         | 3.0 (3.0)                        |
| Completeness (%)       | 95.3 (89.6)       | 99.1 (99.0)                      |
| Rmerge                 | 0.065 (0.379)     | 0.029 (0.464)                    |
| Rpim                   | 0.065 (0.379)     | 0.022 (0.351)                    |
| CC1/2                  | 0.997 (0.638)     | 0.999 (0.716)                    |
| Wilson B-value (Å²)    | 11.2              | 21.7                             |

Refinement

| R          | 0.148 | 0.176 |
| Rfree      | 0.194 | 0.206 |

Number of atoms/B-factor Å²

| Overall    | 4798/15.6 | 4699/25.4 |
| Protein    | 4091/13.7 | 4201/24.4 |
| Ligand     | 2629.9 (TNB-adduct) | 633.5 ($\beta$-OH-butyryl adduct) |
| Water      | 672/26.7  | 492/33.5  |

r.m.s.d. from ideal geometry

| Bond length (Å) | 0.011 | 0.016 |
| Bond angles (deg.) | 1.453 | 1.759 |

Ramachandran plot (%)

| Residues in preferred regions | 433 (95.8%) | 427 (95.5%) |
| Residues in allowed regions | 19 (4.2%)   | 20 (4.5%)   |
| Outliers                  | 0 (0%)     | 0 (0%)      |

Values in parenthesis are for the highest resolution shell.
in 10 mM ammonium bicarbonate buffer (pH 7.0) for 10 min allowing the completion of the acylation event as confirmed by a sample withdrawn for DTNB derivatization and absorbance measurement at 412 nm. The filtered and concentrated small-molecule fraction was analyzed with a Q Exactive mass.
spectrometer. These mass spectra displayed a major peak at 147.11 Da (Fig. 6C), corresponding to a calculated fragment C\text{H}_{14}O_{3} of 146.09 Da, possibly derived from faropenem and released by Ldt\text{M}_{2}.

**Stability of the faropenem-derived covalent adduct**

The small faropenem-derived β-OH-butyryl moiety (Figs 5F and 6A,B) resembles the acyl-enzyme intermediate occurring during the reaction (Scheme 1) hence the question arises whether acyl-acceptor substrates could complete the catalytic cycle of transpeptidation in Ldt\text{M}_{2}. The acyl-acceptor in the PG peptide stem during 3–3 cross-link formation, meso-Dap, was tested in the DTNB derivatization-based assay for the release of the faropenem fragment that would result in a free Cys354 at the enzyme active site. The presence of high excess (2 mM) meso-Dap did not change either the kinetics of the formation or the long-term stability of the faropenem-derived adduct (Fig. 6D). Similar results were achieved when exposing preformed Ldt\text{M}_{2} faropenem adduct to meso-Dap (2 mM) (Fig. 6E).

Alternatively, amino acids (L-aminopimelate, D-aminopimelate, L-lysine, or L-alanine) did not result in the release of the adduct leading to the conclusion that this small hydroxyl-butyryl moiety is inaccessible to potential acceptors and thus a stable modification of the Ldt\text{M}_{2} active site.

**Discussion**

Antibiotics of the β-lactam family were not considered effective against *M. tuberculosis* because of the β-lactamases present in this pathogen. However, the recent findings using a mouse model of TB infection [9,20,21] and the results from the experimental treatment of drug-resistant TB [22–24] using β-lactam compounds triggered a number of studies to establish the biochemical basis of action of this antibiotic class on potential *M. tuberculosis* targets including the classical β-lactam targets, the D,D-transpeptidases [32]. One of the few validated target from the cell wall maintenance apparatus of this organism is the essential D,D-transpeptidase Ldt\text{M}_{2} (Rv2518c, LppS) [26]. Carbapenems were found to be effective on L,D-transpeptidase enzymes in general [19,33–35] and binding of imipenem and meropenem was demonstrated on Ldt\text{M}_{2}, including a crystal structure of the meropenem-bound state [14,15].

Our analysis evaluating a larger panel of 16 β-lactam compounds addressed a comparative analysis based on the kinetics of the acylation reaction (e.g., adduct formation), mass spectrometry and high-resolution structures allowing the detailed analysis of the covalent adducts formed at the Ldt\text{M}_{2} active site. The antibiotics chosen for this work (Fig. 1) included also tebipenem, a β-lactam recently introduced into clinical use [36], and faropenem that showed impressive bactericidal effect on cultures and also on macrophage-resident *M. tuberculosis* [25] and in the mouse model of TB infection [37]. The kinetics of the acylation reaction reported here (Fig. 4) is comparable to the rate of β-lactam action on their classical targets, the D,D-transpeptidases (penicillin-binding proteins) [38–40].

