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High-Resolution Structure of ClpC1-Rufomycin and Ligand Binding Studies Provide a Framework to Design and Optimize Anti-Tuberculosis Leads

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Abstract

Addressing the urgent need to develop novel drugs against drug-resistant *Mycobacterium tuberculosis* (*M. tb*) strains, ecumicin (ECU) and rufomycin I (RUF1) are being explored as

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Author Contributions

N.M.W., Hyun L., M.P.C., and C.A.-Z. performed experiments, analyzed data, and wrote the manuscript. R.P., J.R.A., W.G., J.R., B.D.S., Hanki L., N.A.H., N.M.D., J.-W.S., J.C., and Y.-Y.J. performed experiments. G.F.P. and S.C. designed experiments. All authors contributed to manuscript revision.

ASSOCIATED CONTENT

Supporting Information

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Additional SPR and MIC data for ClpC1 mutants and ecumicin analogue binding, mass spectrometry and NMR data for ecumicin and its analogues, time course analysis of the covalent bond formation between RUF1 and ClpC1-NTD by mass spectrometry monitoring, structural information of ClpC1-NTD-RUF1 B-factors, RMSD between ClpC1-NTD-RUF1 and ClpC1-NTD-CYMA and superposition of CYMA, free cyclomarin analogue and proposed binding of RUF1 on the hexameric ClpC1 (PDF)

Accession Codes

The coordinates of the final refined model and the structure factors have been deposited in the protein data bank (PDB: 6CN8).

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promising new leads targeting cellular proteostasis via the caseinolytic protein ClpC1. Details of the binding topology and chemical mode of (inter)action of these cyclopeptides help drive further development of novel potency-optimized entities as tuberculosis drugs. ClpC1 *M. tb* protein constructs with mutations driving resistance to ECU and RUF1 show reduced binding affinity by surface plasmon resonance (SPR). Despite certain structural similarities, ECU and RUF1 resistant mutation sites did not overlap in their SPR binding patterns. SPR competition experiments show ECU prevents RUF1 binding, whereas RUF1 partially inhibits ECU binding. The X-ray structure of the ClpC1-NTD-RUF1 complex reveals distinct differences compared to the previously reported ClpC1-NTD-cyclomarin A structure. Surprisingly, the complex structure revealed that the epoxide moiety of RUF1 opened and covalently bound to ClpC1-NTD via the sulfur atom of Met1. Furthermore, RUF1 analogues indicate that the epoxy group of RUF1 is critical for binding and bactericidal activity. The outcomes demonstrate the significance of ClpC1 as a novel target and the importance of SAR analysis of identified macrocyclic peptides for drug discovery.

Graphical Abstract

Keywords

Mycobacterium tuberculosis; ClpC1; rufomycin; ecumicin; cyclomarin A; AAA+ protein family; molecular chaperone

Approximately 30% of the world's population is infected with latent *Mycobacterium tuberculosis* (*M. tb*), the etiologic agent of tuberculosis (TB). The prevalence of multidrug resistant (MDR), extensively drug resistant (XDR), and totally drug resistant (TDR) *M. tb* is especially alarming, making development of effective anti-TB therapeutics a significant priority. Bedaquiline is the only new FDA-approved drug in 40 years.¹ The high fatality of tuberculosis and inefficiency of current treatment options also calls for novel potent drug candidates. The actinomycete secondary metabolites cyclomarin A (CYMA), lassomycin,² and ecumicin (ECU) show anti-TB activity toward replicating and non-replicating MDR *M. tb* by targeting the caseinolytic protein C1 (ClpC1).³ The *clpC1* gene was identified as essential for *M. tb* cell growth⁴ by knockout studies; however, ClpC1 is not essential in most bacteria.⁵

ClpC1 description ClpC1 is a hexameric AAA⁺ATPase^{6,7} important for protein homeostasis known to participate in substrate recognition. *M. tb* harbors the ATPase proteins ClpC1, ClpC2 (ClpX'), ClpX, and ClpB along with ClpP1 and ClpP2 proteases.⁸ When ClpC1

forms a complex with ClpP1/ClpP2, that complex becomes an ATP-dependent functional proteolytic unit.⁶ The ClpC1 protein is composed of three domains: the N-terminal helical domain (NTD) and the nucleotide-binding domains D1 and D2 (Figure 1A). Both D1 and D2 contain an ATP-binding domain motif (GXXXXGKT/S) known as the Walker A domain.

In 2011, three X-ray crystal structures of MecA121⁶ were reported, illustrating it as a protein facilitating ClpC hexamerization, in complex with ClpC-NTD, ClpC-NTD-D1, and full-length (FL) ClpC from *Bacillus subtilis*.⁶ Structures of the wild-type (WT) and mutant ClpC1-NTD from *M. tb* have also been solved by X-ray crystallography in the absence and presence of the cyclic peptide, CYMA (Figure 1B).⁹ Using cyclomarin-derived-linked sepharose, the target of CYMA was identified as ClpC1 by analysis of bound proteins from *M. tb* lysates.¹⁰ The *M. tb* ClpC1-NTD structure consists of two α -helical domains with CYMA binding to a hydrophobic platform and loop region connecting the two helical domains. The *M. tb* ClpC1-FL structure has not yet been characterized.

Actino Cyclopeptides.

A high-throughput, cell-based screening of actinomycete extracts against *M. tb* revealed ECU as a potent (sub- μ M) and selective hit compound.¹¹ *M. tb* was grown in the presence of ECU to obtain spontaneous resistant strains (rECU). Genome sequencing revealed mutations in three genes, of which only the *clpC1* gene product was found to directly bind ECU. The ECU-resistant strains exhibited mutations in two leucine residues of the *clpC1* gene (Figure 1C). ECU activates ATPase activity while inhibiting the proteolytic activity of the ClpC1/ClpP1/ClpP2 complex.¹¹ ECU has been proposed to inhibit proteolytic activity by enhancing hexameric association of ClpC1 and blocking substrate recognition.¹² Caseinolytic proteases like ClpP are tightly regulated to avoid uncontrolled proteolysis, making them viable antibiotic targets.¹³ To date, there are no publications identifying ClpC1 as the target of a drug development strategy. However, recently, the ClpC1/ClpP1/ClpP2 complex has been identified as a promising drug target.^{13–16} Targeting the proteostasis network could have a three-pronged effect, preventing damage repair, sensitizing the bacteria to stress, and preventing formation of drug-tolerant persister cells.¹⁵ The two currently identified natural peptides have some undesirable properties with respect to drug development; CYMA has high hepatic clearance and a short half-life in mice, and the depsipeptide ECU has poor solubility and poor intestinal absorption.¹³

