Functional characterisation of new antimicrobial compounds

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Abbreviations

ABC	ATP-binding cassette
AGP	peptidoglycan-arabinogalactan complex
AIDS	Acquired Immunodeficiency Syndrome
AMR	antimicrobial resistance
ATP	adenosine triphosphate
BCG	Bacillus Calmette-Guérin
BDQ	bedaquiline
CA	community-acquired
CISH	cytokine-inducible SH2-containing protein
CoA	coenzyme A
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
Da	dalton
D-Ala	D-alanine
DEL	delamanid
DHPS	dihydropteroate synthase
D-Lac	D-lactose
DNA	deoxyribonucleic acid
D-Ser	D-serine
e.g.	*exempli gratia*
Eap	extracellular adhesion protein
ECDC	European Centre for Diseases Prevention and Control
Efb	fibrinogen-binding protein
EMA	European Medicines Agency
EMB	ethambutol
EPS	extracellular polymeric substances
ESBL	extended-spectrum ß-lactamases
ESKAPE	group of highly drug-resistant human pathogens
ESX	6-kDa early secreted antigenic target (ESAT-6) system
et al.	*et aliae*
ETC	electron transport chain
etc.	*et cetera*
EU	European Union
FAD	Flavin adenine dinucleotide
FDA	Food and Drug Administration
gDNA	genomic DNA

GFP	green-fluorescent protein
HA	hospital-acquired
HDAC	histone deacetylase
HIV	Human Immunodeficiency Viruses
i.e.	id est
IGRA	interferon-γ
IL	interleukin
INH	isoniazid
LA	livestock-acquired
LAM	lipoarabinomannan
LM	lipomannan
LPS	lipopolysaccharides
ManLAM	mannosylated lipoarabinomannan
MDR	multidrug-resistant
MHC	major histocompatibility complex
MRSA	methicillin-resistant Staphylococcus aureus
Mtb	Mycobacterium tuberculosis
MTBC	Mycobacterium tuberculosis complex
NADH	nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate
NIH	National Institutes of Health
NO	nitric oxide
OM	outer membrane
PABA	4-aminobenzoic acid
PAMP	pathogen-associated molecular pattern
PBP	penicillin-binding protein
PCR	polymerase chain reaction
PDIM	phthiocerol dimycocerosate
pH	pondus hydrogenium
PIM	phosphate idylinosisol mannoside
PMF	proton motive force
(p)pGpp	guanosine-3',5'-bispyrophosphat
(p)ppGpp	guanosine pentaphosphate
PVL	Panton-Valentine-leucocidin
PZA	pyrazinamide
qPCR	quantitative polymerase chain reaction
QQ	quorum quenching

QS	quorum sensing
RIF	rifampicin
RNA	ribonucleic acid
RND	resistance-nodulation-division
RR	rifampicin-resistant
RT-qPCR	Realtime-quantitative polymerase chain reaction
SCCmec	staphylococcal cassette chromosome mec
SH	Src-homology
ssp	subspecies
STIKO	Ständige Impfkommission
TB	tuberculosis
TDM	trehalose-6,6-dumycolate
TLR	Toll-like receptor
TNF	tumour necrosis factor
tRNA	transfer-RNA
TSST	toxic shock syndrome toxin
UK	United Kingdom
US	United States
UTI	urinary tract infection
WHO	World Health Organisation
XDR	extensively drug-resistant

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1. Introduction

1.1. Antibiotics

Since the discovery of penicillin by Alexander Fleming in 1929 [1], a variety of new antibiotics were discovered throughout the last decades. With his discovery, Fleming started the era of antibiotics in the treatment of infectious diseases apart from the first antimicrobial compounds, such as Arsphenamin. Arsphenamin is classified as a chemotherapeutic with severe side effects, like rashes or liver damage. The mechanism of action was based on the toxicity of three arsenic atoms in the core of the molecule [2]. Penicillin, however, causes only mild side effects and could be used as a broad-spectrum antibiotic for example during World World II where it saved thousands of lives [3]. Until today dozens of families of antibiotics have been discovered, developed and optimised to fight infectious diseases worldwide [4-7]. Many antibiotics are found on the list of essential medicines of the World Health Organisation (WHO), underlining their great impact on humanity [8].

1.1.1. Penicillins

Penicillins, discovered in the mould fungus *Penicillum notatum*, are the first class of natural-sourced antibiotics that have been isolated and are still in use since the 1940s. The central part of all penicillins is a ß-lactam-ring that is variously substituted to synthesise derivatives [9], and many have been developed over the past decades, such as penicillin G (Figure 1 A), penicillin V or ampicillin [10, 11]. The mechanism of action relies on the binding of penicillins to the DD-transpeptidase which is responsible for cross-linking peptidoglycan layers in the bacterial cell wall via D-alanyl-D-alanine linkage. If penicillin binds the enzyme, the cross-linking reaction cannot be catalysed and the bacteria are dying because of the loss of a stable, protective cell wall [12, 13]. Various bacteria, however, developed resistances against penicillins because of extensive application rather fast [14-16]. In general, there are two widespread modes of resistance against ß-lactam-antibiotics. Bacteria developed a penicillinase, a ß-lactamase, that is able to cleave the essential ß-lactam-ring of penicillins via hydrolysis [17, 18]. During the following years even more ß-lactamases have been developed by bacteria leading to a new classification of those bacteria to so-called extended-spectrum beta-lactamase (ESBL) producers that are virtually resistant to all penicillins [19, 20]. Therefore, ß-lactamase-resistant penicillins have been developed, such as methicillin [21]. Resistant bacteria occurred also against methicillin which are found to have a second copy of a transpeptidase, *mecA*, a low-affinity penicillin-binding protein (PBP2a) resulting in resistance to all penicillins [22]. To date, especially the methicillin-resistant *Staphylococcus aureus* (MRSA) is the most prominent bacterium harbouring pan-penicillin resistance [23].

1.1.2. Sulfonamides

Sulfonamide-based antimicrobials were first developed in Germany during World War II. They represent the first group of synthetic antimicrobials and are used against diverse microorganisms, such as bacteria or parasites [24, 25]. Sulfonamides are based on the sulphonamide functional group (Figure 1 B) and various derivatives were synthesised like sulfadimidine or sulfadoxine that exhibit (bacterio)static activity [26, 27]. Sulfonamides belong to the group of dihydropteroate synthase (DHPS) inhibitors. They mimic 4-aminobenzoic acid (PABA) and are able to bind to the active centre of the DHPS competitively. Thus, the synthesis of folic acid from PABA, 2-amino-4-oxo-6-methylpteridine and L-glutamic acid is inhibited. Since folic acid is an essential compound of the nucleic acid synthesis, more precisely thymidine and purines, the bacterial DNA can not be replicated [28]. Because mammalian cells lack a DHPS and are not dependent on endogenous folic acid synthesis, sulfonamides can be used in humans without interrupting the folic acid metabolism. Resistances against sulfonamides, however, emerge because of mutations in the *folP* gene, encoding for the DHPS, leading to an enzyme that can not be inhibited. Other than this, resistance can be obtained by plasmid-borne expression of a second *folP* gene copy or parts of the gene via horizontal gene transfer among bacteria [29].

1.1.3. Tetracyclines

Tetracyclines are a group of antibiotics that are based on natural products. In 1948, Duggar was able to isolate chlortetracycline from *Streptomyces aureofaciens* and named it Aureomycin [30]. Tetracyclines have broad-spectrum activity against Gram-positive and as well as Gram-negative bacteria, among others chlamydia, *Vibrio cholerae* and mycoplasma [31]. The molecules are synthesised via the polyketide-pathway in which acyl-CoA is the basic precursor molecule that provides the carbon atoms for the characteristic structure of tetracyclines consisting of four 6-carbon-rings (Figure 1 C) [32]. Besides the natural tetracyclines, semisynthetic tetracyclines have been developed, such as doxycycline and minocycline [33, 34]. The bacteriostatic effect of tetracyclines is based on the inhibition of protein biosynthesis. The antibiotic binds to the 30S ribosomal subunit and prevents binding of aminoacyl-tRNA and therefore peptide extension. Because a 30S ribosomal subunit is not found in mammalians, tetracyclines can be used with little side effects in humans [35, 36]. However, resistance against tetracyclines can emerge by different mechanisms. The most frequently found mechanisms of resistance are efflux pumps [37] and ribosomal protection proteins that are able to replace the ribosomal subunit or trap the antibiotic [38-40]. Less

common is the enzymatic inactivation of tetracyclines. Only one gene, *tetX*, has yet been found to alternate the structure of tetracyclines in presence of oxygen and NADPH [41].

1.1.4. Quinolones

Nalidixic acid, discovered by George Lesher in 1962, was the first quinolone antibiotic [42]. Based on the structure of quinoline, a heterocyclic aromatic organic ring system, many derivatives have been synthesised but only a few exhibited antimicrobial activity. Quinolones and its derivatives fluoroquinolones are having broad-spectrum bactericidal activity against Gram-positive and Gram-negative bacteria [43, 44]. Quinolones can be divided into four generations with different characteristics. The first generation of quinolones provides the basic structure as described above (Figure 1 D). The second generation was modified to have a fluorine atom at its central ring system typically at position C-6 or C-7, known as fluoroquinolones (Figure 1 E). Generation three shows additional activity against streptococci and the fourth generation of fluoroquinolones have a dual mode of action acting on DNA gyrase and topoisomerase IV (Figure 1 F+G) [45]. In general, quinolones act on topoisomerases which are needed for proper DNA replication in bacteria. The enzymes are essential in bacteria to remove supercoils during DNA synthesis and separate the bacterial chromosome afterwards [46]. Quinolones hamper the ligase function of topoisomerases while leaving the nuclease function intact. This leads finally to the fragmentation of the bacterial chromosome [47, 48]. Resistance against fluoroquinolones, however, emerges quite fast for the first three generations. The first generation of quinolones is no longer used because of this issue. Quinolone-resistant strains show three different mechanisms of resistance. Firstly, mutations in the DNA gyrase can occur and reduce the binding affinity of the antibiotics to the enzyme. Secondly, efflux pumps can reduce the drug concentration in the cells. And thirdly, an aminoglycoside acetyltransferase, AAC(6′)-Ib-cr, can be expressed from a plasmid and is able to modify certain fluoroquinolones [49-51].

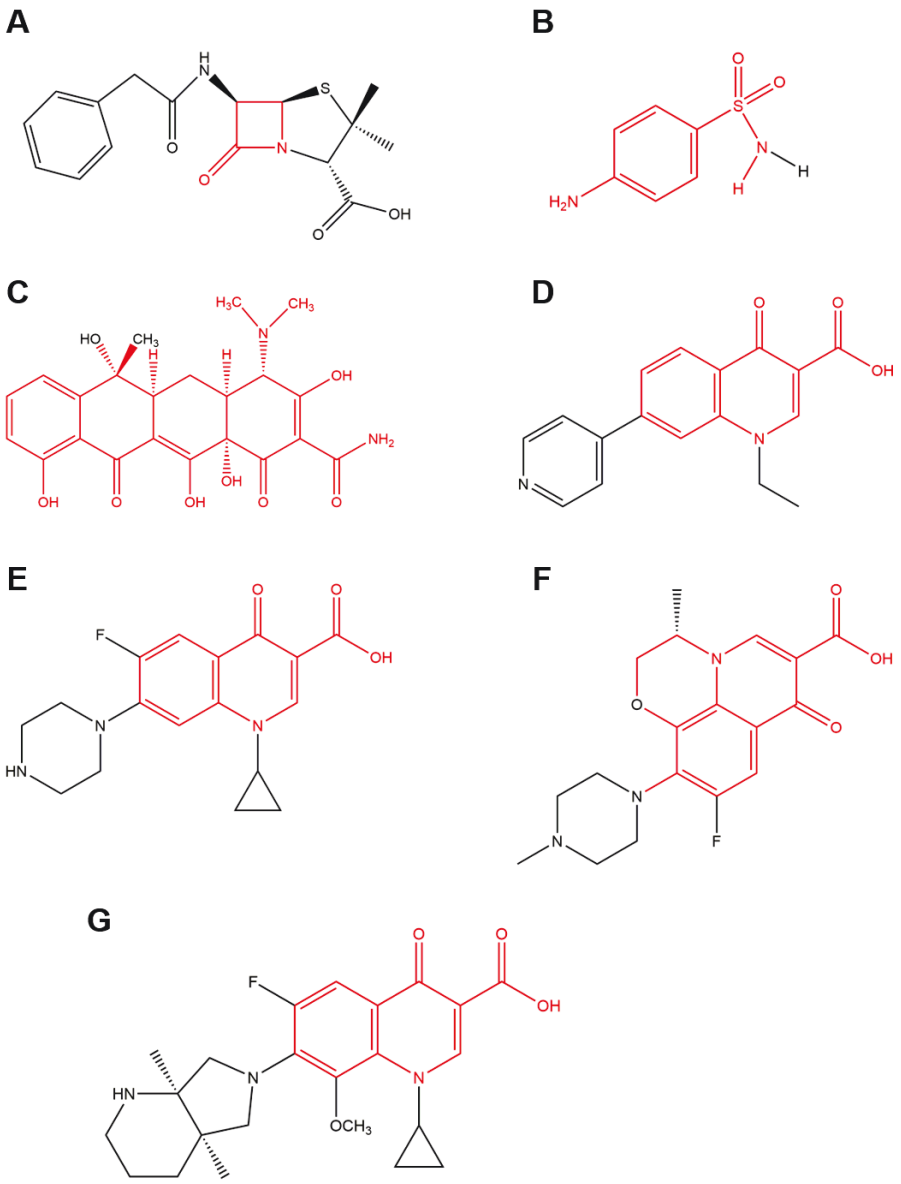

Figure 1 – Exemplary structures of basic antibiotics. The basic structure for antibiotic classes mentioned in this chapter is shown in red. Penicillin G (A) with its essential ß-lactam ring, sulfonamide (B) with the sulfonamide group and tetracycline (C) consisting of four 6-carbon rings. To illustrate the diversity in certain antibiotic classes, fluoroquinolones from each generation are depicted: first-generation rosoxcin (D), second-generation ciprofloxacin (E), third-generation levofloxacin (F) and fourth-generation moxifloxacin (G).

1.1.5. Antibiotic resistance crisis

The antibiotic resistance crisis is a matter which has great impact on science, economy and society alike. Antibiotic resistance started to occur almost simultaneously with the use of penicillin during World War II. Although it was deployed in hospitals and the open market in 1943, first penicillin-resistant staphylococci emerged already in 1940 [15, 16]. The same is true for methicillin, another ß-lactam-antibiotic introduced in 1960. Resistances occurred in 1962

first in the UK and some years later in the US [52]. During the following decades, new antibiotics and new classes of antibiotics were developed and introduced, such as rifampicins, fosfomycin, vancomycin or lipopeptides, but resistances against these new antimicrobial drugs emerged within a few years (Figure 2) [53-57]. Since the end of the 1980s, however, the development and research of new antimicrobials declined and no new antibiotic classes have been discovered [58]. Although 13 new antibiotics belonging to established structural scaffolds were approved by the Food and Drug Administration (FDA) in the US during 2000 and 2015 [59], it is just a matter of time until new resistances will occur.

Different factors play a role in the emergence of new resistances. First of all, overuse or misuse of antibiotics can contribute to the development of resistance, and it has been shown that there is a clear link between consumption and resistance [60]. Single point mutations can potentially lead to resistance against antibiotics, and if these genes are part of mobile genetic elements such as plasmids, horizontal gene transfer can spread resistance even among different bacteria species. The resistant bacteria will survive the antibiotic therapy while susceptible bacteria will die. This will allow the resistant bacteria to spread. Overuse of antibiotics is connected to their availability. In many countries, antibiotics can be bought without prescription which leads to higher consumption [60, 61]. Between 2000 and 2015, the consumption of antibiotics increased worldwide by 65%. Moreover, the use of antibiotics of last resort, such as glycylcyclines or carbapenems, also increased dramatically [62].

Another important factor that is driving the antibiotic resistance crisis is the inappropriate prescription of antibiotics. Although it is unnecessary to use antibiotics for infections that are caused by viral pathogens, they are often prescribed or the patients can buy them even without prescription. Furthermore, the choice of the correct antibiotic is the most important factor when an infection is severe. Studies in the US have revealed that in up to 50% of cases the wrong antibiotic was chosen to treat infections [63]. To circumvent this, microbiological screening and susceptibility testing have to be performed first. Otherwise, the treatment with a wrong antibiotic or a too short regiment is driving the evolution of antimicrobial resistance *via* mutations, enrichment of tolerant subpopulations or alternations of the virulence of bacteria [64].

Agricultural use of antibiotics is a driving force of the antibiotics resistance crisis as well. This issue is known for a long time and frequently discussed. However, supplying animals with antibiotics is widely common since antibiotics became available [65-68]. In the UK, for instance, 36% of antibiotics are administered to farm animals [69]. Often antibiotics are only used as prophylaxis to protect the animals from eventual infections. These antibiotics are then enriched

in the products we gain from the animals, such as milk, meat or eggs [68, 70-72]. Therefore, the antibiotics are also administered to humans, and this regular uptake of sub-inhibitory antibiotic concentrations drives the emergence of resistance in bacteria in our body. Moreover, rapidly occurring side effects such as allergic reactions or carcinogenicity have been observed among consumers [67]. In addition to that, the animals are also undergoing constant antibiotic treatment leading to a suppression of the natural microbiome, allowing resistant bacteria to accumulate and subsequently transfer to humans causing zoonoses [73, 74].

Finally, the low rate of new antibiotic development is an economic problem. Costs for discovery, optimisation and clinical trials of antibiotics are often exceeding the profit margin of pharmaceutical companies. Antibiotics are used for a rather short period of time, whereas drugs for chronic diseases are possibly used over years and are, therefore, more profitable [75]. In 2019, however, two new antibiotics have been approved by the FDA tackling the problem of antimicrobial resistance. Lefamulin belongs to the class of pleuromutilin antibiotics and is used to treat pneumonia. It inhibits bacterial growth via inference with protein biosynthesis and provides activity against MRSA among others [76, 77]. The other one, pretomanid, is used for therapy of multi-drug resistant tuberculosis. It is classified as a nitroimidazole antibiotic, like delamanid, and inhibits the mycolic acid synthesis of *Mycobacterium tuberculosis* [78]. For Pretomanid, the European Medicines Agency (EMA) received an application of approval in 2018.

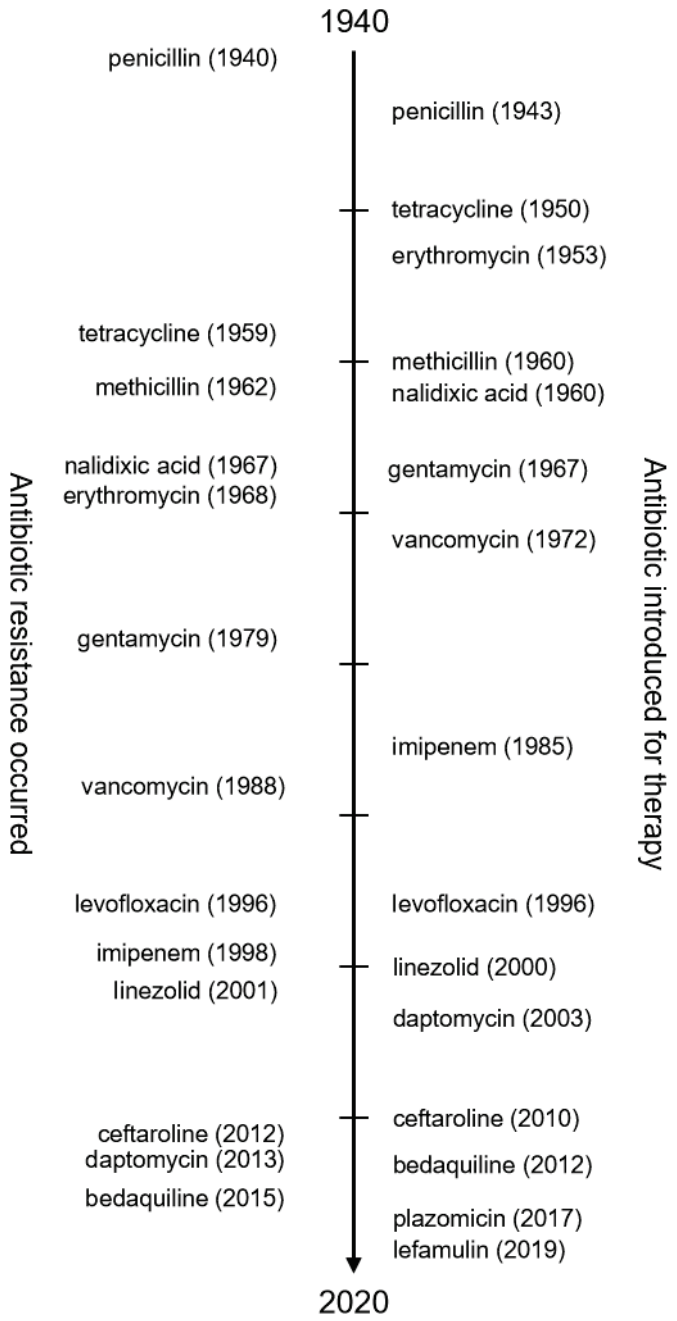

Figure 2 – Rapid emergence of antibiotic resistances since 1940. In the past 80 years, various antibiotics have been introduced for the therapy of infectious diseases (right side). The most significant drugs are displayed in the graph as well as the emergence of resistance against them (left side). Shortly after their introduction, resistance to the vast majority of antibiotics was described. The graphic has been adapted from [79] and updated [77, 80-85].

1.2. ESKAPE pathogens

Multidrug resistance is a problem of great concern for humanity. In the EU alone, approx. 25,000 patients die annually because of antimicrobial resistance (AMR) [86]. Globally, the numbers are even more alarming as the number of deaths caused by AMR exceeded 700,000 and is often hospital-acquired [87]. Therefore, the World Health Organization (WHO) started to publish a priority list of antibiotic-resistant pathogens against which new antibiotics with new modes of action are urgently needed (Figure 3). It is striking that priority 1 bacteria are all Gram-negative bacteria which are especially hard to treat because of their additional outer membrane (OM) which is missing in Gram-positive bacteria. Therefore, all of these pathogens are an immense health threat to humans, especially to immunosuppressed or elderly people.

Many of the pathogens from Figure 3 are also members of the ESKAPE group. This acronym is commonly used to describe bacteria that have a strong ability to adapt to antibiotic pressure through different mechanisms. The ESKAPE group comprises ***E****nterococcus faecium,* ***S****taphylococcus aureus,* ***K****lebsiella pneumoniae,* ***A****cinetobacter baumannii,* ***P****seudomonas aeruginosa* and ***E****nterobacter* spp. All of these bacteria are resistant to a large variety of antibiotics except for a small number of antibiotics of last resort. It is, however, just a matter of time until resistance mechanisms also against the antibiotics of last resort appear and spread throughout bacteria *via* horizontal gene transfer [88, 89].

S. aureus (see chapter 1.3.) and *E. faecium* are the only Gram-positive bacteria in the ESKAPE group. Both are highly resistant to different classes of antibiotics, such as vancomycin or ß-lactam-antibiotics. Especially vancomycin-resistant *E. faecium* is known to transfer its resistance on a virulence plasmid to other bacteria [90]. Most infections with *E. faecium* are nosocomial infections and therefore hospital-acquired. Furthermore, endocarditis, cystitis or urosepsis are common infections caused by *E. faecium* [91, 92]. Because of a very high intrinsic resistance to cephalosporins, aminoglycosides and ß-lactam-antibiotics, *E. faecium* is a hard-to-treat bacterium, especially when further resistances are acquired [85, 93].

K. pneumoniae, *A. baumannii*, *P. aeruginosa* and *Enterobacter* spp. are all Gram-negative bacteria. The main difference between Gram-positive and Gram-negative bacteria regarding AMR is a second cell membrane which is surrounding Gram-negative bacteria. In contrast to the Gram-positive cell membrane, the Gram-negative OM consists of phospholipids on the inner and lipopolysaccharides (LPS) on the outer leaflet. Formerly known as a stimulator of the immune response in humans, LPS are contributing to resistance against various antibiotics by increasing the negative charge of the OM. In addition to that, the OM contains porins that are the limiting factor for antibiotic passage over the membrane, because they can not transport

molecules larger than 600 Da [94-97]. This contributes to a general intrinsic resistance against large antibiotics such as vancomycin, rifampicin or daptomycin [98, 99].

The Gram-negative members of the ESKAPE group are all of clinical relevance as they are causing a wide range of infections. Urinary tract infections (UTI), skin infections and sepsis up to toxic shock syndrome are just a few afflictions [100-106]. The great challenge remains the treatment of these bacteria, because of the high intrinsic resistance and their ability to adapt quickly to environmental changes in multiple ways. *K. pneumoniae* and *P. aeruginosa*, for instance, have a strong ability to form biofilms and withstand even high doses of antibiotics. Furthermore, *K. pneumoniae* is able to easily take up foreign DNA to gain resistances [103, 107-110]. *A. baumannii* has the ability even to survive in harsh environments and is one of the most common pathogens in hospitals. It was recently shown that *A. baumannii* is able to survive sterilisation processes in intensive care units and has, therefore, emerged to an important health threat [111, 112]. *Enterobacter* ssp., a family of 14 different bacteria, are usually part of the human microbiome located in the human gut. Outside their natural environment, *Enterobacteriaceae* are causing UTIs and sepsis [104]. The most important species are *Escherichia coli* and *Enterobacter cloacae* from which a strain virtually resistant to all clinical antibiotics was isolated. This, however, is not the result of intrinsic resistances but of further acquisition of resistance mechanisms [113-115].

Figure 3 – Global priority list for research and development of urgently needed antibiotics. Annually, the WHO publishes a list of multidrug-resistant pathogens against which antibiotics are urgently needed. *M. tuberculosis*, the causative agent of tuberculosis, is missing in this list although multidrug-resistant and extensively drug-resistant tuberculosis is a global health problem as well. Research and development on antitubercular drugs, however, have already started worldwide to tackle this issue. Graphic adapted from [116].

1.2.1. Mechanisms of resistance in ESKAPE pathogens

Bacteria are fast adapting organisms, especially because of their short generation time and their ability to exchange DNA ranging from plasmids to parts of their chromosomes. The translocated DNA can contain genes or gene clusters that confer resistance to one or multiple antibiotics [117]. The spread of resistance mechanisms among pathogens even among different species is therefore common. There is a wide range of resistance mechanisms apart from intrinsic resistance, such as efflux pumps, antibiotic-modifying enzymes or modifications of antibiotic targets, to name a few [118, 119].

The best-evaluated mechanism regarding the inactivation of antibiotics is the production of ß-lactamases. These enzymes mediate resistance against ß-lactam antibiotics as described in section 1.1.1. by hydrolysis of the essential ß-lactam ring. Furthermore, ß-lactamases provide resistance not only to penicillins but to many other ß-lactam antibiotics. The resistance spectrum also includes monobactams, cephalosporins and carbapenems. The diversity might be directly linked to the rather high mutation rate of the enzymes. These ß-lactamases are found in many different bacteria, amongst others the ESKAPE pathogens *K. pneumoniae*, *A. baumannii*, *P. aeruginosa* and *Enterobacter* ssp., which are therefore classified as extended-spectrum ß-lactamase producers (ESBL) [120-122].

Antibiotic resistance can further be achieved by modification of the antibiotic target. Besides penicillins, glycopeptides are another class of antibiotics targeting the cell wall. Vancomycin or teicoplanin are two examples of glycopeptide antibiotics. They target the glycosyltransferase which establishes the synthesis of acyl-D-alanyl-D-alanine (D-Ala-D-Ala) during peptidoglycan biosynthesis [123]. Alteration of the linkage between peptidoglycan layers from D-Ala-D-Ala to D-Ala-D-Lac or D-Ala-D-Ser, however, will result in resistance to glycopeptides [124]. Another way of target modification is connected to the property of some antibiotics to be prodrugs that need to be transformed into their active form inside the bacteria. Isoniazid (INH), an anti-tuberculosis (anti-TB) drug, needs to be activated in the bacterium by the catalase peroxidase KatG. The enzyme catalyses the reaction of INH to isonicotinic acid that needs NADH to form an isonicotinic acid-NADH adduct. Finally, the latter inhibits mycolic acid biosynthesis by binding covalently to the active centre of InhA. The most common resistance mechanism is a mutation in the gene *katG* resulting in no activation of INH. This is, however, not a modification of a drug target *per se* but represents another mechanism that is directly linked to alterations of cellular drug-binding proteins [125, 126].

Decreasing the intracellular drug concentration is another strategy of bacteria to counter antibiotic treatment. In general, there are two possibilities to achieve that. On the one hand, bacteria can reduce the uptake of antimicrobial drugs by decreasing the expression of certain membrane proteins. It is, for instance, known for *P. aeruginosa* that a lower expression of the OM porin, OprD, leads to imipenem resistance [127]. The same is true of other ESKAPE pathogens. For *K. pneumoniae*, it was shown that lower expression of the outer membrane protein (OMP) OmpK35 confers resistance to cephalosporins, carbapenems, fluoroquinolones and chloramphenicol. *E. cloacae* was shown to be resistant against carbapenems when OmpF expression was reduced, and *E. coli* was resistant to ß-lactam antibiotics when either OmpE or OmpF were down-regulated [128-132]. On the other hand, the intracellular concentrations of antimicrobials can be reduced by efflux pumps. These are certain membrane proteins that

function as exporters at high rates. Two efflux pump families, in particular, are important. The resistance-nodulation-division (RND) family is the most common type of efflux pumps in Gram-negative bacteria. It plays a crucial role in multidrug resistance of Gram-negative bacteria because it is able to pump various antibiotics out that are not structurally related [133]. *P. aeruginosa* has evolved a number of efflux pumps that are coupled with proton antiporters. These type of pump is using the proton motive force as energy source. The efflux pump MexAB-OprM is responsible for resistance to aminoglycosides, ß-lactams, chloramphenicol, macrolides, novobiocin, tetracycline and trimethoprim, because of the ability to expel antibiotics unspecifically [134, 135]. Other examples for efflux pumps of the RND family are AdeFGH in *A. baumannii* conferring resistance to fluoroquinolones, tetracycline, chloramphenicol and sulfamethoxazole among others [136]. For *E. cloacae*, AcrAB is known to be an RND efflux pump that confers resistance to aminoglycosides, ß-lactams, chloramphenicol, erythromycin, fluoroquinolones, tetracycline and tigecycline [137]. The other important family of efflux pumps are ATP-binding cassette (ABC) pumps, which use an energy-related process depending on the hydrolysis of ATP. ABC efflux pumps are more specific and are not conferring resistance to a wide range of different antibiotics. Nonetheless, for *E. coli* MacAB-TolC can effectively remove macrolides from the bacterial cytoplasm preventing their concentration to reach effective levels [138, 139]. MsrC leads also to resistance against macrolides but is found in the Gram-positive ESKAPE pathogen *E. faecium* showing that also Gram-positive bacteria can interfere with antibiotic treatment using efflux pumps [140]. It has also been shown that the Gram-positive species *M. tuberculosis* is able to use efflux pumps effectively against antibiotics. *Rv0194* is one example for an ABC family efflux pump that allows resistance against ampicillin, erythromycin, novobiocin and vancomycin [141, 142].

Taken together, bacteria have developed a wide range of different resistance mechanisms against antibiotics. The gain, loss or mutation of a certain gene can be enough to confer resistance to one or multiple antibiotics. Especially the high intrinsic resistance of Gram-negative bacteria combined with the gain of resistances via the exchange of interspecies DNA is providing a perpetual challenge regarding effective antimicrobial treatment.

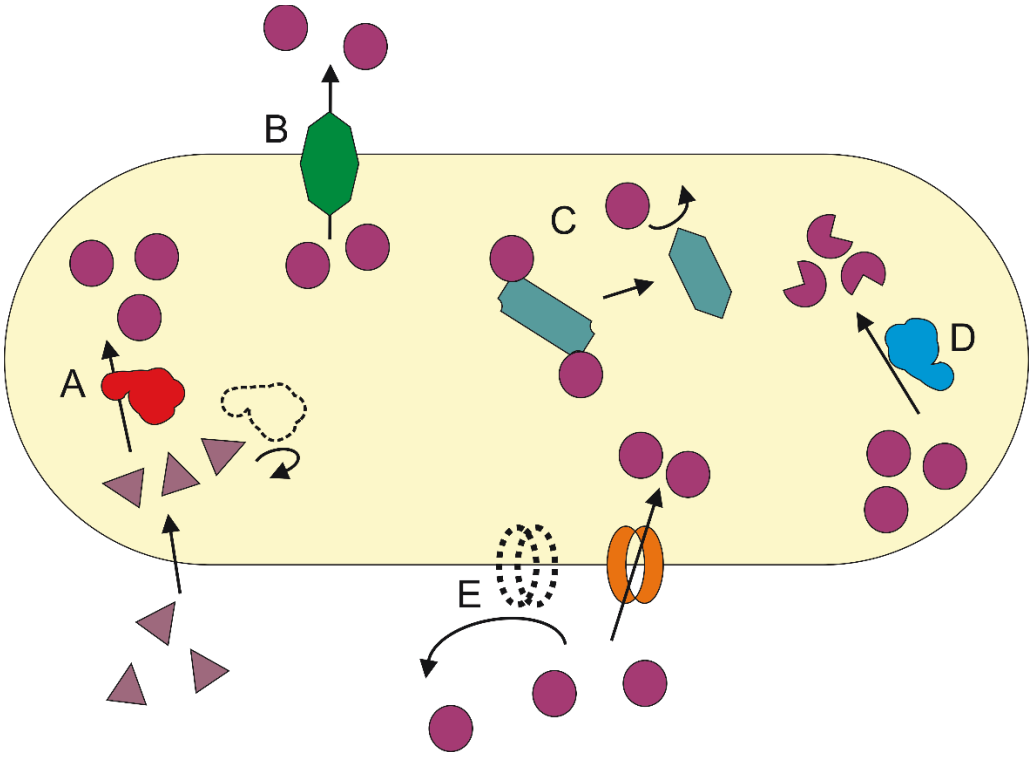

Figure 3 – Resistance mechanisms in bacteria. Bacteria are fast adapting organisms and have the ability to exchange parts of their genomic DNA. Therefore, resistance mechanisms are spreading even among species. A couple of different resistance mechanisms have evolved, such as loss or alteration of prodrug activating enzymes (A). A prodrug (triangle) enters the cell and is modified by a bacterial enzyme to its active form (circle). Alteration or loss of the activating enzyme results, therefore, in resistance to the respective drug. A wide range of efflux pumps is known (B). Efflux pumps are exporters or symporters integrated into the bacterial cell membrane. They are, for instance, utilising ATP to expel antibiotics. The variation of the antibiotic target can also lead to resistance because the antibiotic is not able to bind anymore (C). Alteration of the promotor region of an antibiotic target might also lead to resistance when the mutation results in overexpression of the target gene. The modification of the antibiotic itself is also a widespread resistance mechanism (D). The antibiotic is altered (open circles) and loses its antibacterial effects. To circumvent antibiotic effects, the drug can be hampered to enter the cell (E). As a result of antibiotic therapy, transporters and/or porins can be lower expressed or even lost completely leading to increased resistance against the antimicrobial.

1.3. *Staphylococcus aureus*

Staphylococcus aureus is a coccoid Gram-positive bacterium. As a natural human commensal *S. aureus* can be found on the skin and in the throat and nose of approx. 30% of humans. It is an opportunistic pathogen that causes mild infections such as respiratory tract infections or food poisoning. The risk of infection for immune-compromised people, however, is unevenly higher. In those, S. *aureus* can cause life-threating infections, such as pneumonia, endocarditis, meningitis or the toxic shock syndrome, to name a few [143-146]. The most prominent variety of *S. aureus* is MRSA, the methicillin-resistant *Staphylococcus aureus* [147].

1.3.1. Methicillin-resistant *Staphylococcus aureus* (MRSA)

MRSA is a member of the ESKAPE group (chapter 1.2). It is a variant of *S. aureus* and differs genetically from it because of multiple acquired resistance mechanisms and virulence factors. Therefore, every *S. aureus* that acquired multidrug resistance and resistance to ß-lactam antibiotics, especially oxacillin and methicillin, is called MRSA. Thus, MRSA is a hard-to-treat pathogen and is listed as a critical pathogen to find new antibiotics against by the WHO [116]. MRSA is a ubiquitous problem for many years. Since its first discovery in 1962 in the UK, MRSA spread globally [52]. Especially in hospitals, MRSA is one of the five most common reasons for infections and is associated with invasive medical devices, such as prosthetic joints or catheters. As MRSA remains an opportunistic pathogen, although it gained virulence factors, it is frequently passed on from one person to another and remains undetected. Elderly people, in particular, are more often hospitalised with an acute MRSA infection than younger people. The European Centre for Diseases Prevention and Control (ECDC) published data with MRSA incidence rates for Europe in 2017 [148]. For the European Union (EU) the mean MRSA incidence rate was 16.9% and 9.1% in Germany. Southern and Eastern Europe, however, are facing a severe MRSA problem with incidence rates up to 50%. MRSA infections are not uniquely hospital-acquired (HA) but they are, moreover, also community- (CA) and livestock-acquired (LA). The tremendous use of antibiotics in agriculture is, as previously described, not only a driving force for AMR but also for MRSA prevalence. That way LA-MRSA has become a severe problem beside HA-MRSA and CA-MRSA [149-152]. However, they differ in a certain type of a certain mobile genetic element, the staphylococcal cassette chromosome mec (SCCmec) and the presence of the Panton-Valentine-leucocidin (PVL) exotoxin that is characteristic for CA-MRSA [153].

Initially, MRSA gained resistance against penicillins due to expression of a second copy of the penicillin-binding protein, PBP2a, which is encoded by *mecA*. The gene is located on SCCmec along with various other genes conferring resistance to erythromycin, fluoroquinolones or carbapenems to name a few. Furthermore, it contains two recombinases, *ccrAB*, that mediate high mobility and enable transfer to other staphylococci. The recombinases allow SCCmec to integrate into the bacterial chromosome at specific sites, attSCCs, in close proximity to *orfX*, an ORF with unknown function. Additionally, SCCmec contains a third recombinase, *ccrC*, that allows reorganisation of the mobile element itself [154]. To date, eight different SCCmec have been identified and all confer resistance in special patterns [154-162]. SCCmec is one of the most, if not the most, important element regarding the maintenance and transfer of resistances among staphylococci.

The virulence potential of MRSA is based on intrinsic factors and the acquisition of new virulence factors. Those are encoded on mobile genetic elements and differ among MRSA strains. In general, MRSA binds to epithelial cells via SasG or SasX (surface protein G or X) or ClfAB (clumping factor A and B). Afterwards, the transcription of virulence factors starts that are usually under the control of transcription factors like *agr* or *sarA* [163]. The bacteria can enter the bloodstream after damaging the epithelial cells and then start to secrete further virulence factors to circumvent the host's immune response. At least three major fibrinogen-binding proteins are secreted by the bacteria. The extracellular adhesion protein (Eap) inhibits the recruitment of leucocytes, the fibrinogen-binding protein (Efb) prevents aggregation of thrombocytes and coagulase converts fibrinogen to fibrin in which the bacteria can be coated and escape the host's immune response [164-167]. Other factors are further contributing to virulence but in addition are also important for antibiotic tolerance. Usually, these genes are controlled by the same transcription factor as other virulence factors [168]. The bacteria are able to organise their growth in micro-colonies and mature to biofilms that are up to 1000-fold more tolerant to antibiotics and provide a source for resurgence of infections.

Pathogenicity of MRSA is based on toxins. PVL is an important exotoxin and is associated with reoccurring soft tissue infections and necrosis [169]. PVL consists of two secreted proteins, LukF-PV and LukS-PV, and forms a pore in the membrane of the target cell. It predominantly targets leucocytes and macrophages, which further contributes to the inflammatory effect and weakens the immune response [170-172]. Moreover, PVL is connected to necrotising pneumonia that is fatal in more than 50% of the cases [173, 174]. Besides PVL, the pathogenicity of MRSA is based on enterotoxins, such as enterotoxin B, that causes food poising, and other exotoxins, like toxic shock syndrome toxin-1 (TSST-1) [175, 176]. TSST-1 stimulates T-cells non-specifically. As a result, large amounts of interleukins, IL-1 and IL-2 in particular, are released along with tumour necrosis factor (TNF) to the bloodstream. A systematic immune response is a consequence that finally leads to multiorgan failure [177].

1.3.2. Antibiotic-tolerant subpopulations

The resurgence of infections that were thought to be overcome after initial treatment is a problem that is often attributed to false antibiotics or too short therapy. Moreover, bacteria are able to circumvent antimicrobial therapy by resistance mechanisms, but also due to dormancy or biofilm formation. Bacteria are entering the stationary phase when they are challenged with environmental changes they are not able to compensate or adapt to. The bacteria persist in the human body in dormancy and are therefore known as persisters [178]. Persisters are a particularly hard-to-treat bacterial subpopulation because they become tolerant to antibiotics,

not to be mistaken as resistant. Resistance is an active process in which antibiotics are, for example, inactivated (chapter 1.2.1). Tolerance, however, is no active process and nothing that can be easily passed on because regulators of persistence are commonly not encoded on mobile genetic elements. The regulators of persistence, such as the stringent response and (p)pGpp signalling or toxin-antitoxin systems, like *mazEF* in *E. coli* or *M. tuberculosis*, remain undiscovered in *S. aureus* although their contribution to persistence is highly probable [179-182]. During persistence, however, the bacteria downregulate their metabolism. The bacteria do not synthesise DNA, RNA, proteins or cell wall compounds, the generation and consumption of ATP are quiesced and the uptake of metabolites is massively reduced [183, 184]. Since almost all antibiotics in clinical use are targeting processes that are involved in the mentioned cellular processes, persisters are phenotypically resistant to antibiotics. Strategies for fighting persisters range from persister formation inhibitors such as relacin, which is a (p)pGpp analogue and blocks the stringent response, to metabolite-enabled killing where metabolites like glucose or mannitol are meant to restart the bacterial metabolism to allow antibiotics such as daptomycin or glycopeptides to kill the reactivated persisters [185-187].

Another important bacterial subpopulation, which is antibiotic tolerant and therefore hard-to-treat, are biofilms. Biofilms are organised colonies of actively replicating and persistent bacteria that are attached to a biological or artificial surface and are embedded in extracellular polymeric substances (EPS), a matrix consisting of polysaccharides, DNA and proteins [188]. As for persistence, biofilm formation is widely spread and well documented for Gram-positive and Gram-negative bacteria [189]. A multitude of infections is associated with biofilm formation, for instance, endocarditis, chronic pneumonia or chronic skin infections. Moreover, medical devices, such as catheters or pacemakers, offer an excellent surface for the initial attachment of bacteria to form biofilms [190, 191]. Embedded in the biofilm, however, the bacteria are able to withstand harsh antibiotic treatment and provide a source for the resurgence of infections. The EPS forms a physical barrier around the cells that does not allow large molecules, such as glycopeptides, to pass and enables the bacteria to become up to 1000-fold more tolerant than planktonic cells [192]. The therapy of biofilms is therefore even more challenging than the therapy of persisters. Eradication of biofilms with antibiotics is often impossible and surgical removal is often the final choice. In the last decade, new strategies have emerged to tackle biofilm-related infections. Quorum sensing is an essential process for biofilm formation and was targeted by various groups that developed quorum quenching molecules to inhibit biofilm formation, such as hordenine or quercetin [193-196]. In the majority of cases, biofilms are already established and quorum quenching molecules are almost ineffective. The dispersal of the biofilm, followed by exposure of the bacteria to antibiotics is also a field of vivid research. Dispersin is a promising compound that disperses *S. aureus*

biofilms by degrading the EPS and enables tobramycin to eradicate the bacterial population [197].

1.4. *Mycobacterium tuberculosis*

Mycobacterium tuberculosis (Mtb), discovered by Robert Koch in 1882, is a rod-shaped, slow-growing bacterium with a proliferation time of approx. 20 hours. It belongs to the *Mycobacterium tuberculosis* complex (MTBC) of which, among others, *Mycobacterium bovis* is also a member. The Mycobacteria of the MTBC are able to cause tuberculosis (TB) in humans, an infectious disease often associated with the lungs although other organs, such as the bone marrow, liver, gut and meninges can be affected as well. It is estimated that around 2 billion people worldwide are latently infected with Mtb. In 2018, the number of TB-deaths exceeded the number of deaths caused by HIV/AIDS, making TB the leading cause of death caused by a single pathogen worldwide. Approx. 1.45 million people died because of TB, among them 250,000 HIV positive people. Moreover, 10 million new TB cases have been reported, and 3.4% of these are multidrug-resistant TB (MDR-TB) [198]. The TB incidence rates (Figure 4 A) are particularly high in southern and central Africa and Southeast Asia, especially in Indonesia and the Philippines (>300 cases per 100,000 population per year). Moreover, the percentages of new TB cases with MDR- or rifampicin-resistant TB (RR-TB) are increasing in Russia and the former Sowjet Union to more than 18%. Since new MDR-/RR-TB cases are also alarmingly high in the Baltic states, the TB problem has spread into the EU. This demonstrates that TB is not only a problem in developing countries but also for industrialised countries such as EU countries. Although TB research is a vivid field for studies since decades to elucidate mechanisms of pathogenicity and to develop new antibiotics and vaccines, TB remains a global threat.

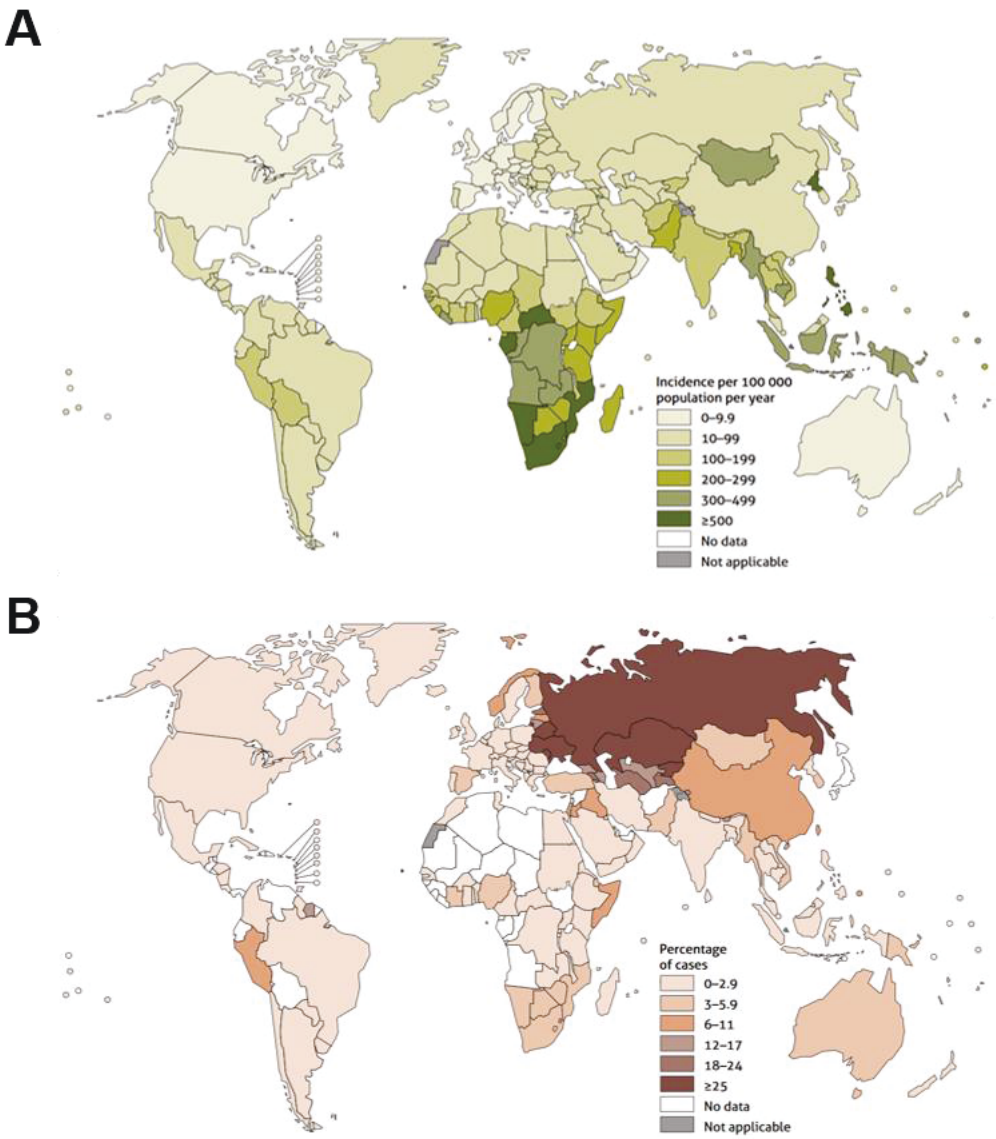

Figure 4 – Global TB and MDR-TB incidence rates. Data collected by the WHO showing the estimated incidence rates of TB (A) and the percentage of new TB cases with MDR/RR-TB (B) worldwide. While the TB incidence rates are higher in south and central Africa and Southeast Asia, MDR-/RR-TB cases are raising in the former Sowjet Union and eastern Europe. The data has been adapted from [198].

1.4.1. Pathogenicity and Infection

The pathogenicity of Mtb is closely associated with the unique and complex structure of its cell envelope. Mtb's cell envelope can roughly be divided into four compartments: the inner membrane, the peptidoglycan-arabinogalactan complex (AGP), an asymmetrical mycomembrane that is covalently linked to AGP via mycolic acids, and an external capsule that mainly consists of neutral polysaccharides and lower amounts of proteins and lipids. The mycomembrane, in particular, contains several mycolic acids, glycolipids and lipoglycans, such as phosphatidylinositol mannoside (PIM), phthiocerol dimycocerosate (PDIM), lipomannan

(LM), lipoarabinomannan (LAM), mannosylated LAM (ManLAM) and trehalose-6,6-dimycolate (TDM), that mediate the adaptability of Mtb [199]. The organisation of these constituents and compartments, as well as the lipophilic properties of the mycomembrane, contribute to the low permeability of the cell envelope. In consequence, Mtb has high intrinsic resistance against antibiotics, but also against physical and chemical challenges like temperature or pH changes [200]. Another important quality of the cell envelope is the interference with the human immune response during infection. PDIMs and LAMs are, among other factors, responsible for the inactivation of macrophages and the phagosomal escape of Mtb during infection [199, 201, 202].

For its pathogenicity, it is essential for Mtb to persist inside macrophages. As part of the innate immune response, pathogens are ingested by macrophages. Therefore, recognition of Pathogen-Associated Molecular Patterns (PAMPs) is necessary. Receptors on the surface of macrophages, such as Toll-like Receptor 2 (TLR-2), interact with the PAMPs and the bacteria are internalised. In the macrophages, bacteria are disassembled in the phagosome and lysosome to present the bacterial membrane components *via* MHC-II-molecules. These antigens are recognised by T-helper cells that activate the adaptive immune response which finally eradicates the pathogens. Mtb is transmitted by droplet infection with two to five bacteria being enough to start a TB infection. The bacteria reach the lungs and the alveolar macrophages recognise the bacteria [201]. Interaction and recognition of Mtb and macrophages are, among others, established by LAM and PIM *via* TLR-2 whereafter Mtb is phagocytosed [203]. In contrast to other bacteria, Mtb has evolved strategies to prevent maturation of the phagosome, which is essential for later fusion with the lysosome. Mtb interferes early in the maturation of the phagosome and recruits Rab, a marker of early endosomes. As a consequence, the phagosome stays tagged as early endosome and maturation is hampered [204-207]. Furthermore, ManLAM has been shown to deplete the uptake of lysosomal enzymes and molecules as well as hydrolytic enzymes from Golgi. Acidification of phagosomes is essential for phagosomal maturation, and because of this Mtb blocks the maturation process. The interaction of TDM, also known as Mtb's cord factor, with the Mincle receptor, has been shown to defer acidification. In addition, Mtb targets the H+V-ATPase proton pump, which is responsible for rapid acidification of the phagosome and marks it for ubiquitination employing the macrophage's CISH protein. These mechanisms ensure the survival of Mtb inside macrophages [208-210].

The internalisation of Mtb by macrophages, however, induces an immune response including cytokine, interleukin, interferon- γ, TNF and NO production [211]. Due to the immune response, immune cells are recruited to the infected macrophages and an organised cell aggregate is

formed, known as granuloma. Mtb avoids phagosomal fusion to the lysosome but granuloma formation traps the bacteria in a harsh environment of constant chemical and physical stress. The bacteria become dormant and a latent TB infection is established that shows no clinical symptomatology. If the immune system is suppressed, the latent TB infection can turn into an active TB infection. It has been shown that Mtb can escape the phagosome employing the ESX-1/T7S system which leads to rupture of the phagosomal membrane. Furthermore, DIM and PDIM production and translocation across the Mtb mycomembrane are crucial for interaction with the phagosomal membrane to promote rupture as well as death of the macrophage itself [212-215]. This finally leads to necrosis of macrophages and the entire granuloma. Thus, Mtb can be transmitted to the surrounding tissues and enters the bloodstream to reach other organs. Most importantly, the bacteria can be transmitted to other people via droplet infection if an active TB infection has been established [216, 217].

1.4.2. Tuberculosis Therapy

Although approx. 2 billion people are infected with TB globally, TB therapy is only prescribed when an active TB infection is established. After confirmation of a TB infection *via* cultivation of sputum, molecular tests or interferon-γ (IGRA) testing complemented by X-ray diagnosis, the standard six-month TB therapy, which is always a combination therapy, consists of the four first-line drugs rifampicin (RIF), isoniazid (INH), ethambutol (EMB) and pyrazinamide (PZA) [218-220]. This drug combination is administered since the 1960s unalteredly and resistances occurred in Mtb leading to the emergence of MDR-TB as a consequence. MDR-TB is characterised by resistance against RIF and INH and is therefore treated with second-line drugs, such as fluoroquinolones, kanamycin, amikacin and/or capreomycin for at least 18 months. Resistance, however, also emerged against the second-line drugs resulting in extensively-drug resistant TB (XDR-TB) [221]. In 2014, bedaquiline (BDQ) and delamanid (DEL) were introduced to the market and are part of the XDR-TB therapy. In 2019, pretomanid was introduced in the US and is still pending its accreditation in the EU [78, 222, 223]. Vaccination against TB, however, was deployed for many years utilising the attenuated vaccine *M. bovis* strain BCG. Immunisation was rather poor and TB vaccination is therefore no longer part of the recommendations of the “Ständige Impfkommission” (STIKO) in Germany since 1998 [224].

Although the cure rates of drug-susceptible TB are very high, the fast adaptability of Mtb against antitubercular drugs renders it imperative to develop new drugs, vaccines and diagnostic methods to stop the emerge of MDR- and XDR-TB around the world.

2. Aim

As the spread of multidrug-resistant pathogens is a global problem new antimicrobial drugs are urgently needed. The aim of this thesis was the identification and characterisation of new antimicrobial compounds able to tackle this problem. Libraries of natural and synthetic compounds have been screened for their activity against nosocomial pathogens as well as Mtb, the etiologic agent of tuberculosis.

Two natural products, both brominated phenoxyphenols, were used for experiments in the nosocomial pathogens MRSA and *P. aeruginosa* to characterise their spectrum. A second focus of this studies were antibiotic-tolerant subpopulations that are phenotypically resistant to classic antibiotics, such as glycopeptides or fluoroquinolones. Persisters, dormant bacteria with a massively downregulated metabolism, and biofilm-incorporated cells are the major source of reoccurring bacteraemia during hospitalisation. Because brominated phenoxyphenols provide high activity against these subpopulations, the deciphering of the mode of action and resistance was of particular interest to reveal new potential drug targets.

The characterisation of anti-tuberculosis drugs and their respective targets was the second topic of this thesis. A library of synthetic compounds was screened to identify new classes of antibiotics that adress urgently needed new drug targets in Mtb. Different methods were employed to verify the drug target and characterise the mechanism of action, such as isolation of spontaneously resistant mutants and whole-genome sequencing.

Furthermore, the analysis of the anti-TB potential of human interleukin-26 (IL-26) was also part of this work. As the antimicrobial activity of human IL-26 against nosocomial pathogens was already proven, the focus was on the evaluation of its anti-TB potential and the elucidation of the mode of action.

Additionally, chitosan, a natural compound isolated from marine shell waste, was analysed in terms of its properties to prevent biofilm formation. The aim was to evaluate whether chitosan and its derivatives provide antimicrobial activity against Gram-positive and Gram-negative bacteria and, moreover, are able to efficiently prevent formation or induce dispersal of biofilms *in vitro*.

3. Summary

Antimicrobial resistance (AMR) has become a global problem. The increased use of antibiotics in hospitals and agriculture is a driving factor for the emergence of new resistances. Antibiotics of last resort are often not effective anymore and, as a consequence, more patients die because of multidrug-resistant pathogens each year. In this thesis, the antimicrobial potency of new antimicrobial compounds has been evaluated to tackle the rising problem of AMR.

The group of ESKAPE pathogens is of special interest because this group comprises Gram-positive and Gram-negative multidrug-resistant bacteria that cause life-threatening systemic infections. Natural brominated phenoxyphenols have been isolated from the marine sponge *Dysidea granulosa* and provide high bactericidal potential against MRSA and *Pseudomonas aeruginosa* (chapter 5). Furthermore, broad-spectrum activity was proven against all ESKAPE pathogens, including multidrug-resistant *Enterococcus faecium*, *Klebsiella pneumoniae*, *Acinetobacter baumannii* and *Enterobacter cloacae*. Antibiotic-tolerant subpopulations, such as persisters and biofilm-incorporated cells, are an increasing problem because these bacteria survive antibiotic therapy with conventional antibiotics in clinical use. Their tolerance against antibiotics is up to 1000-fold higher than the tolerance of their planktonic counterparts. Brominated phenoxyphenols, however, also provide activity against these hard-to-treat bacteria and are of particular interest as lead-structures for new antibiotics.

Mycobacterium tuberculosis (Mtb), the causative agent of tuberculosis (TB), is a global threat and kills approx. 1.45 million people annually. While incidence rates are particularly high in Southern Africa and Southeast Asia, the emergence of multidrug-resistant TB is high in Eastern Europe and the former Soviet Union. To tackle the rising number of multidrug- or extensively drug-resistant TB, we screened a library of synthetic molecules and found two molecules that exhibit high anti-TB activity *in vitro* without detectable cytotoxicity against various human cell lines (chapter 6). The alkoxyamide-based molecules KSK-104 and KSK-106 kill Mtb in nanomolar concentrations while the frequency for resistance-mediating mutations is extremely low. In addition, both molecules provide anti-TB activity against internalised bacteria within THP-1 derived macrophages which is an essential property for anti-TB drugs because Mtb is an intracellular pathogen that survives within macrophages.

In chapter 7, we have shown that human interleukin-26 (IL-26) provides anti-TB activity. We demonstrated that IL-26 causes cell wall damage in Mtb most likely by binding to mannosylated lipoarabinomannan as revealed by microscale thermophoresis. Consequently, this leads to morphological cell wall changes and “bleb” formation resulting in membrane disruption and cell death as we have proven employing scanning electron microscopy.

As biofilm formation is a huge problem during hospitalisation, the natural compound chitosan, which has been isolated from marine shell waste, was tested for its capability to prevent formation of biofilms or induce dispersal of pre-formed biofilms (chapter 8). We demonstrated that chitosan and its derivatives are able to prevent biofilm formation *in vitro* when plastic surfaces were coated which the compounds. It is likely that chitosan is preventing the initial attachment of bacteria to the surface and, therefore, prevents biofilm formation.

4. (Some) Current Concepts in Antimicrobial Drug Discovery

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5. Natural brominated phenoxyphenols kill persistent and biofilm-incorporated cells of Methicillin-resistant *Staphylococcus aureus* and *Pseudomonas aeruginosa*

Manuscript submitted

Overall contribution to the paper: 65%

- Writing of first version of complete manuscript draft
- Determination of minimal inhibitory concentrations (MICs) against nosocomial pathogens
- Determination of MICs against persisters and biofilms of nosocomial pathogens
- Determination of cytotoxicity against human cell lines
- Determination of time-kill curves *in vitro*
- Isolation of spontaneously resistant mutants
- Various genetic manipulations of methicillin-resistant *Staphylococcus aureus* (MRSA)
- Isolation of whole-cell protein lysates of MRSA

Natural brominated phenoxyphenols kill persistent and biofilm-incorporated cells of pathogenic bacteria

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Abstract

Due to a high unresponsiveness to chemotherapy, biofilm formation is an important medical problem that frequently occurs during infection with many bacterial pathogens. In this study, the marine sponge-derived natural compounds 4,6-dibromo-2-(2',4'-dibromophenoxy)phenol and 3,4,6-tribromo-2-(2',4'-dibromophenoxy)phenol were found to exhibit broad antibacterial activity against medically relevant Gram-positive and Gram-negative pathogens. The compounds were not only bactericidal against both replicating and stationary-phase persistent planktonic cells of Methicillin-resistant *Staphylococcus aureus* (MRSA) and *Pseudomonas aeruginosa*; they also killed biofilm-incorporated cells of both species while not affecting biofilm structural integrity. Moreover, these compounds were active against carbapenemase producing *Enterobacter* bacteria. This simultaneous activity of compounds against different growth forms of both Gram-positive and Gram-negative bacteria is rare. Genome sequencing of spontaneous resistant mutants and proteome analysis suggest that resistance is mediated by downregulation of the bacterial EIIBC phosphotransferase components *scrA* and *mtlA* in MRSA likely leading to a lower uptake of the molecules. Due to their only moderate cytotoxicity against human cell lines, phenoxyphenols provide an interesting new scaffold for development of antimicrobials with activity against planktonic cells, persisters and biofilm-incoporated cells of ESKAPE pathogens.

Keywords

Natural products, ESKAPE pathogens, Antimicrobials, Anti-biofilm

Introduction

Staphylococcus aureus is a Gram-positive bacterium, which is part of the human skin flora but most commonly known as an opportunistic pathogen causing different life-threating diseases such as endocarditis or toxic shock syndrome (Boswihi and Udo 2018; Haysom et al. 2018; Lowy 1998; Wolk et al. 2009). The most prominent member in the group of pathogenic staphylococci is Methicillin-resistant *S. aureus*, commonly known as MRSA. It first occurred almost 80 years ago in the 1940s just a few years after the introduction of penicillin, but about 20 years before methicillin was first used as an antibiotic (Harkins et al. 2017; Jevons 1961; Kirby 1944; Rammelkamp and Maxon 1942). A single mutation in the gene *mecA*, located on the staphylococcal cassette chromosome *mec* (SCC*mec*) coding for a low-binding affinity penicillin-binding protein (PBP), is the source of MRSA's resistance not only to methicillin but to plenty of members of ß-lactam antibiotics (Hartman and Tomasz 1984; Lakhundi and Zhang 2018; Matsuhashi et al. 1986). This example shows that antimicrobial resistance (AMR) and cross-resistance can emerge very fast and can spread among a population *via* mobile genetic elements like SCC*mec*. Nowadays, clinical isolates of MRSA frequently show multi-drug resistance against several classes of antibiotics like macrolides, lincosamides, fluoroquinolones and aminoglycosides to name but a few (Chambers and Deleo 2009; Foster 2017; Jayaweera and Kumbukgolla 2017; Kaur and Chate 2015). In addition to MRSA, multidrug resistance (MDR) is also widespread among other members of the so-called ESKAPE pathogens such as the Gram-negative bacterium *Enterobacter cloacae*. Recently, we have isolated and characterized clinically relevant MDR *E. cloacae* strains harboring the metallo-β-lactamase (MBL) bla_{GIM-1} (German imipenemase-1) (Wendel et al. 2013; Wendel et al. 2016), conferring resistance against imipenem and meropenem, and which are additionally resistant against aztreonam, ciprofloxacin, trimethoprim-sulfamethoxazole, tigecycline, and chloramphenicol [17].

MRSA, like other bacteria, is able to change its growth behaviour. Persistence and biofilm formation render bacteria phenotypically resistant against most antibiotics, despite being

genetically identical to their replicating counterparts. Persistence is widely spread in bacteria and known for both Gram-positive and Gram-negative bacteria alike (Barraud et al. 2013; Bigger 1944; Stewart and Rozen 2012). The state of persistence is mainly characterised by a dramatically slowed metabolism as well as the stop of cell division, replication of their genome or protein-synthesis. Via different mechanisms, such as toxin-antitoxin systems, bacteria are able to adapt very quickly to environmental changes like antibiotic treatment and consequently switch to persistence (Balaban 2011; Balaban et al. 2004; Conlon et al. 2016). Biofilms are often the reason for the resurgence of infections. Surfaces like plastic catheters or prosthetic joints provide an appealing scaffold for biofilm-forming bacteria like *S. aureus* and *Pseudomonas aeruginosa*, which are two of the most common biofilm-forming bacteria. Biofilms are composed of replicating and persistent cells, which are surrounded by extracellular polymeric substances (EPS) such as eDNA, proteins and polysaccharides, forming a physical barrier to protect bacteria from antibiotics or other harmful abiotic and biotic stressors (Hoiby et al. 2010),(Flemming 2016). Therefore, almost every antibiotic in clinical use, such as broad-spectrum penicillins as the most prescribed antibiotic class in 2015, is inactive against persisters and especially biofilm-incorporated cells (Klein et al. 2018). Both growth forms are able to survive antibiotic treatment with up to 1000-fold of the minimal inhibitory concentrations (MIC) against actively growing cells, and surgical removal of infected tissues and devices is often the last choice (Barraud et al. 2013; Mermel et al. 2009).

To address the growing problem of AMR, it is essential to find and develop new lead structures and antibiotics aiming at new targets in the bacterial cell. Several approaches have been used to tackle the problems of rising AMR, persistence and biofilm growth, unfortunately with little success to challenge bacteria in all growth forms simultaneously (Allison et al. 2011; Prax et al. 2016; Zipperer et al. 2016). A rich source for new lead structures are natural products (reviewed in (van Geelen et al. 2018)). Here, we investigated two structurally related compounds isolated from the marine sponge *Dysidea granulosa*,

which was collected in the Andaman Sea (Thailand) in 2007. The compounds, 4,6-dibromo-2-(2',4'-dibromophenoxy)phenol (referred to as 2-bromo-PP) and 3,4,6-tribromo-2-(2',4‘-dibromophenoxy)phenol (referred to as 3-bromo-PP) are both brominated phenoxyphenols (PPs) and only differ in the additional bromide atom in 3-bromo-PP at position 3 in the phenol moiety (Figure 1). Both compounds are active against replicating, persistent and biofilm-incorporated cells of Gram-positive and Gram-negative bacteria and show a promising therapeutically window for further investigation.

Material & Methods

Bacterial strains, plasmids and growth conditions.

Strains and plasmids used in this study are listed in Supplementary Table 1 and 2, respectively. Cultures were, if not stated differently, incubated at 37 °C with aeration and shaking (150 rpm) in Mueller-Hinton broth (MHB). Bacteria were freshly incubated at the next morning from an overnight pre-culture for actively growing bacteria to an OD_{600} of approx. 0.6. To ensure persistent growth form, the bacteria were grown overnight until a density at OD_{600} > 5.0 was obtained. For biofilm growth, some colonies from a Mueller-Hinton agar (MHA) plate were picked and resuspended in MHB. Cell density has been adjusted to OD_{600} = 0.8 and cells were sown into a flat-bottom 96-well plate to be incubated statically at 37 °C with aeration. Studies were mainly performed with *S. aureus* Mu50.

Compounds.

2-bromo-PP and 3-bromo-PP were initially isolated from an undescribed *Dysidea* sp. from Satawan Atoll, Chuuk State, Federal States of Micronesia (Fu and Schmitz 1996). The compounds have been re-isolated from the marine sponge *Dysidea granulosa* harvested in the Andaman Sea in 2007 and were identified based on their NMR and MS spectroscopic data and comparison to the literature. The molecules are now part of a compound library of

the Institute of Pharmaceutical Biology and Biotechnology of the Heinrich Heine University Düsseldorf, Germany. The compounds were freshly prepared as 10 mM stock in DMSO and stored at -20 °C until further use.

MIC determination.

The minimal inhibitory concentration (MIC) has been determined according to the CSLI guidelines (CLSI 2012). Briefly, in a sterile polystyrene U-bottom 96-well plate, a two-fold serial dilution ranging from 100 µM to 0.78 µM of testing compounds has been prepared in 50 µL MHB. A freshly inoculated *S. aureus* culture was grown until $OD_{600\ nm}$ of approx. 0.6 and diluted to 10^6 CFU/mL in MHB. Finally, 50 µL of the latter cell suspension was added to each well and then incubated statically for 18 – 24 h at 37 °C. MIC was determined using BacTiterGlo ATP assay (Promega, Madison, Wisconsin, USA) and TECAN Infinite F200 Pro (TECAN, Männedorf, Switzerland). Moxifloxacin and DMSO were used as positive and negative control, respectively. Sublethal concentrations of colistin (0.1 µM) were used in some experiments to increase the permeability of the Gram-negative cell envelope for compound testing. For *E. cloacae*, a broth microdilution assay was performed using a serial two-fold dilution in 96-wells flat-bottomed plates (Falcon, BD Bioscience, Heidelberg) of both PPs in broth LB medium (Roth, Karlsruhe) at a concentration range of 50-0.024 µM.

Anti-persister activity assay.

Sterile U-bottom 96-well polystyrene plates were used for the assay. The wells were prepared with a final volume of 50 µL phosphate-buffered saline (PBS, NaCl = 137 mM, KCl = 2.7 mM, $NaHPO_4$ = 10 mM, KH_2PO_4 = 1.8 mM, pH = 7.4) containing a two-fold serial dilution of compounds ranging from 100 µM to 0.78 µM. *S. aureus* was cultivated as described above and washed three times with PBS before adjusting the cell density to

approx. 2 x 10^7 cells/mL ($OD_{600\ nm}$ = 0.08). To the prepared 96-well plates 50 µL of the cells suspension was added to each well. The plate was statically incubated for 24 h at 37 °C.

Viability of bacterial cells was estimated employing the Resazurin dye reduction assay. Briefly, 10 µL of a 100 µg/mL Resazurin solution was added to each well and were resuspend carefully. After several hours of incubation at 37 °C, the cells were inactivated with 10 % formalin solution. Finally, the fluorescence was quantified in a plate reader with 535 nm excision and 590 nm emission wavelengths. Moxifloxacin, DMSO and NH125 were used as negative and positive controls, respectively.

Anti-biofilm activity assay.

Sterile flat-bottom 96-well polystyrene plates were used for the assay. Some *S. aureus* colonies were picked from an MHA plate and resuspend in MHB + 10 % glucose and cell density was adjusted to 0.8 cells per mL. 100 µL of cell suspension was added to each well and the plate was incubated statically for 24 h to induce growth and mature of biofilms. After 24 h compounds were added to the wells to check for anti-biofilm activity. To check for inhibition of biofilm formation, compounds were added immediately after adding of cells to the wells in sub-lethal concentrations. Lysostaphin, Moxifloxacin and DMSO served as positive and negative controls, respectively.

Viability was measured as described above utilising the Resazurin reduction assay. Biofilm formation was measured using crystal violet staining as described elsewhere (Christensen et al. 1985; Stepanovic et al. 2007). Briefly, biofilms were washed three times with PBS and allowed to air dry before 0.1 % (v/v) crystal violet solution (Waldeck GmbH & Co., Münster, Germany) was added to each well. The biofilms were stained for 15 min at room temperature (RT). Afterwards, the plates were washed three times again with PBS and 30 % acetic acid was added for 30 min at RT to solubilise the dye. Finally, 100 µL were transferred to a fresh 96-well plate to measure absorption at 600 nm with a plate reader.

Killing kinetics.

S. aureus cells were freshly incubated in MHB until a density of approx. 0.6 was reached. Next, the cells were diluted to 10^6 CFU/mL in 5 mL MHB. Then compounds were added to the cultures as follows: 2-bromo-PP 0.78 µM (4x MIC), 3-bromo-PP 0.39 µM (4x MIC), Moxifloxacin 20 µM (2x MIC), DMSO 50 µM. At certain time points (0, 0.5, 1, 3, 6, 8, 24 h) 100 µL were removed from the culture, diluted and plated onto MHA plates. After 24 h incubation, colonies were counted and viability (expressed as colony forming units per mL, CFU/mL) was calculated considerung the dilution factor.

For persister killing kinetics, cells were grown overnight until a density of $OD_{600\ nm}$ > 5.0 was reached and then washed three times with PBS. Next, cell density was adjusted to 10^8 CFU/mL in 5 mL PBS. Compounds were added as follows: 2-bromo-PP 12.5 µM (4x MIC), 3-bromo-PP 6.25 µM (4x MIC), Moxifloxacin 100 µM, DMSO 50 µM, NH125 10 µM. Analysis was performed as described above.

Quantitative Realtime-PCR.

Quantitative Realtime-PCR (qPCR) was performed as described (Lewis and Rice 2016). Briefly, cells were lysed using bead beating in a tissuelyser Precellys 24 (Bertin Instruments, Montigny-le-Bretonneux, France). RNA was isolated using the Qiagen RNeasy kit (Qiagen, Hilden, Germany) and quality was checked with an RNA 6000 Nano Chip in a Bioanalyzer (Agilent, Santa Clara, California, USA) performed by the BMFZ in Duesseldorf. cDNA was synthesised using SuperScript IV First-Strand Synthesis kit (ThermoFisher, Meerbusch, Germany). qPCR was performed using GoTaq qPCR Master Mix from Promega. The experiment was performed as given in the manufacture's protocol. As template 5 µL of 1:10 diluted cDNA samples were used. Results have been normalised to 16S rRNA using the Livak method. All experiments have been performed in triplicates.

Cytotoxicity assay.

Human cell lines were grown in their respective growth medium. Cells have been counted using a haemocytometer and cell density was adjusted to 10^6 cells/mL. A two-fold serial dilution was prepared in 96-well-U-bottom plates and cells were sow into the wells to a final density of $5x10^5$ cells/well. After two days at 37 °C and 5 % CO_2 10 µL of a 100 µg/mL Resazurin dye solution were added to each well and was incubated for several hours. The reaction was inactivated with 100 µL of a 10 % formalin solution per well. Finally, the fluorescence was quantified in a plate reader with 535 nm excision and 590 nm emission wavelengths. Cell lines are shown in Supplementary Table 3.

Fluorescence microscopy.

Biofilms were pre-grown as described above. Alcian blue staining was performed to stain the EPS. A 1 % Alcian blue staining solution was prepared in 3 % glacial acidic acid. Staining solution was added to the biofilm in sufficient amount to cover the whole biofilm and incubated for 30 minutes at RT. Next, the biofilm was washed twice with PBS and 200 µL 10 % formalin solution was added. Fluorescence microscopy was performed using a Nikon Eclipse TS100 fluorescence microscope.

Sample clean-up for LC-MS.

After in-solution digestion (ISD) peptides were desalted on home-made C18 StageTips (Rappsilber et al. 2007). Briefly, the peptide solution was passed over the MeOH pre-conditioned and 0.5% FA equilibrated StageTip. Immobilized peptides were then washed twice with 0.5% (v/v) FA. Washed peptides were eluted from the StageTips with 80% (v/v)

ACN 0.5% (v/v) FA and dried using a vacuum concentrator (Eppendorf). Before LC-MS peptide samples were resuspended in 10 µl 0.1% (v/v) FA.

LC-MS/MS.

LC–MS/MS experiments were performed on an Orbitrap Elite instrument(Michalski et al. 2012) (Thermo) that was coupled to an EASY-nLC 1000 liquid chromatography (LC) system (Thermo). The LC was operated in the one-column mode. The analytical column was a fused silica capillary (75 µm × 45 cm) with an integrated PicoFrit emitter (15 µm, New Objective) packed in-house with Reprosil-Pur 120 C18-AQ 1.9 µm resin (Dr. Maisch). The analytical column was encased by a column oven (Sonation) and attached to a nanospray flex ion source (Thermo). The column oven temperature was adjusted to 45 °C during data acquisition. The LC was equipped with two mobile phases: solvent A (0.1% formic acid, FA, in water) and solvent B (0.1% FA in acetonitrile, ACN). All solvents were of UPLC grade (Sigma). Peptides were directly loaded onto the analytical column with a maximum flow rate that would not exceed the set pressure limit of 980 bar (usually around 0.5–0.6 µl min^{-1}). Peptides were subsequently separated on the analytical column by running a 140 min gradient of solvent A and solvent B at a flow rate of 300 nl min^{-1} (gradient: start with 7% B; gradient 7 to 35% B for 120 min; gradient 35–100% B for 10 min and 100% B for 10 min). The mass spectrometer was operated using Xcalibur software, Thermo Fischer Scientific, UK (version 2.2 SP1.48) and was set in the positive ion mode. Precursor ion scanning was performed in the Orbitrap analyzer (FTMS; Fourier Transform Mass Spectrometry) in the scan range of m/z 300–1,800 and at a resolution of 60,000 with the internal lock mass option turned on (lock mass was 445.120025 m/z, polysiloxane)(Olsen et al. 2005). Product ion spectra were recorded in a data dependent fashion in the ion trap (ITMS) in a variable scan range and at a rapid scan rate. The ionization potential (spray voltage) was set to 1.8 kV. Peptides were analyzed using a repeating cycle consisting of a full precursor ion scan (1.0×10^6 ions or 50 ms) followed by 15 product ion scans (1.0×10^4 ions or 100 ms), where

peptides are isolated based on their intensity in the full survey scan (threshold of 500 counts) for tandem mass spectrum (MS2) generation that permits peptide sequencing and identification. Collision-induced dissociation (CID) energy was set to 35% for the generation of MS2 spectra. During MS2 data acquisition, dynamic ion exclusion was set to 120 s with a maximum list of excluded ions consisting of 500 members and a repeat count of one. Ion injection time prediction, preview mode for the FTMS (the orbitrap), monoisotopic precursor selection and charge state screening were enabled. Only charge states higher than 1 were considered for fragmentation.

Peptide and Protein identification using MaxQuant and Perseus.

RAW spectra were submitted to an Andromeda(Cox et al. 2011) search in MaxQuant (version 1.5.3.30) using the default settings(Cox and Mann 2008). Label-free quantification was activated(Cox et al. 2014). MS/MS spectra data were searched against the Uniprot *Staphylococcus aureus* strain Mu50 reference proteome database (UP000002481_158878.fasta ; 2714 entries; downloaded 23.02.2017). All searches included a contaminants database (as implemented in MaxQuant, 245 sequences). The contaminants database contains known MS contaminants and was included to estimate the level of contamination. Andromeda searches allowed oxidation of methionine residues (16 Da), acetylation of the protein N-terminus (42 Da) as dynamic modifications and the static modification of cysteine (57 Da, alkylation with Iodoacetamide). Enzyme specificity was set to “Trypsin/P”. The instrument type in Andromeda searches was set to Orbitrap and the precursor mass tolerance was set to ±20 ppm (first search) and ±4.5 ppm (main search). The MS/MS match tolerance was set to ±0.5 Da. The peptide spectrum match FDR and the protein FDR were set to 0.01 (based on target-decoy approach). Minimum peptide length was 7 amino acids. For protein quantification unique and razor peptides were allowed. Modified peptides with dynamic modifications were allowed for quantification. The minimum score for modified peptides was 40. Further data analysis and filtering of the MaxQuant

output was done in Perseus v1.5.5.3(Tyanova et al. 2016). MS/MS counts were loaded into the matrix from the proteinGroups.txt file and potential contaminants as well as reverse hits, hits only identified by site and protein groups with less than 2 identified unique peptides were removed. For the statistical calculations samples technical replicates were grouped in categorical groups and filtered. Only those protein groups were kept that contained three valid values in a minimum of one categorical group. The missing values in the remaining protein groups were then imputed and the t-test perfomed (number of randomizations 250; initial FDR 0.05 and S0 0.1).

Data Availability.

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE(Vizcaino et al. 2016) partner repository (https://www.ebi.ac.uk/pride/archive/) with the dataset identifier PXD016373. During the review process the data can be accessed via a reviewer account (Username: reviewer86883@ebi.ac.uk; Password: GiXzRDPj).

Genome sequencing.

DNA was extracted from individual colonies using protocol from Krausz and Bose (Krausz and Bose 2016). The samples were prepared for sequencing using the standard Illumina whole-genome sample prepartion kit (Illumina, Inc.; San Diego, CA) and sequenced on an Illumina HiSeq instrument. Paired-end reads with a read-length of 150 bp were collected. The mean depth of coverage ranged from 165 to 390. Genome sequences for the isolates were assembled using a comparative assembly approach(Ioerger et al. 2010). Reads were mapped to the genome sequence of the parent S. aureus strain (either ATCC 25923 or ATCC 700699, GenBank accession numbers CP009361.1 and NC_002758.2) using BWA v0.7.12 (Li and Durbin 2009), and insertions and deletions (indels) were identified

using local contig building. Polymorphisms were identified by aligning each genome to the reference sequence (using MUMmer v3.20 (Kurtz et al. 2004)) and tabulating single-nucleotide polymorphisms (SNPs) and indels according to the following criteria: coverage by at least 10 reads, and not heterogeneous (>=70 % conversion to the nonreference nucleotide). SNPs in repetitive regions were also filtered out (defined as sites for which an overlapping 35-bp window matched a sequence elsewhere in the genome with at most 2 mismatches).

Results

In vitro antibacterial activity of 2-bromo-PP and 3-bromo-PP.

In an initial screening with an in-house library of natural compounds, 2-bromo-PP and 3-bromo-PP have been found to inhibit growth of *S. aureus* and *Pseudomonas aeruginosa*. Further experiments have been performed with both PPs in microbroth dilution assay for activity tests against different MRSA strains and various nosocomial pathogens, most representing the ESKAPE group (Table 1). In general, the activity of 3-bromo-PP was better against Gram-positive (MIC range 0.1 - 0.78 µM), whereas that of 2-bromo-PP was better against Gram-negative bacteria (MIC range 0.78 – 3.125 µM), including a multidrug-resistant clinical isolate of *E. cloacae* harbouring the metallo-ß-lactamase (MBL) bla_{GIM-1} as well as resistance genes against aminoglycosides, fluorochinolons, thrimethoprin/sulfamethoxazole, tetracylines, tetracylins, fosmomycin, and chloramphenicol (Wendel et al. 2013; Wendel et al. 2016). Activity of both compounds against Gram-negative bacteria could be substantially increased when used in combination with a sublethal concentration of colistin (0.1 µM) to increase the permeability of the outer membrane (Table 1). Furthermore, PPs were tested against various human cell lines to assess their general cytotoxicity (Figure 2a+b; Table 1; Supplementary Figure 1; Supplementary Table 3). 2-bromo-PP showed moderate cytotoxicity (IC_{50} = 6.25 µM) against THP-1 cells, but only minor cytotoxic effects against the other tested cell lines (IC_{50} = 12.5 – >100 µM) . In contrast, virtually no cytotoxic effects were observed for 3-bromo-PP (IC_{50} = 25 - >100 µM) with the exception of HEK293 cells (IC_{50} =

3.125 µM). These data are in accordance with a previous study that reported on low cytotoxicity of both compounds for human PBMNCs (Mayer et al. 2019). Next, the activity of PPs was tested against persisters and preformed biofilms of MRSA Mu50 and of *P. aeruginosa* POA1. Both compounds showed activity against persisters with an MIC of 12.5 µM for 2-bromo-PP for both MRSA and *P. aeruginosa* as well as 6.25 µM and 50 µM for 3-bromo-PP against MRSA (Figure 2a, b) and *P. aeruginosa* (Supplementary Figure 1a), respectively. For MRSA biofilms, 2-bromo-PP inhibited viability of cells in biofilms completely at 100 µM, while 3-bromo-PP was more potent and showed a maximal effect already at 12.5 µM. For *P. aeruginosa* biofilms, 2-bromo-PP showed an increased effect compared to MRSA biofilms (complete inhibition at 25 µM), whereas 3-bromo-PP was less potent with complete inhibition of cell viability at 50 µM (Supplementary Figure 1b). Although both PPs showed a substantial effect on the viability of biofilm-incorporated cells, neither of the PPs influenced integrity of preformed biofilms (Figure 2c+d).

PPs have bactericidal activity against both replicating and persistent MRSA cells.

For further characterisation of PPs, killing kinetics were performed with cells of MRSA strain Mu50 cultivated in Mueller-Hinton broth (MHB) containing compounds at 4x MIC. Viability was monitored for 8 or 24 hours for actively growing or stationary phase-induced persistent cells, respectively, by plating of serial dilutions and determinating colony forming units (CFU) (Figure 3). For actively replicating MRSA, moxifloxacin as a positive control exhibited a strong and rapid bactericidal effect as expected with viability reaching the detection limit after 3 hours of incubation. In contrast, 2-bromo-PP and 3-bromo-PP acted much slower with first antibacterial effects occuring after 3 and 6 hours, respectively. Notwithstanding, both compounds also showed a slow but strong bactericidal effect, and viability eventually reached the detection limit after 8 hours (Figure 3a). Furthermore, a comparable bactericidal effect of both 2-bromo-PP and 3-bromo-PP was observeable against replicating cells of multidrug-resistant *Acinetobacter baumannii* (Supplementary Figure 2).

In contrast to this potent bactericidal activity on actively growing MRSA cells, moxifloxacin did not show any effect against stationary phase-induced persistent cells even after 24 h of incubation. On the other hand, NH125, which has been reported to be active against persister cell populations (Kim et al. 2016), was used as a positive control in this assay and was able to reduce cell viability almost down to the detection limit after 3 h of incubation (Figure 3b). Compared to NH125, 2-bromo-PP acted with a slight delay but eventually reduced viability dramatically down to the detection limit after 8 h of treatment. Thus, this compound is affecting both actively replicating and persister cells of MRSA, although substantially higher concentration were required for achieving effects on persister cells. 3-Bromo-PP had a weaker effect on persistent cells and steadily decreased viability by 2 logs after 24 h (Figure 3b).

The bacterial phosphotransferase system is crucial for PP activity.

Spontaneous resistant mutants have been generated with 2-bromo-PP and 3-bromo-PP to further investigate the mechanism of action and resistance. Since isolation of spontaneous resistant mutants in one step was not successful on solid media containing 2-bromo-PP or 3-bromo-PP (i.e. resistance frequency < 10^{-7} at 5-fold MIC), enrichment was done by passaging bacteria daily in liquid media containing increasing sublethal concentrations of PPs. After 15 passages, mutants were obtained that showed a 4- (2-bromo-PP) or 32-fold (3-bromo-PP) increased MIC, respectively (Table 2). Moreover, we observed cross-resistance between PPs since 2-bromo-PP-resistant mutants were also resistant against 3-bromo-PP and vice versa (Figure 4a, Table 2).

Genomic DNA of six different resistant mutants raised against either 2-bromo-PP or 3-bromo-PP, respectively, was isolated and genome sequencing was performed to analyse the mechanism of resistance. As a result of the serial sub-passaging at sublethal compound concentrations, the mutants had accumulated multiple genetic alterations as expected. However, most of these mutations occurred only once among the mutants, indicating they

were likely not relevant for resistance (Supplementary Table 2). However, a SNP in the gene *tetR* coding for a transcription regulator was found in 5 of the 6 analyzed mutants, strongly suggesting that this mutation is causally involved in the resistance phenotype (Table 2). In close chromosomal proximity to *tetR*, genes of the magnesium transporter *corA* and the phosphotransferase system (PTS) component *scrA* can be found (Figure 4b). ScrA is predicted to be the EIIBC component of a sucrose-specific PTS (Deutscher et al. 2014; Wagner et al. 1993). Interestingly, one mutant (M5) harboured a SNP in the conceivable promoter region upstream of the *mtlA* gene as part of another PTS system (Figure 4b). MtlA is an essential component of the mannitol-specific PTS (Reiche et al. 1988; UniProt 2019). These observations pointed toward a potential role of PTS systems in the resistance mechanisms

In order to investigate how the mutations identified in the spontaneous resistant mutants affected expression levels of *scrA* and *mtlA*, qPCR analyses were performed. We found a ca. 0.75-fold decrease in the expression of *mtlA* in mutant M5 that carries a G to A substitution in the putative promotor region of *mtlA* (Figure 4c). For mutant M4, which harbours a SNP in the *tetR* gene leading to an R197L amino acid exchange in the TetR protein (Table 2), we observed a strong and statistically significant downregulation of *scrA* expression (Figure 4c). A similar trend was found for mutant M6, which carries an identical SNP, although differences in expression levels did not reach statistical significance (Figure 4c).

For further investigation of the resistance mechanism against PPs, Mu50 cells were treated with a sublethal concentration of 3-bromo-PP that allowed ca. 50 % residual growth compared to the untreated control. Subsequently, whole protein extracts were prepared to perform global proteome analysis of the elicited stress response. This revealed that MtlA was the most downregulated protein (approx. 2.5-fold) in sublethally 3-bromo-PP-stressed cells (Figure 4d). In combination, these results suggest that reduced expression of EIIBC

components of certain sugar-specific PTS such as MtlA or ScrA mediate resistance towards PPs.

To corroborate the role as potential determinants of resistance, *mtlA* and *tetR* were overexpressed under control of an anhydrotetracycline (Atc)-inducible promoter, and the effect on sensitivity towards 3-bromo-PP was analysed (Figure 4e). The vector control strain harbouring the empty plasmid pLA03 showed an MIC of approx. 0.625 µM irrespective of the presence or absence of Atc. Non-induced strains containing the expression plasmids pLA03::*mtlA* and pLA03::*tetR* showed a marginal shift of the MIC to 0.3125 µM. However, induction of *mtlA* or *tetR* expression in presence of Atc strongly rendered the cells more susceptible to 3-bromo-PP resulting in MICs of 0.019 µM for *mtlA* and 0.078 µM for *tetR* overexpression, respectively. This translates into a 33-fold and 8-fold increased sensitivity during *mtlA* or *tetR* overexpression, respectively, compared to the empty vector control (Figure 4e). This characteristic differential susceptibility pattern in mutants and recombinant strains under- or overexpressing *mtlA* or *tetR* strongly indicates that both genes are causally involved in the mechanism of resistance against PPs.

Discussion

In this study, we demonstrated that both 2-bromo-PP and 3-bromo-PP are potent inhibitors of bacterial growth of various ESKAPE pathogens. Whereas 2-bromo-PP is more potent against Gram-negative bacteria such as *P. aeruginosa* or *A. baumannii*, 3-bromo-PP is more active against Gram-positive bacteria like MRSA. However, the most interesting feature of these compounds is their activity against persistent and biofilm-incorporated cells. The majority of antibiotics in clinical use show only good activity against actively replicating cells, while the available therapeutic options against persisters and especially biofilms are very limited (Hoiby et al. 2015). The effect on persistent bacteria is diminished because several antibiotics target processes that are down-regulated during persistence or shut off

completely. Fluorchinolones such as moxifloxacin for example target the bacterial DNA-gyrase and have no activity against MRSA persisters at physiologic relevant concentrations (Drlica and Zhao 1997). This is also true for other entire families of antibiotics such as sulphonamides interfering indirectly with the nucleotide synthesis, and tetracyclines that are inhibiting protein biosynthesis (Chukwudi 2016; Henry 1943). Biofilm-incorporated cells, in particular, are even more tolerant to antibiotics although the biofilm is composed of persisters as well as actively replicating cells (Barraud et al. 2013). Thus, it is an important trait that PPs are not only killing Gram-positive and Gram-negative persisters, but also biofilm-incorporated cells *in vitro*.

The biofilm matrix is a strong physical barrier against large molecules like rifamycins and glycopeptides. This might be one reason for the high tolerance against these classes of antibiotics. Small molecules, such as PPs, are promising candidates to pass the complex biofilm matrix and to reach biological active concentrations inside the biofilm. In agreement with this, we observed a slow but constant reduction of viability so that eventually the majority of biofilm-incorporated bacteria were killed within 24 h by PPs. The biofilm matrix itself, however, was not affected in its integrity. In consequence, we could show that PPs are bactericidal against actively replicating MRSA Mu50 and *P. aeruginosa* cells as well as against their dormant counterparts and biofilm-incorporated cells.

During our studies, we were able to generate spontaneously resistant mutants performing liquid enrichment and genome sequencing to elucidate the mode of resistance. We found a consistent single nucleotide polymorphism (SNP) in several independent mutants in the transcription factor *tetR* resulting in an amino acid exchange (R197L). Interestingly, TetR appears to be a positive regulator of the neighboring gene *scrA*, which is the EIIBC component of sucrose-specific PTS, since we found that the $TetR^{R197L}$ mutation led to a lower expression of *scrA.* Furthermore, a G to A substitution in the putative promotor region of *mtlA* was found in one mutant that did not have the SNP in the *tetR* ORF. This promoter mutation led to a lower expression of *mtlA* as revealed by RT-qPCR analysis. MtlA is the EIIBC

component of the mannitol-specific PTS. It catalyses the phosphorylation of incoming D-mannitol to D-mannitol-1-phosphate by translocating a phosphate group of an N-phospho-L-histidine residue to the sugar. D-mannitol-1-phosphate is then passed through the membrane. Likewise, ScrA is a sucrose-specific EIIBC PTS-component that catalyses a similar reaction for sucrose. This strongly pointed toward a specific role of these EIIBC components and PTS in resistance to PPs. This hypothesis was further corroborated by the strong increase in sensitivity to 3-bromo-PP in recombinant MRSA strains overexpressing either *mtlA* or *tetR*, and by the strong downregulation of MtlA in cells treated with a sub-lethal concentration of 3-bromo-PP as revealed by proteome analysis. This differential susceptibility pattern (i.e. decreased sensitivity at reduced expression, increased sensitivity during overexpression) is in agreement with a potential role of the identified PTS for uptake of PPs. Based on the regular mechanism of EIIBC components in sugar uptake by PTS it is conceivable that uptake of PPs is coupled to phosphorylation of the hydroxyl-moiety in the left part of the molecule, potentially resulting in intracellular activation. Downregulation of MtlA in 3-bromo-PP treated cells might represent a specific stress response aiming at reducing the uptake of the antibacterial compound. Although we did not observe a similar stress response for ScrA, it is likely that both PTS have a redundant or additive role in uptake and/or activation of PPs. This might explain why we were unable to isolate single-step spontaneous PP-resistant mutants and obtained a low level of resistance only after serial subpassaging at escalating sublethal doses of the compounds. It is possible that further EIIBC PTS-component might also be able to phosphorylate and subsequently take up PPs to some extent. Sofar, however, we have no evidence for the involvement of other PTS, e.g. the fructose PTS, in resistance to PPs.

With respect to the clinical potential of the studied PPs, a medicinal chemical optimisation regarding the mitigation of cytotoxic effects would be a major point besides the derivatization to increase the antibacterial potency. Most importantly optimisation of the anti-biofilm activity should be in the focus because the current therapeutical window is too small for proper clinical use by now. The synthesis of polybrominated diphenyl ethers (PBDEs), PP-like

molecules, is well understood (Lin et al. 2014), which will greatly facilitate such medicinal chemistry efforts and will also support mode-of-action studies. For instance, synthesis of phosphorylated PPs could help to test the postulated requirement of phosphorylation by EIIBC components for activating PPs

In summary, PPs are small bioactive molecules that are able to kill Gram-positive and Gram-negative bacteria. Most importantly, PPs kill not only planktonic bacterial cells but also their persistent counterparts, biofilm-incorporated and MDR enterobacteria cells as well. Some compounds are in development showing promising results in either preventing the attachment of bacteria to surfaces as the first step in biofilm formation (Elshaarawy et al. 2017) or the maturation of the biofilm *via* quorum quenching (Gopu et al. 2015), but these molecules are incapable to sterilise preformed biofilms. In this regard, PPs provide interesting new hit structures in the quest for new anti-biofilm antibiotics. Advantages of PPs are their low potency to develop resistances (resistance rate < 10^{-8}) as well as their broad-spectrum activity against ESKAPE pathogens. To elucidate the mechanism of action, further experiments with derivatives of PPs are necessary in the future.

Figures

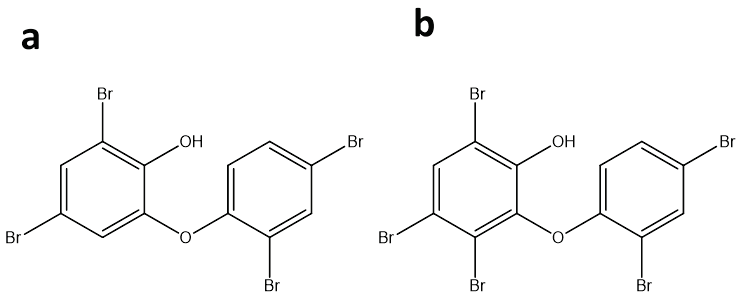

Figure 1. Structures of (a) 2-bromo-PP and (b) 3-bromo-PP Both molecules were isolated from the marine sponge *Dysidea granulosa* and are highly active against ESKAPE pathogens like MRSA. The difference in both molecules is a single bromide atom at position 3 in the phenol moiety.

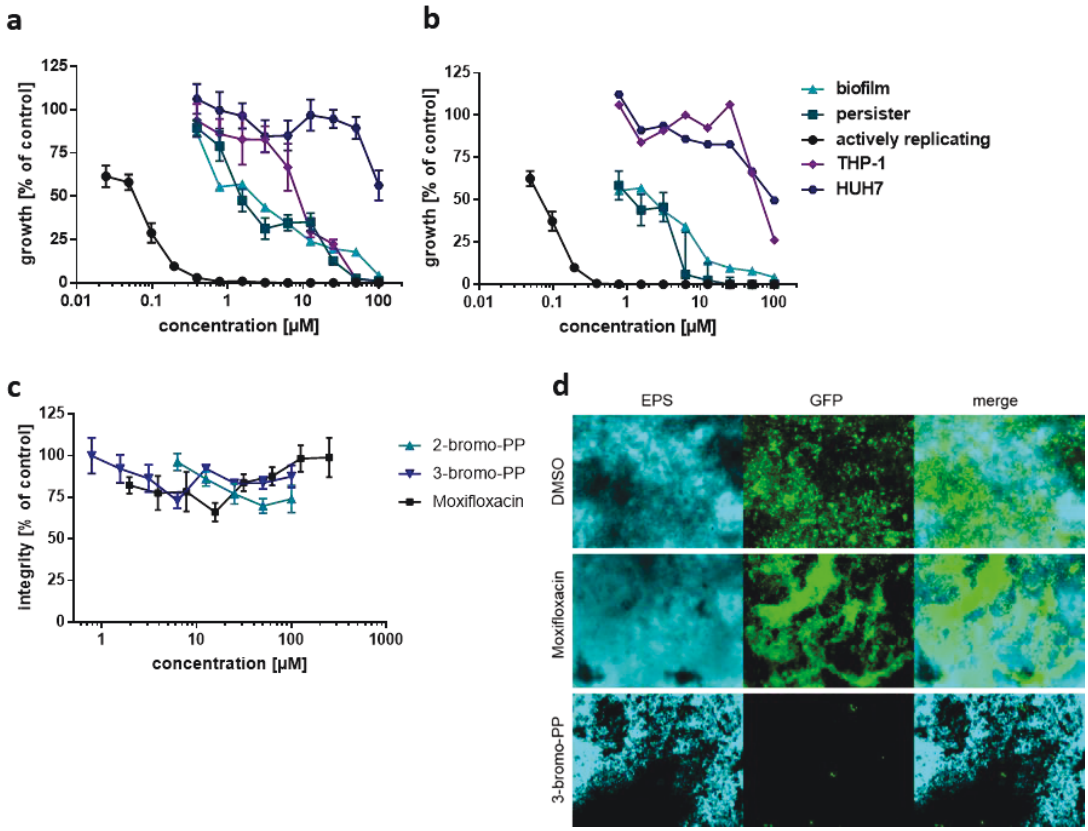

Figure 2. Antimicrobial activity of brominated phenoxyphenols Minimal inhibitory concentrations and cytotoxicity data for (a) 2-bromo-PP and (b) 3-bromo-PP against actively replicating (black circle), dormant (turquoise square) and biofilm incorporated (light blue triangle) MRSA cells. THP-1 monocytes (purple rhombus) and Huh7 hepatocytes (blue hexagon) were used to determine cytotoxicity. For persister, biofilm and eukaryotic viability assays, the Resazurin dye reduction assay was used. For actively replicating cells the BacTiter Glo assay was used. To measure the biofilm integrity (c), crystal violet staining was used. Neither 2-bromo-PP (light blue triangle) nor 3-bromo-PP (blue inverted triangle) disperse biofilms. Moxifloxacin (black square) was used as control. Calculations were made with respect to the controls. Values are means of triplicates ± SEM. Fluorescence microscopy was used to visualise the impact of 3-bromo-PP on preformed biofilms (d). EPS was stained with Alcian blue, GFP was expressed by MRSA Mu50 GFP-reporter strain. DMSO and Moxifloxacin were used as controls.

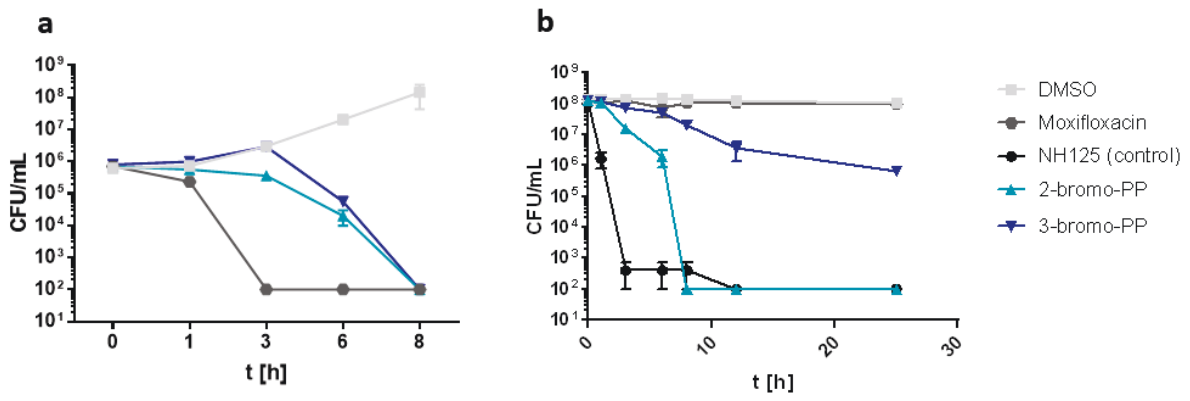

Figure 3. Bactericidal effects of 2-bromo-PP and 3-bromo-PP on cells of MRSA strain Mu50 Actively replicating (a) or stationary phase-induced persistent cells (b) were cultivated in MHB with the indicated compounds, and viability was monitored by CFU plating at various time points. Samples were inoculated at a starting cell density of 10^6 CFU/mL (a) or 10^8 CFU/mL (b), respectively. Limit of detection was 10^2 CFU/mL. Values are means of triplicates ± SEM. Compounds have been added at the following concentrations: DMSO 50 µM (solvent control, light grey square); moxifloxacin 20 µM (positive control for actively replicating cells, grey rhombus); NH125 10 µM (positive control for persistent cells,black circle); 2-bromo-PP 0.76 µM (a) and 50 µM (b) (= 4x MIC, light blue triangle) ; 3-bromo-PP 0.4 µM (a) and 25 µM (b) (= 4x MIC, blue inverted triangle).

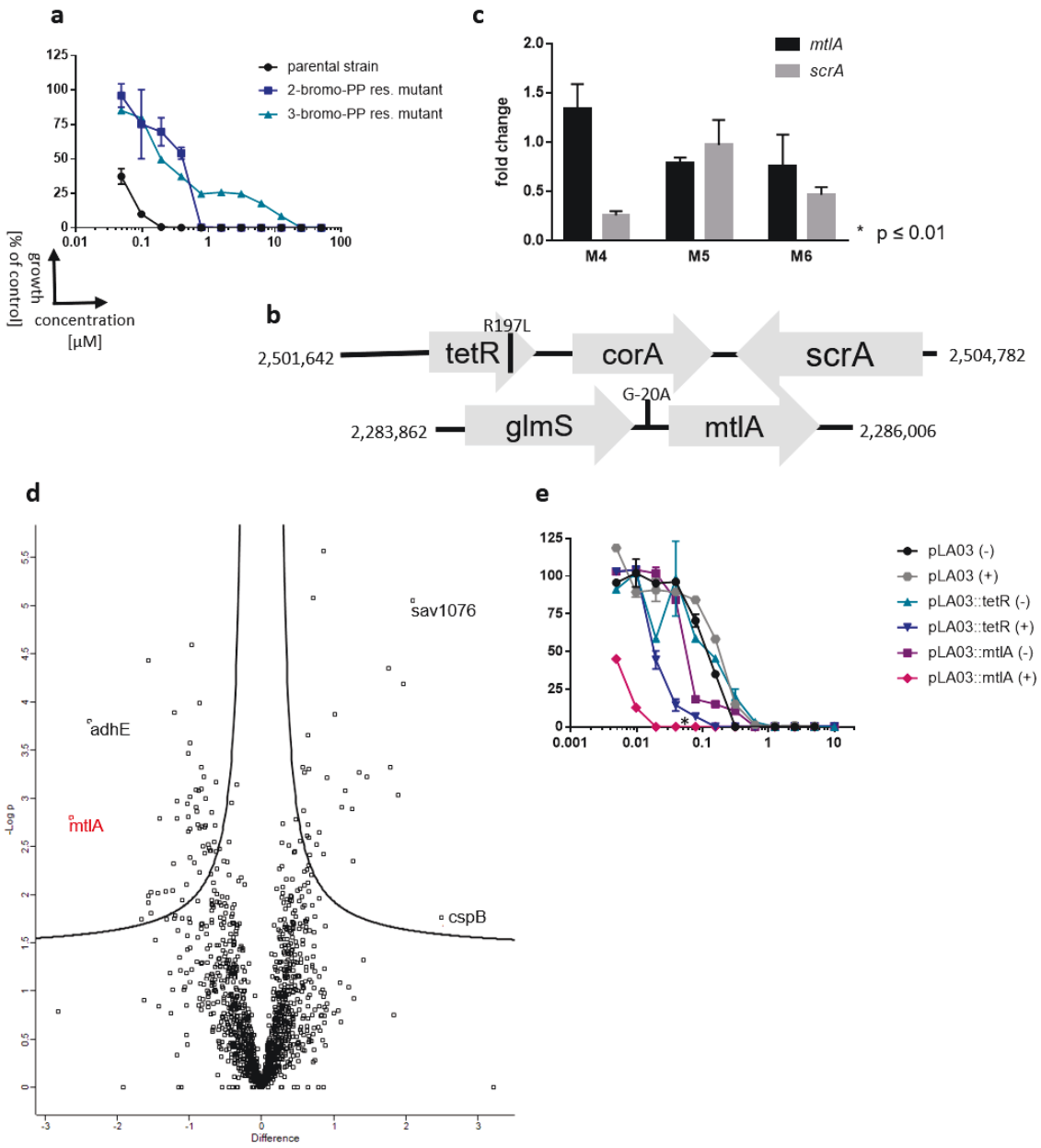

Figure 4. Analysis of spontaneously resistant *S. aureus* mutants (a) Dose-response curve against 3-bromo-PP of the MRSA parental strain Mu50 (black circle), spontaneously resistant mutant M1 raised against 2-bromo-PP (blue square) and spontaneously resistant mutant M4 raised against 3-bromo-PP (turquoise triangle) showing a 4x and 32x MIC shift, respectively. Moreover we observe cross-resistance between PPs. (b) The genomic regions of SRM containing mutations. SNPs are marked with a black line and amino acid changes or nucleotide changes are annotated. (c) RT-qPCR of SRMs to check expression levels of *mtlA* (black) and *scrA* (grey). Expression was normalised to wildtype expression levels for each gene (set to 1). Statistical significance was determined using the student's t-test. Error bars

show standard error of mean; n = 3. (d) Proteome analysis of MRSA Mu50 cells treated with a sublethal concentration of 3-bromo-PP. Red marked protein MtlA is downregulated by approx. 2.5-fold. (e) Overexpression of *tetR* and *mtlA* render the cells more susceptible to 3-bromo-PP. Induced expression is indicated with a plus (grey hexagon, blue inverted triangle, magenta rhombus), non-induced expression (black circle, turquoise triangle, violet square) is depicted with a minus. The expression was induced using 1 µg/mL anhydrotetracycline.

Table 1. Activity of 2-bromo-PP and 3-bromo-PP against various nosocomial pathogens. The antimicrobial activity of PPs is notable good against multi-drug resistant bacteria. Asterisks indicate multi-drug resistant strains. The MIC of colistin against *A. baumanni* ATCC 747 and *E. coli* ATCC 25922 was 0.78 µM.The growth of Gram-negative bacteria was not inhibited at a colistin concentration of 0.1 µM.

Organism	MIC_{90} [µM]	
	2- bromo-PP	3- bromo-PP
Staphylococcus aureus ATCC 25923	0.39	0.78
S. aureus Mu50*	0.19	0.1
S. aureus COL*	0.78	0.78
S. aureus USA300*	0.78	0.39
S. aureus TCH1516*	0.78	0.78
Enterococcus faecium ATCC 35667	0.78	0.39
E. faecium ATCC 700221*	0.78	0.78
E. faecalis ATCC 29212	0.78	0.78
E. faecalis ATCC 51299*	0.78	0.78
Bacillus subtilis 168 trp C2	0.39	0.39
Acinetobacter baumannii ATCC 747	0.78	3.125
A. baumannii ATCC 1605*	0.78	1.56
A. baumannii ATCC 1605* + 0.1 µM Colistin	0.39	0.78
Pseudomonas aeruginosa PAO1	0.78	1.56
Escherichia coli ATCC 25922	3.125	6.25
Escherichia coli ATCC 25922 + 0.1 µM Colistin	0.04	0.04
Klebsiella pneumoniae ATCC 700603	1.56	3.125
Enterobacter cloacae isolate 3678*	12.5	12.5

Table 2. SNPs in spontaneously resistant mutants raised against 2- or 3-bromo-PP.

S. aureus strain ATCC 25923 was used to generate resistant mutants against 2-bromo-PP and *S. aureus* strain Mu50 was used to generate resistant mutants against 3-bromo-PP. A full list of identified SNPs can be found in Supplementary table 4.

Strain	Parental strain	MIC 2-bromo-PP (μM)	MIC 3-bromo-PP (μM)	Relevant SNP
ATCC 25923	-	0.39	0.78	
Mu50	-	0.19	0.1	
M1	ATCC 25923	1.56	3.125	KQ76_12400: S155F (*tetR*)
M2	ATCC 25923	1.56	3.125	KQ76_12400: S155F (*tetR*)
M3	ATCC 25923	0.78	6.25	KQ76_12400: S155F (*tetR*)
M4	Mu50	1.56	3.125	SAV_RS12945: R197L (*tetR*)
M5	Mu50	1.56	3.125	C to G substitution between SAV_RS11755/11760 (5' to *mtlA*)
M6	Mu50	0.78	3.125	SAV_RS12945: R197L (*tetR*)

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Author contributions

L.v.G. and R.K.conceived the study; L.v.G., F.K., S.S.S., E.T.A., D.M., K.P., M.K. and T.R.I.designed and conducted experiments; L.v.G., F.K., S.S.S., E.T.A., D.M., K.P., M.K., T.R.I. and R.K.analyzed the data; P.P. provided compounds; L.v.G. and R.K. wrote the first draft of the manuscript; all authors contributed to the final version of the manuscript.

Competing interests

The authors declare no conflict of interest.

Supplementary Data

Natural brominated phenoxyphenols kill persistent and biofilm-incorporated cells of pathogenic bacteria

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Supplementary Table 1 – Strains used in this study

Strain	Relevant properties	Origin
Staphylococcus aureus Mu50 (ATCC 700699)	Multidrug-resistant screening strain, parental strain for spontaneously resistant mutants M4 - M6	American Type Culture Collection (ATCC)
S. aureus Mu50 + pLA02::GFP	Constitutively expressing GFP-reporter strain	This study
S. aureus USA300	Multidrug-resistant screening strain	Biodefense and Emerging Infections Research Resources Repository (BEI Resources)
S. aureus TCH1516	Multidrug-resistant screening strain	BEI Resources
S. aureus COL	Multidrug-resistant screening strain	BEI Resources
S. aureus ATCC 25923	Screening strain, parental strain for spontaneously resistant mutants M1 - M3	ATCC
S. aureus RN4220	Cloning intermediate for *S. aureus* plasmids	German Collection of of Microorganisms and Cell Cultures GmbH (DSMZ)
Enterococcus faecium ATCC 35667	Screening strain	ATCC
E. faecium ATCC 700221	Multidrug-resistant screening strain	ATCC
E. faecalis ATCC 29212	Screening strain	ATCC
E. faecalis ATCC 51299	Multidrug-resistant screening strain	ATCC
Bacillus subtilis 168 trp C2	Screening strain	ATCC
Acinetobacter baumannii ATCC 747	Screening strain	ATCC
A. baumannii ATCC 1605	Multidrug-resistant screening strain	ATCC
Enterobacter cloacae isolate 3678	Multidrug-resistant screening strain	Clinical isolate [16,17]
Klebsiella pneumonia ATCC 700603	Screening strain	ATCC
P. aeruginosa PAO 1	Screening strain, does not produce alginate	DSMZ
Escherichia coli ATCC 25922	Multidrug-resistant screening strain	ATCC
E. coli NEB 5-alpha	Cloning strain for plasmids	New England Biolabs (Cat.-No. C2987I)

Supplementary Table 2 – Plasmids used in this study

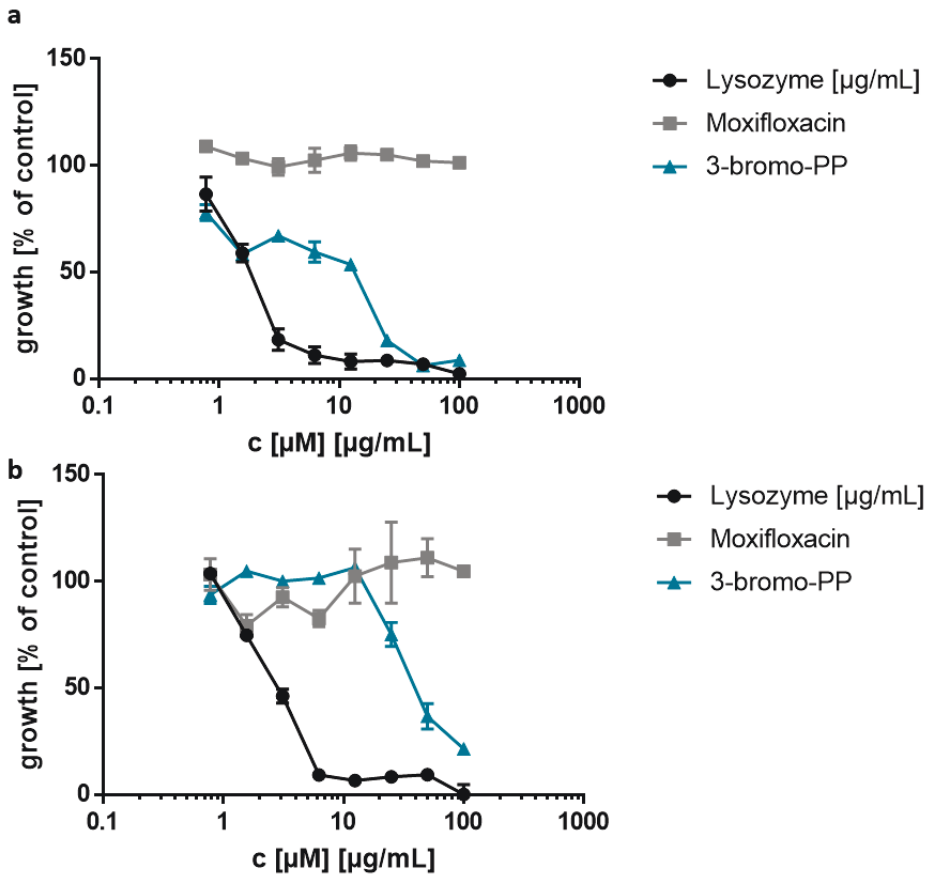
Plasmid	Properties
pLA03::-	Chloramphenicol resistance, Atc-inducible expression vector for *S. aureus*
pLA03::tetR	Overexpression plasmid for tetR
pLA03::mtlA	Overexpression plasmid for mtlA
pLA02::GFP	Chloramphenicol resistance (cmR), GFP-reporter plasmid, generated from pCN57 [61] by ligation of cmR into *Apa*I- and *Xho*I-cut plasmid

Supplementary Table 3 - Cytotoxicity data and cell lines

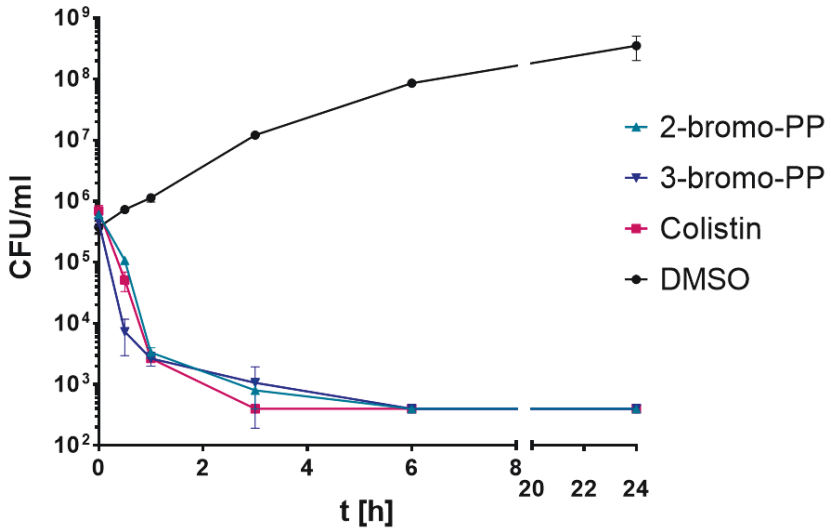
Cell line	IC_{50} [µM]	
	2- bromo-PP	3- bromo-PP
THP-1 (ATCC TIB-202)	6.25	50
MRC-5 (ATCC CCL-171)	>100	100
HEK293 (CLS 300192)	12.5	3.125
HepG2 (CLS 300198)	50	25
CLS-54 (CLS 300227)	>100	>100
HuH7 (CLS 300156)	>100	>100

Supplementary Table 4 – Single nucleotide polymorphisms (SNPs) in spontaneously resistant mutants

Strain	SNPs
M1	KQ76_12400:S155F
M2	KQ76_12400:S155F
M3	KQ76_12400:S155F
M4	SAV_RS00960:G304W, SAV_RS05415:c-47a, SAV_RS05435:L182H, SAV_RS07330:C204*, SAV_RS07940:L96L, SAV_RS12945:R197L, SAV_RS14075:D296E, SAV_RS14630/hisZ:a-80t
M5	SAV_RS00525:S400F, SAV_RS01095:V461L, SAV_RS03035:G44V, SAV_RS07965:D209N, SAV_RS07970:S265F, SAV_RS13840:V23L, c>g between SAV_RS11755/11760
M6	SAV_RS00960:G304W, SAV_RS05415:c-47a, SAV_RS05435:L182H, SAV_RS07330:C204*, SAV_RS07940:L96L, SAV_RS12945:R197L, SAV_RS14075:D296E, SAV_RS14630/hisZ:a-80t



Supplementary Figure 1 - 3-bromo-PP activity against *P. aeruginosa* PAO1 (a) persister and (b) biofilm. Lysozyme has been used as a positive and moxifloxacin as a negative control, respectively. 3-bromo-PP shows activity against *P. aeruginosa* PAO1 persisters and biofilm, with slightly weaker effects compared to MRSA Mu50 persisters and biofilm (see Figure 2).



Supplementary Figure 2 – Bactericidal effects of 2-bromo-PP and 3-bromo-PP against replicating cells of *A. baumannii* ATCC 747. Colistin was used as positive control (3.125 µM). 2-bromo-PP and 3-bromo-PP were used at concentrations of 3.125 µM and 6.25 µM, respectively. The limit of detection was 500 CFU/mL.

6. Alkoxyamid-based molecules interfere with energy metabolism of *Mycobacterium tuberculosis* to kill bacteria at nanomolar concentrations

Unpublished

Overall contribution to the paper: 65%

- Writing of first version of complete manuscript draft
- Determination of minimal inhibitory concentrations (MICs) against *M. tuberculosis*, other *Mycobacteriaceae* and nosocomial pathogens
- Determination of cytotoxicity against human cell lines
- Structure-activity relationship analysis of anti-TB drug derivatives
- Determination of time-kill curves *in vitro*
- Isolation of spontaneously resistant mutants
- Genetic manipulation of *M. tuberculosis* and *M. smegmatis*
- Isolation of whole-cell protein lysates of *M. tuberculosis*
- Infection of THP-1 derived macrophage-like cells with *M. tuberculosis*
- Analysis of ATP depletion in *M. tuberculosis*
- Isolation and analysis of mycolic acid composition of *M. tuberculosis*
- Determination of membrane integrity of *M. tuberculosis*

Alkoxyamid-based molecules interfere with energy metabolism of *Mycobacterium tuberculosis* to kill bacteria at nanomolar concentrations

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Abstract

KSK-104 and KSK-106 are synthetic alkoxyamide-based molecules, which exhibit potent antibacterial activity against *Mycobacterium tuberculosis*, the causative agent of human tuberculosis. The molecules kill *M. tuberculosis* cells at nanomolar concentrations (MIC_{90} 0.048 µM and 0.098 µM) whereas no cytotoxic effects could be monitored against various human cell lines, such as THP-1 or HepG2. Compared to the first-line drug rifampicin or the second-line drug streptomycin, we demonstrated that both KSK compounds provide better activity against internalised bacteria within macrophages. Both KSK compounds have a strong bactericidal effect in monotreatment resulting in 100- to 1000-fold reduction in viability within 10 days and show additive effects in combination with first- and second-line antibiotics except for bedaquiline. Furthermore, whole-genome sequencing of spontaneously resistant mutants revealed that loss-of-function mutations in certain amidohydrolases are involved in resistance. This suggests that the KSK compounds represents prodrugs that need to be hydrolysed by these amidohydrolases to intracellulalrly release an active hydroxamic acid moiety. The hydroxamic acid functionality might be able to bind metal ions and interfere with the electron transport chain, which causes a collapse of the proton motive force and ATP depletion eventually leading to cell death.

Introduction

Mycobacterium tuberculosis, the causative agent of tuberculosis (TB), was responsible for around 1.5 million deaths in 2017. Therefore, TB is the leading cause of death from a single infectious disease, even more deathly than HIV/AIDS. Despite several decades of research and development of new anti-tubercular drugs and vaccines, TB is still not eradicated. It is estimated that approx. 1.7 billion people worldwide are infected with latent TB, and 10 million new cases of active TB occur each year. Among these new infections, around half a million are multidrug-resistant (MDR-)TB, and approx. 10 % of those are estimated to be extensively drug-resistant (XDR-)TB which are even more difficult to treat [1]. Although two new anti-TB drugs, bedaquiline [2] and delamanid [3], have been introduced recently to treat MDR- and XDR-TB in particular, the general TB therapy has not changed since the 1960s [4,5]. The therapy consists of a 2-month combination treatment with isoniazid, rifampicin, pyrazinamide and ethambutol followed by a 4-month treatment with isoniazid and rifampicin [6,7]. Since resistances to bedaquiline and delamanid have already emerged very quickly after introduction to the clinic (REFERENCE), it is imperative to find, develop and optimise new anti-TB drugs that can support the treatment and prevent the further emergence of MDR- and XDR-TB. This quest is challenging because of the distinctive properties of *M. tuberculosis*, such as its unique cell wall composition and the ability to survive within macrophages [8-10].

In this study, we introduce a group of anti-TB molecules which are based on an alkoxyamide-structure which forms the central part of the molecules (figure 1 A). KSK-104 and KSK-106 (Figure 1 B+C) are both molecules based on this alkoxyamide structure carrying a benzyl moiety in region C. Region A, however, differs in these molecules. KSK-104 has a *para*-substituted biphenyl moiety, whereas KSK-106 has a phenyl moiety substituted with a penthylether in *para*-position. A patent by Kisfaludy *et al.* from 1971 described α-aminooxyhydroxamic

acid derivatives as compounds with antimycobacterial activity including the main chemotypes 1-5 (Figure S1). However, no data with regard to their antimycobacterial activity or their molecular target have been published [11]. A follow up publication by the same authors reported on additional α-aminooxy-hydroxamic acid derivatives of type 1b, 6 and 7 (Figure S1) that also exhibit antimycobacterial effects [11]. The INH derivative (type 7) represents a hybrid of INH and aminoxyacetic acid and displayed the most potent antimycobacterial *in vitro* activity (MIC 0.07 µg/mL ≈ 0.33 µM) [11]. The compound was also active *in vivo* in animal infection models and inhibited "*the generalization of M. tuberculosis infection in a 3-month experiment on guinea pigs*". "*Good results*" were also obtained in mice, and the INH derivative demonstrated "*fairly low toxicity*" [11]. In two additional patents from 1975 and 1979 the authors reported on antitubercular α-aminooxyhydrazides and on α-aminooxyamide derivatives with terminal aldoxime moieties (e.g. type 8) [15-16]. However, to the best of our knowledge no further reports on compounds of type 1-8 have been published, and the development was discontinued due to unknown reasons.

The structures of our hit compounds KSK-104 and KSK-106 differ from the previously reported antimycobycterial α-aminooxyhydroxamic acid derivatives shown in Figure S1. KSK-104 and KSK-106 are *para*-substituted benzoylated derivatives of 2-aminoxy-*N*-(benzyloxy)acetamide. In contrast to KSK-104 and KSK-106, the previously reported anti-TB α-aminooxyhydroxamic acid derivatives contain: (i) a non-acylated, free terminal aminoxy group, (ii) an only acetylated/aminoacetylated terminal aminoxy group, (iii) an aralkoxy/alkoxycarbamate moiety, (iv) an alkoxyurea moiety or (v) an oxime moiety (Figure S1). Surprisingly and to the best of our knowledge, *para*-substituted benzoylated derivatives of 2-aminoxy-*N*-(benzyloxy)acetamide such as KSK-104 and KSK-106 have not been reported in the literature before; neither their preparation nor their antimycobacterial properties are known. No

information is available regarding the mode-of-action (molecular target) and resistance mechanism of the previously reported α-aminooxyhydroxamic acid derivatives.

Material and Methods.

Media and strains.

M. tuberculosis H37Rv wildtype was used for most experiments unless otherwise indicated. Additionally, the *M. tuberculosis* strains mc^{2}6230, CDC1551 and Erdman as well as a number of XDR strains (KZN06, 07, 13, 14, 15, 16) obtained from KwaZulu-Natal, South Africa, were used. Other mycobacterial species that were used are *M. abscessus* clinical isolate CF001, *M. smegmatis* mc²155, *M. bovis* BCG Pasteur and *M. marinum* DSM 443344. All mycobacteria were grown in Middlebrook 7H9 broth or on Middlebrook 7H10 agar (Difco) supplemented with 10% ADS (0.81% NaCl, 5% BSA, 2% dextrose), 0.5% glycerol and 0.05% tyloxapol. Mycobacteria were incubated at 37 °C, 5% CO_2 and 80% humidity. For cloning purposes, *E. coli* NEB-5alpha cells (New England Biolabs) were used and grown in lysogeny broth (LB)-medium or LB agar containing antibiotics for selection if required at 37 °C (100 µg/mL ampicillin, 150 µg/mL hygromycin, 40 µg/mL kanamycin, 20 µg/mL apramycin). *Staphylococcus aureus* Mu50 (ATTC 700699), *Pseudomonas aeruginosa* (ATCC 27853) and *Acinetobacter baumannii* (BAA-1609) were grown in Mueller-Hinton broth (Difco) at 37 °C.

Determination of minimal inhibitory concentration against mycobacteria.

Microbroth dilution assays were performed according to the CLSI guidelines[12] to determine the minimal inhibitory concentration (MIC). Briefly, a two-fold serial dilution of compounds was prepared in a sterile 96-well U-bottom polystyrene plate (Greiner) starting with 100 µM in growth medium. A cell suspension with 10^6 CFU/mL was prepared and 50 µL were added to each well. DMSO was added to the growth control and rifampicin (RIF) was used as positive control. After 5 days incubation, 10 µL of a 100 µg/mL resazurin solution was added to each well and carefully resuspended. The plates were incubated for further 24 h at room temperature (RT) and inactivated with 100 µL 10% formalin per well. After 30

minutes incubation in formalin, growth was quantified in a TECAN plate reader with 535 nm excitation and 590 nm emission wavelength. Relative growth was calculated with respect to the growth control. Experiments were performed in triplicates.

Cytotoxicity assay.

To determine the cytotoxicity of KSK-104 and KSK-106 *in vitro*, human cell lines deriving from different tissues were used. THP-1 cells (monocytes), CLS-54 (lung) and HUH7 (liver) were grown in RPMI supplemented with 10% fetal bovine serum (FBS). H4 (brain) and SH-SY5Y (neuroblasts) cell lines were cultivated in DMEM supplemented with 10% FBS. MRC-5 (lung fibroblasts), HEK293 (kidney) and HEPG2 (liver) cells were grown in EMEM supplemented with 1% non-essential amino acids, 1 mM sodium pyruvate and 10% FBS. Sterile 96-well flat-bottom polystyrene plates (Greiner) were prepared with a two-fold serial dilution of compounds with 100 µM as the highest concentration. Approx. $5x10^4$ cells were seeded in each well in a total volume of 100 µL per well. The cells were incubated for 48 h at 37 °C and 5% CO_2 before viability was quantified using the resazurin reduction assay as described above.

Generation of spontaneous resistant mutants

After determination of the MIC, spontaneous resistant mutants were generated using 2-, 4-, 8- and 10-fold MIC of KSK-104 and KSK-106, respectively. For each compound and concentration, 50 mL Middlebrook 7H10 square agar plates supplemented with 10% ADS and 0.5% glycerol were prepared containing the indicated concentrations of KSK-104 or KSK-106, respectively. *M. tuberculosis* H37Rv wildtype cells were grown in 7H9 broth containing 10% ADS, 0.5% glycerol and 0.05% tyloxapol. Cell density was adjusted to plate 10^8, 10^9 and 10^{10} cells on each plate. The plates were incubated at 37 °C, 85% humidity and 5% CO_2 for four

to twelve weeks. Single emerging colonies were picked and checked for increased resistance against KSK-104 and KSK-106 by microbroth dilution assay. A change in the MIC of at least 4-fold was referred to as resistant.

Extraction of genomic DNA.

M. tuberculosis was grown in Middlebrook 7H9 broth supplemented with 10% ADS, 0.5% glycerol and 0.05% tyloxapol to late log phase. After centrifugation, the pellet was resuspended in 450 µL GTE solution (50 mM glucose; 25 mM Tris-Cl, pH 8.0; 10 mM EDTA) and 50 µL of a 10 mg/mL lysozyme solution was added. The mixture was incubated overnight at 37 °C. 150 µL of a 2:1 solution of 10% SDS and 10 mg/mL proteinase K was added and incubated for 30 minutes at 55 °C. Next, 200 µL 5 M NaCl and 160 µL preheated (65 °C) CTAB solution (4.1 g NaCl; 10 g hexadecyltrimethylammonium bromide in 90 mL ddH_2O) was added and incubated at 65 °C for 10 minutes. One volume of 24:1 (v/v) chloroform : isoamyl alcohol was added, mixed and centrifuged for 5 minutes. The aqueous layer was transferred and the procedure was repeated once. Isopropanol (0.7 volume) was added and the solution was centrifuged after precipitation of gDNA was observed. The samples were washed with 70% ethanol and resuspended in ddH_2O. Until further use gDNA was stored at -20 °C.

Whole-genome sequencing.

To identify the resistance mediating mutations, genomic DNA of five independent mutants was isolated as described previously[13]. Libraries were prepared for sequencing using the standard paired-end genomic DNA sample prep kit from Illumina. Genomes were sequenced using an Illumina HiSeq 2500 next-generation sequencer (San Diego, CA, USA) and compared with the parent *M. tuberculosis* H37RvMA genome (GenBank accession GCA_000751615.1). Paired-end sequence data was collected with a

read length of 106 bp. Base-calling was performed using Casava software, v1.8. The reads were assembled using a comparative genome assembly method, using *M. tuberculosis* H37RvMA as a reference sequence[14].

Protein extraction.

M. tuberculosis H37Rv was grown in 20 mL Middlebrook 7H9 medium supplemented with 0.5% glycerol, 0.2% glucose, 0.085% NaCl and 0.05% tyloxapol. An equal number of cells were centrifuged at 4 °C and washed thrice with PBS (137 mM NaCl; 2.7 mM KCl; 10 mM Na_2HPO_4; 1.8 mM KH_2PO_4; pH 7.4). Cells were finally resuspended in 2 mL PBS and lysed by bead beating using 100 µm silica-zirconium beads at 50 Hz for 3x3 minutes. Afterwards, 200 µL of a 10% SDS solution was added to each sample, vortexed carefully and incubated for 30 minutes at 4 °C. After centrifugation, the clear supernatant was collected and used as protein solution. To ensure safety, the protein solution was thrice sterile filtered through a bacteria-tight 0.2 µM cellulose acetate filter. For each filter step, a fresh filter was used. Protein concentration was measured with BCA assay (Merck Millipore).

Sample clean-up for LC-MS.

After in-solution digestion (ISD) peptides were desalted on home-made C18 StageTips [15]. Briefly, the peptide solution was passed over the MeOH pre-conditioned and 0.5% FA equilibrated StageTip. Immobilized peptides were then washed twice with 0.5% (v/v) FA. Washed peptides were eluted from the StageTips with 80% (v/v) ACN 0.5% (v/v) FA and dried using a vacuum concentrator (Eppendorf). Before LC-MS peptide samples were resuspended in 10 µl 0.1% (v/v) FA.

LC-MS/MS.

LC–MS/MS experiments were performed on an Orbitrap Elite instrument[16] (Thermo) that was coupled to an EASY-nLC 1000 liquid chromatography (LC) system (Thermo). The LC was operated in the one-column mode. The analytical column was a fused silica capillary (75 µm × 45 cm) with an integrated PicoFrit emitter (15 µm, New Objective) packed in-house with Reprosil-Pur 120 C18-AQ 1.9 µm resin (Dr. Maisch). The analytical column was encased by a column oven (Sonation) and attached to a nanospray flex ion source (Thermo). The column oven temperature was adjusted to 45 °C during data acquisition. The LC was equipped with two mobile phases: solvent A (0.1% formic acid, FA, in water) and solvent B (0.1% FA in acetonitrile, ACN). All solvents were of UPLC grade (Sigma). Peptides were directly loaded onto the analytical column with a maximum flow rate that would not exceed the set pressure limit of 980 bar (usually around 0.5–0.6 µl min^{-1}). Peptides were subsequently separated on the analytical column by running a 140 min gradient of solvent A and solvent B at a flow rate of 300 nl min^{-1} (gradient: start with 7% B; gradient 7 to 35% B for 120 min; gradient 35–100% B for 10 min and 100% B for 10 min). The mass spectrometer was operated using Xcalibur software, Thermo Fischer Scientific, UK (version 2.2 SP1.48) and was set in the positive ion mode. Precursor ion scanning was performed in the Orbitrap analyzer (FTMS; Fourier Transform Mass Spectrometry) in the scan range of m/z 300–1,800 and at a resolution of 60,000 with the internal lock mass option turned on (lock mass was 445.120025 m/z, polysiloxane)[17]. Product ion spectra were recorded in a data dependent fashion in the ion trap (ITMS) in a variable scan range and at a rapid scan rate. The ionization potential (spray voltage) was set to 1.8 kV. Peptides were analyzed using a repeating cycle consisting of a full precursor ion scan (1.0×10^6 ions or 50 ms) followed by 15 product ion scans (1.0×10^4 ions or 100 ms), where peptides are isolated based on their intensity in the full survey scan (threshold of 500 counts) for tandem mass spectrum (MS2) generation that permits peptide sequencing and identification. Collision-induced dissociation (CID) energy was set to 35% for the generation of MS2 spectra. During MS2 data acquisition, dynamic ion exclusion was set to 120 s with a maximum list of excluded ions

consisting of 500 members and a repeat count of one. Ion injection time prediction, preview mode for the FTMS (the orbitrap), monoisotopic precursor selection and charge state screening were enabled. Only charge states higher than 1 were considered for fragmentation.

Peptide and Protein identification using MaxQuant and Perseus.

RAW spectra were submitted to an Andromeda[18] search in MaxQuant (version 1.5.3.30) using the default settings[19]. Label-free quantification was activated[20]. MS/MS spectra data were searched against the Uniprot *Staphylococcus aureus* strain Mu50 reference proteome database (UP000002481_158878.fasta ; 2714 entries; downloaded 23.02.2017). All searches included a contaminants database (as implemented in MaxQuant, 245 sequences). The contaminants database contains known MS contaminants and was included to estimate the level of contamination. Andromeda searches allowed oxidation of methionine residues (16 Da), acetylation of the protein N-terminus (42 Da) as dynamic modifications and the static modification of cysteine (57 Da, alkylation with Iodoacetamide). Enzyme specificity was set to “Trypsin/P”. The instrument type in Andromeda searches was set to Orbitrap and the precursor mass tolerance was set to ±20 ppm (first search) and ±4.5 ppm (main search). The MS/MS match tolerance was set to ±0.5 Da. The peptide spectrum match FDR and the protein FDR were set to 0.01 (based on target-decoy approach). Minimum peptide length was 7 amino acids. For protein quantification unique and razor peptides were allowed. Modified peptides with dynamic modifications were allowed for quantification. The minimum score for modified peptides was 40. Further data analysis and filtering of the MaxQuant output was done in Perseus v1.5.5.3[21]. MS/MS counts were loaded into the matrix from the proteinGroups.txt file and potential contaminants as well as reverse hits, hits only identified by site and protein groups with less than 2 identified unique peptides were removed. For the statistical calculations samples technical replicates were grouped in categorical groups and filtered. Only those protein groups were kept that contained three valid values in a minimum

of one categorical group. The missing values in the remaining protein groups were then imputed and the t-test performed (number of randomizations 250; initial FDR 0.05 and S0 0.1).

Time killing kinetics.

M. tuberculosis H37Rv was pregrown in Middlebrook 7H9 supplemented with 10% ADS, 0.5% glycerol and 0.05% tyloxapol to exponential phase. This pre-culture was used to prepare cultures containing 10^6 CFU/mL which were incubated either with KSK-104 or KSK-106 (0.25 µM) individually or in combination with the anti-tubular drugs isoniazid (10 µM), rifampicin (1 µM), bedaquiline (0.5 µM), delamanid (0.5 µM) or ethambutol (10 µM). Furthermore, for each antibiotic individual cultures were prepared. The cultures were incubated shaking at 37 °C for 35 days. At certain time points, samples were taken, diluted and plated on Middlebrook 7H10 agar plates supplemented with 10% ADS and 0.5% glycerol for colony counting to determine the effects on the growth of *M. tuberculosis*. After 3 weeks colonies were counted. All experiments were performed as triplicates.

RNA isolation.

M. tuberculosis H37Rv cells were incubated in 10 mL Middlebrook 7H9 broth in sub-lethal concentrations of anti-tubercular drugs (0.01 µM isoniazid, 0.78 µM ethambutol, 0.02 µM bedaquiline, 0.02 µM delamanid, 0.2 µM rifampicin) and KSK-104 (0.024 µM) and KSK-106 (0.012 µM), respectively. Cultures were grown at 37 °C with shaking until the late exponential growth phase of the DMSO control was reached. The cultures were centrifuged and resuspended in 5 mL RNA protect bacteria reagent (QIAGEN) and incubated overnight at RT. To isolate the total RNA, the QIAGEN RNeasy kit (QIAGEN) was used following the manufacturer's protocol. Until further use RNA was stored at -80 °C.

Quantitative PCR.

For qPCR analysis, total RNA samples were used to generate cDNA using the SuperScript III First-Strand Synthesis System (Invitrogen) for reverse transcription. Each primer was used in a final concentration of 250 nM and 5 µL of the template reactions were used in a 1:10-dilution to set up 20 µL reactions with GoTaq qPCR Master Mix (Promega). Samples were run in a CFX96 Real-Time System (Bio-Rad). Thresholds of triplicate samples were normalised to 16S rRNA.

ATP assay.

To determine the relative ATP concentration, 10^6 CFU/mL of *M. tuberculosis* H37Rv cells were added to a previously prepared 96-well U-bottom polystyrene plate (Greiner) with a two-fold serial dilution of compounds. The cells were incubated for 24 h at 37 °C, 5% CO_2 and 85% humidity. A 96-well flat-bottom white plate (ThermoFisher) containing 25 µL of BacTiter Glo reagent (Promega) was used to transfer 25 µL of each well into it. The plate was incubated for 5 minutes in the dark and luminescence was measured in a TECAN plate reader. Bedaquiline and Carbonylcyanid-*m*-chlorphenylhydrazon (CCCP) were used as positive controls, DMSO was used as growth control and negative control, respectively. Experiments were performed in triplicates.

Propidium iodide assay.

A sterile 96-well U-bottom polystyrene plate (Greiner) was prepared and seeded in for microbroth dilution assay as described earlier. *M. tuberculosis* H37Rv cells were incubated for 5 days at 37 °C, 5% CO_2 and 85% humidity. To each well, 10 µL of a 2 mM propidium iodide solution was added and mixed thoroughly. Afterwards, the plate was incubated in the dark for 5 hours and inactivated with a final formalin concentration of 5% for 1 h. Fluorescence was quantified in a TECAN plate reader

with 535 nm excitation and 635 nm emission wavelength. DMSO and Triton-X-100 were used as positive and negative control, respectively. All experiments were performed as triplicates.

Macrophage infection.

THP-1 cells were grown in RPMI medium supplemented with 10 % FBS. Cells were counted using a haemocytometer and 10^5 cells were seeded into each well of a sterile 96-well flat-bottom polystyrene microtiter plate (Greiner) in a total volume of 100 µL. To differentiate the cells to adherent macrophage-like cells, the medium was supplemented with 50 nM phorbol-12-myristate-13-acetate (PMA). After differentiation to adherent cells overnight at 37 °C, 5% CO_2 in a humid atmosphere, the macrophage-like cells were washed twice with PBS. An *M. tuberculosis* H37Rv reporter strain was used for infection which was grown in Middlebrook 7H9 broth containing 150 µg/mL hygromycin, harvested, washed and resuspended in RPMI supplemented with 10% FBS to a density of $3x10^6$ CFU/mL. 100 µL of this cell suspension was added to each well. After 3 h, the cells were washed twice with PBS to remove non-phagocytised bacteria. PBS was replaced with 100 µL RPMI + 10% FBS containing 0.5 µM KSK-104 or KSK-106 or different antibiotics (3 µM rifampicin, 20 µM streptomycin). After 5 days at 37 °C, 5% CO_2 and 85% humidity, the macrophage-like cells were fixed with formalin (5% final concentration) and fluorescence was documented using a Nikon Eclipse TS100 fluorescence microscope. Additionally, colony counting was performed. Therefore, macrophages were lysed with ddH_2O for 30 minutes. Dilutions of each well were plated on Middlebrook 7H10 plates and colonies were counted after 3 weeks of incubation at 37 °C.

Isolation of mycolic acid methyl esters (MAME) from *M. tuberculosis* H37Rv and thin-layer chromatography (TLC).

M. tuberculosis H37Rv was cultivated in sub-lethal concentrations of antibiotics (0.01 µM isoniazid, 0.78 µM ethambutol, 0.02 µM bedaquiline, 0.02 µM delamanid, 0.2 µM rifampicin) and KSKs (0.12 µM KSK-104, 0.24 µM KSK-106) for several days until all cultures reached an $OD_{600\ nm}$ of at least 0.2. Cultures were washed twice with PBS and heat-inactivated for 2x 30 minutes at 100 °C. The resulting pellets have been dried in a heatblock and nitrogen stream and the fatty dry mass was weighed. The fatty dry mass was transferred to Pyrex glass tubes (Corning) and 7 mL $CHCl_3$:MeOH (2:1 v/v) was added. The tubes were incubated with shaking overnight and were centrifuged for 15 minutes at 4000 RPM. The supernatant has been discarded and the pellet was dried under nitrogen flow to weigh the fat-free dry mass. Thereafter, 2 mL of a 15 % aqueous tetra butyl ammonium hydroxide solution (TBAH) was added to the pellet and heated overnight at 100 °C. After cooling down, 2 mL H_2O, 1 mL CH_2Cl_2 and 250 µL CH_3I were added per 50 mg fat-free dry mass. The tubes were shaken for 30 minutes and centrifuged for 1 h at 4000 RPM. The aqueous phase was discarded and the organic phase was transferred into an HPLC vial. The solvent was evaporated under a nitrogen stream and the MAMEs were finally solved in 125 µL CH_2Cl_2.

The applied volume was adjusted to the fat-free dry mass of each sample and transferred to a pre-heated TLC silica 60 plate (Merck). Petroleum spirit : diethyl ether (95:5) was used as solvent in a saturated chamber. The TLC was run 6 times and the solvent was removed between runs. Following this, the plate was stained with 5% molybdato phosphoric acid in ethanol by dipping the plate shortly into the solution. Finally, the TLC plate was heated for 15 minutes at 110 °C for consistent staining.

Results.

***In vitro* activity of alkoxyamide-based molecules against *M. tuberculosis*.**

During screening of an *in-house* compound library for potential anti-TB molecules, KSK-104 and -106 have been found to exhibit activities against the laboratory strain *M. tuberculosis* H37Rv *in vitro*. KSK-104 showed a MIC of 0.096 µM and KSK-106 of 0.048 µM (figure 1 D). Thus, both molecules show a more potent activity against *M. tuberculosis* H37Rv than the first-line antibiotic rifampicin. In addition, also other *M. tuberculosis* strains including various XDR clinical isolates originating from KwaZulu-Natal, South Africa, were susceptible to KSKs, although the MICs were higher than observed for H37Rv but still in the low micromolar range (Table 1, