Antibiotics

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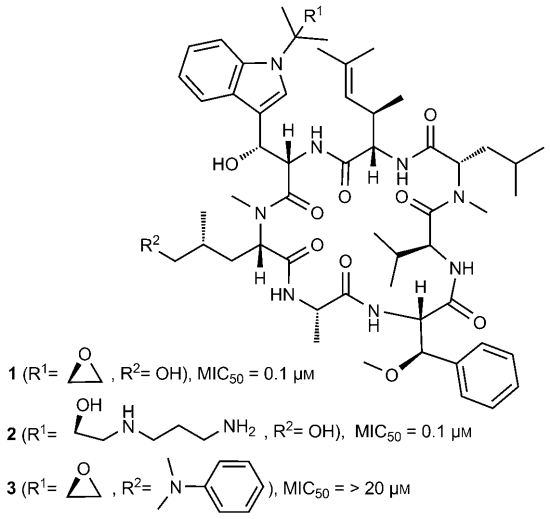
The Natural Product Cyclomarin Kills Mycobacterium Tuberculosis by Targeting the ClpC1 Subunit of the Caseinolytic Protease**

Esther K. Schmitt, Meliana Riwanto, Vasan Sambandamurthy, Silvio Roggo, Charlotte Miault, Christian Zwingelstein, Philipp Krastel, Christian Noble, David Beer, Srinivasa P. S. Rao, Melvin Au, Pornwaratt Niyomrattanakit, Viviam Lim, Jun Zheng, Douglas Jeffery, Kevin Pethe, and Luis R. Camacho**

In the course of a natural product whole-cell screen, the cyclic peptide antibiotic cyclomarin A was identified as a potent antitubercular compound (Scheme 1). Cyclomarin A (**1**)[1] proved to be bactericidal against *Mycobacterium tuberculosis* (*Mtb*) replicating in culture broth medium and in human-derived macrophages. The cidal concentration was determined as 0.3 and 2.5 μM, respectively (Figure 1 in the Supporting Information). In addition, **1** was active against a panel of multidrug-resistant clinical isolates of *Mtb* (Table 1 in the Supporting Information), thus indicating that it acts through a novel mechanism. No bactericidal activity of **1** was found in a panel of Gram-positive and Gram-negative organisms including *Staphylococcus aureus* and *Pseudomonas aeruginosa* (Table 1 in the Supporting Information). This interesting antibiotic profile of **1** prompted us to investigate its effect on *Mtb* in more detail.

Antituberculosis drugs should rapidly kill replicating mycobacteria, but also have a sterilizing activity on persistent, nonreplicating cells. These nongrowing bacilli are phenotypically drug-resistant and therefore lengthy tuberculosis drug regimens are required to obtain curative effects.[2] Importantly, **1** was highly active against hypoxic nonreplicating *Mtb* in the Wayne model[3] and killed more than 90% of the initial inoculum in five days at 2.5 μM concentration. The ability to kill both growing and nonreplicating mycobacteria is shared with only few recently described antituberculars.[4] Thus the antibiotic effect of 1 has to be a novel mechanism essential for bacterial growth in replicating and dormant cells of *Mtb*. The identification of the molecular target would enable further antitubercular drug discovery.



Scheme 1. Cyclomarin A (**1**) and the aminoalcohol **2** inhibit the growth of *Mtb*, whereas the methyl-phenylamine derivative **3** is inactive. Aminoalcohol **2** is made in a one-step reaction of **1** with 1,3-diaminopropane. The tosylate of **1** reacts readily with methyl-phenylamine to yield **3**.

Typically, spontaneous resistant mutants against an antibiotic can be raised and the mutations can be mapped to potential target genes by genome sequencing. However, in the case of **1** no spontaneous resistant mutants of *Mtb* could be isolated, thus suggesting a plausible frequency of mutation $<10^{-9}$. Next, an affinity chromatography approach was undertaken with the goal to identify the target through chemical proteomics. The *Mtb* active aminoalcohol derivative of cyclomarin **2** (Scheme 1) was covalently linked to sepharose beads and co-incubated with a mycobacterial cell lysate both in the presence and absence of free **2** or **1**. Bound proteins were eluted and separated by SDS-PAGE. A protein of an apparent molecular weight of 93 kDa was identified in

[*] Dr. E. K. Schmitt, Dr. S. Roggo, C. Miault, C. Zwingelstein, Dr. P. Krastel
Novartis Institutes for BioMedical Research, Novartis Campus
4056 Basel (Switzerland)
Fax: (+41) 61-43-279
E-mail: esther.schmitt@novartis.com
M. Riwanto, Dr. V. Sambandamurthy,[+] Dr. C. Noble, Dr. D. Beer, S. P. S. Rao, M. Au, P. Niyomrattanakit, V. Lim, Dr. J. Zheng, Dr. K. Pethe, Dr. L. R. Camacho
Novartis Institutes for Tropical Diseases
10 Biopolis Road, 05-01 Chromos, Singapore 138670 (Singapore)
Fax: (+65) 6722-2917
E-mail: luis.camacho@novartis.com
Dr. D. Jeffery
Novartis Institutes for BioMedical Research Inc.
500 Technology Square, Cambridge MA 02139 (USA)
[+] Current address: AstraZeneca India Pvt Ltd.
Bellary Road, Hebbal, Bangalore 560024 (India)