In addition to CYMA and ECU, rufomycin I (RUF1) was identified as a sub- μ M hit compound through an actinomycetes extract screening campaign.¹⁷ RUF1 was first identified as an active anti-*M. tb* hit compound in 1962.¹⁸ The rufomycin peptides are also known as ilamycins.¹⁹ The ability of RUF1 to target ClpC1 was demonstrated by identification of whole genome sequencing of RUF1 resistant *M. tb* strains (rRUF1), revealing mutations at V13F, H77R, and F80X¹⁷ (Figure 1c). RUF1 is a macrocyclic heptapeptide with structural similarities to CYMA, containing seven amino acids, including a 1-methyl-1-oxiranylethyltryptophan and a second aromatic group (3-nitro-tyrosine in RUF1, β -methoxy-tyrosine in CYMA, Figure 1D).

This study presents the structure of the ClpC1 N-terminal domain in complex with RUFI, refined by X-ray crystallographic methods. Furthermore, cyclopeptide interactions with ClpC1 were evaluated by surface plasmon resonance (SPR). Both competition SPR and combination growth inhibition studies provided evidence for antagonistic effects between RUFI and ECU on ClpC1. The structure of the complex identified specific residue interactions and a time-dependent covalent bond formation between the Met1 of ClpC1 and the RUFI epoxide ring opening.

RESULTS

Evaluation of Direct Binding by Surface Plasmon Resonance.

To determine which domains of the ClpC1 protein are required for peptide recognition and binding to ClpC1, four ClpC1 constructs were prepared (Figure 2A). Subsequently, quantitative binding analysis by SPR was used to determine the binding affinity (K_D), the association rate (k_a), and the dissociation rate (k_d) constants between the ClpC1 constructs and the inhibitors. ECU, RUFI, and analogues were isolated from actinomycete extracts as previously reported, and NMR and mass spectrometry data were reported (Figures S1–S7).²⁰ The K_D values of ECU to ClpC1-FL and ClpC1-NTD were similar at 71.7 and 48.0 nM, respectively, while ECU bound slightly weaker to the ClpC1-NTD-D1 at 147 nM (Figure 2B). ECU was unable to bind to ClpC1-D1-D2, indicating only NTD is required for binding. A similar pattern of binding was observed with RUFI with K_D values of 85.1, 175, and 63.4 nM for FL, NTD-D1, and NTD, respectively (Figure 2C). Sensorgrams of ECU and RUFI binding to the ClpC1-FL and ClpC1-NTD proteins are shown (Figure 2D–I). K_D values of ECU and RUFI to the ClpC1-FL at equilibrium were similar. However, their k_a and k_d constants were very different (Figure 2D, E). The k_a and k_d values of RUFI to ClpC1-FL were ~50-fold faster than those of ECU (2.98×10^6 vs 6.12×10^4 M⁻¹ s⁻¹ for k_a and 3.01×10^{-1} vs 6.14×10^{-3} s⁻¹ for k_d). The k_d of CYMA for both the FL and NTD ClpC1 constructs (Figure 2F, I) had similar values, and its association and dissociation rates were intermediate compared to RUFI and ECU. The k_d values of an amino-alcohol derivative of CYMA (cyclomarin A1) are 6–8 fold lower than the values determined previously with a CYMA analogue using isothermal titration calorimetry (ITC).⁹

Binding Affinity Comparison of WT and Drug-Resistant *M. tb* Mutants.

ECU and RUFI produced two distinct sets of resistant strains each with a single mutation site. Three protein constructs with sites resistant to ECU (L92F, L92S, L96P) and seven constructs resistant to RUFI (V13F, H77R, F80I, F80V, F80S, F80L, F80C) were produced. The binding affinities of all ten drug-resistant mutant ClpC1-NTD constructs with the two lead peptides were compared (Figure 3A, B). The binding affinity of ECU to the three rECU mutant constructs was 70–230 times weaker than to the WT (K_D of 47.6 nM compared to 3320–11 600 nM), while binding of all rRUFI variants showed similar K_D values to the WT within a 2.3-fold range (Figure 3A and Table S1). This indicates that none of the rRUFI variants affected ECU binding to the ClpC1-NTD. In the case of RUFI interacting with its seven mutants, six variants bound from 20- to 80-fold weaker than the WT, while only one variant, H77R, remained at a similar binding affinity (Figure 3B and Table S1). H77R is also the only mutation site that did not have reduced affinity for either peptide. RUFI was able to

bind to three rECU variants with binding affinity similar to the WT. Collectively, the data suggested the lack of cross resistance between the two sets of variants generated by ECU and RUFI.

Binding Profiles of RUFI and ECU Analogues.

Next, chemical analogues of ECU and RUFI were evaluated to investigate critical functional groups for binding. Binding affinities of a RUFI analogue (compound **2** in Table 1) plus RUFI were determined in parallel. RUFI (compound **1**), containing an epoxy functional group on the tryptophan, gave a minimum inhibitory concentration (MIC) value against *M. tb* of 10 nM and binding affinity at 63.4 nM. RUFVII (**2**), also containing an epoxy group, was active against *M. tb* suggesting its possible importance in ClpC1-NTD binding. However, the MIC value of RUFVII is 100-fold higher than that of RUFI. The only difference between RUFI and RUFVII is at R₁ and R₂ positions: RUFVII has H at R₁ and 4-methyl-glutamic acid at R₂, whereas the R₂ residue of RUFI is bound to the amide N at R₁.

The MIC and K_D values of three ECU analogues (compounds **3–6** in Table S2) were also determined. The MIC value of deoxyECU (**5**) was ~1.5-fold enhanced at 130 nM, in comparison to that of ECU (**3**) and norECU (**4**) at 190 nM, whereas nordeoxyECU (**6**) was 1.3-fold weaker at 250 nM. Binding affinities of **4** and **5** were similar, while compound **6** had a K_D value of 168 nM. Therefore, K_D and MIC values of three ECU analogues were comparable within a 3-fold range, within the error of measurement. However, the K_D value of ECU is ~4-fold smaller than its MIC value.

Structure of ClpC1-NTD in Complex with RUFI.

Co-crystals of RUFI and ClpC1-NTD grew after two months and were small (10–30 μm) rhombic dodecahedra with well-defined faces and edges. The ClpC1-NTD structure in complex with RUFI was initially solved at 2.0 Å and later refined at 1.4 Å in the cubic space group $P4_132$, containing one ClpC1-NTD-RUFI complex in the asymmetric unit. Collection data and refinement statistics are presented in Table 2.

The structure of the ClpC1-NTD RUFI complex contains ten helices, including a core of two compact four-helical domains. The two orthogonal domains are connected by a flexible loop (residues 67–81), exhibiting weaker electron density in the final refined map (Figure S8A). Two additional helices are present, one at each end of the protein. The 3_{10} -helix at the N-terminus provides a hydrophobic anchor for Phe2, facilitating the position and orientation of Met1 next to the epoxy ring of RUFI (Figure 4A). Notably, this one-turn 3_{10} -helix is present in the complex with CYMA but not in the apo structure (PDB: 3WDB). The C-terminal α -helix is visible to residue Glu152, while the terminal six histidines of the affinity tag are not visible.

Monomeric ClpC1-NTD was bound to a single RUFI molecule (Figure 4A). The macrocyclic heptapeptide adopted an antiparallel β -sheet fold, bending sharply over a hydrophobic ridge created by Phe2 and Phe80 (Figure 4B). One hydrogen bond (3.1 Å) was present from the amide backbone of Phe80 to the carbonyl of the Tyr of RUFI. Nonbonding contacts existed between RUFI and 11 residues, including Met1, Phe2, Val13, His77, Phe80,

and Glu89. RUFI hydrogen bonded with eight water molecules (Figure 4C) and exhibited three intramolecular hydrogen bonds. A ring at R₁/R₂ locked the peptide into a rigid, compact structure (Figure 4C). Superposed structures of ClpC1-NTD in complex with CYMA and RUFI revealed that the peptides bind to the same location on the surface of ClpC1-NTD (Figure 4D).

The crystal structure of ClpC1-NTD-RUFI shows electron density that connects the sulfur of Met1 and the carbon of the RUFI epoxy moiety with a short (1.7 Å) distance between the two atoms. The position of the Met1 sulfur was confirmed by an omit map, where Met1 was replaced by Ala. Density in the $F_o - F_c$ map indicated the location of the sulfur atom with weak density for the terminal carbon of Met1 (Figure 5A and Figure 5D, IV). Interestingly, the nitro group on tyrosine of the 3-fold symmetry related RUFI molecule is nearby (3.7 Å) this covalent bond. In addition, a chloride ion detected at 4.4 Å from the Met1 sulfur is within range to help stabilize a positive charge.²¹

To confirm experimentally that a covalent bond can form between ClpC1-NTD and RUFI, the protein was incubated overnight at 4 °C in the presence and absence of RUFI. Molecular mass determination by mass spectrometry (MS) found the mass of ClpC1-NTD to be 17 501.3 ± 0.1 Da, 0.2 Da less than the calculated mass (17 501.1 Da) (Figures S9–S15). The sample incubated with RUFI contained a mixture of unbound ClpC1-NTD and a second species with a molecular weight of 18 543.7 Da (17 501.1 + 1042.6 = 18 543.7 ± 0.1 Da; < 0.1), thus confirming the presence of a covalent adduct. Further exploration of this time dependent bond formation resulted in discovery of a 63% formation after 12 h and 92% formation after 5 days (Figures S9–15, Table S3).

A phosphate ion (orange tetrahedra in Figure 4A) was found at the N-terminus of helices H3, H6, and H8 of ClpC1-NTD and showed hydrogen bonding to residues T30, T81, R83, V119, and two water molecules. This site is conserved in the ClpC1-NTD-CYMA complex, with acetate occupying the phosphate site. Additionally, a phosphate moiety of a phospho-arginine was found bound in the same pocket in the ClpC1-NTD structure from *B. subtilis* (PDB: 5HBN).²² ClpC1-NTD recognizes phospho-arginine of proteins as a degradation tag.²³ More recently, it has been suggested that the binding of phospho-arginine induces movement in ClpC1-NTD, while binding of phospho-arginine/CYMA prevented these dynamics.²⁴

Comparison of ClpC1-NTD in Complex with RUFI and CYMA.

The structures of ClpC1-NTD bound to RUFI and CYMA exhibit several important differences. Containing 142 equiv atom pairs, the two structures show a root-mean-square deviation (rmsd) of 0.71 Å. Comparison of rmsd of C_α pairs resulted in less than 1.5 Å differences throughout the protein except at residues 96–99 and the C-terminus (Figure S8B) due to different composition of the C-terminal His-tag.

As macrocyclic heptapeptides, the differences in RUFI and CYMA lie in the identity of their individual amino acids. One such alteration is the displacement of an aromatic group by a 3-nitro-tyrosine or β-methoxy-phenylalanine residue (Figure 1D). Importantly, both cyclopeptides contain a 1-methyl-1-oxiranylethyl-L-tryptophan. Although the binding of

CYMA and RUFI is topologically similar (Figure 4D), there are variations in the atomic interactions, in part due to the minor differences in the constituent amino acids. There is a minor conformational change of the loop containing H77, an rRUFI site. In the CYMA complex, the subsequent helix contains K85, which hydrogen bonds to a carbonyl backbone of CYMA at Wlu2 and its corresponding side chain.

The crystallographic structure of a free CYMA analogue (Cambridge Structural Database (CSD) ID 1169867)²⁵ is essentially the same (rmsd 0.27 Å of 27 pairs of main chain atoms, Figure S16) as that of CYMA in ClpC1-NTD complex, with only minor differences in the side chain orientations and a closed epoxide ring. However, like RUFI, CYMA in complex with ClpC1-NTD (PDB: 3WDC) appears to have its tryptophan's epoxide moiety open and bound to Met1;⁹ the distance between the sulfur of Met1 and the C5 atom of the epoxide ring is 1.7 and 2.6 Å for the two possible conformations.

