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

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General Introduction

CHAPTER 1

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ANTIMICROBIAL DISCOVERY

Antimicrobial resistance

The average life expectancy was around 47 years in the pre-antibiotic era, partly due to the high mortality as a result of infectious diseases [1]. The discovery of penicillin in 1928 has written a new chapter in the book of medicine and was marked as the beginning of the antibiotic era [2]. The worldwide use of penicillin started in the year 1945, since when antimicrobials have been essential drugs to modern healthcare. After penicillin, many more antimicrobials were discovered, especially between the 1950s and 1970s. In this period, more than half of the antibiotic classes in use today was isolated; therefore, it was named "the golden era" in antibiotic discovery [3,4]. As a result, the leading medical reason causing death has shifted from infectious diseases to non-infectious diseases such as cancer and stroke, and the average life expectancy has risen [1]. In addition, the application of antimicrobials is expanded, from only for treating bacterial infections, to a large variety of uses such as protecting patients with compromised immune systems or cancers, and even to preventing infections in agriculture and livestock.

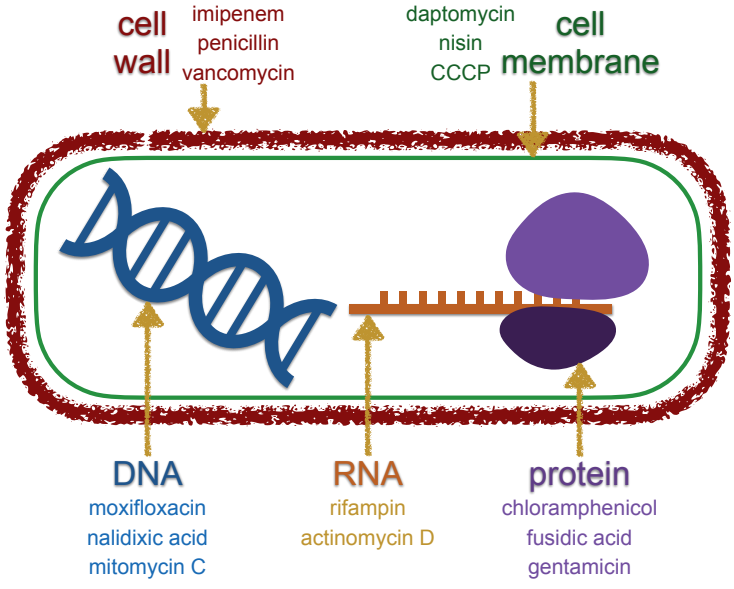

Fig. 1. Most common classes of antimicrobials. Antimicrobials can be classified based on their target. Examples of antimicrobials of each class are listed.

Nevertheless, with the abuse of antimicrobials, an issue has developed, which is antimicrobial resistance [5–7]. Actually, the emergence of antimicrobial resistance is due to the natural evolutionary response of bacterial strains to antimicrobials that are naturally produced by microorganisms. For example, in 1940, an *Escherichia coli* strain was reported to produce penicillinase, which inactivates penicillin [8]. This was even one year before the clinical use of penicillin [9]. However, large scale applications of antimicrobials in health care and agriculture have driven bacterial selection, resulting in spreading of antimicrobial resistance into human society [10]. Despite the discovery of a variety of antimicrobial classes with different targets (Fig. 1), no antimicrobial could avoid development of resistance against it in hospitals. Fig. 2 exhibits a timeline for some antimicrobials, depicting the year of their first clinical application and the year when resistance was identified. Because of the emerging antimicrobial resistance issue, bacterial infections have become a serious threat again, requiring a more complicated therapy to be cured [11]. The resistant bacteria, especially the multidrug resistant "superbugs", have led to higher patient mortality and rising costs [12,13]. Therefore, we are in a strong need for new antimicrobials with novel mechanism of action (MoA) to combat the continuously emerging antimicrobial resistance.

