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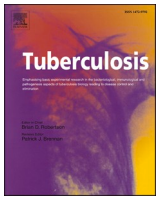

Identification of the inhibitory mechanism of ecumicin and rufomycin 4-7 on the proteolytic activity of *Mycobacterium tuberculosis* ClpC1/ClpP1/ClpP2 complex

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ABSTRACT

Ecumicin and rufomycin 4-7 disrupt protein homeostasis in *Mycobacterium tuberculosis* by inhibiting the proteolytic activity of the ClpC1/ClpP1/ClpP2 complex. Although these compounds target ClpC1, their effects on the ATPase activity of ClpC1 and proteolytic activity of ClpC1/ClpP1/ClpP2 vary. Herein, we explored the ClpC1 molecular dynamics with these compounds through fluorescence correlation spectroscopy. The effect of these compounds on the ATPase activity of ClpC1-cys, the recombinant protein for fluorescence labeling, and proteolytic activity of ClpC1-cys/ClpP1/ClpP2 were identical to those of native ClpC1, whereas the intermolecular dynamics of fluorescence-labelled ClpC1 were different. Treatment with up to 1 nM ecumicin increased the population of slower diffused ClpC1 components compared with ClpC1 without ecumicin. However, this population was considerably reduced when treated with 10 nM ecumicin. Rufomycin 4-7 treatment resulted in a slower diffused component of ClpC1, and the portion of this component increased in a concentration-dependent manner. Ecumicin can generate an abnormal ClpC1 component, which cannot form normal ClpC1/ClpP1/ClpP2, via two different modes. Rufomycin 4-7 only generates slower diffused ClpC1 component that is inadequate to form normal ClpC1/ClpP1/ClpP2. Overall, we demonstrate that ecumicin and rufomycin 4-7 use different action mechanisms to generate abnormal ClpC1 components that cannot couple with ClpP1/ClpP2.

1. Introduction

M. tuberculosis ClpC1 recognizes unstructured proteins as substrates and unfolds substrates for maintenance of protein homeostasis in prokaryotes [1,2]. It also plays a pivotal role to maintain the protein homeostasis which is essential for the survival of *M. tuberculosis* through protein degradation by the ClpC1/ClpP1/ClpP2 complex [1,2]. This complex is assembled with the hexameric ClpC1 and the heptameric ClpP1 and ClpP2 [1,2]. Therefore, the intermolecular interaction of each component as well as the interaction among the ClpC1, ClpP1 and ClpP2 are important for protein degradation by this complex [3,4]. In addition, the characteristics of this complex provide insights for screening and developing novel antituberculosis agents [5].

Recently, ecumicin, rufomycin 4-7, and lassomycin, the antituberculosis compounds produced by actinomycetes, were found to inhibit the proteolytic activity of ClpC1/ClpP1/ClpP2 complex resulting in the death of *M. tuberculosis* [6–10]. The target protein of these compounds is ClpC1 [6–8]. Ecumicin and rufomycin 4-7, produced by *Nonomuraea* sp.

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MJN5123 and *Streptomyces atratus* MJN3502, respectively [6,8], are macrocyclic compounds that are biologically synthesized by non-ribosomal peptide synthetase (NRPS) but lassomycin is synthesized by the ribosome [6–8]. Ecumicin and rufomycin 4-7 bind to the *N*-terminal domain (NTD) of ClpC1 and inhibit the proteolytic activity of ClpC1/ClpP1/ClpP2 complex in a concentration-dependent manner [6,8]. Interestingly, ecumicin significantly stimulates the ATPase activity of ClpC1, whereas rufomycin 4-7 did not affect this activity [6,8]. This result raises the possibility of ecumicin and rufomycin 4-7 cause different effects on ClpC1 structure and ultimately lead to changes in the formation of ClpC1/ClpP1/ClpP2 complex. Previous studies either examined the oligomeric structure of ClpC1 and investigated the function of each domain [3] or the structural change of the *N*-terminal domain [11,12]. However, there are no publications discussing the entire ClpC1 structure in detail.

Therefore, the aim of this study was to investigate the intermolecular interaction of the fluorescence conjugated ClpC1 component by fluorescence correlation spectroscopy in the presence of either ecumicin or rufomycin 4-7. This was performed after constructing a recombinant ClpC1 through introducing a cysteine residue at the ClpC1 *C*-terminal for fluorescence labelling to confirm the biochemical activity of the ClpC1-cys ATPase.

2. Materials and Methods

2.1. Materials

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) when mentioned specifically.

2.2. Purification of ClpC1-cys for labelling by Atto 647 N

The *clpC*1 gene from *M. tuberculosis* H37Rv, which was cloned in pET28a, was kindly provided by the University of Illinois, Chicago (USA) [6]. ClpC1 was labelled with Atto 647 N maleimide and fluorescence labelled ClpC1 was monitored through Fluorescence correlation spectroscopy (FCS). The *clpC*1-cys gene was amplified to incorporate ACA nucleotides encoding cysteine using degenerated primers (Forward primer: 5′-TTCACATATG GCTTCGAACGATTTACCGACC-3′, Reverse primer: 5′-GTAAAAGCTTACACGCTCCAGCCTTGGCCAGATC-3′) by PCR and the *clpC*1-cys gene was retrieved using a PCR clean-up kit (BIONEER, Daejeon, South Korea). The *clpC*1-gene was cloned into pET21a (+) digested with *Nde*I (New England Biolals, Ipswich, MA) and *Hind*III (New England Biolals, Ipswich, MA). And the incorporation of cysteine into *clpC*1 gene was confirmed by DNA sequencing.

For ClpC1-cys expression, transformation of pET21(a)-*clpC*1-cys was performed in *Escherichia coli* BL21(DE3) (Invitrogen, Waltham, MA) as the expression host and clones were selected on an LB agar (Merck, Rahway, NJ) containing 100 μg/mL ampicillin. The clones were inoculated in LB medium containing 100 μg/mL ampicillin and left to grow by incubation at 37 °C while shaking with 200 rpm until the optical density of the culture broth reached 0.6 at A600 nm. Then, IPTG was added to final concentration of 1 mM into the culture broth for induction of ClpC1-cys and it was incubated overnight at 16 °C while shaking with 150 rpm.

To purify ClpC1-cys, an Ni-TED column kit (MACHEREY-NAGEL, Düren, Germany) was used following the instruction manual. Briefly, the cell pellet was collected by centrifuging the culture broth at 6,000×*g* for 10 min at 4 °C followed by suspension in Lysis-Equilibration-Wash buffer (LEW) containing a protease inhibitor cocktail. Subsequently, cells in the suspension were disrupted by sonication for 10 min on a 50% duty cycle at 50% amplitude. The supernatant was then retrieved by centrifuging the cell suspension at 12,000×*g* for 30 min at 4 °C. For removal of the cell debris, the supernatant was clarified by filtration using a 0.2-μm syringe filter. After loading the filtrate, the Ni-TED column was washed five times with 4 mL of LEW buffer. Finally, ClpC1-cys was purified with the elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, pH 8.0) and purity was verified by 10% SDS-PAGE (Supplementary Fig. 1).

2.3. Purification of ClpP1 and ClpP2 for the biochemical assay

The *clpP*1 and *clpP*2 gene from *M. tuberculosis* H37Rv, which was cloned into pET21(a), were kindly provided by the University of Illinois Chicago (USA) [6]. For the expression of *clpP*1 and *clpP*2, *E. coli* BL21 (DE3) were used as the expression host. Expression and purification of these proteins were carried out using the aforementioned method and purity was verified by 10% SDS-PAGE.

2.4. Labelling the purified ClpC1-cys with Atto 647 N maleimide

To label the purified ClpC1-cys with Atto 647 N maleimide, the thiol-maleimide crosslinking method was employed and the purified ClpC1-cys was labelled with Atto 647 N maleimide using a labelling kit (Jena Bioscience, Thuringia, Germany), according to the instruction manual [13]. Briefly, 10 mg/mL purified ClpC1-cys and Atto 647 N maleimide were prepared for dissolution in Tris buffer (pH 7.5) and dimethylformamide (DMF), respectively. Thereafter, 33 μL of the dye solution was added to 100 μL of the ClpC1-cys solution and incubated for 2 h at room temperature in the dark. After incubation, Atto 647 N maleimide-labelled ClpC1-cys was selectively collected by size exclusion chromatography with a Sephadex G-25 resin (Sigma-Aldrich, St. Louis, MO). The concentration of the conjugates was calculated following the instruction manual.

2.5. Determination of ClpC1-cys ATPase activity and ClpC-cys/ClpP1/ClpP2 proteolytic activity in presence of ecumicin and rufomycin 4-7

ClpC1-cys ATPase activity was measured using a BIOMOL Green reagent (Enzo Life Sciences, Farmingdale, NY) as modified Malachite green reagent [14,15]. Briefly, 1 μM of ClpC1-cys and 100 μM of ATP were added to the reaction buffer (pH = 7.5) containing 100 mM Tris, 200 mM KCl, and 8 mM $MgCl_2$ (final concentration). The 50 μL reaction mixture was incubated for 1 h at 37 °C. Afterwards, the content of free phosphate was detected at *A*620 nm after adding 100 μL of BIOMOL Green solution in a multi-well plate reader (Infinite® 200 PRO, TECAN, Männedorf, Switzerland). And the sample contained only ATP with the reaction buffer was used as the negative control in this assay.

To measure the ClpC1-cys/ClpP1/ClpP2 proteolytic activity, a reaction mixture containing 1 μM of ClpC1-cys and 2 μM of ClpP1 and ClpP2 was prepared. The substrate, fluorescein Isothiocyanate (FITC)-casein (final concentration = 1 μM, Thermo Fisher, Waltham, MA), was added followed by the addition of ATP (final concentration = 100 μM). The reaction buffer for the ClpC1/ClpP1/ClpP2 proteolytic activity was identical to the buffer for ClpC1-cys ATPase activity. We incubated 100 μL of the reaction mixture for 1 h at 37 °C. Afterwards, FITC-casein degradation was detected in a multi-well plate reader (Infinite® 200 PRO, TECAN, Männedorf, Switzerland) conditioned at ex485 nm/em538 nm. The sample containing only FITC-casein and another sample containing only protein with reaction buffer were used as the blank. And 1 μM of trypsin containing Pierce™ Fluorescent Protease Assay Kit (Thermo Fisher, Waltham, MA, US) was used as the positive control. Relative fluorescence unit was calculated through subtraction the fluorescence level of sample from the fluorescence level of sample containing only FITC-casein.

To examine the effect of ecumicin and rufomycin 4-7 on ClpC1-cys ATPase activity and ClpC1-cys/ClpP1/ClpP2 proteolytic activity, three different concentrations of ecumicin and rufomycin 4-7 were used (10 folds lower, the same, and 10 folds higher than those of the previously used ClpC1-cys). Ecumicin and rufomycin 4-7 were dissolved in dimethyl sulfoxide (DMSO) and treated with a volume equivalent to 1/50 of the total reaction volume to avoid protein denaturation.

Additionally, the effect of ampicillin as a non-binding molecule was investigated in ClpC1-cys ATPase activity and ClpC1-cys/ClpP1/ClpP2 proteolytic activity.

2.6. Monitoring the molecular dynamics of the ClpC1-Atto 647 N conjugate by fluorescence correlation spectroscopy (FCS)

The highly sensitive and versatile technique, FCS, is generally used for monitoring the dynamics of fluorescently labelled molecules in solution [16]. In the present study, FCS measurements were performed using an inhouse built confocal microscopy system. An A633 nm He–Ne laser (25-LHP-925, Melles-Griot, Carlsbad, CA) was used as the light source (1.3 kW/cm^2). The laser beam was reflected using a dichroic mirror (Z532bcm, Chroma, Bellows Falls, VT) on an inverted microscope Ti-U (Nikon Corporation, Tokyo, Japan) and focused at 20 μm from the sample chamber of the bottom coverslip through a 60x objective lens (NA = 1.2, CFI Plan Apochromat, Nikon Corporation, Tokyo, Japan). The fluorescence emissions of Atto 647 N were passed through a 100-μm pinhole to reduce the out of plane fluorescence for detection by the silicon avalanche photodiode detectors (APD) (SPCM AQR-13, EG&G Perkin Elmer). To remove any scattered laser light, a notch filter (NF03-633E-25, Semrock, NY) was placed in front of the APD. Sample measurements were performed in an imaging buffer (20 mM Tris-HCl, pH 7.5, 50 mM NaCl, 0.1 mg/mL NaCl, and 1 mM Cysteamine) at a 1 μs time resolution. We also measured the FCS signal of 10 nM Cy5 for the calibration of the system. The diffusion coefficient of a Cy5 has been measured to be 360 μm^2/s at 25 °C [17]. As a result, the structural parameter κ as the ratio of radial to axial in detection volume is determined as 6.53. The molecular dynamics of the sample were measured as described below. Briefly, 1 nM ClpC1-Atto 647 N conjugate and 100 nM ATP (final concentrations) were added to the reaction mixture. Thereafter, ecumicin and rufomycin 4-7 dissolved in DMSO were treated with 0.01, 0.1, 1, 10, and 100 nM, which 100 folds lower, 10 folds lower, same, 10 folds higher and 100 folds higher than the ClpC1 concentration, of the reaction mixture. When ecumicin and rufomycin 4-7 dissolved in DMSO were used as treatments, they were treated with a volume equivalent to 1/50 (2% DMSO final concentration) of the total reaction volume to avoid protein denaturation by DMSO. Only 11 μL of the 20 μL total reaction mixture was used for this experiment. Additionally, the effect of ampicillin as a non-binding molecule was investigated in this experiment.

For this experiment, a microfluidic chamber was manually assembled as described. Briefly, two coverslips (24′50 mm, No. 1.5 and 24′30 mm, No. 1) were soaked in acetone and treated for 30 min in a sonic bath. After washing each component with deionized water, they were soaked in 1 M of potassium hydroxide and treated for 30 min in a sonic bath. After a second washing with deionized water, the components were finally soaked in deionized water and treated for 30 min in a sonic bath. Subsequently, the components were completely dried with nitrogen gas by an air gun. To assemble the microfluidic device, a coverslip (24′50 mm, No. 1.5) was attached to one side of the Press to Seal Silicone (Thickness 2.5 mm) with a hole (diameter 6 mm) generated using a stationery single hole punching plier. Thereafter, 11 μl of the reaction mixture were added into the hole before attachment of the coverslip (24′30 mm, No. 1) to the other side of the Press to Seal Silicone without the occurrence of leakage.

When fluorophores diffuse in a small confocal volume by 3D Brownian motion, temporal fluctuations in the fluorescence emission occur due to changes in the number and kinetics of molecules [18]. By analyzing the fluorescence fluctuation autocorrelation, valuable information regarding molecular dynamics can be retrieved in the microsecond to second time range. The autocorrelation of the time trajectories of a fluorescent fluctuation can preserve information, such as the number of molecules in the probe volume and diffusion time related to the size of the molecule. A simplified three-dimensional form of the autocorrelation function is calculated as [18]:

$$G(t) = 1 + \frac{1}{N_p}\sum_{i=1}^{M}\frac{f_i}{(1 + t/\tau_{Di})(1 + t/\kappa^2\tau_{Di})^{1/2}}$$

where N_p is the number of molecules in the detection volume, τ_{Di} is the diffusion time of i_{th} molecule in detection volume, f_i is the fraction of i_{th} molecule, κ is the ratio of radial to axial in detection volume and M is the number of kinds of particles. The autocorrelation signal was fitted using the OriginPro 9.0 (OriginLab Corp., Northampton). First, the measured fluorescence intensity fluctuations of bare ClpC1 are fitted to above autocorrelation function G(t) with one particle version. And then, we calculated G(t) in presence of ecumicin and rufomycin 4-7 and could not fit with one particle version. For fitting with two particle version, we fixed one diffusion time as 0.45 msec obtained from bare ClpC1 and fitted the other diffusion time and numbers of molecules.

