Screening for novel antimicrobial agents and elucidation of the corresponding mechanisms

Inaugural dissertation

for the attainment of the title of doctor
in the Faculty of Mathematics and Natural Sciences
at the Heinrich Heine University Düsseldorf

presented by

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from Schuchinsk

Düsseldorf, June 2020

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Faculty of Mathematics and Natural Sciences at
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Date of the oral examination: 05.08.2020

" EVERYTHING IS THEORETICALLY IMPOSSIBLE, UNTIL IT IS DONE."

- Robert A. Heinlein

ACKNOWLEDGEMENTS

First of all, I wish to express my sincere thanks to my supervisor Prof. Dr. Rainer Kalscheuer for giving me the opportunity to graduate in his lab. I am very grateful for the guidance, support and encouragement during all the time and for such a relevant topic I was working on.

I also want to thank my co-supervisor Prof. Dr. Peter Proksch for the professional and constructive collaboration within the institute and for providing the extraordinary big library of compounds which were fundamental for my work.

A big thanks to all members of my working group for such a kind and familiar atmosphere, especially to my office members Dr. Lasse van Geelen, Heike Goldbach-Gecke, Steffen Schindler, Dr. Yvonne Gröner, Anna-Lene Ilse Rosemarie Kiffe-Delf and the former colleagues Dr. Jan Korte and Dr. Nidja Rehberg I spent the most time with. Additional thanks to Lasse and Heike for the professional support and answering my stupid questions.

Also, a big thanks to the working group of Prof. Dr. Proksch for the cooperation and the support during the practical courses especially Dr. Nam Michael Tran-Cong, Dr. Marian Frank and Simone Miljanovic. Also, a thanks to Claudia Eckelskemper for the administrative support. A special thanks goes to Imke Form (†) for the support during the practical course. It was a pleasure to work with you, rest in peace.

Additional thanks to Prof. Dr. Julia Bandow for such a constructive and successful cooperation.

And last but not least, a big thanks to my parents for the love, patience and support over countless years and to give me the opportunity to study. And a big thanks to my girlfriend Cora Benita Mooren for the patience, love and encouragement.

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ABBREVIATIONS

A. baumannii	*Acinetobacter baumannii*
ABCs	Active Bacterial Core surveillance
ATCC	American Type Culture Collection
ATP	Adenosine Tri-Phosphate
B. subtilis	*Bacillus subtilis*
BDE	Brominated Diphenyl Ether
blaOXA	Beta-Lactamase of type Oxacillinase
C. difficile	*Clostridium difficile*
CCCP	Carbonyl Cyanide m-Chlorophenyl Hydrazone
CDC	Centre for Disease Control and Prevention
CF	Cystic Fibrosis
DM	Didymellanosine
DNA	Deoxyribo-Nucleic Acid
DOI	Digital Object Identifier
E. coli	*Escherichia coli*
E. faecalis	*Enterococcus faecalis*
E. faecium	*Enterococcus faecium*
e.g.	*Exempli gratia,* for example
ECDC	European Centre for Disease Prevention and Control
EEA	European Economic Area
ESBL	Extended Spectrum Beta-Lactamase
ESCAPE	Abbreviation of a group of pathogens including ***E**nterococcus faecium, **S**taphylococcus aureus, **C**lostridium difficile, **A**cinetobacter baumannii, **P**seudomonas aeruginosa and **E**nterobacter* spp.
ESKAPE	Abbreviation of a group of pathogens including ***E**nterococcus faecium, **S**taphylococcus aureus, **K**lebsiella pneumoniae, **A**cinetobacter baumannii, **P**seudomonas aeruginosa and **E**nterobacter* spp.
et al.	*Et alii*, and others
etc.	*Et cetera*, and so on

EU	European Union
Gen.	Generation
GFP	Green Fluorescent Protein
GISP	Gonococcal Isolate Surveillance Project
HAIC	Healthcare-Associated Infections-Community Interface
IC	Inhibitory concentration
K. pneumoniae	*Klebsiella pneumoniae*
LPS	Lipo-Poly-Saccharide
MATE	Multi-drug And Toxic compound Extrusion
MDR	Multi-Drug Resistant
MeO-PBDE	Methoxylated Poly-Brominated Diphenyl Ether
MERS-CoV	Middle East Respiratory Syndrome Corona-Virus
MFS	Major Facilitator Superfamily
MIC	Minimal Inhibitory Concentration
MRSA	Methicillin-resistant *Staphylococcus aureus*
NF-κB	Nuclear Factor 'kappa-light-chain-enhancer' of activated B-cells
NMP	1-(1-Naphthyl-Methyl)-Piperazine
NTSS	National Tuberculosis Surveillance System
OH-PBDE	Hydroxylated Poly-Brominated Diphenyl Ether
Omp	Outer membrane protein
Opr	Outer-membrane porin protein
P. aeruginosa	*Pseudomonas aeruginosa*
PAβN	Phenylalanine-Arginine *β*-Naphthylamide
PBDD	Poly-Brominated Dibenzo-*p*-Dioxin
PBP	Penicillin binding protein
POP	Persistent Organic Pollutant
PP	Phenoxy-Phenol
PSM	Phenol-Soluble Modulin
PTP	Protein-Tyrosine Phosphatase
PTS	Phosphor-Transferase system
PVL	Panton-Valentine Leucocidin
QS	Quorum Sensing

RNA	Ribo-Nucleic Acid
RND	Resistance-Nodulation cell Division
RT-qPCR	Reverse Transcription quantitative Polymerase Chain Reaction
S. aureus	*Staphylococcus aureus*
SCC	Staphylococcal Cassette Chromosome
SI	Selectivity Index
SMR	Small Multi-drug Resistance
SNP	Single Nucleotide Polymorphism
sp.	Species
spp.	Several species
TSST	Toxic Shock Syndrome Toxin
US	United States (of America)
UPEC	uropathogenic *Escherichia coli*
USDA	US Department of Agriculture
VRE	Vancomycin-resistant *Enterococci*
WHO	World Health Organization
*µ*M	Micromolar

ABSTRACT

Drug resistant pathogens have become a big threat to human health in the past decades since new antimicrobials are rare. Fast resistance evolution is owed to the misuse in healthcare and livestock that has led to the current antimicrobial resistance crisis. The most prevalent drug-resistant bacterial pathogens involved in hospital- and community-associated infections are designated as ESKAPE pathogens and comprise, among others, *Staphylococcus aureus* and *Acinetobacter baumannii*.

In chapter 4 of this thesis, different current strategies are reviewed to tackle antibacterial drug resistance including exploration of new antimicrobial sources, targeting subpopulations such as persisters and biofilms, anti-virulence approaches or the use of antimicrobial conjugates and nanocarriers. Chapter 5-7 of this thesis describe my contributions to research on new antimicrobial structures based on three publications.

In chapter 5, the plant-derived geranylated chalcone Xanthoangelol was identified to exhibit antibacterial activity against Gram-positive bacteria including Methicillin-resistant *Staphylococcus aureus* (MRSA) in low micromolar concentration. In this study, we could demonstrate that Xanthoangelol treatment leads to a leakage of intracellular metabolites by affecting bacterial membrane potential and membrane integrity resulting in cell lysis. Treatment with sublethal compound concentrations in *Bacillus subtilis* revealed indications of cell wall and/or membrane damage and oxidative stress in a proteomic stress profiling approach. In contrast, Xanthoangelol revealed only low hemolytic and cytotoxic effects at higher concentrations. Therefore, Xanthoangelol is a promising antimicrobial lead structure but requires further medicinal chemical optimization before its clinical potential can be assessed.

In chapter 6, natural sponge-derived brominated phenoxyphenols were identified with a broad-spectrum activity against Gram-positive and Gram-negative pathogens including the ESKAPE group. Additionally, the phenoxyphenols revealed activity against MRSA subpopulations such as persisters and biofilms. Resistant mutants were isolated harbouring mutations in Phosphotransferase system involved in uptake of sugars. Proteomic stress profiling of sublethally treated cells revealed a strong downregulation of a specific Phosphotransferase component. Additionally, overexpression of this Phosphotransferase component resulted in hypersensitivity

towards brominated phenoxyphenols. In combination, these findings corroborate the involvement of Phosphotransferase systems in resistance to brominated phenoxyphenols and suggest that these compounds might serve as a surrogate substrate and hijack Phosphotransferase systems to enter the cell. However, while brominated phenoxyphenols are interesting scaffolds to tackle the hard-to-treat MRSA subpopulations and multi-drug resistant Gram-negative bacteria, further investigations are needed to improve their potency and selectivity.

Endophytic fungi are a promising source of active compounds. In chapter 7, several active compounds were isolated from the endophytic fungus *Didymella* sp. IEA-3B.1, originated from leaves of *Terminalia catappa* (*Combretaceae*) from Bali, Indonesia. The new compound Didymellanosine and the previously described compound Ascomylactam C showed activity against murine and human cancer cell lines and against Gram-positive pathogens including MRSA, Vancomycin-resistant *Enterococcus faecium* and Vancomycin-resistant *Enterococcus faecalis*. Furthermore, both compounds were active against Gram-negative drug-resistant *Acinetobacter baumannii* in combination with sublethal Colistin concentrations. In contrast, the new compound Didymellanosine was tested on non-malignant human fetal lung fibroblasts MRC5 revealing a 5-6-fold lower cytotoxicity. Due to the increasing emergence of drug-resistant Gram-negative pathogens, Didymellanosine could be a promising lead structure. Nevertheless, further investigations on natural and/or synthetic derivatives are needed to develop congeners with reduced cytotoxicity and improved antimicrobial selectivity.

