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Summarizing Discussion

CHAPTER 6

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Revisited insight into the traditional antimicrobial discovery strategy

Despite all the benefits from antimicrobial therapies, we cannot ignore the issue that these therapies have contributed to antimicrobial resistance [1–4]. In **Chapter 1**, we have introduced this issue briefly. The infections by multidrug resistant "superbugs" are causing higher patient mortality and rising costs, resulting in high pressure on healthcare systems around the world [5,6]. To facilitate lifting this issue, our lab was motivated to establish a project on searching for novel antimicrobials, preferably ones with a new mechanism of action (MoA). We have chosen fungal natural products as the source for this purpose, with one of the reasons being the adjacency to the Westerdijk Fungal Biodiversity Institute, which is one of the biggest fungal collection centers in the world. Additionally, natural products have had long and important applications in medical treatments through history [7,8], although pharmaceutical companies have been less interested in natural antimicrobial discovery recently [9]. We want to attract their attention back to the promising field of fungal natural products by exhibiting some encouraging antimicrobial candidates from our screen.

In **Chapter 3**, we have described our antimicrobial discovery strategy in detail. In spite of the popularity of genomics and bioinformatics in biosynthetic gene cluster screening [10], we have still chosen to take advantage of the traditional cultivationbased screening method, because this method is straightforward and provides direct insight into antimicrobial activity, and genome data were not always available particularly for less studied species, which actually have a high chance to produce novel metabolites. After screening for antimicrobial activity of more than 10,000 fungal species on several pathogenic bacteria, including both Gram-positive strains and Gram-negative strains, the positively scored fungi were then ready for further analysis. Among this fungal library, we have found hundreds of hits, including potent antimicrobial hits that inhibit the growth of multiple pathogenic strains and selective antimicrobial hits that only inhibit one of these bacterial strains. However, only approximately 3% of the fungi scored positive. This might due to the resistance of the tested pathogenic bacteria. Another, perhaps more convincing reason might be the low expression level of antimicrobial gene clusters, which might have led to low amount of antimicrobial activity in the fungal supernatant. Low levels of antimicrobial compounds hampered the downstream identification process as well, but we increased the antimicrobial yield for many strains by changing the culture conditions, e.g. using plate culture. Fungi regulate their secondary metabolite production under different conditions [11–13]. Therefore, to establish a new fungal library by plate extractions, might be a good future plan to explore the hidden sites of the fungal kingdom. Another promising plan might be to start new screens by activating the silent genes regarding secondary metabolite producing by co-culturing with inhibitors, e.g. suberoylanilide hydroxamic acid or anacardic acid. These inhibitors were also proven to generate new metabolite maps in fungi [14,15]. With increasing sequencing data being available in the future, genomics will certainly become powerful tools regarding fungal metabolite discovery. However, the cultivation-based strategies seem to be promising and readily available tools to explore antimicrobials among fungal natural products at the moment.

Next, to identify the antimicrobial activity from the obtained hits, we chose the model organism of Gram-positive bacteria, Bacillus subtilis, for the purpose of rescreening with the following reasons: In **Chapter 1**, we have discussed the distinctive structures between Gram-positive and Gram-negative bacteria, which makes Grampositive strains to be more sensitive to antimicrobial compounds [16,17]. Thus, using a Gram-positive organism as a read-out might reduce the chance to omit potential antimicrobials. Furthermore, with the development of antibiotic adjuvants [18], such as inhibitors of the efflux pump or outer membrane permeabilizers, it might be possible to apply Gram-positive antimicrobials on Gram-negative pathogens in the future. In addition, treatment of infections with Gram-positive multi-resistant pathogens is also a considerable issue to be resolved. In **Chapter 3**, it was revealed that *B. subtilis* was inhibited by more than 80% of the rescreened fungi, suggesting B. subtilis is a well-qualified read-out. Notably, instead of testing the supernatant, we only tested the ethyl acetate extractions on *B. subtilis*, because of the inaccurate results from the test with supernatant according to the unstable compound yield of large-scale cultures. Therefore, the remaining 20% of inactive hits might due to the resistance of *B. subtilis*, or to the invalidation of ethyl acetate extraction of these specific active compounds. For the latter problem, we have not established an easy protocol as an appropriate solution yet. The compounds that are not extractable by ethyl acetate, do not rank high on our priority list currently, but rather may be potential candidates for the future.