2.7. Hydrodynamic size of ClpC1-cys in the presence of either ecumicin or rufomycin 4-7

Dynamic light scattering method (DLS) is one of quick and useful methods to monitor the size of protein according to the diffusion behaviors of proteins so ClpC1-cys size was analyzed by DLS (Zetasizer Nano S, Malvern Panalytical, Malvern, UK) in presence of either ecumicin or rufomycin 4-7. Briefly, 200 nM of purified ClpC1-cys in the reaction buffer for ClpC1 ATPase activity was added in the 5 ml sample cuvette. For monitoring the effect of either ecumicin or rufomycin 4-7 on the size of ClpC1-cys, five different concentrations of ecumicin and rufomycin 4-7 were used (100 folds lower, 10 folds lower, the same, 10 folds higher and 100 folds higher than 200 nM of ClpC1-cys). The scattering was recorded at 25 °C for 50 s with 653 nm. A total of ten autocorrelation functions were recorded for samples, and intensity-weighted hydrodynamic diameters determined. And the effect of ampicillin as a non-binding molecule on hydrodynamic size of ClpC1-cys was investigated. In addition, the effect of ampicillin as a non-binding molecule was investigated in this experiment.

3. Results

3.1. ATPase activity of ClpC1-cys and proteolytic activity ClpC1-cys/ClpP1/ClpP2 complex in presence of ecumicin and rufomycin 4-7

For labeling ClpC1 with a fluorescence molecule, cysteine was introduced as an amino acid to the *C*-terminal of ClpC1. This protein was designated as ClpC1-cys. The introduction of an amino acid in a protein may affect its structure and activity. Thus, ClpC1-cys ATPase activity was determined according to ATP concentrations. Firstly, the purity of each protein was verified in 10% SDS-PAGE (Supplementary Fig. 1). The K_M values of ATP binding to ClpC1-cys and ClpC1 were 22.82 ± 2.73 μM and 24.34.76 ± 2.24 μM, respectively (Fig. 1A), and their V_{max} value was 0.0238 ± 0.001 μM/min and 0.237 ± 0.11 μM/min, respectively (Fig. 1A). Next, we monitored the ATPase activity of ClpC1-cys and the proteolytic activity of ClpC1-cys/ClpP1/ClpP2 complex in presence of ecumicin and rufomycin 4-7. Ecumicin stimulated the ClpC1-cys ATPase activity but rufomycin 4-7 did not affect this activity (Fig. 1B). Moreover, both compounds repressed the proteolytic activity of ClpC1-cys/ClpP1/ClpP2 complex (Fig. 1C). Thus, the addition of cysteine does not affect the wild type ClpC1. In addition, to test whether ampicillin as the compound not binding to ClpC1 can affect ClpC1-cys ATPase and ClpC1-cys/ClpP1/ClpP2 proteolytic activity, these activities of ClpC1-cys was determined in presence of ampicillin. Treatment of ampicillin did not affect ClpC1-cys ATPase and ClpC1-cys/ClpP1/ClpP2 proteolytic activity (Supplementary Figs. 2A and 2B). And, to examine whether ecumcin and rufomycin 4-7 selectively interact with ClpC1-cys in the proteolysis of ClpC1-cys/ClpP1/ClpP2 complex, we monitored the proteolytic activity of trypsin in presence of ecumicin and rufomycin 4-6. As a result, the proteolytic activity of trypsin was not changed regardless of

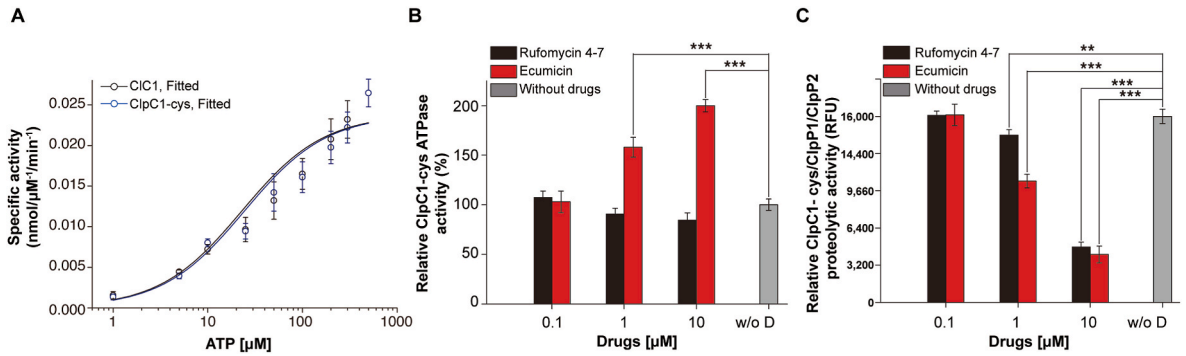

Fig. 1. Biochemical characteristics of ClpC1-cys. A) The specific ATPase activity of ClpC1-cys and ClpC1. The line was fitted by Michaelis-Menten equation. B) ATPase activity of ClpC1-cys in the presence of ecumicin and rufomycin 4-7. C) The Proteolytic activity of ClpC1-cys/ClpP1/ClpP2 in the presence of ecumicin and rufomycin 4-7. For this experiment, 1 μM of ClpC1 and ClpC1-cys were used individually. ** and *** indicate $P < 0.01$ and $P < 0.001$, as assessed using the paired *t*-test, respectively and all error bars indicate standard deviation. All experiments were carried out in triplicates.

treatment of ecumicin and rufomycin 4-6 (Supplementary Fig. 3).

3.2. Intermolecular dynamics of fluorescence conjugated ClpC1 in the presence of either ecumicin or rufomycin 4-7

After the conjugation of a fluorophore to ClpC1-cys, the intermolecular dynamics of the fluorescence conjugated ClpC1 was analyzed in the presence of either ecumicin or rufomycin 4-7. Results revealed that the monophasic ClpC1 component diffused with a diffusion time of 0.46 ms calculated by the simplified three-dimensional form of the autocorrelation function (Fig. 2 and Supplementary Table 1).

In the presence of ecumicin, the ClpC1 component diffused with a diffusion time of 0.46 msec and new component of ClpC1 as Component N2 were generated (Supplementary Table 1). In the presence of 0.01, 0.1 and 1 nM of ecumicin, the diffusion time corresponding to the new component of ClpC1 as Component N2 increased, compared with Component N1 (Supplementary Table 1). In particular, in the presence of 0.1 and 1 nM of ecumicin, the diffusion times of the Component N2 of ClpC1 were 97.5 ± 2.84 and 64.73 ± 5.46 msec, respectively (Supplementary Table 1), and the portions were 69.3% and 81.8%, respectively (Fig. 3A). These results indicate that the slower diffused ClpC1 component as Component N2 mainly inhibit the proteolytic activity of this complex as it may not be coupled to ClpP1/ClpP2 to form the ClpC1/ClpP1/ClpP2 complex. However, in presence of 10 nM, which is 10 folds higher concentration than ClpC1 in FCS experiment, of ecumicin, the diffusion time and the portion of the Component N2 of ClpC1 were dramatically reduced to 13.64 ± 1.54 msec and 16.5%, respectively (Fig. 3A). Interestingly, in Figs. 1 and 10 μM, which is 10 folds higher concentration than ClpC1in proteolytic activity of ClpC1-cys/ClpP1/ClpP2, of ecumicin was able to further reduce the proteolytic activity of the ClpC1/ClpP1/ClpP2 complex to over 80%. This indicates that the inhibition of the proteolytic activity of the ClpC1/ClpP1/ClpP2 complex mainly occurred due to the ClpC1 component with a diffusion time of 0.46 msec in the condition that the ecumicin concentration of ecumicin is 10 folds higher than ClpC1 concentration. Based on these results, ecumicin might abnormally influence the intermolecular association and dissociation of ClpC1 in a certain concentration and thus generate an abnormal component of ClpC1 that is unable to couple with ClpP1/ClpP2. Therefore, the interaction between ecumicin and ClpC1 induce the repression of the proteolytic activity of ClpC1/ClpP1/ClpP2 complex.

In the presence of rufomycin 4-7, the ClpC1 component diffused with a diffusion time of 0.46 msec and new component of ClpC1 as Component N2 were generated. In presence of 0.01, 0.1 and, 1 nM, which is 100 folds lower, 10 folds lower and same to the ClpC1 concentration, of rufomycin 4-7, the diffusion time and portion corresponding to the Component N2 of ClpC1 increased in a concentration dependent manner (Fig. 3B), similar to the effect of ecumicin. However, in presence of 10 nM, which is 10 folds higher than the ClpC1 concentration, of rufomycin 4-7, the diffusion time and portion of the Component N2 of ClpC1 component were 94.37 ± 3.2 msec and 62.1%, respectively (Fig. 3B). Based on these results, rufomycin 4-7 is only able to only generate an abnormal association of ClpC1 that is difficult to couple with the ClpP1/ClpP2 and consequently induce the repression of the proteolytic activity of ClpC1/ClpP1/ClpP2 complex, which differs from the effect of ecumicin. In addition, in the presence of 100 nM of ecumicin and rufomycin 4-7, the ClpC1 was nonspecifically aggregated by the hydrophobicity of these compounds so the amount of ClpC1 which was detected by FCS was dramatically reduced. Moreover, the diffusion time and portion of the Component N2 of ClpC1 component increased (Supplementary