1 INTRODUCTION

Bacteria are considered the oldest living organisms that colonizes different habitats. They can be found in soil and water, inside and outside of plants and animals [1-3]. Furthermore, many bacteria are adapted to live under extreme environmental conditions e.g. under acidic, high salt or high temperature conditions [4, 5]. Logically, also the human body is colonized by bacteria at the outer and inner surfaces, including skin, oral and nasal mucosa and the gastrointestinal tract. The total community of microorganisms including bacteria, fungi, viruses, archaea and protozoa inhabiting specific environment such as humans is called microbiome. Especially commensal bacteria are known to be strongly connected to human health [6]. In contrast, pathogenic bacteria can cause minor to serious and also deadly infections. Additionally, opportunistic pathogens associated with the human microbiome are able to infect humans and lead to a high morbidity and mortality.

After the industrial revolution in the 19th century, the economical productivity increased and the biological and medicinal research changed. In the 20th century, the world population increased exponentially [7]. One of the reasons is the modern healthcare that includes sanitation standards, vaccination and usage of antibiotics. Consequently, the mortality rates caused by pathogens decreased dramatically and the population growth increased. While hygiene and vaccination are preventive measures to avoid infections, antibiotics are commonly used during the infection.

After Alexander Fleming coincidentally discovered Penicillin in 1928 and published the results in 1929 [8], no further progress was made first as Fleming was not able to establish an efficient method for purification and stability of the active compound. Interestingly, in the same year when World War II had started in 1939, Howard Florey and Ernst Chain picked up the Penicillin project and established a robust method of Penicillin isolation. In the 1940s, the commercial production started in the US and Penicillin became an important tool during World War II. In consequence, the antibiotic era was started and Fleming, Florey and Chain became the Nobel price of medicine in 1945 [9, 10].

1.1 ANTIBIOTIC ERA

The first antibiotic that was used in hospitals was Pyocyanase from *Pseudomonas aeruginosa* described by Emmerich and Löw in 1899 over 40 years before Penicillin was commercially available. It was active against cholera, typhoid, diphtheria and anthrax [11]. Pyocyanase is not used today due to its toxicity. Additionally, the drug Salvarsan was the antimicrobial of choice in syphilis treatment discovered by Ehrlich and Hata in 1909 *via* large scale screening. Further, Neosalvarsan was discovered that was more soluble and less toxic [9, 12]. Also the drug Prontosil synthesized by Bayer and tested by Domagk in the 1930s [13] was applied in cases of infection but later replaced by Penicillin after a few years [9]. While Salvarsan and Prontosil were synthetic compounds, Pasteur and Joubert had already noticed that also microorganisms can have an antagonistic effect on other microorganisms in animal infection models in 1877 [14, 15].

After the discovery of Penicillin, the research on new antibiotics was intensified revealing many new classes of antibiotics including aminoglycosides, tetracyclines, chloramphenicols and macrolides in the 1940s, glycopeptides, oxazolidinones, rifamycins in the 1950s and quinolones and streptogramins in the 1960s [16]. Especially the screening methods established by Paul Ehrlich (large scale screening; in the 1900s) and Alexander Fleming (inhibition zone screening; in the 1930s) were used [9]. However, the pathogens were able to adapt and develop resistance against different antibiotics.

1.2 DRUG RESISTANCE

Already in the year 1945, A. Fleming warned about the evolution of resistance to Penicillin by misuse [17, 18]. Indeed, in the following decades many antibiotics were developed and many resistances occurred to these antibiotics, in some cases, only after a very short time following their introduction into the clinics (e.g. Levofloxacin: same year, Linezolid: after one year, Methicillin: after two years). The successive emergences of drug resistances over the last decades have culminated into today´s antibiotic resistance crisis [17, 19, 20]. The occurrence of drug resistant bacteria can have many different origins and these were identified and described [17, 21].

1.2.1 CAUSES OF DRUG RESISTANCE EVOLUTION

First, the overuse:

In many countries worldwide antibiotics are available without prescription. Many people are able to buy and use it inappropriate e.g. in case of virus infections but also in inefficient concentrations. Lower concentration of antibiotics can drive the resistance evolution by selective pressure [21]. Furthermore, the use of antibiotics increased with the occurrence of resistance.

Second, inappropriate prescribing:

Antibiotics are prescribed without identification of the pathogen in many cases. Consequently, many prescriptions are suboptimal or unnecessary and have a questionable therapeutic benefit [17, 21, 22]. Subinhibitory concentrations of antibiotics can cause genomic alterations in pathogens that can lead to resistance [17, 23].

Third, extensive agricultural use:

The biggest part of antibiotic usage is agricultural. In the US, for example, 80% of all antibiotics that are used are administered to food animals as infection prophylaxis but also for growth promotion [21]. Furthermore, this antibiotics are excreted by the animals with a profound impact on environmental microbiome [24]. Also the consumption of treated animals can lead to an acquisition of resistant bacteria to human body [17, 21].

Fourth, economic and regulatory barriers:

The availability of new antibiotics in the last decades was reduced due to the withdrawal of many companies out of antibiotic research, because it is economically not profitable. While research, development, clinical trials and drug approval are taking much time and money, the duration of antibiotic application is quiet short due to a rapid resistance evolution and antibiotics are low priced compared to the more profitable cancer therapeutics [17, 21, 25].

Nevertheless, new strategies are necessary but also available to reduce the resistance evolution and to overcome the antibiotic resistance crisis [19, 21].

1.2.2 MECHANISMS OF DRUG RESISTANCES

To identify and understand different resistance mechanisms, the variety of antibiotic targets should be described first. Antibiotics are divided into various classes that have different targets. β-lactam antibiotics such as penicillins, cephalosporins or carbapenems are targeting the cell wall synthesis. Polymyxins, cyclic lipopeptides and polyenes are interfering the with the cell envelope affecting the permeability. Other antibiotics can inhibit the protein biosynthesis targeting the subunits of the ribosomes such as macrolides, tetracyclines, Chloramphenicol, aminoglycosides or lincosamides. Bacterial DNA metabolism is also targeted by antibiotics such as quinolones, sulphonamides or nitroimidazoles (Figure 1) [26, 27].

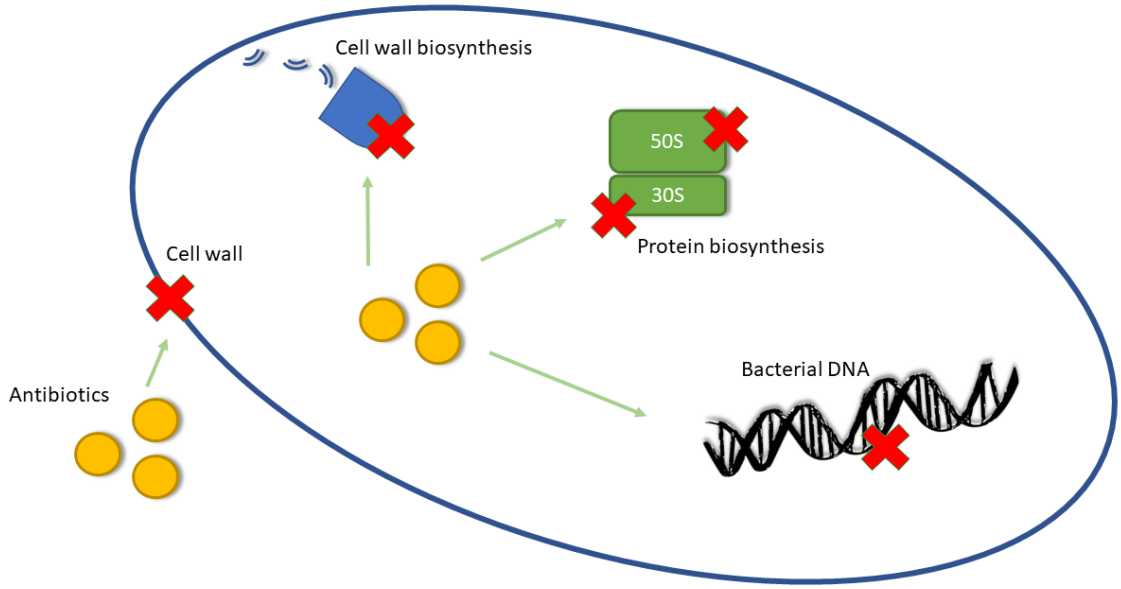

Figure 1: Different antibiotic targets in bacteria. Antibiotics can interfere with the cell envelope, with intracellular enzymes of cell wall synthesis, protein biosynthesis or with nucleic acids (from left to right).

To overcome antibiotic treatment, bacteria use three different strategies to become resistant: preventing the drug reaching the target, alter the target or inactivate the antibiotic (Figure 2) [18, 26, 28-30]. Further, the resistance mechanisms are divided into three classes: the intrinsic, the acquired and the mutational (adapted) resistance [26].

In case of intrinsic resistance, e.g. bacteria are lacking enzymes that are involved in conversion of pro-drugs and the active form of the antibiotics is not available.

Acquired resistance refers to a horizontal acquisition of genetic elements that encode antibiotic resistance. For instance, β-lactamases can be acquired to become resistant to different β-lactam antibiotics. Horizontal acquisition occurs *via* conjugation, transformation or transduction. In case of conjugation, the most common acquisition type, the genetic elements are exchanged by cell-to-cell contact *via* pili or bridges. In case of transformation, commonly used in the laboratories, the bacteria acquire genetic elements as naked DNA from the environment. And in case of transduction, bacteriophages are involved in translocation of genetic element from one bacterium to another [26].