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the absence of free **2** or **1**. The protein was extracted from the gel, trypsin digested, and subjected to mass spectrometry. Proteomic analysis identified it as ClpC1 (Rv3596c), an ATPase belonging to the Clp/Hsp100 AAA+ superfamily (ATPases associated with various cellular activities). The ClpC proteins function as regulatory subunits of the caseinolytic Clp protease. It is a chaperone that unfolds protein substrates in an ATP-dependent manner before channeling them to the ClpP protease subunit of the complex.[5] The Clp complex typically consists of a sandwich of two heptameric rings of ClpP protease units flanked by two hexameric rings of Clp ATPases, thus structurally and functionally resembling the eukaryotic proteasome. In bacteria, AAA+ proteins play a crucial role in protein quality control by removing unfolded proteins from the cell. In addition, AAA+ proteins have been described to specifically control the turnover of regulatory proteins and thus play a key role in developmental processes and virulence in pathogenic bacteria such as *S. aureus*.[6] In *Mtb* the Clp complex has not been studied extensively.[7] Activators and inhibitors of the proteolytic subunit ClpP have been described in *S. aureus* and other Gram-positive bacteria.[8] To the best of our knowledge cyclomarin A is the first small molecule described to date to modulate the activity of the regulatory subunit ClpC of the Clp complex.

The specificity of the protein–compound interaction was analyzed by affinity chromatography combined with competition experiments using free compounds preincubated with cell lysate. Both free **1** and **2** reduced the amount of captured protein by 50% at a concentration of 10 nM, thus suggesting a specific and high affinity binding of both compounds (Figure 1a). Unrelated cyclic peptides like thiopeptide GE2270 and cyclosporin had no influence even at concentrations of 10 μM. Neither ATP nor the nonhydrolyzable γ-S-ATP competed for binding at a concentration of 10 mM. To the contrary, γ-S-ATP increased the amount of captured ClpC1 protein, probably owing to stabilization of the hexameric complex in the protein lysate.[7] From these experiments it can be concluded that **1** binds specifically and with high affinity to ClpC1 and does not interfere with ATP binding by the two ATPase domains of ClpC1.

The compound–protein interaction was confirmed using purified, recombinant ClpC1 in isothermal titration calorimetry experiments (Figure 1b). The data fitted to an equilibrium dissociation constant of 16 nM, thus indicating that the aminoalcohol **2** binds to ClpC1 with high affinity. The stoichiometry of the interaction was 1:1 (Table 2 in the Supporting Information). Importantly, the antimycobacterial activity of cyclomarin-derivatives correlated well with binding to ClpC1. Binding to ClpC1 was not detected for the inactive methyl-phenylamine derivative **3**.

To further study the effect of **1** on the Clp complex we employed a protein degradation assay in *Mycobacterium smegmatis*. This fast growing mycobacterium is sensitive to **1** ($MIC_{50} = 0.6$ μM) and possesses Clp proteins exhibiting >90% identity to the *Mtb* Clp proteins. The Clp ATPases recognize their substrates by binding to an 11 amino acid peptide sequence, which was used to tag green fluorescent protein (GFP-LDD).[9] A reduction of fluorescence by 40%