To assess the validity of the open epoxide in the existing ClpC1-NTD-CYMA complex (PDB: 3WDC) critically, the position of the sulfur in the Met1 side chain was examined. Refining the structure further involved replacing the existing Met1 by Ala to have an unbiased electron density map and establish the chemical relationship of epoxide atoms to the side chain of Met 1 (Figure 5B). The $F_o - F_c$ map confirmed the presence of a very high electron density peak within covalent bonding distance (1.7 Å) of the exposed carbon upon epoxide ring opening. In addition, the map showed the corresponding electron density of the C_ε atom of Met1 and additional electron density within adequate bonding distance of the sulfur, suggesting the presence of a sulfonium ion (Figure 5D).

SPR Assessment of Competitive Binding of Cyclopeptides.

According to the newly solved ClpC1-NTD-RUFI and already available ClpC1-NTD-CYMA complex structures, RUFI and CYMA bind to the same site of the ClpC1-NTD. Because an experimentally determined structure of the ClpC1-ECU complex is unavailable, competition between either RUFI or CYMA and ECU was investigated by SPR. The binding of RUFI was abolished in the presence of an excess amount of ECU at 10 μM (Figure 6A, B), while the binding affinity of ECU in the presence of 10 μM RUFI was weakened by ~5-fold (Figure 6B). CYMA and RUFI had similar behavior in competition experiments; therefore, only RUFI data are shown. In addition, the total amount of ECU bound (max response, R_{max}) was decreased to almost half of the original R_{max} of ECU alone, indicating that RUFI interferes with ECU binding without completely abolishing it. Considering the weakened binding affinities and significantly reduced R_{max} values, RUFI and ECU can be considered (partially) competitive binding agents. Although the overall binding affinities of ECU and RUFI alone are very similar, ECU has a slower off-rate (Figure 6A), remaining bound ~50-fold longer than RUFI. This likely prevents RUFI binding when both ECU and RUFI are present and suggests that the binding site of ECU may overlap in part with that of RUFI and CYMA. Moreover, while the rECU mutation sites (L92 and L96) are different from the rRUFI sites (V13, H77, and F80), they are located nearby (Figure 6C). Hence, the core binding sites of ECU and RUFI may be slightly different but could be overlapped due to the 1.5-fold larger size of ECU. ECU was docked to ClpC1-NTD domain to provide a possible structural framework to interpret the competition studies. ChemPLP scores from

molecular docking studies predicted the binding pose of ECU with a partial overlap of ECU and RUFI as shown in Figure 6C.

Evaluation of Competitive Effects of Cyclopeptides by *M. tb* Growth Inhibition Measurements.

To assess competitive effects on bacterial growth, a luciferase-expressing strain of *M. tb* was utilized in a checkerboard assay at several concentrations and time points. Results yielded a fractional inhibitory concentration index of 0.8–2.0,²⁰ indicating that the compounds were possibly additive or have no effect when dosed in combination.²⁶ From the assay, the concentrations of 140 nM of ECU and 190 nM of RUFI alone produced at least a two-log drop in bioluminescence signal after a two-week incubation when compared to the untreated bacterial control (Figure 6D). Therefore, these two concentrations were plotted to graphically compare the combination of ECU and RUFI for their cumulative effects on the growth of *M. tb*. Evidence of competitive effects were observed by day 5, where the magnitude of signal dropped by roughly 20-fold from day 3 for ECU alone; however, at the same concentration of ECU in combination with RUFI, this magnitude of drop was 2-fold or 5-fold in the presence of 190 and 7 nM RUFI, respectively. While both peptides lead to cell death, there appears to be a trend in which the magnitude of bacterial killing is greater when ECU is combined with lower concentrations of RUFI (pink vs blue) or when dosed alone.

DISCUSSION

Four known natural actinomycete-derived cyclic peptide inhibitors target ClpC1: CYMA, ECU, lassomycin, and RUFI. Importantly, they are all highly active against and selective for mycobacteria while being inactive against other microorganisms such as *E. coli* and *Staphylococcus aureus*.^{2,17}

While all four cyclic peptides interact with the N-terminal domain of ClpC1, their mechanisms of action differ. CYMA binds to NTD of ClpC1 and prevents movement of the NTD, accelerating its ATPase turnover. On the other hand, ECU and lassomycin² bind to NTD probably at two different locations (presumed by different resistant mutation sites) and not only activate ATPase activity, but also uncouple ClpP1/ClpP2 peptidase from ClpC1. RUFI also interacts with the NTD and significantly decreases the proteolytic capabilities of the complex ClpC1/ClpP1/ClpP2 to degrade casein while having no significant effect on the ATPase activity of ClpC1.¹⁷

Measurement of k_a and k_d parameters are critical in hit-to-lead and lead optimization studies. A slow k_d determines the *in vivo*²⁷ lifetime of a drug and is the most significant for its effectiveness. Slow binding is often due to an induced rate-limiting conformational change of the protein. Longer dissociation rates of inhibitors have been correlated with preferred bactericidal effects, as opposed to less favorable bacteriostatic effects in antibiotic action models.²⁸ ECU has the slowest k_d , making it a promising lead, but also demonstrates poor solubility and intestinal absorption. The slow k_d rate of ECU may be due to its larger size and/or increased flexibility. In contrast, RUFI is more sterically restrained forming a more compact heptapeptide, minimizing entropic effects and resulting in faster k_a and k_d . Like RUFI and CYMA, the free ECU (CSD 940680) involves intramolecular hydrogen

bonding, but its larger tail and overall size gives it additional flexibility that could result in a different binding mechanism. CYMA has the tightest binding affinity of the three peptides, possibly due to the dual interactions of K85 (the NZ amine with the carbonyl and hydroxyl oxygens) with the 5-hydroxy-Leu residue, and Met1 (the N-terminal amine with the β -hydroxyl) with the β -hydroxy-Trp of CYMA (Figure 1D). The 3-nitro-tyrosine of RUFI is displaced by one residue losing the interaction with K85 and causing weaker affinity and inhibitory effects with ClpC1.

The covalent adduct between ClpC1-NTD and RUFI could be important for biological activity and makes it a reasonable hypothesis that the same covalent adduct will form in the full-length functional protein. Superposition with the full length ClpC from *B. subtilis*²² (Figure S17) elucidated the location of the RUF molecule on the outer surface, which could be accessible even in the hexameric complex. The reaction of methionine with epoxides²⁹ by nucleophilic attack of sulfur was shown to result in a positively charged sulfonium ion. There is weak density for this terminal carbon in the RUFI complex, but electron density is much stronger (2.7-fold) in the refined CYMA complex. It is quite probable that the hydrophobic ridge created by Phe2-Phe80 is critical for optimal positioning of the epoxide next to the sulfur of Met1. SPR and ITC⁹ data support this assumption by showing significantly decreased affinity for Phe80 and Phe2 mutants. Additionally, CYMA has a stable epoxide in other structures.³⁰ While the covalent bond formation appears to be critical for activity of RUFI/CYMA, the S-methyl group may be neutral for activity. In contrast, the X-ray structure of a catalase-peroxidase with a stable sulfonium ion contained a conserved triad of residues creating an ionic cage to stabilize the positive charge.³¹ No such ionic cage was found in the ClpC1-NTD-CYMA structure as would be expected for the stabilization and functional significance of the sulfonium ion. Although similar sulfonium ions have been shown to be reversible, they are expected to be reasonably stable under neutral conditions.³² The cause of the chemical difference between the suggested thioether linkage and the sulfonium adduct is uncertain but could be influenced by the chemical environment of the crystal packing (i.e., interaction with 3-nitro-Tyr in RUFI), different components in the crystallization media (high NaCl), and/or the time for crystal growth (days vs months).

Further investigation in the Phe2-Phe80 hydrophobic ridge in ClpC1-NTD-CYMA was accomplished by mutation of Phe to Tyr.⁹ In both cases, the aromatic ring was rotated slightly 8–13° from the original position. The data indicate that the additional polar interaction in F2Y weakens the affinity more than that of F80Y. However, it is unlikely that the small alteration of contact area observed can account for the 300-fold difference in affinity.⁹ Alternatively, it is likely that Phe2 (in the H3₁₀) plays a critical structural role in forming the hydrophobic ridge and orienting Met1. Spontaneously derived F80 protein variants measurably disrupted the binding of RUFI, regardless whether the variants were polar or nonpolar. F80C had the weakest binding affinity to RUFI, suggesting that the thiol group altered the formation of the hydrophobic ridge dramatically, hindering the anchoring of the RUFI macrocyclic scaffold.

As measured by SPR, RUFI has rapid k_a and k_d values with ClpC1-NTD; therefore, it is highly probable that the covalent bond is not detected by this method. SPR experiments involve the constant flow of ligand over the protein surface, without sufficient time for a

covalent bond to form. In addition, the covalent bond is only formed for a population of protein molecules over 24 h. It is likely that this bond forms slowly and persists thereafter. Previously, it was shown that RUF1 had a concentration dependent bactericidal effect, requiring approximately 10× higher concentration than the MIC value for a significant signal drop in CFU (minimum bactericidal concentration 400 nM).¹⁷ In addition, the initial inhibition of *M. tb* by RUF1 is slower compared to ECU but has a lower MIC value, indicating a delayed event contributing to its activity. Thus, a higher concentration of the cyclopeptide could be needed for the equilibrium to shift and form the covalent adduct in a bioactive-relevant manner. The time required for crystal growth is also consistent with this hypothesis. The concept of dynamic residual complexity may help explain differences in RUF1 and CYMA binding and covalent adduct formation.³³ If the peptide side chains exist in different conformations, and only a particular conformation binds to ClpC1, the peptide is only available for binding in that conformation. It is also possible that a conformational change occurs upon binding that is required for the chemical reaction to occur delays the reaction.

RUF1 analogues have significant differences in binding to ClpC1-NTD. The two RUF1 analogues, both with low MIC values, contain an epoxy group, suggesting that this moiety or a polar group at this position may be important for predicting binding affinity. When comparing the higher MIC value of **2** and **1**, varying at R₁ and R₂, the difference in potency is suggested by an increased flexibility of **2** and/or increased polarity. The increased flexibility may decrease cell permeability or reduce the binding affinity to ClpC1 *in vivo*. The RUF1 binding site is hydrophobic and the carboxylic acid moiety may be repelled by the hydrophobic surface. It was previously stated that the epoxide was not required for binding based on experiments with cyclomarin C, lacking an epoxide.³⁴ However, unlike RUF1, CYMA has a lower K_D , k_a and k_d values with ClpC1, structural differences and, most significantly, differences in activity against resistant *M. tb* strains.

A time kill experiment was used to determine competitive effects between ECU and RUF1. In the presence of RUF1, the efficiency of bacterial killing of ECU is decreased, confirming that ECU and RUF1 are competitive compounds when dosed together against *M. tb*. SPR competition experiments found that RUF1 weakened binding affinity of ECU and reduced the total number of molecules of ECU binding to ClpC1-NTD. A very high concentration of ECU prevented RUF1 binding to ClpC1-NTD, likely due to slow dissociation of ECU. In addition, as ECU increases ATPase activity, reduces proteolytic activity, and binds to an allosteric site. An induced conformational change³ has been proposed to explain the disruption of RUF1 binding.

SPR indicated no cross resistance between the two sets of protein variants, i.e., ECU resistant variants do not have significantly weaker affinity for RUF1 and vice versa. Comparison of the drug-resistant mutants for ECU and RUF1 strongly suggest that residues at the carboxy end of H5 (L92, L96) are critical for ECU binding, extending from the binding site occupied by the CYMA/RUF1. This is in contrast to the critical role played by the residues forming the “hydrophobic ridge” (F2–F80) in binding of the heptacyclopeptides. MIC values also indicate no cross resistance of RUF1 and ECU.¹⁷ This suggests that these two cyclopeptides do not bind in the same pocket, which was also

suggested by different downstream effects of activities. However, behavior in SPR and growth inhibition experiments between RUFI/CYMA and ClpC1 suggested overlapping binding sites for these two cyclopeptides, and X-ray crystallography results confirmed this conclusively. The MIC data for CYMA further indicate that there are different modes of action, as CYMA activity was decreased in only some of rRUFI and unaffected in all rECU.¹⁷ A larger binding area for binding ECU on the surface of the ClpC1 protein, extending from the surface occupied by CYMA/RUFI, would be consistent with the results of the competition experiments between CYMA/RUFI and the larger ECU.

CONCLUSION

In summary, the results indicate that the potent growth inhibitory effects of RUFI consist of two parts: (i) a specific and high affinity binding provided by the cyclic scaffold of the macrocyclic peptide to the ClpC1-NTD and (ii) the formation of a covalent adduct between the opened epoxide of the tryptophan and the sulfur atom of the N-terminal Met residue. The slower formation of the covalent adduct will be consistent with long-term bactericidal effects of this class of cyclic antibiotics. The formation of the covalent adduct provides the chemical framework for the suggested blocking of ClpC1-NTD dynamics upon the binding of phospho-arginine.²⁴ The biological effect resulting from the formation of the covalent adduct with the active heptapeptide antibiotics CYMA/RUFI on the regulation of ClpC1 activity should be investigated further to distinguish them from those of the longer and more flexible depsipeptide ECU. Understanding the role of the critical functional groups of these macrocyclic peptides will allow for the design of optimized, bioavailable compounds against ClpC1 for antituberculosis therapy.¹⁴

METHODS

Plasmid Preparation.

The coding sequence of *clpC1* (Rv3596c) was amplified by polymerase chain reaction (PCR) from genomic DNA of the wild-type *M. tb* strain H37Rv. The *clpC1* gene was cloned into pET-30a+ plasmid using *Hind*III and *Nde*I restriction sites. The final full length ClpC1 plasmid contained residues 1–842, omitting the terminal 6 residues due to difficulty amplifying the GC rich C-terminus. After transformation, the plasmid was amplified by PCR and cloned into a pET-30a+ vector to create ClpC1-NTD (residues 1–145), ClpC1-NTD-D1 (residues 1–493), and ClpC1-D1-D2 (residues 165–842) recombinant vectors. These wild-type plasmids were used for site-directed mutagenesis to create single mutation sites on the gene.

Protein Expression and Purification.

Previous data determined that affinity for CYMA did not change whether the protein was tagged at the N or C-terminus,⁹ but the diffraction quality of crystals obtained with the N-tagged protein was inferior. Therefore, the C-terminally tagged protein was chosen. The recombinant ClpC1 protein¹¹ was transformed into BL21C⁺ cells, grown in LB or XYT media, and induced by IPTG. The His-tagged protein was purified with a Ni-NTA column equilibrated with 20 mM phosphate and 300 mM NaCl pH 8.0 buffer. Wash steps included

20 mM imidazole, while the elution buffer had 500 mM imidazole. SDS-PAGE was used to determine protein purity. Protein was buffer exchanged into PBS containing 15% glycerol and stored at -80°C . For cocrystallization, the ClpC1-NTD protein was injected to a Superdex 75 column via an FPLC (AKTA, GE Health Sciences) equilibrated with 50 mM TRIS, 150 mM NaCl, pH 7.5. The ClpC1-NTD-RUFI complex was obtained by mixing protein (15 mg/mL, 0.85 mM, 17501 g/mol, $\epsilon_{280} = 4470 \text{ M}^{-1} \text{ cm}^{-1}$) with 1.2 mM RUFI and incubating overnight at 4°C . The complex was mixed 1:1 with solutions from several screening kits (Microlytic, Qiagen). Crystals grew by vapor diffusion after two months at 18°C in precipitant of 0.2 M $\text{Na}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$ pH 6.2 and 2.5 M NaCl.

X-ray Structure Data Collection, Processing, Structure Solution, and Refinement.

Initially a data set was collected to $2.0 \text{ \AA}$ resolution. Molecular replacement was carried out by MOLREP in CCP4 (v7.0)³⁵ using ClpC1-NTD (PDB: 3WDB) as the search model. A second data set was collected on a Dectris Eiger 9 M detector with 0.2° oscillation and 0.02 s per frame for 360° using a single crystal, flash frozen in liquid nitrogen. Data were processed using XDS (version June 17, 2015). The solution to the $2.0 \text{ \AA}$ data set was used to refine the structure using the $1.4 \text{ \AA}$ data set. Refinement was completed in COOT, REFMAC, and Phenix (1.12–2829).

Further refinement of the 3WDC PDB entry was performed using the protein structural coordinates and structure factors derived from the deposited model, where Met1 was substituted with Ala. Three cycles of restrained coordinate refinement followed by occupancy refinement were performed. The final R_{work} and R_{free} were 0.115 and 0.142, respectively, with appreciable improvement in the structural restraints (rmsd bonds = 0.016 to $0.011 \text{ \AA}$, rmsd angles = 1.71 to 1.37° , clash score of 19 to 7.8). The clash score improved dramatically upon removal of the close contact between the sulfur of Met1 and the carbon of the open epoxide of CYMA. The difference electron density map ($F_o - F_c$) located the position of the terminal atoms of the Met side chain ($\text{C}_{\gamma}\text{-S}_{\delta}$) unambiguously (sulfur peak $>25\sigma$, C_{γ} peak 15σ). The terminal carbon atom of Met1 had unambiguous density to 8σ .

Molecular Docking Studies.

The structure of ClpC1-NTD in complex with CYMA (PDB: 3WDC) was prepared by the Protein Preparation Wizard in Schrodinger (Release 2016–1, Epik v 3.5, Impact v 7.0) to remove crystallographic waters and ions, fill missing chains and loops, fix bond orders, add hydrogens, assign partial charges with the OPLS3 force field³⁶ and minimize the added hydrogens. The 3D structure of ECU (CSD 940680) was processed by LipPrep module v 3.7 of Schrödinger. ECU was then docked into the binding pocket of ClpC1-NTD without CYMA using a radius of $12 \text{ \AA}$ centered near L92 and L96 at ($x = -24.3$, $y = -0.75$, $z = -10.17 \text{ \AA}$) using GOLD v 5.2.2.³⁷ A rigid receptor and flexible ligand were used during the docking, while default settings were used for other parameters. The binding poses of ECU were evaluated using the ChemPLP scoring function,³⁸ and a conformation with the top ChemPLP score was selected.

Mass Spectrometry for Covalent Adduct Formation.

To test formation of a covalent bond between ClpC1-NTD and RUF1, the protein (30 μM) was exchanged into PBS pH 7.4 with 15% glycerol and incubated 0–120 h at 4 °C with and without RUF1 (90 μM). The samples were centrifuged and injected into an Agilent 6550 iFunnel QTOF MS coupled to an Agilent 1290 UPLC system. Proteins were resolved on a C3 300SB C3 2.1 $\times$ 75 mm 5 μm column at a flow rate of 400 $\mu\text{L}/\text{min}$ by a linear gradient of formic acid and acetonitrile. Data were acquired via AJS-ESI (drying gas 290 °C, flow 14 L/min, nebulizer pressure 60 psi, sheath gas 400 °C, flow 11 L, Vcap 5500 V, and nozzle voltage 2200 V). Data were acquired at 1 spectra/sec from 100 to 3000 m/z . Percent species was analyzed by area under the curve in HPLC spectrum. The measurements were performed by the Mass Spectrometry, Metabolomics, and Proteomics Facility in the UIC Research Resources Center.

Surface Plasmon Resonance Binding Assays.

Each ClpC1 enzyme was immobilized on a CM5 sensor chip using standard amine-coupling at 25 °C with running buffer PBS-P (20 mM Phosphate, 137 mM NaCl, 27 mM KCl, 0.05% surfactant P-20, pH 7.4) using a Biacore T200 instrument. Unmodified blank surface was used on flow channel 1 as a control. ClpC1 enzymes were diluted with 10 mM sodium acetate (pH 4.0 or pH 5.0) to 50 $\mu\text{g}/\text{mL}$ and immobilized to flow channels 2, 3, and 4 after sensor surface activation with EDC/NHS, followed by ethanolamine blocking on unoccupied surface area. Compound solutions with a series of increasing concentrations (0–5 μM at 2-fold dilution) were applied to all four channels in SPR binding buffer (PBS-P, 0.5 mM TCEP, 2% DMSO) at a 30 $\mu\text{L}/\text{min}$ flow rate at 25 °C. For competition experiments, each titration of RUF1 also contained 10 μM ECU and vice versa. Sensorgrams were analyzed using the Biacore T200 evaluation software 3.0, and data were double-referenced with reference channel and zero concentration of compound response unit values. Kinetic rate constants (k_a and k_d) were determined by fitting the double-reference data globally to the 1:1 Langmuir model embedded in the Biacore T200 evaluation software (v3.0). K_D values were then calculated from the two rate constants ($K_D = k_d/k_a$).

RUF and ECU Analogues.

RUF1 and RUFVII were isolated as described previously²⁰ (these compounds are of the same lot used in these studies). The anti-*M. tb* compound ECU and its analogues were isolated from *Nonomuraea sp.* MJM5123 using multiple chromatographic fractionation steps as published previously.^{39,40} Preparative scale purification of ECU, deoxy-ECU, nor-ECU, and nor-deoxy-ECU was carried out using preparative HPLC instrument by Shimadzu (LC-20AP) using a 250 $\times$ 21.2 mm Kinetex 5 μm Evo C18 column. A mobile phase composition consisting of a 40/60 mixture of triethylammonium acetate pH 8.1/acetonitrile as solvent A and acetonitrile as solvent B was used. Separation was carried out in an isocratic mode using 88% A and 12% B at a flow rate of 21 mL/min. Samples were dried on a rotary evaporator to remove acetonitrile, followed by resuspension in water and lyophilization to obtain white amorphous powders. For NMR analysis, lyophilized samples were dissolved in 200 μL of methanol- d_4 and transferred to 3 mm NMR tubes. Data were acquired on an 800 MHz Bruker Avance spectrometer equipped with a 5 mm triple

resonance inverse TCI RT probe. Mass spectrometry data were acquired on a Bruker impact II with ESI source. Compounds were dissolved in water:acetonitrile 1:1 with 0.1% formic acid at approximately 10 $\mu\text{g/mL}$.

***M. tb* Growth Inhibition Assays.**

MICs for ECU, RUFI, and analogues were determined by Microplate Alamar Blue Assay⁴¹ and were defined as the lowest concentration reducing fluorescence signal by 90% relative to untreated controls. For time kill combination experiments, a recombinant *M. tb* strain containing pMV306G13+Lux⁴² from frozen stock was reconstituted into 7H12 liquid medium⁴¹ and incubated at 37 °C for 24 h prior to addition to white-bottomed 96-well assay plates. The 96-well plates were prepared in checkerboard, 6 × 6 format with 3-fold concentration dilutions. The highest starting concentration of RUFI and ECU was 190 nM and 1250 nM respectively. Compounds were dissolved and prediluted in DMSO in a separate plate, then transferred by 1 μL aliquots to the assay plate. Bioluminescence was measured using a Berthold Centro XS3 LB 960 (Berthold Technologies GmbH & Co. KG, Bad Wildbad, Germany). Measurements were taken at T_0 (after bacterial addition to assay plate) and 1, 2, 3, 5, 7, 10, 12, and 14 days after addition to the assay plate. The experiment was run in triplicate with standard deviation reported.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

CYMA	cyclomarin A
ECU	ecumicin
HTS	high-throughput screening
ITC	isothermal titration calorimetry
rECU	ecumicin resistant <i>Mycobacterium tuberculosis</i> mutation sites
R_{max}	max response
rRUFI	rufomycin I resistant <i>Mycobacterium tuberculosis</i> mutation sites
RUFI	rufomycin I
SPR	surface plasmon resonance

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Figure 1.

(A) A schematic representation of the ClpC1 protein domains and motifs. The consensus ATP binding motif and *M. tuberculosis* ClpC1 sequences are shown. (B) X-ray structure (PDB: 3WDC) of ClpC1-NTD in complex with CYMA. (C) X-ray structure (PDB: 3WDC) of ClpC1-NTD with two mutation sites L92 and L96 (pink) against ECU and three mutation sites V13, H77, and F80 (blue) against RUFI. (D) Structures of the three macrocyclic peptides.

Figure 2.

SPR binding analyses. (A) Various ClpC1 constructs to determine where compounds bind. Bar graph of binding affinities (K_D values) between four WT ClpC1 constructs and two inhibitors, ECU (B) and RUFI (C). Sensorgrams of ECU (D), RUFI (E), and CYMA (F) binding to the ClpC1-FL protein from *M. tuberculosis*, immobilized on a CM5 sensor chip. Sensorgrams of ECU (G), RUFI (H), and CYMA (I) binding to the ClpC1-NTD protein from *M. tuberculosis*. Experimental sensorgrams (colored lines) were fitted by the 1:1 Langmuir model (black dotted lines).

Figure 3. Binding affinity comparison. K_D values of various ClpC1-NTD mutant constructs and two inhibitors, ECU (A) and RUF1 (B), in comparison to the WT (green). Drug resistant mutation sites against ECU and RUF1 are shown in pink and blue, respectively.

Figure 4.

Structures of ClpC1-NTD CYMA and RUF1 complexes. (A) Structure of ClpC1-NTD in complex with RUF1 with core helices labeled. Purple/green spheres indicate the sodium/chloride ions identified in the refined structure and present in the crystallization media. (B) The polar surface depiction of the ClpC1-NTD RUF1 complex (green sticks) illustrates a prominent hydrophobic ridge over which the ligand bends, fitting the indole epoxide in a deep, narrow cavity. Phe2 and Phe80 form the hydrophobic ridge. The polar surface representation covers hydrophobic (orange-red) to hydrophilic (blue) properties. (C) Intramolecular hydrogen bonding (red lines) in RUF1 (green) in the complex structure and the R₁/R₂ ring stabilize the heptapeptide, giving it a more rigid structure within the complex. (D) The superposed structures of ClpC1-NTD in complex with CYMA vs RUF1 show binding at approximately the same position and orientation for the heptapeptides.

Figure 5.

Epoxide of RUFI bound to ClpC1-NTD at Met1. (A) The structure of the ClpC1-NTD in complex with RUFI is shown after refinement of the Met1 to Ala replacement to create an unbiased assessment of atom locations near the epoxide ring. The $F_o - F_c$ map in green at 3.0σ ($2F_o - F_c$ map at 2σ) shows clear density for the Met1 side chain in close proximity to the open epoxide ring of RUFI. Notably, the refined coordinates show weak ($<2\sigma$) electron density for the terminal carbon of the side chain of Met1, suggesting a loss of the methyl group and a thioether bond (IV). (B) The structure of ClpC1-NTD in complex with CYMA (PDB: 3WDC) was refined with a Met1 to Ala substitution to determine the definitive location of the Met1 sulfur by the $F_o - F_c$ map in green at 3.0σ ($2F_o - F_c$ map at $1.2\text{ \AA}$) and strongly suggested covalent bond formation. (C) The R_1/R_2 groups of RUFI were found as a closed ring with a γ -S-carbon based on the $2F_o - F_c$ map at 2.0σ . Figures were prepared with COOT. (D) Proposed mechanism for the opening of the epoxy ring in the CYMA/RUFI complex structures via a nucleophilic attack of the sulfur in the side chain of the N-terminal Met1 (I). Subsequent protonation and nucleophilic attack could lead to a thioether product (IV).

Figure 6.

Competitive effects of cyclopeptides. (A) Sensorgrams of RUFI (green) and ECU (red) alone in comparison to RUFI in the presence of ECU at 10 μ M (blue) and ECU in the presence of RUFI at 10 μ M (pink). (B) Steady-state fitting curves of RUFI alone (green), ECU alone (red), RUFI with ECU at 10 μ M (blue), and ECU with RUFI at 10 μ M (pink). (C) The predicted binding pose of ECU (yellow) by molecular docking. ChemPLP score was used to select the best binding pose of ECU. (D) Bioluminescence signal over time of the bacterial control (BC) of *M. tb* H37Rv strain growth without any compound (black), ECU alone at 140 nM (red), RUFI alone at 190 nM (green), the combination of ECU at 140 nM and RUFI at 190 nM (pink), and the combination of ECU at 140 nM and RUFI at 7 nM (blue). Each data point represents the mean of three measurements. Error bars represent the standard deviation.

Table 1.

RUF1 Analogues Tested against ClpC1-NTD in SPR^a

Compound	R ₁	R ₂	MIC(nM)	K _D (nM)	k _a (M ⁻¹ s ⁻¹)	k _d (s ⁻¹)
Rufomycin I (RUF1) (1)			10 ± 1	63.4 ± 6.0	1.71 × 10 ⁶ [±0.21 × 10 ⁶]	1.08 × 10 ⁻¹ [± 0.16 × 10 ⁻¹]
Rufomycin VII (RUFVII) (2)			1,000 ± 400	3,530 ± 134	1.59 × 10 ³ [± 0.06 × 10 ³]	5.59 × 10 ⁻³ [± 0.01 × 10 ⁻³]

^aFor compound 1, R₁ and R₂ form a ring with the peptide backbone, as can be seen in Figure 1D.

Table 2.**Data Collection and Refinement Statistics^a**

ClpC1-NTD-RUF1	
wavelength (Å)	1.0781
resolution range (Å)	19.40–1.40 (1.43–1.40) ^a
space group	<i>P</i> ₄ 32
unit cell (Å, °)	109.758, 109.758, 109.758, α , β , γ = 90
total reflections	1708 016
unique reflections	44919 (4391)
multiplicity	38.0 (24.1)
completeness (%)	99.90 (99.73)
mean $I/\sigma(I)$	58.7 (2.5)
Wilson B-factor	18.12
R_{meas}	0.099 (1.362)
CC _{1/2} (%)	99.9 (84.0)
reflections used in refinement	44 888 (4391)
reflections used for R-free	2359 (227)
R_{work}	0.159 (0.231)
R_{free}	0.185 (0.250)
number of nonhydrogen atoms	1544
macromolecules	1171
ligands	85
solvent	288
protein residues	152
RMS (bonds, Å)	0.008
RMS (angles, °)	1.112
Ramachandran favored (%)	100.0
clash score	5.45
average B-factor (Å ²)	23.05
macromolecules	19.82
ligands	18.41
solvent	37.57

^aData collection and refinement statistics for the 1.4 Å data set are shown with highest-resolution shell in parentheses.