Last but not least, the mutational or adapted resistance occurs when the genetic material is altered by mutation and the antibiotic target is altered and cannot be inhibited by antibiotics.

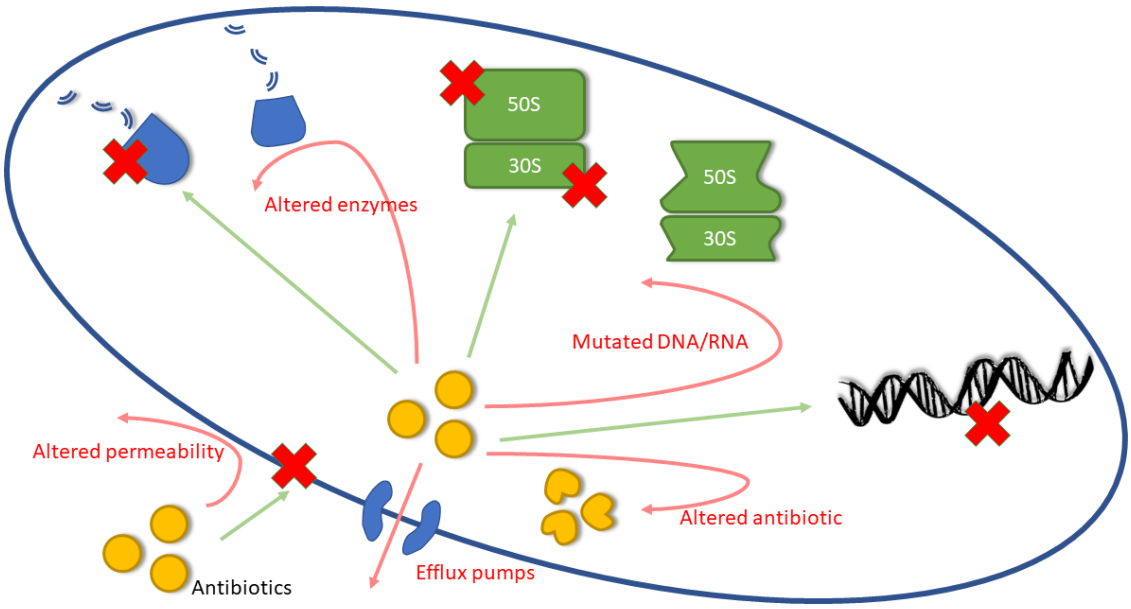

Figure 2: Antibiotic targets (green arrow) and corresponding resistance mechanisms (red arrow). Antibiotic targets such as enzymes or DNA/RNA are altered in resistant bacteria. Acquired enzymes can alter the antibiotics and altered cell envelope (incl. efflux mechanisms) can prevent antibiotics to bind to the target [18, 30].

1.3 CRITICAL NOSOCOMIAL PATHOGENS

Since the beginning of the antibiotic era, many different resistances occur against all the variety of antibiotics. Furthermore, multi-drug resistant pathogens are causing increasing death rates worldwide. Based on the locations where most infections occur, two main types can be distinguished: community-acquired infections and hospital-acquired infections. Especially the hospital-acquired infections are alarming, since resistant pathogens occur in hospitals due to inappropriate antibiotic treatment of the patients and can easily spread due to sometimes suboptimal hygienic conditions and the gathering of patients with compromised health conditions and often impaired immune status [31-33]. Additionally, surgery patients can be infected by resistant pathogens after their surgical procedure.

In general, the most relevant pathogens with regard to the need of new drug development were identified and listed by the World Health Organization (Table 1) [33]. Gram-negative bacteria such as *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and different *Enterobacteriaceae* such as *Klebsiella pneumoniae* and *Escherichia coli* are very prominent in the list constituting all critical and also most of the high priority pathogens (*Helicobacter pylori*, C*ampylobacter* sp., *Neisseria gonorrhoeae*, *Salmonella* sp.).

Table 1: WHO priority list of drug-resistant pathogens from “2019 antibacterial agents in clinical development” publication [33].

Critical priority
Carbapenem-resistant *Acinetobacter baumannii*
Carbapenem-resistant *Pseudomonas aeruginosa*
Carbapenem-resistant and 3rd Gen. Cephalosporin-resistant *Enterobacteriaceae*
High priority
Vancomycin-resistant *Enterococcus faecium*
Vancomycin-resistant and Methicillin-resistant *Staphylococcus aureus*
Clarithromycin-resistant *Helicobacter pylori*
Fluoroquinolone-resistant *Campylobacter* sp.
Fluoroquinolone-resistant *Salmonella* sp.
Fluoroquinolone-resistant and 3rd Gen. Cephalosporin-resistant *Neisseria gonorrhoeae*
Medium priority
Penicillin-non-susceptible *Streptococcus pneumoniae*
Ampicillin-resistant *Haemophilus influenzae*
Fluoroquinolone-resistant *Shigella* sp.

Nevertheless, two Gram-positive drug-resistant commensal bacteria are listed to the high priority level: *Enterococcus faecium* and *Staphylococcus aureus*. Furthermore, Methicillin-resistant *S. aureus*, also known as MRSA, has one of the highest morbidity and mortality rates among nosocomial pathogens in the EU [31, 34]. Overall infection and death rates in the EU attributed to these pathogens increased by the factor 2.5 between 2007 and 2015 [34].

The prevalence of Gram-negative pathogens is attributed to its different cell envelope compared to the Gram-positive bacteria. Gram-negative bacteria exhibit an additional outer membrane and are consequently less permeable due to porins and efflux systems [30, 35, 36].

1.4 ESKAPE PATHOGENS

A few years before the WHO compiled the priority list of resistant pathogens, critical pathogens were grouped together due to their ability to become resistant. The group is called ESKAPE pathogens.

This group consists of ***E**nterococcus faecium, **S**taphylococcus aureus, **K**lebsiella pneumoniae, **A**cinetobacter baumannii, **P**seudomonas aeruginosa* and ***E**nterobacter* spp. [37, 38]. Compared to the WHO list, all members of this group are listed as critical and high priority pathogens with a variety of resistances. Furthermore, the ESKAPE members are the most common opportunistic pathogens causing the majority of nosocomial infections [39]. Indeed, the ESKAPE group is still prominent in current studies [39-43]. Extending, Lance R. Peterson offered in 2009 to change the term to ESCAPE, since *Klebsiella pneumoniae* is related to *Enterobacteriaceae* and proposed to add ***C**lostridium difficile* to the group. *C. difficile* cause severe hospital-associated infections [44]. While the clinical importance and treatment of *C. difficile* is undisputed among clinicians and researchers alike, the term ESCAPE has not yet found wide acceptance among the research community.

Nevertheless, to get more into detail, the ESKAPE pathogens should be extensively described focusing on *S. aureus, E. coli*, *A. baumannii* and *P. aeruginosa* that are relevant for this thesis.

1.4.1 *STAPHYLOCOCCUS AUREUS* AND MRSA

Staphylococcus aureus is a Gram-positive bacterium with coccoid shape and is a human commensal that colonizes skin and nasal mucosa in up to 30% of humans without causing any infections [45, 46]. However, *S. aureus* is also an opportunistic pathogen causing different infections such as pneumonia, endocarditis, meningitis, skin, urinary tract, blood stream and soft tissue infections [45, 47-50]. Furthermore, *S. aureus* has an extensive host range especially among domestic animals such as cats, dogs, horses, goats, sheep, cattle, rabbits, pigs, and poultry [49, 51]. Frequent occurrence of drug resistant *S. aureus* strains such as Methicillin-resistant *S. aureus* (MRSA) in hospital-acquired, livestock-acquired and community-acquired infections makes *S. aureus* a prominent pathogen with a hard-to-treat ability [49].

After Penicillin introduction in the 1940s, first resistant *S. aureus* strains occurred in 1942 [51, 52]. The resistance was driven by the enzyme Penicillinase that hydrolyses the β-lactam ring of Penicillin and inactivates the drug. To overcome the Penicillin resistance, the semisynthetic drug Methicillin was developed in 1959, which was Penicillinase resistant while still possessing a β-lactam ring. Nevertheless, after one year also first Methicillin-resistant *S. aureus* strains were reported in the UK [51, 53, 54]. The mechanism behind the Methicillin resistance is the acquisition of a staphylococcal cassette chromosome *mec* (SCC*mec*). SCC*mec* is a genetic fragment with various length depending on SCC*mec* type that harbours *inter alia* the PBP2a gene *mecA* encoding for a Penicillin-binding protein that is responsible for Methicillin resistance. β-lactam antibiotics are not able to inhibit PBP2a, in contrast to native PBPs in *S. aureus* [51, 54].

The virulence and pathogenicity of MRSA is based on different toxins, immune invasion and adhesion factors. Since *S. aureus* is able to acquire different genetic elements, virulence factors such as toxins varies between different MRSA strains. While the Toxic Shock Syndrome Toxin (TSST), Panton-Valentine Leucocidin (PVL) and Exfoliative Toxins are mostly acquired, alpha- and gamma Hemolysins and Phenol-soluble Modulins (PSM) are produced by most strains. In contrast, the most surface proteins involved in host adhesion, immune invasion or internalization are encoded on the core genome except for the SasX protein that is acquirable *via* mobile genetic elements. Furthermore, SasX has a significant impact on virulence in SasX-positive MRSA [55]. The virulence of MRSA is also genetically regulated *via* two-component systems such as Agr or accessory regulators such as *sarA* [54, 56].