Following the acylation reaction at the catalytic cysteine residue, the bound adducts may undergo degradation or partial decomposition. Mass spectrometry results indicated the loss of a 44-Da fragment consistent with the release of the ring-bound carboxylate moiety in the cases of meropenem, etrapenem, and doripenem (Table 1), while the mass of the unmodified adduct was found for tebipenem where the carboxylate group is substituted by a methyl-tert-butylryl moiety (Fig. 1, Table 1). The loss of the carboxylate moiety is a typical event in the reaction pathway of serine-β-lactamases following ring opening [30].

Binding of faropenem and faropenem daloxate to Ldt\text{M}_{2} on the other hand, resulted in a smaller 87 Da β-hydroxyl-butyryl adduct indicated by mass spectrometry (Fig. 3) and subsequently captured in the crystal structure of Ldt\text{M}_{2} (Figs 5 and 6). The same fragment was observed from the recent investigation of the homolog Ldt\text{M}_{1} [25,37] suggesting a general mechanism of faropenem action. We show here that the formation of this small adduct derived from faropenem is dependent on a functional enzyme active site, since the H336A mutant displayed a decrease in the faropenem-driven acylation reaction by several orders of magnitude. The high stability of this adduct is indicated by the facts that it could be trapped in the crystal structure and by kinetic data as it could not be released by the high access of the acyl-acceptor substrate meso-Dap (or other potential substrate analogs; Fig. 6). The analysis of this crystal structure and comparison to other enzyme complexes, for example, Ldt\text{M}_{2}-TNB and Ldt\text{M}_{2}-meropenem adducts (4GSU, 3VYP), showed that the faropenem-derived adduct is bound with a closed lid conformation (Fig. 5) and that the faropenem-derived thioester is also the least accessible when comparing different adducts of Ldt\text{M}_{2}. The observed kinetics of the faropenem-driven acylation reaction and the remarkable stability of the covalent adduct may well be the reason for the efficacy of this β-lactam as an antimycobacterial compound [25,37,41].
Fig. 6. Formation and stability of the FARO-derived adduct at the LdtM2 active site. (A) The FARO structure indicating the trapped \( \beta \)-OH-butyryl fragment in blue is depicted with the mass spectrum in which mass of the FARO-sodium adduct was detected (308.06 m/z). (B) Following the acylation reaction at the LdtM2 active site, (green cartoon) the resulting covalent adduct trapped at the catalytic Cys354 is shown as a stick model (blue carbons) with the 2F_o-F_c electron density map contoured at 1\( \sigma \) as gray mesh. (C) The mass-spectrometric analysis of the protein-free solution following FARO exposure to LdtM2 revealed a dominant peak at 147.1 Da, corresponding to a compound with chemical composition C_7H_14O_3 (calculated mass 146.09 Da) as a possible degradation product of FARO after the acylation reaction. (D) Formation of the FARO-derived adduct in the presence of a potential acyl-acceptor substrate meso-Dap recorded using the DTNB-based assay. The reaction LdtM2 with FARO (blue) and with FARO in the presence of 2 mM of meso-Dap (red) shows fast modification of the sulfhydryl group. The effect of meso-Dap on the free sulfhydryl reaction of the native protein (gray) and the signal of the native protein (black) is shown as reference. Error bars indicate the standard deviation of three individual measurements, \( n = 3 \). (E) Exposure of a preformed FARO-derivatized LdtM2 to 2 mM meso-Dap (red) in comparison to the nonperturbed adduct (blue). The native protein signal with free cysteine (black) is shown as reference.
Materials and methods

Gene expression and protein purification

The expression construct of LdtMt2 (UniProt code O53223; Rv2518c), comprising the B (immunoglobulin-like) and C (catalytic) domains (the BC module, residues 149–408) was used for protein production and purification as previously reported [12].

The coding sequences for active site mutants C354A, H336A, and H352A were amplified by the QuickChange method using Pfu-Turbo polymerase (Stratagene, La Jolla, CA, USA) with the wild-type LdtMt2 DNA as a template. The mutant protein was purified as the wild-type construct except that pellets were washed in 0.3 M NaCl, 10 mM Tris-HCl pH 8.0, followed by flash-freezing, treatment with BugBuster Protein Extraction Reagent (Novagen, Merck group, Darmstadt, Germany) and DNase I (2 μg·mL⁻¹). The cleavable N-terminal His₆ tag (H2N-MHHHHHHH SSVDLGTFNYFQ*SM) was not removed for the H336A and H352A mutant protein constructs. The purified proteins were concentrated to 30 mg·mL⁻¹, flash-frozen in liquid nitrogen and stored at −80 °C until further use.