In the end, we achieved our goal of successful identification of several antimicrobials, including both well-known and poorly-described ones. This suggests that our screening strategy works for antimicrobial discovery and our fungal collection is a promising source for bioactive compounds.

Rapid prediction of antimicrobial MoA

Identification of antimicrobial targets is one of the vital parts in antimicrobial discovery and development. However, the target candidates in bacterial cells are very broad, making this MoA identification process complex and laborious [19,20]. As discussed in **Chapter 2**, there is no universal method intended for all antimicrobials. Direct approaches (target-based screenings) are able to show direct interactions between antimicrobials and their targets, but these processes are time-consuming and require prerequisite hypotheses. Although these are old approaches that were established several decades ago, they are the only commonly used methods that may provide the direct targets and therefore are still in use today. Improvements of these methods are generally along two avenues. One is the combination of the old strategies with the continuously developing high-end techniques and modernize them into fast and high-throughput approaches, e.g. applying next-generation sequencing onto genetic approaches, or developing a non-selective universal labeling ligand in affinity chromatography. The other avenue is to lower the workload by providing appropriate and accurate hypotheses about the pathways of the antimicrobial targets, which are normally generated by indirect approaches, i.e. the phenotype-based screenings. In this manner, it is important to establish rapid and accurate phenotype-based approaches. Because the time spent and cost involved in the omics-approaches is relatively high, we have developed a simple, cost-effective method for the initial stage of target identification, which is an imaging-based strategy, dynamic bacterial cvtological profiling (DBCP).

DBCP is similar to cytological profiling of eukaryotic cells and the details were described in **Chapter 4**. It is an imaging strategy to track the morphological changes of fluorescently labeled bacterial cells in response to antimicrobials for 30 to 60 min using time-lapse imaging and quantitative image analysis. Using this strategy, we successfully distinguished 14 known antimicrobial compounds into five main classes: DNA, RNA, protein, cell membrane and cell wall. The concept of DBCP was established on the basis of bacterial cytological profiling (BCP), a static imaging strategy which has been applied to classify antimicrobials against Gram-negative bacteria since 2013 [21]. We successfully used DBCP on Gram-positive bacteria. In comparison, DBCP provides more information than BCP without extra elaborate preparation. DBCP allows recording of the direct response from single cells to specific antimicrobial agents over time. Hence the differences between treated and untreated are compared from the same cell, which minimizes the chance to compare differences because of changing circumstances. DBCP also provides the first indication of the

timepoints when a certain antimicrobial starts to have effects, cell growth starts to be inhibited and cells are killed. These are also important parameters that distinguish antimicrobial MoA.

To facilitate DBCP, we developed a bacterial time-lapse imaging protocol, which is based on a previously described technique [22]. We included several improvements in the imaging technique that we developed. Our imaging technique is less timeconsuming and drugs do not have to be pre-added to bacteria. Our syringe-made agarose pad is robust and can be used within 10 min from preparation. Drugs for experiments may be added to the agarose pad during preparation, or to the top well of the agarose pad at any stage of the imaging process. This proved to be important for time-lapse imaging of drug-treatment of bacteria, because it allowed us to image bacteria before treatment, establish normal growth and then image bacteria during drug treatment. With these improvements, DBCP may be used to rapidly distinguish the MoA class and subclass even without prior knowledge of the antimicrobial. In addition, this time-lapse imaging protocol may also be a promising tool for other studies like antibiotic combination, protein interaction or gene function.

Compared to the omics approaches, DBCP provides reliable MoA information, but with a less tedious workflow and lower amount of compound required. But it also has limitations. This imaging-based strategy results in the 'most similar' signatures, and thereby has an intrinsic danger of false positive matches. Therefore, subsequent validation experiments are fundamental to this approach. Nevertheless, considering the ease of accessibility and the affordability, DBCP is an excellent approach for the initial stage of MoA identification.

