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Dual mechanism of action of the atypical tetracycline chelocardin

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ABSTRACT

Classical tetracyclines targeting the protein biosynthesis machinery are commonly applied in human and veterinary medicine. The development and spread of resistance seriously compromise the successful treatment of bacterial infections. The atypical tetracycline chelocardin holds promise as it retains activity against tetracycline-resistant strains. It has been suggested that chelocardin targets the bacterial membrane, thus differing in mode of action from that of classical tetracyclines. We investigated the mechanism of action of chelocardin using global proteome analysis. The proteome profiles after sublethal chelocardin stress were compared to a reference compendium containing antibiotic response profiles of *Bacillus subtilis*. This approach revealed a concentration-dependent dual mechanism of action. At low concentrations, like classical tetracyclines, chelocardin induces the proteomic signature for peptidyl transferase inhibition demonstrating that protein biosynthesis inhibition is the dominant physiological challenge. At higher concentrations *B. subtilis* mainly responds to membrane stress indicating that at clinically relevant concentrations the membrane is the main antibiotic target of chelocardin. Studying the effects on the membrane in more detail, we found that chelocardin causes membrane depolarization but does not lead to formation of large pores. We conclude that at growth inhibiting doses chelocardin not only targets protein biosynthesis but also corrupts the integrity of the bacterial membrane. This dual mechanism of action might prove beneficial in slowing the development of new resistance mechanisms against this atypical tetracycline.

1. Introduction

Tetracyclines are a commonly applied class of antibiotics. These broad-spectrum antibiotics inhibit protein biosynthesis by preventing binding of aminoacyl-tRNAs to the acceptor site of ribosomes [1]. The widespread resistance mechanisms against classical tetracyclines are efflux pumps, ribosome protection proteins, drug modification mechanism, and rRNA mutations [2]. Much effort is put into the development of tetracyclines as reflected by new tetracycline derivates for specific infections, which recently entered clinical trials, such as omadacycline, sarecycline, and eravacycline [3]. The latter one belongs to the new fluorocycline tetracycline subclass with activity against bacteria with tetracycline-specific efflux pumps and ribosomal protection proteins [4].

Another group of tetracyclines, namely atypical tetracyclines, also feature broad-spectrum activity. These atypical tetracyclines have virtually been neglected due to suboptimal pharmacological profiles. One member of this group is chelocardin. Chelocardin retains most of its activity against strains with efflux pumps and ribosome protection proteins [5,6]. It proved non-toxic in rats and dogs [7] and was efficacious in a small clinical study involving twelve patients suffering from urinary tract infections [7]. Only one minor adverse event was reported and all patients were successfully treated, including three patients with tetracycline resistant infections. First attempts are being made to improve the pharmacological profile of this class. The biosynthetic gene cluster for chelocardin in the producing organism *Amycolatopsis sulphurea* was recently identified [8] and the strain was engineered to produce the first novel chelocardin derivates [9].

To date the mode of action of chelocardin and other atypical tetracyclines is discussed controversially in the literature. While classical tetracyclines have a bacteriostatic mode of action, chelocardin is bactericidal [10]. It was first suggested that chelocardin and other atypical tetracyclines share the same mechanism of action as classical tetracyclines. Chelocardin for instance showed significant binding to the rRNA of the ribosomal subunits, especially the 30S ribosomal subunit [11]. Similar conclusions were drawn from another more recent study, which analyzed the induction of biosensors for various types of stress to determine the mechanism of action of different antibiotics. Another atypical tetracycline, anhydrotetracycline, strongly activated biosensors for protein biosynthesis inhibition. However, a slight activation of the cell envelope stress markers was also observed [12]. The idea that atypical tetracyclines primarily affect the membrane arose when no inhibition of cell-free protein biosynthesis by chelocardin or anhydrotetracycline was detectable using *Escherichia coli* or *Bacillus subtilis* cell extracts. Precursor incorporation assays indicated an inhibition of DNA, RNA, and protein biosynthesis *in vivo*, a phenomenon typically observed for membrane-active

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antibiotics, where protein biosynthesis inhibition may be a secondary effect caused by energy limitation due to membrane damage [10,13].