Mass-spectrometric analysis of covalent adduct formation

Samples of LdtMt2, LdtMt2-C354A, LdtMt2-H336A, and LdtMt2-H352A (30 μg) were dispensed in 20 μL of water (corresponding to a concentration of 52 μM) with 4 mM of the β-lactam compound tested and incubated for 30 min at 22 °C. Subsequently, the samples were diluted in 0.5 mL denaturing buffer [5% (v/v) acetonitrile, 0.1% (v/v) formic acid, 0.5 mM TCEP] and loaded onto a CapLC system and an ESI-Q-TOF quadrupole/orthogonal acceleration time-of-flight mass spectrometer (Waters Corp., Milford, MA, USA) using the method described by Sundqvist et al. [42]. The spectra were combined and deconvoluted with the Maximum Entropy 1 algorithm from the MASSLYNX software suite (Waters Corp.) to derive the mass of the protein and protein adducts.

Mass-spectrometry analysis of faropenem decomposition by LdtMt2

Samples of 70 μM LdtMt2 with 70 μM of faropenem were incubated for 10 min in 10 μM ammonium-bicarbonate buffer at pH 7.0 in a total volume of 750 μL. The progress of the acylation reaction was monitored spectrophotometrically at 412 nm as described below. After completion of the reaction, the mixture was filtered through a 3 kDa cut-off filter (Pall centrifugal device; Pall Life Sciences, Portsmouth, UK). The filtrate was concentrated by centrifugal vacuum device, diluted 1 : 2 in 50% acetonitrile and 1% formic acid and injected directly using a NanoMate system (NanoMate Technology Inc., San Jose, CA, USA) coupled on-line with a Q Exactive mass spectrometer (Thermo Fisher Scientific, Bremen, Germany).

Kinetics of antibiotic binding

β-lactam compounds were obtained from Sigma-Aldrich (Saint Louis, MO, USA), faropenem daloxate was obtained from Apex Biotech LLC (Huston, TX, USA). The formation of the enzyme–antibiotic adducts was followed by monitoring the loss of the free cysteine sulphydryl group using 5,5′-dithio-bis-(2-nitrobenzoic acid) (DTNB; Sigma-Aldrich). The 10 mM DTNB development solutions were always prepared freshly from a 100-mM stock solution in 95% ethanol and diluted with assay buffer [0.1 M potassium phosphate pH 8.0 and 1 mM EDTA]. The assay mixture consisted of 50 μL of reaction sample (LdtMt2 in 0.1 M potassium phosphate pH 8.0 and 1 mM EDTA with the β-lactam compounds) and 12 μL of DTNB development solution mixed in CORNING 96-well half area assay plates (No. 3994; Corning, New York, NY, USA). The absorption at 412 nm, corresponding to the released TNB, was monitored using a Bio-tek Synergy HT plate reader (BIO-TEK Instruments Inc., Winooski, VT, USA). A standard curve was generated using LdtMt2 in the 2–300 μM concentration range that compared well with the standard curve generated using 1-cysteine in the 3–250 μM concentration range, confirming the reactivity of a single cysteine in the enzyme. An LdtMt2 protein concentration of 40 μM was chosen that gave an absorbance value of about 0.15–0.2. For ranking the various β-lactam compounds, 1 mM of antibiotics were mixed with LdtMt2 and incubated for 7 min. After adding 12 μL of DTNB working solution the absorption was monitored immediately at 412 nm. Reactions were carried out in triplicates. Exposure of LdtMt2 to the various β-lactams at pH 6.0 were carried out in 0.05 M potassium phosphate pH 6.0, 1 mM EDTA, while the DTNB derivatization reagent contained 0.5 M Tris-HCl pH 8.0, 1 mM EDTA, 10 mM DTNB to ensure the pH 8.0 for TNB derivatization.

Five compounds, faropenem (faropenem sodium hydrate from Sigma), ertapenem (ertapenem sodium salt; Sigma), meropenem (meropenem trihydrate; Sigma), doripenem (doripenem hydrate; Sigma), piperacillin (piperacillin sodium salt; Sigma), and 6-aminoopenicillanicacid (Sigma) were analyzed further regarding the kinetics of the acylation reaction (e.g., formation of the covalent adduct with the catalytic Cys354). The acylation kinetics of these β-lactams was followed using concentrations of antibiotics at 50 μM and LdtMt2 at 40 μM. The protein was incubated with the various β-lactams in the assay buffer at 22 °C in 600 μL reaction mixtures. Aliquots of 50 μL were withdrawn and mixed with 12 μL DTNB working solution at various time points. The kinetic constants kobs of covalent adduct formation (acylation) were derived from the exponential fits of absorbance versus time data using the model.
of a single exponential $A = A_0 + A_1 e^{-kt}$ for fitting in the ORIGINLAB software (OriginLab, Northampton, MA, USA).