Another ability of *S. aureus* is the biofilm formation, that is highly problematic especially in hospital environment regarding catheter and ventilation-associated infections [57]. Biofilms are bacterial agglomerations embedded in an extracellular matrix that is surface-attached [54]. This extracellular matrix consists of DNA, proteins, polysaccharides and teichoic acids and forms an additional barrier against antimicrobials [58]. Bacteria within the matrix have an increased tolerance to antimicrobial agents [57-60]. Furthermore, sublethal antibiotic stress can induce biofilm formation [58, 61].

In the European Union (EU) and European Economic Area (EEA), the percentage of invasive MRSA isolates that were identified between 2015 and 2018 decreased from 19% to 16.4%. In contrast, the MRSA occurrence in the EU/EEA varies between 0% and 57% [31]. Especially in countries with higher MRSA incidence rates the percentage decreased. Countries like The Netherlands or the Scandinavian countries exhibit very low MRSA rates due to strong surveillance policies and restrained prescription handling. Nevertheless, in general, MRSA strains have the ability to acquired additional resistances to other antibiotics such as Erythromycin, Tetracycline or Vancomycin etc. and become multi-drug resistant [54].

1.4.2 *ESCHERICHIA COLI*

Escherichia coli is a Gram-negative rod-shaped human commensal colonizing the intestinal microbiota. In contrast, *E. coli* is an opportunistic pathogen causing severe infections in hospital or community environment [31] and is the most frequent cause of extraintestinal infections such as bloodstream and urinary tract infections [62]. Furthermore, *E. coli* is the most common Gram-negative organism that causes urinary tract infections [63, 64]. Resistant *E. coli* strains become a bigger threat since *E. coli* is able to acquire mobile genetic elements harbouring different resistance genes such as extended spectrum β-Lactamases (ESBLs). Carbapenems are β-lactam antibiotics that are not metabolized by ESBLs and can be used in the treatment of ESBL-positive *E. coli* infections. Nevertheless, Carbapenem-resistant *E. coli* strains were identified harbouring acquired Carbapenemase genes that can metabolize all available β-lactam antibiotics [31, 63, 65]. Furthermore, Carbapenem-resistant Gram-negative strains such as *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* spp. such as *E. coli* are listed as critical priority level pathogens concerning the development of novel antibiotics as published by the WHO (Table 1) [33].

In general, the mechanism behind resistance *via* ESBLs and Carbapenemases is the hydrolysis of the β-lactam ring and subsequently inactivation of the drug. ESBLs are divided into four Ambler classes from A to D where Penicillinases are belonging to class A, Metallo-β-lactamases to class B, Cephalosporinases to class C and Oxacillinases to class D. For Carbapenemases, there are three different classes A, B and D with a hydrolysis activity range between all β-lactam antibiotics and variable activity against Carbapenems and extended spectrum Cephalosporins but also with no hydrolysis of Aztreonam [63, 65].

To treat ESBL-producing organisms, different β-Lactamase inhibitors, such as Sulbactam, Clavulanate, and Tazobactam are applied in combination therapy with β-lactam antibiotics. Another option is using last resort antibiotics Polymyxins B and E (Colistin) to treat severe ESBL and Carbapenemase- bearing Gram-negative infections. Nevertheless, polymyxins have a nephrotoxic effect and should be used only in severe cases of drug resistance [63]. Furthermore, many Colistin resistances were already reported harbouring the *mcr* gene on various mobile genetic elements [66-70]. To tackle the Polymyxin resistance, MCR-1 protein is also a potential target for new antimicrobials [71].

As already mentioned in chapter 1.4.1, biofilm formation is a big threat among nosocomial pathogens due to its increased persistence against antibiotics and this is also true for many Gram-negative pathogens such as *E. coli* [72]. In cases of urinary tract infections, that can be associated to catheters harbouring *E. coli* biofilms, the uropathogenic *E. coli* (UPEC) express a variety of different virulence factors such as surface-associated factors, toxins and iron acquisition systems [64, 73, 74]. Especially the cell envelope of Gram-negative bacteria comprises a broad diversity of surface-associated virulence factors such as pili, flagella, capsules, curli, adhesins, lipopolysaccharides (LPS) and different outer-membrane proteins [64, 74].

For EU/EEA countries 58.3% of the reported *E. coli* isolates in 2018 were resistant to at least one group of antibiotics, among which 34.7% were resistant to one group, 10.9% to two groups, 7.8% to three groups and 5% to four groups. Furthermore, 0.1% of the isolates were resistant to all five groups of antibiotics consistent of aminopenicillins, 3rd-generation cephalosporins, fluoroquinolones, aminoglycosides and carbapenems. Fortunately, Carbapenem resistant strains are strongly underrepresented in the EU and EEA with a value of 0.1% in 2018. The resistance ratio for different antimicrobial groups decreased from 2015 to 2018 for

aminopenicillins (58.9% to 57.4%), carbapenems (0.2% to 0.1%) and aminoglycosides (11.6% to 11.1%) and increased for fluoroquinolones (24.8% to 25.3%) and 3rd generation cephalosporins (14.6% to 15.1%). Furthermore, multidrug resistant strains with resistances to four different classes (aminopenicillins, aminoglycosides, 3rd generation cephalosporins and fluoroquinolones) decreased slightly from 6.3% to 6.2% [31]. Nevertheless, it is still alarming to have over 50% of *E. coli* infections to be drug resistant in the EU/EEA countries.

1.4.3 *ACINETOBACTER BAUMANNII*

Acinetobacter baumannii is a ubiquitous, Gram-negative, non-motile coccobacillus that is predominantly drug-resistant and is involved is different nosocomial infections such as ventilation-associated pneumonia, urinary tract infections, wound infections and bloodstream infections [31, 75-77]. Nevertheless, community-associated *A. baumannii* infections increased over the last decade [78, 79]. Additionally, drug resistant *A. baumannii* was a highly prevalent pathogen during the wars in Lebanon, Iraq and Afghanistan causing many infections among combat casualties [80-82]. Another source for *Acinetobacter* spp. infections are animals that are susceptible for *Acinetobacter* spp. infections such as cats, dogs, horses or bovines [83].

The intrinsic drug resistance of *A. baumannii* is due to a high selectivity and prevention mechanisms to pass the outer membrane. Furthermore, *A. baumannii* possess a variety of different efflux systems to get rid of antimicrobials even if they are able to pass the membranes [75-77]. Antimicrobial groups that are still active in *A. baumannii* despite the high intrinsic resistance are carbapenems, polymyxins, fluoroquinolones, aminoglycosides, Tigecycline and Sulbactam [31]. However, *A. baumannii* is able to acquire mobile genetic elements with different resistances to become multi-drug resistant (MDR) [75, 76, 84]. MDR *A. baumannii* strains are defined to exhibit resistances to three or more of the previously named active antimicrobial classes [84, 85]. Interestingly, *Acinetobacter* spp. is able to survive on dry surfaces longer than other nosocomial pathogens such as *S. aureus* or *P. aeruginosa* [86, 87].

A variety of four different classes of efflux pumps are crucial for drug resistance in *A. baumannii* including the resistance-nodulation cell division (RND) family, small multidrug resistance (SMR) family, major facilitator superfamily (MFS), and multidrug and toxic compound extrusion (MATE) family. RND efflux is one of the most important mechanisms in drug resistant *A. baumannii* due to its extensive substrate variety

including antibiotics, detergents, dyes, antiseptics and biocides. Furthermore, RND is the most common family of efflux pumps in all Gram-negative bacteria [75, 88, 89]. The prominent RND efflux pump AdeABC in *A. baumannii* is associated with a broad range of resistances to antibiotics, including β-lactams, fluoroquinolones, tetracyclines, macrolides, aminoglycosides and Chloramphenicol [75, 87-89]. In addition, porins, channel proteins that allow the passage of hydrophilic substances such as antibiotics, show low permeability in *A. baumannii* and *P. aeruginosa*, getting lost or are lower expressed [90, 91]. Consequently, decreased permeability increases the synergistic effects in combination with efflux systems in case of resistance [89]. Porins that are involved in Carbapenem resistance were recently described [92-94]. Fortunately, few efflux pump inhibitors such as Omeprazole, Verapamil, Reserpine, CCCP, NMP or PAβN are available which can strengthen the activity of antibiotics in resistant strains. Additionally, the flavonoid compound Quercetin was found to inhibit efflux pump as well as Carbapenemase [95]. However, the cytotoxicity of these inhibitors can strongly curb their clinical applicability [75].

An additional threat related to *A. baumannii* infections is the ability of this bacterium to form biofilms. Biofilms are complex communities with an extracellular matrix consisted of DNA, proteins, lipids and polysaccharides that are attached to a biotic or abiotic surface [89]. Furthermore, biofilm formation is found in common nosocomial pathogens such as *S. aureus* (chapter 1.4.1), *E. coli* (chapter 1.4.2), *P. aeruginosa* (chapter 1.4.4), *K. pneumoniae* and *A. baumannii* (this chapter) [89] which are members of the ESKAPE group. Furthermore, catheter-related urinary and bloodstream infections are caused by biofilm-forming *A. baumannii* [79, 96].

A. baumannii exhibits different virulence factors including biofilm formation, stress resistance, surface motility and extracellular components involved in hemolytic, iron chelating, Phospholipase and Protease activities [79, 97]. Iron acquisition systems are crucial for pathogenicity and survival of bacteria. For *A. baumannii*, a variety of endogenous siderophores were identified in clinical isolates. Further, a zinc acquisition system was found in *A. baumannii* involved in pathogenesis [79, 98]. Other virulence factors involved in pathogenesis were identified: the K1 capsular polysaccharide prevents phagocytosis by macrophages [99]; the outer membrane protein 38 (Omp38) induces apoptosis of host cells [100]; inactivation of Phospholipase D diminishes *A. baumannii* pathogenesis [101]; and the outer membrane protein A (OmpA), which is the most abundant surface protein, is involved in apoptosis of epithelial cells and

intrinsic antibiotic resistance [102, 103]. Furthermore, loss of Lipid A, a lipopolysaccharide (LPS) component, leads to Polymyxin resistance in *A. baumannii* [104, 105]. Due to these reasons, *A. baumannii* is listed on top of the WHO priority list of critical pathogens (Table 1) [33].

In the ECDC surveillance report for 2018, *Acinetobacter* spp. were consolidated in a statistical analysis. Overall, 56.4% of reported isolates in the EU/EEA were resistant to at least one antibiotic. The variety of the ratios between different countries is from 0 to 96.1%. The southern, south-eastern and eastern countries in the EU/EEA are the most critical. While the average of Fluoroquinolone resistant cases decreased for the EU/EEA from 2015 to 2018 (38.6% to 36.2%), the reported ratios of resistant isolates were increased in 10 of 30 countries. Especially in Croatia the ratio of resistant isolates increased from 92.3% to 96.1%, concluding that almost all *Acinetobacter* spp. isolates are resistant to fluoroquinolones. In case of Aminoglycoside resistant isolates, the average slightly decreased in the EU/EEA (32.4% to 31.9%). Nevertheless, the ratio increased in 13 of 30 countries including Croatia (88.3% to 91.5%). For Carbapenem, one of the most relevant antibiotics in combating drug resistant Gram-negative infections, the average ratio decreased slightly (32.1 to 31.9%) for the EU/EEA, but increased in 17 of 30 countries including Croatia (89% to 95.5%). Regarding pan-resistance to all of the three antimicrobial groups fluoroquinolones, aminoglycosides and carbapenems, the ratio increased within EU/EEA (27.7% to 28.8%) and overall, in 16 of 30 countries. Croatia again showed the highest ratio (90.8% in 2018) [31].

Alternative therapies were recently described [84, 106] and tested including iron chelating therapy [107-109], bacteriophage therapy [110-113] and prophylactic vaccination [114-119]. However, these therapies are still not optimal. Nevertheless, multi-drug resistant *Acinetobacter* spp. is still a major threat especially in nosocomial infection settings.

1.4.4 *PSEUDOMONAS AERUGINOSA*

Pseudomonas aeruginosa is a rod-shaped, encapsulated, Gram-negative bacterium which is ubiquitous in aquatic environments. As an opportunistic pathogen, *P. aeruginosa* causes various healthcare-associated infections such as bloodstream and urinary tract infections and pneumonia [31]. Furthermore, *P. aeruginosa* is also infecting animal and plant hosts [83, 120-123].

There are a few antimicrobial groups that are active against *P. aeruginosa* including polymyxins, some fluoroquinolones, some β-lactams and aminoglycosides [31]. Nevertheless, *P. aeruginosa* is intrinsically resistant to various antibiotics due to its selective permeability and efflux systems associated with the outer membrane composition [124-127]. For instance, the outer membrane porin OprD is involved in diffusion of amino acids and peptides but also in Carbapenem resistance in clinical isolates [128-132]. The constitutively expressed efflux pump MexAB-OprM from the RND family has a broad substrate specificity such as β-lactams (including β-Lactamase inhibitors and some carbapenems), some aminoglycosides, fluoroquinolones, macrolides, tetracyclines, amphenicols, sulfonamides, and few more. Other substrates beside antibiotics are a series of dyes, disinfectants, detergents, solvents and amphiphilic molecules [125, 133-135]. Overexpression of MexAB-OprM in clinical isolates increases the resistance to these different antimicrobials [136-139]. Further, four RND efflux pumps, MexCD-OprJ, MexEF-OprN, MexGHI-OpmD and MexJK, were identified that are involved in drug resistance [125, 133]. Another mechanism conferring intrinsic resistance to β-lactam antibiotics is the inducible expression of chromosomal Cephalosporinase (β-Lactamase) AmpC and of the Oxacillinase Bla_{OXA-50}, which plays a minor role in β-lactam resistance [83, 126, 140, 141]. Specific mutations can lead to an antimicrobial resistance. For instance, the chromosomal gene *aphA* coding for an Aminoglycoside inactivation enzyme was already described in 1983. Mutations can activate the gene resulting in Aminoglycoside resistance [142]. Further, DNA Gyrase, efflux pump and outer membrane protein mutations can result in Quinolone resistance [126, 127, 143, 144]. Another intrinsic resistance mechanism in *P. aeruginosa* is the ability to form biofilms [83]. Biofilms are estimated to be involved in 65% of microbial diseases and in more than 80% of chronic infections. Furthermore, *P. aeruginosa* is the 2nd most common pathogen involved in ventilation-associated pneumonia and catheter-associated urinary tract infections potentially related to biofilms [145, 146]. Furthermore, biofilm formation is involved in

non-device-associated infections such as chronic infections, osteomyelitis, periodontitis and is particularly deadly in cystic fibrosis (CF) patients [146, 147]. Quorum sensing (QS) is a cell-to-cell communication mechanism in bacteria and was shown to be involved in expression of virulence genes and biofilm formation in *P. aeruginosa* [145, 146, 148, 149]. Treatment with macrolides such as Azithromycin resulted in inhibition of the production of QS signals, impaired motility, inhibited biofilm formation and inhibition of synthesis of the virulence factor alginate [150, 151].

Additional resistances can be acquired *via* mobile genetic elements. In *P. aeruginosa*, Carbapenemases (Metallo-β-lactamases) and ESBLs are frequently acquired combined with Aminoglycoside-modifying enzymes [124, 126, 152].

P. aeruginosa has a repertoire of different virulence factors including LPS, alginate, flagella, rhamnolipids, exotoxins, adhesion factors, exoenzymes including Exoproteases, Phospholipase C, Hemolysins and Sialidase [153-155]. For instance, the toxin Pyocyanin is secreted by *P. aeruginosa* in the early colonization phase to establish the infection [148, 156]. Elastase is degrading elastin and collagen e.g. in human lung resulting in destruction of lung tissue [148, 155]. Rhamnolipids are hemolytic glycolipids with detergent-like activity found in the sputum of CF patients [148, 157]. Alginate is an exopolysaccharide that protects the bacterium from host immune response and antibiotics. Furthermore, it plays a role in adhesion to respiratory epithelial cells [153]. Adhesion factors such as pili are involved in adhesion, initiation of colonization of the host and biofilm formation [153, 158, 159]. One of the major virulence factors in lung infections is the exotoxin ExoU that is injected *via* Type III secretory apparatus including the enzymes ExoS, ExoT and ExoY, causing loss of membrane integrity in epithelial cells resulting in necrotic cell death [153, 160, 161]. Further, two different Phospholipases C, PcH (hemolytic) and PlcN (non-hemolytic), hydrolyse phospholipids in pulmonary surfactants [153, 162]. Sialidases or Neuraminidases, which are produced by a wide range of bacteria but also by animals, catalyse the removal of sialic acid out of glycoconjugates. Furthermore, pathogens can use the sialic acid as a carbon source when possessing the corresponding Permeases [163]. However, *P. aeruginosa* is a multi-host opportunistic pathogen, and many of the virulence factors are specific regarding the host and infection model [164].

Among the *P. aeruginosa* isolates, 32.1% were drug resistant at least to one antibiotic in the EU/EEA in 2018. Moreover, 12.9% were resistant to one antimicrobial group, 7.6% to two groups, 4.1% to three groups, 3.4% to four groups, and 4.1% were

reported to be almost pan-resistant with a resistance to five different antimicrobial groups. These groups comprise Piperacillin/Tazobactam combination, fluoroquinolones, Ceftazidime, aminoglycosides and carbapenems [31]. In total, between 2015 and 2018, the invasive isolates of *P. aeruginosa* that are multi-drug resistant to three or more antimicrobial groups decreased from 15.1% to 12.8% in EU/EEA, even though the ratios increased in 9 of 30 countries. Especially in Romania every second infection with *P. aeruginosa* is multi-drug resistant (49.4%). Piperacillin/Tazobactam resistance decreased in EU/EEA from 19.9% to 18.3%, while the ratios increased in 14 of 30 countries. For fluoroquinolones, the mean ratio decreased from 20.9% to 19.7%, while it increased in 16 of 30 countries. A comparable pattern was also observed for Ceftazidime. While the mean ratio decreased from 15.4% to 14.1%, the ratios increased in 14 of 30 countries. For aminoglycosides, the average decreased from 15.3% to 11.8% and increased in only 6 of 30 countries. And last but not least, Carbapenem resistant *P. aeruginosa* isolates decreased in the EU/EEA average from 19.4% to 17.2% and increased in 12 of 30 countries.

Taken together, Carbapenem resistant *P. aeruginosa*, which is listed in the WHO critical priority level, is associated with resistance to other antimicrobial groups and is still prevalent in hospital-associated infections (Table 1) [31, 33].

2 AIM

Antimicrobial resistance among human pathogens has become a big threat resulting in a global resistance crisis due to misuse of antibiotics especially in healthcare and livestock environment. Despite of rapid resistance evolution to new antimicrobials that was shown especially in the 1950s and 1960s, a discovery void of new antimicrobials emerged for the last 30 years. To tackle this issue, many different conservative and innovative strategies were described including exploration of new antimicrobial sources, targeting subpopulations such as persisters and biofilms and virulence factors or the use of antimicrobial conjugates and nanocarriers. These strategies are reviewed in chapter 3 of this thesis. For this thesis, new lead structures were sought by screening plant-derived, endophytic and sponge-derived natural product libraries and testing them for activity against different drug-resistant Gram-positive and Gram-negative bacteria including Methicillin-resistant *S. aureus* and multi-drug resistant *A. baumannii* and *E. coli*. Additionally, a susceptible strain of *P. aeruginosa* was also included in the screening approach. Active compounds with a minimal inhibitory concentration (MIC) ≤12.5 μM were screened for cytotoxicity in different human cell lines. A therapeutic window of ≥8 is required for further investigation of target and mechanism. To elucidate the mode of action, spontaneously resistant mutants were generated followed by genome sequencing to identify the mutations. Mutations in distinct genes can reveal the resistance mechanism.

Taken together, in this thesis new urgently needed antimicrobial lead structures with natural origin were sought, identified and the corresponding mechanism was elucidated in some cases.

3 (SOME) CURRENT CONCEPTS IN ANTIMICROBIAL DRUG DISCOVERY

Published in: Applied Microbiology Biotechnology, April 2018; 102(7): 2949-2963.
Published online ahead of print, 17 February 2018
Impact Factor: 3.670 (2018)
DOI: 10.1007/s00253-018-8843-6
Overall contribution to the paper: 25%

- Chapter: Alternative strategies for antimicrobial compound development

4 THE PLANT-DERIVED CHALCONE XANTHOANGELOL TARGETS THE MEMBRANE OF GRAM-POSITIVE BACTERIA

Published in Bioorg. & Medicin. Chem., Vol. 27, Issue 23, December 2019; 115151
Published online ahead of print, 15 October, 2019
Impact factor: 2.802 (2018)
DOI: 10.1016/j.bmc.2019.115151
Overall contribution to the paper: 75%

- Writing of first version of complete manuscript draft
- Determination of minimal inhibitory concentrations (MICs)
- Growth kinetics
- Time-kill assay
- Cytotoxicity assay
- Hemolysis assay
- GFP and ATP release assay
- Propidium iodide internalization
- Measurement of membrane potential

5 NATURAL BROMINATED PHENOXYPHENOLS KILL PERSISTENT AND BIOFILM-INCORPORATED CELLS OF METHICILLIN-RESISTANT *STAPHYLOCOCCUS AUREUS* AND *PSEUDOMONAS AERUGINOSA*

Published in Applied Microbiology and Biotechnology 2020
Print version not published yet (11 June 2020)
Published online ahead of print, 16 May 2020
Impact factor: 3.67 (2018)
DOI: 10.1007/s00253-020-10654-4
Overall contribution to the paper: 15%

- Screening for Gram-negative bacteria

6 DIDYMELLANOSINE, A NEW DECAHYDROFLUORENE ANALOGUE, AND ASCOLACTONE C FROM *DIDYMELLA* SP. IEA-3B.1, AN ENDOPHYTE OF *TERMINALIA CATAPPA*

Published in Royal Society of Chemistry, 18 February 2020; 10, 7232-7240
Impact factor: 3.049 (2018)
DOI: 10.1039/c9ra10685e
Overall contribution to the paper: 10%

- MIC screenings for gram-negative bacteria
- Cytotoxicity assay for MRC5

7 FURTHER RESEARCH CONTRIBUTIONS

Lienau C., Gräwert T., Alves Avelar L.A., Illarionov B., Held J., Knaab T.C., Lungerich B., van Geelen L., **Meier D**., Geissler S., Cynis H., Riederer U., Buchholz M., Kalscheuer R., Bacher A., Mordmüller B., Fischer M., Kurz T., Novel reverse thia-analogs of fosmidomycin: Synthesis and antiplasmodial activity. Eur J Med Chem. 181(1), (2019).
DOI: 10.1016/j.ejmech.2019.07.058

Yu X., Müller W.E.G., **Meier D.**, Kalscheuer R., Guo Z., Zou K., Umeokoli B.O., Liu Z., Proksch P. "Polyketide Derivatives from Mangrove Derived Endophytic Fungus Pseudopestalotiopsis theae." Marine Drugs 18(2): 129. (2020)
DOI: 10.3390/md18020129

8 DISCUSSION

Natural products are a major source of different pharmaceuticals beside the synthetic and semisynthetic bioactive agents. With respect to antimicrobials, natural products are basically secondary metabolites of fungi, bacteria or plants [165] able to combat different pathogens. Indeed, the variety of natural products is very huge but many sources are still underexplored. In our institute, we are isolating natural compounds for a constantly growing library for bioactivity prospecting focusing on the struggle of fighting drug resistance in nosocomial pathogens and in *Mycobacterium tuberculosis*. The majority of these compounds are marine-, plant- and endophyte-derived.

New natural compounds were identified with activity against Gram-positive and Gram-negative pathogens including drug-resistant strains in this work. Especially the ESKAPE pathogens are of major concern in the global resistance crisis and a central point in this thesis. Xanthoangelol was shown to be active against various Gram-positive pathogens including Methicillin-resistant *Staphylococcus aureus*. Two brominated phenoxyphenols were active against all tested Gram-positive and Gram-negative pathogens such as MRSA, MDR *A. baumannii*, Vancomycin-resistant *E. faecium* and *E. faecalis* and a drug susceptible strain of *P. aeruginosa*. A new compound from an endophytic fungus, Didymellanosine, revealed activity against critical Gram-positive pathogens but also against murine and human cancer cells. Furthermore, combinatory treatment of Didymellanosine with sublethal concentrations of Colistin showed activity against MDR *A. baumannii*. All compounds revealed a moderate therapeutic window. Nevertheless, the compounds should be optimized to reduce the cytotoxic and hemolytic effects.

In detail: Xanthoangelol is a plant-derived geranylated chalcone isolated from fruits of the deciduous shrub *Amorpha fructicosa* [166]. Furthermore, Xanthoangelol has also been isolated from *Angelica keiskei*, a herb from the *Apiaceae* family commonly based in Japan, and was recently described to exhibit anti-cancer [167-172], anti-diabetic [173-176], anti-thrombotic [177, 178], anti-inflammatory [179-181], anti-oxidative [182] and antibacterial [166, 183, 184] activity. However, the mechanism behind the antibacterial activity was not elucidated. In previous studies, Xanthoangelol derivatives Xanthoangelol B, D, E, F and K with various bioactivities such as anti-virulence [185], anti-thrombotic [180, 186], anti-cancer *via* inhibitory effect on NF-*κ*B [187] and anti-diabetic *via* PTP1B inhibition [188] were described. Other bioactive derivatives such

as Bavachalcone and Isobavachalcone were reported with different activities [189-197] including anti-viral activity against corona virus of MERS-CoV type [198].

In this, study the antibacterial effect on various Gram-positive pathogens including Methicillin-resistant *S. aureus*, Vancomycin-resistant *E. faecium* and Vancomycin-resistant *E. faecalis* was confirmed demonstrating an MIC varying between 3.125 and 12.5 *µ*M. Unfortunately, the specific mechanism of Xanthoangelol could not be identified, but the study revealed the bacterial membrane as a target. Furthermore, affected membrane integrity was verified by measurements of membrane potential and leaking metabolites such as GFP and ATP. Lysis of MRSA cells was confirmed by measurement of the optical density. To substantiate the hypothesis that the bacterial membrane is affected, the proteomic stress response was elucidated in the model organism *Bacillus subtilis*. Proteins involved in cell envelope stress, oxidative stress and heat shock stress were induced by treatment with sublethal Xanthoangelol concentrations. In conclusion, the killing effect of Xanthoangelol might be based on forming pores in the membrane after a rapid decrease of membrane potential that leads to a leakage of metabolites and subsequent lysis of the cell. Additionally, cytotoxic (IC_{90}: 27.6 – 85.8 *µ*M) and hemolytic (>100 *µ*M) effects were only observed at concentrations considerably above the MIC. Thereby, the selectivity index (SI) of Xanthoangelol in different human cell lines versus the MRSA strain (ATCC 700699) varies between 10.30 and 44.21 depending on the tested cell line, resulting in an appropriate therapeutic window.