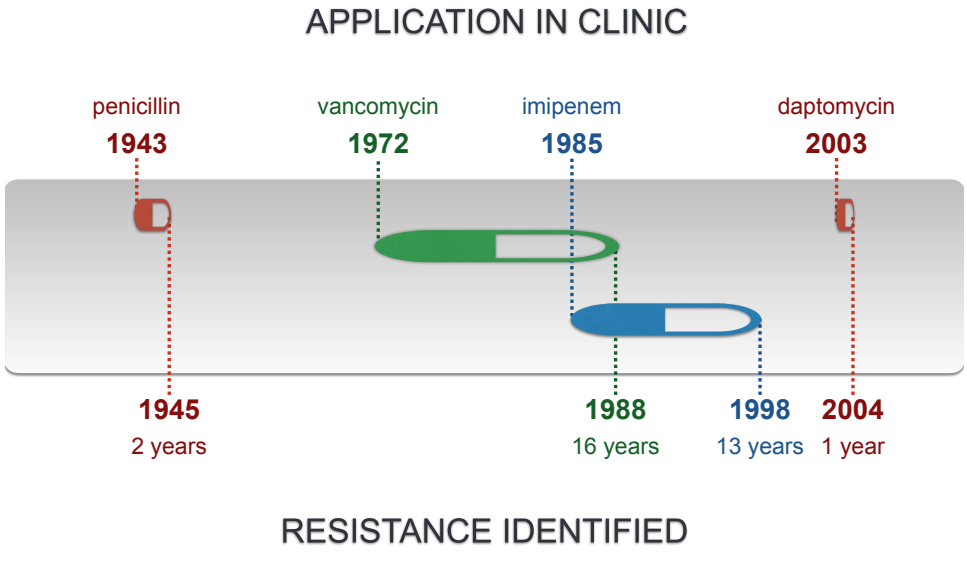

Fig. 2. Antimicrobial usage and resistance. The timeline of antimicrobials is depicted, indicating when they were first used in the clinic and when resistance was identified. Four examples of antimicrobials are shown.

Fungal natural products

To compete with other species or to survive a harsh environment, organisms have good reasons to produce functional compounds, otherwise known as natural products [14]. These compounds have played important roles in medical application through history, and will undoubtedly continue to open up the unknown medical spaces and provide pharmacological benefits [15,16], especially in the field of antimicrobial drug discovery and development [17]. In the Food and Drug Administration (FDA) of the U.S., approximately 59% approved antimicrobials were derived from nature [18], and natural products from microbial origin are privileged in this sphere [17]. The kingdom of fungi contains countless and diverse fungal species, which produce a large variety of bio-active compounds as secondary metabolites [19]. These metabolites are not essential for the growth of fungi, and are different from the metabolites of the primary metabolic pathways [20]. In general, secondary metabolites are small bioactive compounds that are produced at specific stages of fungal growth to alter fungal development or communicate with their environment [21,22]. One example is melanin, a special pigment found in most organisms, including fungi. It provides fungi the ability to defend against environmental stresses like ultraviolet light or oxidizing agents [23]. Another example is variegatic acid, which is a pigment from the rot fungus *Serpula lacrymans* that is induced by bacterial encounters. It has the function to inhibit the biofilm formation of *Bacillus subtilis* [24]. Fungal natural product research is aimed at transferring the natural ecological functions of secondary metabolites to medical application for the benefit of human society.

Fungal natural products have been effectively used in Chinese medicine through the ages, without knowing the identity of the compounds themselves. The study of fungal natural products as we know it, started in 1922 by Harold Raistrick and bloomed after the successful discovery of penicillin. Since then, it has led to the identification of thousands of metabolites containing antimicrobial, antifungal or antitumor activities [19,25]. However, in spite of the successful application of β-lactams in the clinic, which accounts for up to 60% of all clinical antimicrobials, fungal natural products are not the main source of compounds in the antimicrobial discovery process [17]. Instead, actinomycetes and bacteria are the main source of newly identified compounds with antimicrobial activity. Nevertheless, genomic approaches have identified several gene clusters encoding biosynthetic enzymes producing natural products per fungal genome, just like actinomycetes, suggesting that fungal natural products are an excellent source of compounds for the antimicrobial discovery field [17].

GRAM-POSITIVE BACTERIA

Gram-positive pathogens

Traditionally, the first procedure to identify an unknown bacterial strain is to perform Gram-staining and evaluate using a microscope. This approach generates the first-sight information of a bacterium: cell size, cell shape and the distinction between Gram-positive bacteria or Gram-negative bacteria [26]. The name of the Gram-staining comes from Hans Christian Gram, a Danish bacteriologist who found that some bacterial cells are not able to achieve decolorization of gentian violet, later known as Gram-positive bacteria [27]. These have a thick peptidoglycan cell wall topped by teichoic acid and lipoteichoic acid as the outside of their cell envelope, together with a single cytoplasmic membrane [27,28]. Their thick peptidoglycan cell wall retains the violet stain. Gram-negative bacteria have a thin peptidoglycan layer as their cell wall, in between the cytoplasmic membrane and the outer membrane [27,28]. It is easier to decolorize the violet stain from the thin peptidoglycan layer. The different cell envelopes between Gram-positive and Gram-negative bacteria are visualized in Fig. 3.

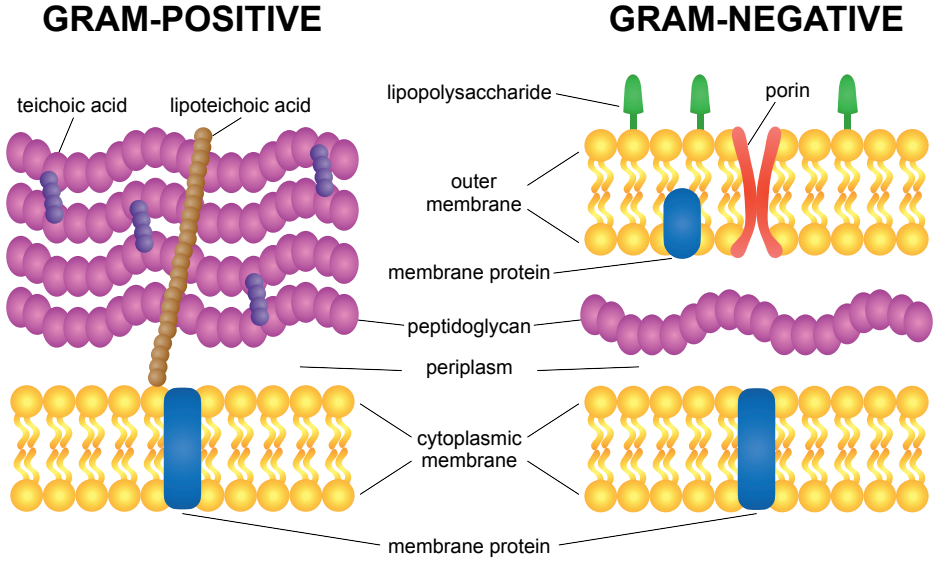

Fig. 3. The most striking difference between Gram-positive and Gram-negative bacteria is the presence of an outer membrane in the cell envelope. The structure of a fraction of the cell envelope of Gram-positive and Gram-negative bacteria is shown.

There are pathogenic bacteria among both the Gram-positive and Gram-negative bacteria. With the development of antimicrobial resistance, multidrug resistant Gram-positive pathogens have become one of the major therapeutic challenges [29]. Three Gram-positive strains were present on the list of global priority pathogens by the World Health Organization (WHO) in 2017: vancomycin-resistant *Enterococcus faecium* (VRE), Methicillin-resistant *Staphylococcus aureus* (MRSA) and penicillin-non-susceptible *Streptococcus pneumonia* [30]. These bacteria cause both community-acquired infections and healthcare-associated infections, forming serious clinical problems. The mechanism underlying resistance is generally acquisition of the ability to degrade antimicrobials, modification of the antimicrobial targets, or overexpression of efflux pumps [31]. For example, *S. aureus* is one of the major human pathogens, which was initially treated with penicillin. Only a few years after the clinical use of penicillin, a penicillin-resistant *S. aureus* strain emerged, which produced a plasmid-encoded penicillinase [32]. Later, a more dangerous methicillin-resistant strain, MRSA, was identified to produce an additional penicillin binding protein PBP2a, which has a reduced affinity to almost all β-lactam antimicrobials [33] and allows bacteria to grow in the presence of these antimicrobials. The outbreaks of MRSA have led to severe problems both in healthcare systems and communities. Another example is VRE that acquired vancomycin-resistance gene clusters through transposons, resulting in the replacement of D-alanyl-D-alanine with D-alanyl-D-lactate termini in the cell wall to lower the binding affinity of vancomycin [29]. Several attempts have been made to deal with multidrug resistant pathogens by modification of existing antimicrobials, but with little success [31]. Searching for innovative antimicrobial classes with novel targets or even antibiotic alternatives, like bacteriophages and probiotics, holds more promise for the future.

Bacillus subtilis

B. subtilis is a strain of rod-shaped Gram-positive bacteria, with cells that are 4–10 μm long and 0.25–1.0 μm in diameter. It is ubiquitous in nature with large habitats, ranging from soil to aquatic environments [34] and therefore is also simple to be cultured in the lab. Hence, *B. subtilis* is the model of choice for many labs studying Gram-positive bacteria, especially the *B. subtilis* strain 168. When culturing this strain, the addition of tryptophan is essential even if the medium contains acid-hydrolyzed proteins, because this strain is a tryptophan auxotroph (trpC2) [35]. Upon nutrient limitation, *B. subtilis* enters the self-protective process of sporulation. Actually, only a portion (typically 10%) of the population differentiates into

endospores. The rest of the cells use a bet-hedging strategy to lyse themselves and provide nutrients for sporulation [36,37]. Unlike the pathogenic organism *Bacillus anthracis* [38], *B. subtilis* is a Generally Regarded as Safe (GRAS) strain by FDA, and thus is used in a wide range of biotechnology industries, including food and medicine [39]. The popularity in bioindustries has helped this strain to attract attention in the studies of physiology and genetics.

The *B. subtilis* genome was sequenced more than two decades ago [40]. The accumulation of detailed knowledge at the level of DNA has generated a library of gene functions. In addition, with the development of transcriptomics and proteomics, the profiles of gene expression in *B. subtilis* have put another dimension to this important strain [39]. All the benefits suggest *B. subtilis* to be a highly amenable model for studying the antimicrobial MoA [41].

SCOPE OF THIS THESIS

The work described in this thesis aims to search for new classes of antimicrobials that interfere with novel cellular targets of Gram-positive bacteria. The den Hertog lab at the Hubrecht Institute has a library of fungal secondary metabolites from 10,207 strains of fungi. This library was successfully applied to screen for novel bioactive compounds using a zebrafish model [42]. Here, we used this library as a source to screen for novel antimicrobials. To this end, we screened and identified the antimicrobial activity using a working pipeline containing library screening, fungal culture optimization, activity purification and compound identification. Finally, we successfully identified several compounds with antimicrobial activity. Next, to further assess the antimicrobial property of the identified compounds, we developed a novel MoA identification strategy named Dynamic Bacterial Cytological Profiling (DBCP). This strategy distinguished antimicrobials from different classes using time-lapse imaging, and was used to determine the MoA of a poorly studied antimicrobial, harzianic acid (HA). In addition, we further described the MoAs of two promising antimicrobials, Berkchaetoazaphilone B (BAB) and HA. We provide evidence that BAB might affect energy metabolism. HA was found to be a multi-targeting antimicrobial that generated pores in the cell membrane when used at high concentration.

OUTLINE OF THIS THESIS

Chapter 2 provides a review of the advances in MoA identification strategies that were commonly used over the years. We first focused on some classic approaches that were established several decades ago and are still in use today with the implementation of novel techniques. Then we discussed the modern approaches that bring high hopes to the antimicrobial field, including omics approaches and imaging-based strategies.

Chapter 3 describes the strategy 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. The identified metabolites consist of both known antimicrobial compounds as well as relatively unexplored compounds. We also studied the antimicrobial property of BAB, an anti-cancer compound identified in 2015 [43], which was identified to contain antimicrobial activity in this screen.

Chapter 4 describes a novel strategy of antimicrobial MoA identification. An important bottleneck in antimicrobial discovery is the time-consuming analysis of the antimicrobial working mechanism. To facilitate breaking the bottleneck, we tried to develop a method 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 DBCP, we observed bacteria over time and established fluorescence intensities qualitatively and quantitatively. It allowed to rapidly distinguish antimicrobials from all of the five main classes. Finally, we used DBCP to establish the MoA of HA, a poorly described secondary metabolite purified from the fungal culture of *Oidiodendron flavum*. Taken together, DBCP is proven to be an excellent tool for the first approach of antimicrobial MoA classification.

Chapter 5 unravels details about the antimicrobial MoA of HA. HA was first isolated as a novel antimicrobial agent from a fungal strain *Trichoderma harzianum* in 1994 [44], but not much data is available regarding its antimicrobial activity yet. In **Chapter 4**, we predicted HA to target the cell envelope using DBCP. Here, we applied several assays to confirm this prediction. In addition, we isolated HA-resistant bacteria and identified four mutated genes, which provides further insight into its MoA.

Chapter 6 summarizes the discussions of the thesis with future perspectives. 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. Hopefully it will contribute some new insights into antimicrobial research.

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