Two poorly-described antimicrobials with interesting MoAs

In addition to a range of well-known antimicrobials, two interesting antimicrobials were successfully identified in our screen, which are berkchaetoazaphilone B (BAB, from *Pleurostomophora richardsiae*) and harzianic acid (HA, from *Oidiodendron flavum*). Since there were not much data available regarding their antimicrobial activities yet, we investigated their antimicrobial MoAs as described in this thesis.

The compound structure and MoA identification of BAB was described in **Chapter 3**. BAB was previously identified in 2015 as an anti-cancer agent [23]. However, no following data was available regarding its activities. Our study showed that it also had strong anti-bacterial activity against Gram-positive bacteria including a methicillin-resistant *Staphylococcus aureus* (MRSA). To determine its MoA, we chose several strategies. Initially, we applied a phenotype-based approach, DBCP,

and it suggested BAB not to be classified into one of the five main classes, because it had non-cell-envelope effects but without inducing abnormal nucleoid length: cell length ratio. Next, we performed a series of bioactivity assays and a genetic screening. Screening of the Nebraska Transposon Mutant Library (NTML) led to the identification of 16 candidate genes that may be involved in the antimicrobial activity of BAB. 10 of these genes have a role in metabolism, suggesting that BAB affects energy metabolism in *B. subtilis*. Furthermore, a cell metabolism assay suggested that BAB inhibited mitochondrial metabolism completely in HepG2 cells. BAB caused a rapid burst of glycolysis, which ran out quickly, which indicates that BAB has a role in energy metabolism in eukaryotic cells as well. Future work should focus on target identification using genetic approaches or affinity approaches. Although the cytotoxicity of BAB on human cells precludes its potential to be a clinical antibiotic, it is still interesting to study its targets because it might provide some new targets for novel antibiotic discovery.

The identification of another interesting antimicrobial, HA, was described in **Chapter 4**, together with a rapid prediction of its MoA by DBCP. A more detailed description of its MoA identification procedure was given in **Chapter 5**. HA was first isolated as a novel antimicrobial agent from a fungal strain Trichoderma harzianum in 1994 [24]. But surprisingly, subsequent research describes HA as a plant promotor rather than an antimicrobial [25,26]. Recently, one study has described its antibacterial activity against Staphylococcus pseudintermedius, but without any detail about its targets [27]. Therefore, we have tried to elaborate HA's MoA in this thesis with a combination of several MoA identification strategies. First, to generate an accurate hypothesis, we applied DBCP and predicted HA to target the cell envelope (Chapter 4). Next in Chapter 5, with several membrane measurements and developing a resistant mutant, we confirmed this prediction. In addition, we unraveled that HA only targets the cell envelope at high concentration (at least 2-fold minimum inhibitory concentration, MIC). Our results also suggested that the membrane pore formation mechanism was not due to binding certain lipids, making it interesting for further study. Although the mechanism of HA was revealed in more detail, the affected pathway(s) at low concentration remain(s) unclear. The HA-resistant strain, B. subtilis M9015, showed cross resistance to rifampin, which might suggest that the RNA synthesis pathway is relevant for HA's MoA. However, this will require more work to elucidate the function of the mutated genes in B. subtilis M9015, for instance by directed inactivation of each of these genes as described in the future plans of Chapter 5. Overall, HA seems to be an interesting and promising antimicrobial candidate that might have new targets, and therefore is interesting for further analysis.

Conclusion and future perspectives

Overall in this thesis, we have described a cultivation-based strategy for novel antimicrobial screen in fungi and an imaging-based strategy for antimicrobial MoA identification. Furthermore, we have also elaborated the compound structure and MoA identification of two example antimicrobial agents revealed in our screen. One of them, BAB, was found to affect energy metabolism, whereas another one, HA, was determined as a multi-target antimicrobial agent.

There will undoubtedly be more antimicrobial agents produced by fungi in our library that we did not cover. In addition, there are many options to modify the screen and/or the fungal library. In case a new compound is discovered, it might be classified within one hour based on its MoA using the MoA identification strategy, DBCP, that we developed. DBCP is rapid, accurate and is easy-accessible for most microbiology labs. Taken together, the combination of a collection of natural compounds (mixtures) and DBCP is a highly promising strategy for antimicrobial compound discovery.

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