Structural differences between classical and atypical tetracyclines are consistent with differences in mechanism of action. The classical tetracyclines can exist in two different forms at physiological pH [14], the lipophilic non-ionized form allows passage through the membrane and the hydrophilic zwitterionic form most likely is important for ribosomal interaction [15]. In contrast, it is reported that chelocardin solely exists in the lipophilic non-ionized form [6,13].

Taken together, the mode of action of chelocardin remains to be fully elucidated and is controversially discussed in the literature. We explored the mechanism of action of chelocardin using a global proteomic approach. This global method utilizes the fact that bacteria respond to sublethal antibiotic stress by expressing proteins that counteract the impact of antibiotics on bacterial physiology. These proteins can be used as marker proteins that mirror antibiotic mechanisms of action [16]. The interpretation of the response profiles is performed by pattern-matching with a proteomic response library based on the Gram-positive model organism *B. subtilis* that encompasses marker proteins for well over 50 antibiotics acting on different cellular pathways including protein biosynthesis and cellular structures like the cytoplasmic membrane [17–20]. In this study, we compared the proteomic response to different concentrations of chelocardin to the reference profiles in the library to investigate what physiological challenge the bacteria primarily respond to. Subsequently, we studied the cellular distribution of chelocardin using autofluorescence and investigated membrane integrity using fluorescent probes for membrane depolarization and a pore formation assay.

2. Materials and methods

2.1. Antibiotics

Antibiotic stock solutions with concentrations of 10 mg/ml were prepared as follows: tetracycline hydrochloride (Serva Electrophoresis, Heidelberg, Germany) was dissolved in 70% ethanol, valinomycin (Sigma-Aldrich, St. Louis, MO, USA) in DMSO, nisin in 0.01 M HCl (Sahl, Bonn, Germany, purified according to Bonelli et al. [21]), and chelocardin hydrochloride (purified according to Lukežič et al. [8]) in methanol. Dilutions were prepared in distilled water.

2.2. Bacterial strains and growth conditions

B. subtilis 168 (*trpC2*) [22] was grown in Belitzky minimal medium (BMM) [23] under steady agitation at 37 °C. Minimal inhibitory concentrations (MICs) were identified in a test tube assay as described before [17]. The MIC is defined as the lowest antibiotic concentration preventing visible growth of 5×10^5 cells/ml in 2 ml BMM after incubation at 37 °C for 18 h.

For growth experiments, logarithmic bacterial cultures were cultivated in BMM to an optical density at 500 nm (OD_{500}) of 0.35 and were subsequently treated with various antibiotic concentrations to identify concentrations that reduce the growth rates of exponentially growing cultures without being lethal. In addition, samples for a determination of colony forming units (cfus) were collected directly before and 15 min after antibiotic stress. Serial dilutions were prepared in BMM, before plating on BMM plates containing 1.5% agar. Colonies were counted after incubation for 2 days at 37 °C.

2.3. Preparation of cytoplasmic L-[^{35}S]methionine labeled protein extracts

Pulse-labeling of newly synthesized proteins was performed as described recently [18] to study the bacterial response to tetracycline and chelocardin. Briefly, after reaching an OD_{500} of 0.35, 5 ml aliquots of bacterial cultures were treated with 5.5 μg/ml tetracycline, or with 3, 6, or 12 μg/ml chelocardin for 10 min. One aliquot was left untreated as a control. Proteins newly synthesized were labeled radioactively during a 5 min pulse of 1.85 MBq L-[^{35}S]methionine (Hartmann Analytic, Braunschweig, Germany). Subsequently, 100 μg/ml chloramphenicol, 1 mM non-radioactive L-methionine and 10 mM Tris, pH 7.5, were added and the aliquots were transferred onto ice to stop protein biosynthesis. Cells were washed twice with 100 mM Tris/1 mM EDTA pH 7.5, then resuspended in 10 mM Tris buffer containing 1.4 mM PMSF and disrupted by ultrasonication using the VialTweeter system (Hielscher, Teltow, Germany). The soluble protein fraction was separated from the cell debris by centrifugation. A Bradford assay (Roti NanoQuant, Roth, Karlsruhe, Germany) was used to analyze the protein concentration of the samples. Protein biosynthesis rates were determined by measuring the incorporation rates of radioactive methionine with a scintillation counter after precipitation of proteins with 20% trichloroacetic acid.

2.4. 2D-PAGE

2D-PAGE experiments were carried out as published recently [18]. In brief, to ensure high reproducibility of protein separation, equal amounts of protein (55 μg of labeled proteins for analytical gels or 300 μg of unlabeled proteins for preparative gels) were loaded onto 24 cm immobilized pH gradient strips pH 4–7 (GE Healthcare, Chalfont St Giles, UK). Isoelectric focusing and 2D-PAGE were performed with the Multiphor II and the Ettan DALTtwelve system (GE Healthcare), respectively. Gels were stained with 0.003% ruthenium(II)tris (4,7-diphenyl-1,10-phenantroline disulfonate) [24] and protein profiles were imaged using a Typhoon Trio$^+$ Variable Mode Imager (GE Healthcare) with an excitation wavelength of 532 nm and a 610 nm emission filter. Analytical gels were dried on Whatman paper and exposed to Storage Phosphor Screens (GE Healthcare), which were scanned using the Typhoon Trio$^+$ with a setting of 633 nm excitation wavelength and a 390 nm emission filter. Exposure times were adjusted based on radioactivity incorporation rates. The Decodon Delta 2D image analysis software (Decodon, Greifswald, Germany) was used for quantitation and visualization as described previously [25]. Signal intensities for each protein spot were quantified relative to the sum signal intensity of all protein spots. These relative synthesis rates reflect the fraction of the cell's entire synthesis capacity that is allocated to the synthesis of the proteins present in a given protein spot. Marker proteins were defined as proteins identified in protein spots with at least two-fold increased relative synthesis rates in each of three independent biological replicates, each of which was represented by one gel.

2.5. Identification of marker proteins by nUPLC–ESI–MS/MS

A Synapt G2-S high definition mass spectrometer equipped with a lock spray source for electrospray ionization and a ToF detector (Waters, Milford, MA, USA) were used to identify protein spots as described recently [26]. Protein spots were excised manually, destained with 20 mM ammonium bicarbonate in 30% acetonitrile, and digested with 6.25 ng/μl or 12.5 ng/μl trypsin (Promega, Fitchburg, WI, USA) depending on the protein abundance for 16 h at 37 °C. Peptides were eluted with 0.1% trifluoroacetic acid in ultrapure water by ultrasonification for 15 min. In the case of low-intensity protein spots, the elution was performed twice and eluates were concentrated by vacuum centrifugation. First, eluates were loaded onto a trap column (C_{18}, pore size 100 Å, particle diameter 5 μm, inner diameter 180 μm, length 20 mm). After that they were eluted from an analytical column at 50 °C (C_{18}, pore size 130 Å, particle diameter 1.7 μm, inner diameter 75 μm, length 150 mm) using a 0.1% formic acid to acetonitrile gradient (350 μl/min, linear gradient 2–5% acetonitrile in 2 min, 5–60% acetonitrile in 20 min) and directly subjected to mass spectrometry. Spectra were recorded in positive resolution mode over a mass range of 50 to 1800 m/z with 0.5 s/scan. The following parameters were used for the NanoLockSpray source: capillary voltage, 2.7 kV; sampling cone voltage, 30 V; source temperature, 70 °C; desolvation temperature, 150 °C; cone