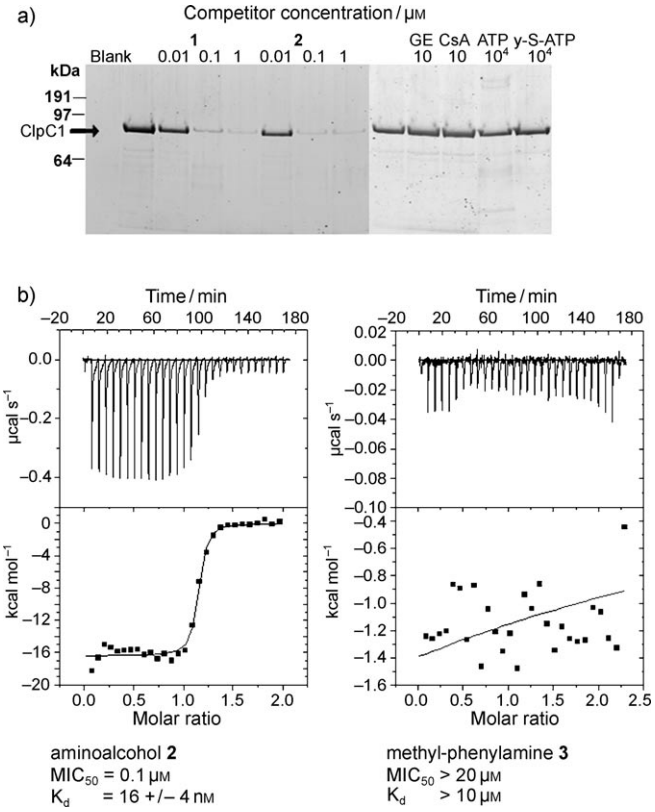

Figure 1. Cyclomarin A (**1**) binds specifically and with high affinity to ClpC1. a) SDS-PAGE gels of mycobacterial protein lysate stained with SYPRO Ruby dye. Lysate (1 mg), prepared from *M. bovis* BCG culture with OD_{600} value of 1.5, was incubated for 2 h at 4°C with different concentrations of free **1**, **2**, thiopeptide GE2270E (GE), cyclosporin A (CsA), ATP, and γ-S-ATP prior to incubation with immobilized **2** (1.4 μmol mL^{-1} NHS-sepharose beads, GE Healthcare). b) Isothermal titration calorimetry experiments were performed using an AutoITC (MicroCal) at 30°C, with 10 μM ClpC1 (in 20 mM Tris-HCl pH 7.8, 100 mM NaCl, 0.5 mM TCEP) in the sample cell. 28 aliquots of 10 μL of 100 μM compound solutions (90% DMSO) were injected into the sample chamber at 6 min intervals. The data were fitted to a single-site binding equation. DMSO = dimethyl sulfoxide, SDS-PAGE = sodium dodecylsulfate–polyacrylamide gel electrophoresis.

was observed in the bacteria expressing GFP-LDD in the presence of cyclomarin, whereas bacteria expressing untagged GFP showed a decrease of only 10% (Figure 2). These results suggest that cyclomarin specifically increases proteolysis mediated by the caseinolytic protease inside the cell.

Previously, *clp*C1 has been indicated as an essential gene in *Mtb* using a genome-wide transposon mutagenesis.[10] This supports our finding that a *clp*C1 knock-out in *Mtb* was successful only when a plasmid-born copy of the *clp*C1 gene was provided in trans (Figure 2 in the Supporting Information). Taken together this observation suggests that ClpC1 is indeed essential for bacterial growth.

In conclusion, ClpC1 is the protein target of the natural product antibiotic cyclomarin in *Mtb*. By using chemical proteomics it could be demonstrated that interference with the function of the ClpC1 ATPase with a noncompetitive small molecule is bactericidal in actively growing and in hypoxic nongrowing mycobacteria. The frequency of sponta-

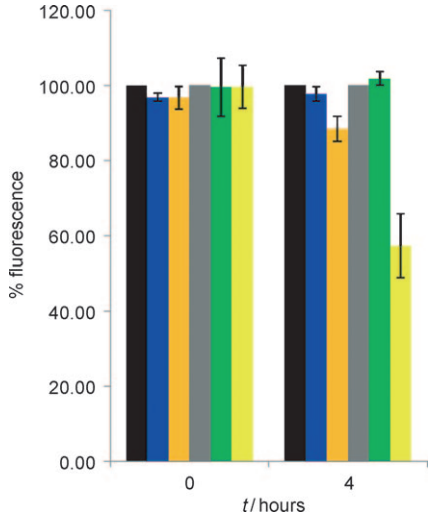

Figure 2. Cyclomarin stimulates intracellular degradation of GFP-LDD. *M. smegmatis* cultures expressing genes for GFP and GFP-LDD were grown to an OD_{600} value of 0.1. At this point, sub-lethal concentrations of **1** (0.3 μM) or Ethambutol (0.4 μM) were added and GFP fluorescence was determined at 0 and 4 h. The fluorescence of the untreated control was set at 100%. The data was obtained in duplicate from three independent experiments and standard deviation calculated after normalization to the control. Black: GFP untreated, blue: GFP ethambutol, orange: GFP **1**, gray: GFP-LDD untreated, green: GFP-LDD ethambutol, yellow: GFP-LDD **1.**

neous mutations rendering *Mtb* resistant to cyclomarin is extremely low and the *clp*C1 gene could be shown to be essential for *Mtb* growth. The exact binding site for **1** on ClpC and the molecular mechanism of action remain to be elucidated. Our results suggest that cyclomarin exerts its action through an allosteric binding site and does not compete with ATP binding. It is tempting to speculate that structural changes in the hexameric ring upon binding of **1** lead to a functional change of the protein complex and an increased proteolysis. Taken together, these results prove that interference with ClpC1 is a promising approach for the development of new antitubercular therapies.

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