The formation of the covalent adducts (the acylation reaction) with faropenem and 6-aminopenicillanic acid was also followed in the presence of $2 \text{ mM meso-Dap}$ using the same conditions as above. In a second set of experiments the preformed covalent adducts (acylation reaction confirmed after 5 min for faropenem and after 25 min for meso-6-aminopenicillanic acid) were challenged by addition of 2 mM meso-Dap. Samples were taken in the range of 300–2400 s for faropenem and in the range of 1500–3600 s for 6-aminopenicillanic acid. Spectrophotometric measurements were carried out as described above.

As a comparison to the DTNB assay the reaction of LdtM2 with faropenem was also followed using a spectrophotometric assay described previously [25]. The reaction mixtures contained 50 μM LdtM2 and 50 μM faropenem in 0.05 M potassium phosphate buffer pH 6.0, containing 1 mM EDTA in 100 μL final volume. The reaction was monitored spectrophotometrically at 299 nm at 22 °C for 400 s.

Protein crystallization

Screening for crystallization conditions was carried out at an initial concentration of 28 mg·mL$^{-1}$ for the wild-type LdtM2 BC module by the vapor-diffusion method using a Mosquito Crystallization Robot (TTP LabTech Ltd, Melbourne, UK) and the commercially available crystallization screens Morpheus® MemStart™ + MemSys™ HT-96, MemGold2™, MD1–63 (Molecular Dimensions, Newmarket, UK), and SaltRx HT™ (Hampton Research Corp., Aliso Viejo, CA, USA).

For cocrystallization with DTNB (LdtM2-TNB adduct) the protein was incubated with 3.3 mM DTNB at 293 K for 30 min prior to mixing the crystallization drops. Crystals of LdtM2-TNB complex were obtained in sitting drops at 293 K by mixing 0.15 μL of protein solution and 0.15 μL of well solution [0.1 M MES/Imidazole pH 6.5, 0.02 M of each carboxylic acid: sodium formate, ammonium acetate; trisodium citrate; sodium potassium DL-tartrate; sodium oxamate; 10% (w/v) PEG 20000, 20% (v/v) PEG 6K] for 60 min and transferred to a drop comprising 0.1 M sodium citrate pH 6.5, 17.5% PEG 6K and 4 mM of faropenem for 360 min and subsequently flash-frozen in liquid nitrogen.

Data collection

The X-ray diffraction datasets were collected at 100 K at beam line ID23-2 (LdtM2-TNB) and beamline ID29 (LdtM2-FARO adduct) of the European Synchrotron Radiation Facility (ESRF) to resolutions of 1.55 and 1.54 Å, respectively. The X-ray datasets were processed using XDS [43] and scaled using AIMLESS from the CCP4 suite [44]. The data collection statistics are summarized in Table 2.

Structure determination and refinement

The structures of the LdtM2 ligand complexes were solved by molecular replacement using MOLREP [45] and the ligand-free structure of the same construct PDB: 4HUC [12] as template. The models were refined by iterative rounds of manual model building including placement of water molecules using COOT [46] and crystallographic refinement with REFMAC5 [47]. The electron density adjacent to the thiol group of Cys354 in both chains in the LdtM2-TNB adduct structure allowed a straightforward fit of 5-mercapto-2-nitro benzoic acid (TNB), covalently linked to the sulfur atom of Cys354. At the end of the refinement, an anisotropic B-factor model was used as implemented in REFMAC5.

The faropenem-derived adduct comprising six atoms (β-OH-butyryl adduct) was fitted to the difference density extending from Cys354 in one of the two protein chains in the asymmetric unit and refined using the CIF dictionary generated for this covalent ligand by PRODRG [48].

Protein models were validated using COOT [46] and MOLPROBITY [49] to monitor the stereochemistry and model quality. A summary of the refinement statistics and model parameters is given in Table 2. Structural comparisons were carried out using superposition by the secondary structure-matching routine in COOT. Molecular contacts, interacting surfaces, and solvent accessibility of the active site residues and the covalent adducts were analyzed using the PISA server [50]. Figures were produced using the program PYMOL (http://www.pymol.org).

The coordinates of LdtM2-TNB adduct and the LdtM2-FARO-derived adduct have been deposited in the Protein Data Bank under accession codes 5LB1 and 5LBG, respectively.

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Author contributions

RS, GS, and EMS designed the experiments, EMS and RS carried out the experiments and wrote the paper with contribution from GS, all authors were included in data analysis.

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