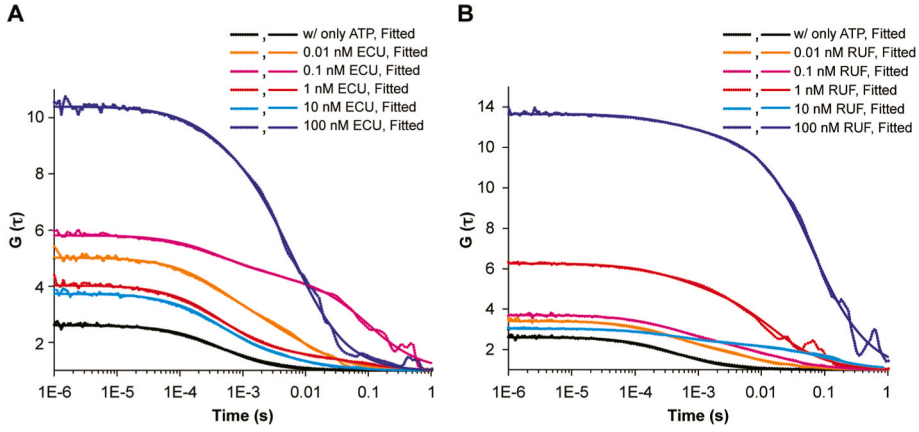

Fig. 2. Intermolecular dynamics of the ClpC1 in the presence of ecumicin and rufomycin 4-7. A) Intermolecular dynamics of the ClpC1 in the presence of ecumicin. B) Intermolecular dynamics of ClpC1 in the presence of rufomycin 4-7. For this experiment, 1 nM of fluorescence labelled ClpC1 was used. All graphs were fitted by the simplified three-dimensional form of the autocorrelation function described in Materials and Methods. ECU and RUF indicate ecumicin and rufomycin 4-7, respectively. This experiment was carried out in triplicates.

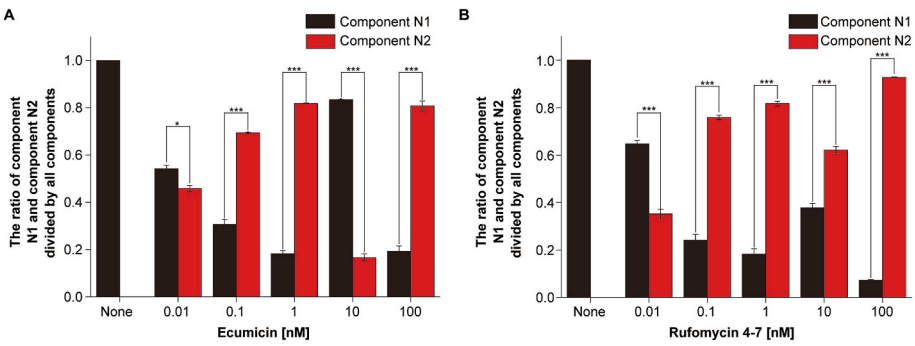

Fig. 3. The analysis of components of ClpC1 in the presence of ecumicin and rufomycin 4-7. A) The ratio of the components of ClpC1 in the presence of ecumicin. B) The ratio of components of ClpC1 in rufomycin 4-7. Component N1 and component N2 indicate that the diffusion time was to 0.045 msec and another time, respectively. All data were revisited from Supplementary Table 1 and the ratio of components were calculated through the division of the number of each component by total number of all components. N1 and N2 indicate the number of the components N1 and N2 of the ClpC1. *, ** and *** indicate $P < 0.05$, $P < 0.01$ and $P < 0.001$, as assessed using the paired *t*-test, respectively and all error bars indicate standard deviation. This experiment was carried out in triplicates.

Table 1 and Fig. 3). In addition, to test whether ampicillin as the compound not binding to ClpC1 can affect intermolecular dynamics of ClpC1, FCS experiment was carried out in presence of ampicillin. In all concentration of ampicillin, the component of ClpC1 diffused with 0.46 msec was over 95% and the other component of ClpC1 diffused with 4.37 ± 3.2 msec was below 5% (Supplementary Figs. 2C and 2D). So, treatment of ampicillin did not affect the intermolecular dynamics of ClpC1.

3.3. Hydrodynamic size of ClpC1-cys in the presence of either ecumicin or rufomycin 4-7

Based on the results through FCS experiment, the hydrodynamic size of ClpC1-cys was determined through dynamic light scattering (DLS) in presence of ecumicin and rufoymcin 4-7. 200 nM of ClpC1-cys was used for this experiment and the compounds like ecumicin and rufomycin 4-7 were treated to 100 folds lower, 10 folds lower, same, 10 folds higher and 100 folds higher than 200 nM of ClpC-cys. As a result, two different peaks of ClpC1-cys were generated without any compound and these peaks were still observed in presence of 2 nM of ecumicin (Supplementary Fig. 4A). However, in presence of 20 and 200 nM of ecumcin, one peak that the hydrodynamic diameter was approximately 1,000 nm was only observed (Supplementary Fig. 4A). Interestingly, in presence of 2 μM, which is 10 folds higher than ClpC1-cys concentration, of ecumicin, only one peak that observed without ecumicin and in presence of 2 nM of ecumicin dramatically increased (Supplementary Fig. 4A). Different of ecumicin, two different peaks were observed until 20 nM of rufomycin 4-7 and one peak peak that the hydrodynamic diameter was approximately 1,000 nm was only observed over 20 nM of rufomycin 4-7 (Supplementary Fig. 4B). And, in presence of 20 μM, which is 100 folds higher than ClpC1 concentration, of ecumicin and rufomycin 4-7, one peak peak that the hydrodynamic diameter was approximately 1,000 nm was only observed (Supplementary Fig. 4B). These results are quite similar to the change of ClpC1 components analyzed by FCS experiment (Fig. 3). In addition, to test whether ampicillin as the compound not binding to ClpC1 can affect hydrodynamic size of ClpC1, DLS experiment was carried out in presence of ampicillin. Treatment of ampicillin did not affect the hydrodynamic size of ClpC1 (Supplementary Fig. 2E).

4. Discussion

The ClpC1/ClpP1/ClpP2 complex corresponding to the ClpXP protease machinery in *M. tuberculosis* maintains protein homeostasis needed for survival [2,19]. The biochemical process of degradation of protein substrate by ClpC1/ClpP1/ClpP2 complex occurs when the ClpC1 recognizes, unfolds, and translocates the protein substrates to the ClpP1/ClpP2 component to degrade them [1,11,20]. Failure of protein homeostasis leads to the death of *M. tuberculosis,* thus, ClpC1 is a candidate drug target for the development of an antituberculosis agent [5]. Several compounds have been reported to target *M. tuberculosis* ClpC1 such as ecumicin, rufomycin 4-7, cyclomarin A, and lassomycin. This leads to the death of *M. tuberculosis* through affecting the ATPase activity of ClpC1 and the proteolytic activity of the ClpC1/ClpP1/ClpP2 complex [6–8,21]. This alternation of ClpC1 related biochemical activity occurs through binding of these compounds to the target protein. The binding induces structural alternation of ClpC1 similar to that induced by cyclomarin [12,22]. ClpC1 should bind with the hexameric form, an essential structure that unfolds and translocates the protein substrates to the ClpP1/ClpP2 component, which leads to the degradation of protein substrates in the ClpC1/ClpP1/ClpP2 complex, [1,11]. This study analyzed the relationship between the ClpC1 component and proteolytic activity of the ClpC1/ClpP1/ClpP2 complex in presence of ecumicin and rufomycin 4-7.

First, the biochemical ATPase activities of the recombinant protein ClpC1-cys and the proteolytic activity of the ClpC1-cys/ClpP1/ClpP2 complex were monitored. Our results conform with the previously reported biochemical activities regarding ClpC1 [6,8]. These results indicate that the introduction of a cysteine does not affect the structure of ClpC1.

Next, the intermolecular interactions of ClpC1 in presence of ecumicin and rufomycin 4-7 were monitored through FCS. The diffusion time of the Component N2 of ClpC1 increased by 19.9, 211.9 and 140.7 folds at 0.01, 0.1, and 1 nM of ecumicin, respectively. Similarly, the portion of the Component N2 of ClpC1 increased in the presence of ecumicin compared to the ClpC1 component without ecumicin and rufomycin 4-7 (Supplementary Table 1 and Fig. 3A). In terms of radius hydrodynamics, the size of ClpC1 component increased proportionally with diffusion time by Stokes-Einstein equation, compared to the component of ClpC1 without ecumicin and rufomycin 4-7 [23]. Accordingly, the repression of the proteolytic activity of the ClpC1/ClpP1/ClpP2 complex by different ecumicin concentrations can be explained. As previously reported, ClpC1 mainly exists in the hexameric form in the absence of ecumicin. Moreover, in the presence of ATP, a large fraction of ClpC1 is shifted towards the hexameric form [1,24]. In addition, in the absence of ecumicin, this hexameric form of ClpC1 can be coupled with the ClpP1/ClpP2 component to unfold and translocate the protein substrate to the ClpP1/ClpP2 component. Finally, this complex initiates the proteolysis of the protein substrate (Fig. 4). However, 0.01, 0.1, and 1 nM of ecumicin generated an abnormal component of ClpC1 which is larger than the hexameric ClpC1. This non hexameric abnormal component of ClpC1 is unable to couple with the ClpP1/ClpP2 component. Therefore, the proteolytic activity of the ClpC1/ClpP1/ClpP2 complex is repressed in the presence of ecumicin concentration lower than and equal to ClpC1 concentration (Fig. 4). Nonetheless, the diffusion time and portion of the Component N2 of ClpC1 were dramatically reduced by 10 nM, which is 10 folds higher than ClpC1 concentration, of ecumicin like the effect on the ClpC1 component in absence of ecumicin (Supplementary Table 1 and Fig. 3A). And the proteolytic activity of the ClpC1/ClpP1/ClpP2 complex was further repressed when ecumcin concentration was 10 folds

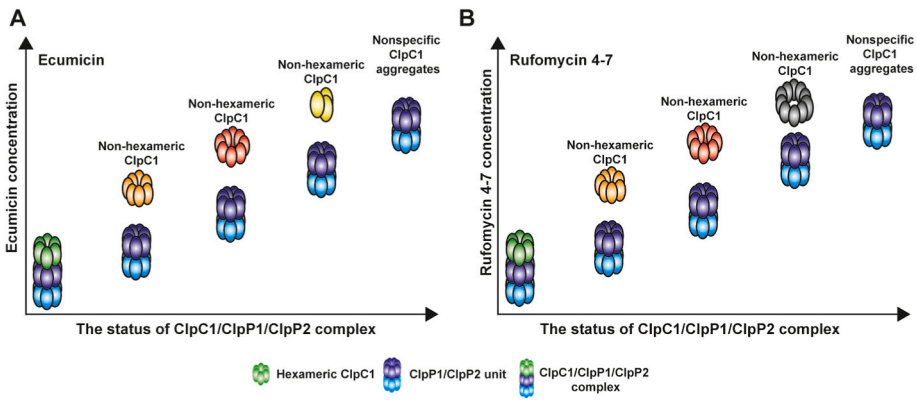

Fig. 4. The proposed mode of action of ecumicin and rufomycin 4-7 on the intermolecular interaction in ClpC1. A) Generation of non hexameric component of ClpC1 according to ecumicin concentration. B) Generation of non hexameric component of ClpC1 according to rufomycin 4-7 concentration. This figure displays how the non hexameric component of ClpC1 id generated by ecumicin and rufomycin 4-7 as shown in Supplementary Table 1 and Fig. 3. The non hexameric ClpC1 indicates ClpC1 that is larger and smaller than hexameric ClpC1 and is unable to couple with the ClpP1/ClpP2 unit and nonspecific ClpC1 aggregates. Nonspecific ClpC1 aggregates indicates ClpC1 aggregated by ecumicin and rufomycin hydrophobicity in extremely higher concentrations of the two compounds.

higher than ClpC1 (Fig. 1A). The coupling of ClpC1 to the ClpP1/ClpP2 component should be inhibited to repress the proteolytic activity of the ClpC1/ClpP1/ClpP2 complex. For this reason, the repression of the proteolytic activity of ClpC1/ClpP1/ClpP2 complex is mediated by the Component N1 of ClpC1, where the diffusion time is similar to the ClpC1 component in the absence of ecumicin, not Component N2 (Fig. 3A). Kar et al. reported that *M. tuberculosis* ClpC1 mainly exists in the monomeric form and it changes to the hexameric form in the presence of ATP, however, the non hexameric form of ClpC1 still exists even in presence of ATP [24]. Similarly, in our FCS experiment, the diffusion times of ClpC1 with or without ATP showed no significant difference even the proteolytic activity of ClpC1/ClpP1/ClpP2 complex is only active in presence of ATP (Supplementary Fig. 5). These results imply that ClpC1 components smaller than hexameric ClpC1 can exist even in presence of ATP. Thus, it can be assumed that the composition of the Component N1 of ClpC1 in the presence of 10 nM of ecumicin is different from that without ecumicin and further, the Component N1 of ClpC1 in the presence of 10 nM of ecumicin consists of the non-hexameric form that is smaller than the hexameric form, which is unable to couple with the ClpP1/ClpP2 component because the proteolytic activity of the ClpC1/ClpP1/ClpP2 complex was repressed in this presence of 10 nM of ecumicin. Alternatively, in the presence of rufomycin 4-7, the diffusion time and portion of the Component N2 of ClpC1 increased in a concentration dependent manner. Thus, the repression of the proteolytic activity of ClpC1/ClpP1/ClpP2 complex by rufomycin 4-7 can be mediated by the larger non hexameric ClpC1 component, which is unable to couple with the ClpP1/ClpP2 component.

Accordingly, the binding of ecumicin and rufomycin 4-7 to ClpC1 have different effects. Our findings are consistent with the findings on the crystallographic structures reported on the complexes of the *N*-terminal domain of ClpC1 (ClpC1-NTD) with ecumicin and rufomycin 4-7 [25]. In brief, the structure of the ClpC1-NTD-ecumicin complex (PDB entry 6 pbs) revealed a unique 1:2 (target:ligand) stoichiometric ratio, where the dual binding was related to the internal symmetry of the ClpC1-NTD target [25]. Based on this study, it can be elucidated that ecumicin binds to ClpC1 according to the ecumicin concentration (1:1 or 1:2) and these bindings can generate a larger or smaller non hexameric ClpC1 form that is hard to couple with the ClpP1/ClpP2 component (Figs. 3A and 4). In contrast, the structure of the ClpC1-NTD-rufomycin 4-7 complex (PDB entry 6cn8) revealed a 1:1 stoichiometric ratio (target:ligand) where the sulfur atom of Met1 was covalently linked to the open epoxide extending out from the indol group of the variant Trp residue in rufomycin 4-7 [25]. This suggests that rufomycin 4-7 only generates a larger non-hexameric ClpC1 form that is unable to couple with the ClpP1/ClpP2 component (Figs. 3B and 4). One of the limitations of this study is that we only monitored the intermolecular interaction of ClpC1 in the presence of ecumicin and rufomycin 4-7. Accordingly, in future studies we will carry out comprehensive analysis of the intermolecular interaction of ClpC1 in the presence of all of the compounds that target *M. tuberculosis* ClpC1. Additionally, the underlying reason behind the interaction of ecumicin and rufomycin 4-7 with ClpC1 with different stoichiometric ratio needs to be further investigated through monitoring the relationship of the intramolecular change of ClpC1 in presence of ecumicin and rufomycin 4-7. In conclusion, this study revealed that ecumicin and **rufomycin 4–7** generate abnormal ClpC1 components that are structurally incompatible with the ClpP1/ClpP2 and eventually repress the proteolytic activity of ClpC1/ClpP1/ClpP2. Both compounds act through different mechanisms of action although they both target ClpC1.

Author contribution

H.L. and J.-W.S. designed the research and conducted all the experiments. J.H. carried out the FCS experiment for monitoring the dynamics of ClpC1 and. N.M.D purified all the proteins and carried out biochemical assay using ClpC1, ClpP1 and ClpP2. G.S. performed cyclic peptides quality control experiment. B.-C.J. supported purification of all of the proteins. S.C., J.C. and H.L. oversee reviewing for this manuscript C.J. maintained FCS microscope and provided the method for analyzing the dynamics of ClpC1. H.L. wrote the manuscript. All of the authors discussed the results and commented on the manuscript.

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Declaration of competing interests

I declare that the authors have no competing interests or other interests that might be perceived to influence the results and/or discussion reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.tube.2022.102298.

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