Witnessing the process of bacterial cell death: novel antimicrobials and their mechanisms of action

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Appendix

SUMMARY

This thesis describes the antimicrobial discovery strategy developed in our group, the den Hertog Group at the Hubrecht Institute. It includes a cultivation-based screening approach for novel antimicrobial agents from the source of fungi, and a bacterial time-lapse imaging approach for antimicrobial mechanism of action (MoA) identification. With this strategy, we have discovered several interesting antimicrobial agents and have demonstrated the detailed antimicrobial property of two of them, berkchaetoazaphilone B (BAB) and harzianic acid (HA).

In **Chapter 1**, we briefly introduce the antimicrobial resistance issue of Gram-positive bacteria. We review information about current status of antimicrobial resistance, which suggests that we have a strong need for novel antimicrobials. Next, we discuss the value of fungal natural products, and their potential for antimicrobial discovery process. We compare the differences between Gram-positive and Gram-negative bacteria, and demonstrate the reasons why we aimed to search for novel antimicrobials against Gram-positive bacteria and why we chose *Bacillus subtilis* as a model for this study. In the end, we provide information on the scope and the outline of this thesis.

In **Chapter 2**, we review the advances in MoA identification strategies that were commonly used over the past 20 years, and provide insight into overcoming this bottleneck step for antimicrobial discovery. We discuss the advantages and disadvantages in both classic and modern approaches. Due to the limitations in almost every single method, the most appropriate strategy has to be selected depending on the expertise of the lab and the properties of the antimicrobial agent.

In **Chapter 3**, we demonstrate the approach that we used to screen fungal natural antimicrobial compounds. We screened for antimicrobial activity using a fungal secondary metabolites library against seven pathogenic bacteria and tried to identify the active compounds using ethyl acetate extraction, HPLC fractionation together with chemical analysis. This approach resulted in 280 antimicrobial hits from 10,207 fungi and the identification of 17 compounds from 26 strains with a re-screening of 56 fungi. The identified metabolites consist of both known antimicrobial compounds as well as relatively unexplored compounds. Among these identified antimicrobials, one compound, BAB is an interesting antimicrobial agent, which was found to affect bacterial energy metabolism.

In **Chapter 4**, we describe a novel antimicrobial MoA identification approach that was developed in our group. This approach is able to rapidly distinguish the effect of anti-Gram-positive bacterial compounds from different classes. To achieve

this, we developed a novel imaging strategy using time-lapse imaging to record dynamic bacterial cytological changes. We improved the imaging protocol to make it simple and functional for bacterial long-term imaging. Using this method, dubbed Dynamic Bacterial Cytological Profiling (DBCP), we observed bacteria over time and established fluorescence intensities qualitatively and quantitatively. It allowed to rapidly distinguish 14 antimicrobials from all of the five main antimicrobial classes. Finally, we used DBCP to establish the MoA of HA, a poorly described secondary metabolite purified from the fungal culture of *Oidiodendron flavum*. We conclude that DBCP is an excellent tool for the first approach of antimicrobial MoA classification.

In **Chapter 5**, we further describe the antimicrobial property of HA on its inhibitory spectrum, MoA and resistance information, discover HA to be a multi-targeting antimicrobial agent against Gram-positive bacteria. It targets the cell membrane, but only at high concentrations. We developed a HA-resistant strain, M9015, and discovered that mutant colonies had a more translucent appearance than wild type, which might be due to reduced cell size. The M9015 strain showed cross resistance to rifampin. However, it remains to be determined whether HA also belongs to the RNA class of antimicrobials. M9015 harbors five mutations in the coding region of four distinct genes. Although further analysis of these genes might still be required to generate more insight into the MoA of HA at low concentrations, the existing data have already revealed the value of HA to be a potential candidate for clinical application.

Finally, we summarize the discussions of this thesis with future perspectives in **Chapter 6**. We have provided a pipeline of antimicrobial discovery with detailed strategies from the upstream of screening fungal natural products to the downstream of MoA identification. This thesis contributes to obtain new insight, valuable for antimicrobial research.