Indeed, in general, chalcones as secondary metabolites are highly interesting natural compounds with a variety of biological activities. Furthermore, chalcones are intermediates of flavonoid synthesis [199] and thereby ubiquitous in plants. That makes chalcones and flavonoids predestine as a scaffold for new phytopharmaceuticals. Nevertheless, beside the fact that chalcones and flavonoids were repeatedly described with antibacterial activity [165, 184, 200-203], none of the commonly used antibiotic groups are chalcone or flavonoid derived. In general, antibacterial activity of flavonoids was estimated considering different publications by Cushnie *et al.* in 2011. Different mechanisms of flavonoids were summarized concluding that flavonoids are very promising due to their broad spectrum of activity including antibacterial and synergistic effects [165]. In addition, chalcone derivatives were examined for structure-activity relationship with respect to antibacterial effects [201, 204, 205] and synergistic effects with antibiotics [200]. Finally, the crucial moieties of chalcones for antimicrobial activity

were identified [203] consisting of a hydrophilic moiety *via* hydroxylation and a lipophilic moiety such as geranyl-group in case of Xanthoangelol. Further, Catechin, a flavonoid (flavan-3-ol), was recently described to have an impact on different virulence factors such as Pyocyanin, Elastase and biofilm in *P. aeruginosa* interfering with the quorum sensing system [206]. Semi-synthetic improvements of the flavonoids revealed enhanced antibacterial activity in different pathogens [165, 207, 208]. A new study published in 2020 revealed ferrocenyl chalcone derivatives with strong antimicrobial activity. Furthermore, bacteriolytic effects were confirmed in this study [202], which is consistent with our study on the geranyl chalcone Xanthoangelol. Another parallel is the lipophilic moiety of the ferrocene group. In contrast, activity in Gram-negative *E. coli* was shown with slightly higher MIC. Nevertheless, hemolytic and cytotoxic effects were not investigated for ferrocenyl chalcones. Taken together, chalcone and flavonoid scaffolds are very promising with respect to different diseases including pathogen-associated infections. Furthermore, chalcones such as Xanthoangelol with lipophilic and hydrophilic moieties should be considered to tackle the drug-resistant pathogens. Nevertheless, the Xanthoangelol structure should be optimized regarding reducing its cytotoxicity and hemolytic effects and lowering the MIC. For instance, already described but also new derivatives should be screened for antimicrobial and cytotoxic activity to improve the compound and hereupon introduce for further studies *in vivo* against drug-resistant pathogens.

For the next project in this thesis, two sponge-derived brominated phenoxyphenols (PPs) from the marine sponge *Dysidea granulosa* were identified with a broad-spectrum activity against Gram-positive and Gram-negative pathogens named 2-bromo-PP and 3-bromo-PP. The sponge samples are originated from the Andaman Sea in Thailand and collected in 2007. In addition, these compounds are also called hydroxylated polybrominated diphenyl ethers (OH-PBDEs or OH-BDEs) in different publications and will be reviewed in the next paragraph. The antibacterial activity was confirmed in almost all pathogens of the ESKAPE group including *E. faecium*, *S. aureus*, *K. pneumoniae*, *A. baumannii, P. aeruginosa* and *E. coli*. The MIC was lower in Gram-positive pathogens with values between 0.1 and 0.78 *µ*M while in Gram-negative the MIC was between 0.78 and 3.125 *µ*M respectively. In addition, PPs were active against subpopulations such as persisters and biofilm-incorporated cells of MRSA and *P. aeruginosa*. While the 2-bromo-PP MIC was consistent at 12.5 *µ*M in

both persistent organisms, the MIC for 3-bromo-PP differs between the organisms with 12.5 *μ*M in MRSA and 50 *μ*M in *P. aeruginosa*. For biofilms, the MIC of 3-bromo-PP was at 12.5 *μ*M and for 2-bromo-PP at 100 *μ*M in MRSA, while the MICs in *P. aeruginosa* were at 25 *μ*M for 2-bromo-PP and 50 *μ*M for 3-bromo-PP. Cytotoxicity of the PPs was evaluated exhibiting IC_{50} values between 6.25 and 100 *μ*M for 2-bromo-PP and between 25 and 100 *μ*M for 3-bromo-PP in various human cell lines, with exception of the kidney cells HEK293 showing an IC_{50} = 3.125 *μ*M. Compared to the MIC values in pathogens, the therapeutic window is appropriate in Gram-positive planktonic pathogens. In contrast, the MIC and IC_{50} values in drug-tolerant subpopulations are quite comparable. Nevertheless, active compounds that able to pass the biofilm matrix or kill bacteria with downregulated metabolism are quite rare since almost all common antibiotics are exclusively interfering with metabolic processes required for active growth. To elucidate the mode of action, spontaneous resistant mutants were generated in *S. aureus*. Thereby, cross-resistance between both agents was found indicating that they have the same target. Two different mutations were identified in various mutants involved in Phosphotransferase systems (PTSs). The *tetR* transcriptional regulator, putatively regulating the sucrose PTS gene *scrA*, and the putative promotor region upstream of *mtlA*, which potentially encodes a component of a mannitol-specific PTS, were mutated *via* single nucleotide polymorphism (SNP). Downregulation of both genes, *mtlA* and *scrA*, was observed in a RT-qPCR assay and proteomic analysis of treated cells with sublethal concentrations of the PPs. *Vice versa*, overexpression of distinct genes led to a higher susceptibility towards PPs in a significant manner. Consequently, the hypothesis that both PTS are involved in PP resistance could be confirmed. Since the PTSs are translocating their sugar substrates *via* concomitant phosphorylation, the same mechanism is suggested for PP uptake *via* PTS. In prospect, further confirmations of the uptake mechanism are necessary. For instance, phosphorylated PPs could be synthesized and analysed for activity also in mutants. The cytotoxicity should be decreased possibly *via* synthesis of new derivatives. Furthermore, the mode of action within the cell that leads to the bactericidal effect should be elucidated, since only the probable uptake was identified in this study. Nevertheless, PP is a suitable lead structure for treatment of difficult subpopulations such as biofilms or persisters.

Marine-derived natural products are of increasing interest for the last two decades [209, 210]. In 2014, Mehbub *et al.* reviewed marine sponge derived natural products

that were described with different bioactivities between 2001 and 2010. Over 1600 compounds from different sponges were listed including 828 compounds with anticancer/cytotoxicity effects, 31 with anti-tuberculosis effects and 145 with antibacterial effects. Gribble *et al.* reviewed halogenated marine natural products that were recently discovered between 2011 and 2015 with various bioactivities [209].

Hydroxylated polybrominated diphenyl ethers (OH-PBDEs) are probably ubiquitous compounds found in different biotic and abiotic environments [211-215]. The sources of OH-PBDEs are still not understood since PBDEs were industrially used as flame retardants in plastic, paper and textile manufacturing and are considered to be a precursor of OH-PBDEs [211, 212] even though OH-PBDEs were never synthesized for industrial purpose [215]. Nowadays PBDEs are not used industrially anymore because of their cytotoxicity but they are still present in the environment as persistent organic pollutants (POPs) due to their stability [215]. For instance, the cytotoxicity was associated with neuronal, thyroid and liver activity [212, 216-227]. Furthermore, it was shown that PBDEs are environmentally transformed and biotransformed to OH-PBDEs and MeO-PBDEs after accumulation in different organisms [212, 215, 223, 226-231] and can be retransformed to even more toxic compounds such as polybrominated dibenzo-*p*-dioxins (PBDDs) [215, 232-234]. Notwithstanding, PBDDs were described as natural substances in red algae, cyanobacteria samples, blue mussels and fish [233, 235]. Also, OH-PBDEs were reported as natural compounds in different marine organisms [212-214, 229, 236, 237]. Interestingly, PBDEs isolated from the sponge *Dysidea sp.* were probably produced by the endosymbiotic cyanobacterium *Oscillatoria spongeliae* and the *Dysidea*-associated marine bacterium *Vibrio* sp. [238, 239]. Corroborating a natural origin, biosynthetic gene clusters of PBDE within sponge-microbiome-associated cyanobacterial endosymbionts [240] and biosynthesis clusters of PBDE in marine bacteria *Pseudoalteromonas* spp. [236] were discovered recently. Regardless of the natural or (semi)synthetic origin, PBDEs and OH-PBDEs were detected in human blood and maternal milk [226, 241, 242]. Furthermore, halogenated flame retardants were identified in European seafood with increased values for PBDEs and most contaminated location was the Mediterranean Sea. In addition, the cooking process accumulates PBDEs in seafood. Nevertheless, the risk assessment revealed that there is no health risk after seafood consumption [227].

OH-PBDEs used in our study, 2-bromo-PP (OH-BDE-68) and 3-bromo-PP (OH-BDE-123), were already described in different studies. Radiocarbon analysis revealed that OH-BDE-68 isolated from *Dysidea granulosa* is naturally produced [243]. Antimicrobial activity of different OH-BDEs including OH-BDE-68 against *B. subtilis* was already described in 1997 by the group of Professor Dr. Proksch in Würzburg [244]. Bioactivity of these compounds against *Streptomyces* sp. and anti-proliferative activity in human cancer cell line MCF-7 were described in 2008 [245] and antibacterial activity against MRSA, and VRE strains was already described in 2009 [246]. Nevertheless, OH-BDE-68 was frequently found in different aquatic organisms with different sources of origin [243]. Further studies on OH-BDE-68: OH-BDE-68 formation occurs under irradiation of sunlight in aqueous solution using one of the most abundant flame retardant BDE-47 or 2,4-Dibromophenol [212, 247]. Bioaccumulation of OH-BDE-68 was shown in liver and kidney of carps [248]. Increased OH-PBDE concentrations, including OH-BDE-68, were found in herring from Baltic Sea between 1980 and 2010 with decreased fat content of the fish concluding the disruption of oxidative phosphorylation *via* OH-PBDEs [249, 250]. Direct photolysis of OH-BDE-68 leads to different products including Tribromodibenzo-*p*-dioxin [251]. In contrast, a recently published study with participation of Prof. Dr. Proksch revealed anti-cancer activity of both compounds (called P1F03 and P01F08 in this study) [252].

Taken together, brominated phenoxyphenols such as OH-PBDEs are bioactive compounds with a variety of bioactivities including antibacterial, anti-cancer and cytotoxic effects. With respect to described cytotoxic effects and its ability as contaminant to accumulate in different organisms, a balance should be found in new antimicrobials or anti-cancer agents with PP (OH-PBDE) scaffold between antimicrobial activity and cytotoxicity. Since PPs are active also against difficult-to-treat bacterial subpopulations such as persisters and biofilms, derivatives of PPs should keep a low molecular weight to maintain a high permeability in case of biofilms. Compounds with a larger molecule size may not be able to penetrate the extracellular biofilm matrix. In case of planktonic pathogens, also bulkier substituents are possible in screening assays. Furthermore, only few agents are available that are able to tackle biofilm formations and therefor PP lead structure should not be discriminated due to its cytotoxic effects.

Another proliferative source of natural products are endophytes. The new alkaloid Didymellanosine (DM), a decahydrofluorene analogue, was isolated from the endophytic fungus *Didymella* sp. IEA-3B.1 inhabiting *inter alia* leaves of the plant *Terminalia catappa* (Combretaceae). Antimicrobial activity was revealed against Gram-positive pathogens such as MRSA and VRE strains. Furthermore, the Gram-negative drug-resistant pathogen *A. baumannii* was also affected in combination with sublethal concentrations of the last resort antibiotic Colistin. In contrast, cytotoxic effects were shown for human (Jurkat and Ramos) and murine (L5178Y) cancer cells compared to moderate cytotoxicity in non-malignant human cells MRC-5 hinting to a higher specificity for cancer cells. Nevertheless, the cytotoxic effects in cancer cells are comparable to its antimicrobial MIC counterpart revealing only a very narrow therapeutic window. Additionally, NF-*κ*B inhibition was found for DM illustrating the cytotoxic effects to all mammalian cells. Further investigations are necessary to improve the DM compound as a lead structure against ESKAPE pathogens, because the target is putatively different in mammalian cells and in bacteria.

Other compounds with a decahydrofluorene scaffold were already previously shown to exhibit various bioactivities. For instance, Hirsutellones A-E from the insect pathogenic fungus *Hirsutella nivea* were described with antitubercular effects [253] and exhibiting moderate cytotoxicity in different cell lines [254]. In addition, hirsutellones are closely related to the antifungal agents GKK1032A and GKK1032B [254]. Furthermore, GKK1032C and GKK1032A2 were identified recently in an endophytic *Penicillium* sp. fungus with antibacterial activity against MRSA [255]. Pyrrocidines A and B were described with antibacterial activity against Gram-positive pathogens including drug resistant strains [256]. Years later, Pyrrocidine C was identified with antibacterial and cytotoxic effects [257]. Cytotoxic effects of Pyrrocidine A was observed in cancer cell lines inducing apoptosis *via* Caspase activation [258]. Unfortunately, only different synthesis approaches were described for these decahydrofluorene compounds in the following years without any substantial investigation of its bioactivities [259-266]. However, another additional decahydrofluorene analogue, HY253, was described with anti-cancer activity inducing apoptosis in two different studies and different cell lines [267, 268].

In general, endophytic fungi are of major interest in exploration of new bioactive agents. Furthermore, endophytes are symbiotically co-existing in plants with various benefits for the plants such as growth enhancement and pathogen resistance. It is also

important to understand the relationship between medicinal plants and their endophytes. Many medicinal plants may have beneficial effects on health due to endophytic metabolites. Therefore, the relationship of endophytic fungi and their host plants were reviewed considering research papers published between 1986 and 2016 [269]. Additionally, anti-fungal agents that were identified between 2013 and 2018 from endophytic fungi were recently reviewed [270]. Different anti-cancer agents with endophytic origin were reviewed for the period between 1990 and 2010 exhibiting a variety of chemical classes [271], and also antimicrobial agents were reviewed in 2015 [272]. Endophytic fungi were also described to synthesize clinical anti-cancer drugs and their precursors [273].

Taken together, endophytic fungi are rich of natural bioactive compounds with different anti-cancer, antibacterial and antifungal activities and are a suitable source for new lead structures to tackle the ESKAPE pathogens. In addition, decahydrofluorene analogues from endophytic fungi were identified with different bioactivities. Our new compound DM with a decahydrofluorene core exhibits antibacterial and cytotoxic effects and must be optimized with respect to its cytotoxicity in case of antibacterial activity. In case of anti-cancer activity, the compound should be improved with regard to reducing cytotoxicity to non-malignant cells.

In conclusion, many different sources of natural products were investigated during this work. On the one hand, plants, an almost unlimited source of metabolites involved in various bioactivities, and their endophytes with also broad-spectrum bioactive metabolites with high potential for new antimicrobials, and on the other hand marine organisms such as cyanobacteria, sponges or algae are suitable sources for new antimicrobials and are also still underexploited. Furthermore, plants were used for thousands of years for medicinal applications and are still a reliable source of new natural compounds able to tackle the drug-resistant pathogens. Nevertheless, newly investigated compounds presented in this work have to be improved to enhance the therapeutic window and to make further steps towards *in vivo* trials. The antimicrobial drug resistance crisis led to an enhancement of research on new drugs to combat the increasing occurrence of drug-resistant strains, but it is still inadequate with respect to the private sector. But the plethora of different natural sources and newly investigated agents are very promising. Even though, it is still a race against time.

Prospective

Different strategies to tackle the drug resistance and research on new antimicrobial agents were intensively described in chapter 3 (page 25). General strategies to manage the antibiotic resistance crisis beside the research on new antimicrobials are preventive provisions and appropriate and well-balanced application of antibiotics. The most promising and practicable steps with respect to the health care sector were summarized by C.L. Ventola in 2015 [19]. The described steps are: tracking and prescription practices, improving diagnosis, optimizing therapeutic regimens, adopting an antibiotic stewardship program and preventing infection transmission.

In the US, tracking programs were established such as the Healthcare-Associated Infections-Community Interface (HAIC), the Active Bacterial Core surveillance (ABCs), the National Tuberculosis Surveillance System (NTSS) and the Gonococcal Isolate Surveillance Project (GISP) supported by the Centre for Disease Prevention and Control (CDC). Also, the National Antimicrobial Resistance Monitoring System (NARMS) was established in collaboration of CDC, the Food and Drug Administration (FDA) and the US Department of Agriculture (USDA). Additionally, the CDC implemented the National Healthcare Safety Network (NHSN) where health care facilities can report infections, antibiotic use and occurrence of resistant strains. In the digital era, it is possible to establish networks worldwide with a database where the facilities can report infections and resistances. With respect to antimicrobial resistant pathogens, surveillance reports with statistics are published frequently in the US, EU and other countries.

To improve the prescription practices, ideally the pathogen should be identified before treatment with antibiotics is initiated. In every second case, the prescribed antibiotics are unnecessary or incorrect. Moreover, in cases of virus infections antibiotics were misleadingly prescribed in 40% to 75%. The prescription practice is still of concern today. Frequent education in handling of antibiotics and its prescription is needed.

Screenings after arrival of infected patients in healthcare facilities such as practised in The Netherlands should be performed to improve diagnosis and to prevent a resistance outbreak within the facility. For instance, in the US only 7.6% of patients with community-acquired pneumonia that were hospitalized were screened for the pathogen. That leads to empiric use of antibiotics, and the treatment with different antibiotics harms the human microbiota including selective pressure towards the resistant strains. Indeed, there is an improvement necessary in diagnosis of bacterial

infections to guarantee appropriate antibiotic treatment. Toward this end, many different molecular diagnostic tools are already established.

Application regimens of antibiotics are in many cases suboptimal. It was shown that shorter courses of therapy can be as effective as long-term treatment. Prolonged application of antibiotics can drive the resistance evolution and occupy the healthcare unit for a longer period of time. Consequently, the standard regimens that are usually used since decades are not up to date in all cases and should be optimized. Nevertheless, non-clinical application of antibiotics e.g. at home, can lead to a shorter treatment after the patient feels better and stops application of the drug before the regimen. Remaining antibiotics will be used for future self-treatments of unknown infections or thrown away and contaminate the environment that can drive resistance evolution. Therefore, an antibiotic recycling system should be established for instance in drug stores.

Antibiotic stewardship programs are also a promising tool to tackle drug resistance. These are educational procedures to improve a proper antibiotic administration in an interdisciplinary manner. Studies over the programs revealed an improvement in the hospitals regarding the reduced hospital stay, reduced usage and dosage of antibiotics and decreased inappropriate use of antibiotics that particularly decreases the pharmacy costs.

To prevent the transmission of infectious pathogens, guidelines are established for health care units. Most of the measures are probably well known such as hand hygiene and disinfection. Additionally, disinfection of healthcare environment and equipment is necessary. Isolation and contact tracing are also promising tools in such cases that is practiced in large-scale during the Ebola crisis and the current corona (COVID-19) crisis 2020. Vaccination is another tool to prevent transmission.

In conclusion, we are able to overcome the antimicrobial resistance crisis with suitable tools as described previously combined with effective research on new antimicrobial agents and strategies. Only the time is the limiting point.

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EIDESSTATTLICHE ERKLÄRUNG

Hiermit erkläre ich, Herr M. Sc. Dieter Meier, gemäß §5 Absatz 1 der Promotionsordnung der Mathematisch-Naturwissenschaftlichen Fakultät der Heinrich-Heine-Universität Düsseldorf des Eides Statt, dass die Dissertation von mir selbstständig und ohne unzulässige fremde Hilfe unter Beachtung der „Grundsätze zur Sicherung guter wissenschaftlicher Praxis an der Heinrich-Heine-Universität Düsseldorf“ erstellt worden ist und dass ich diese in der vorgelegten oder in ähnlicher Form noch bei keiner anderen Institution eingereicht habe.

Düsseldorf, den 23.06.2020 _____

Dieter Meier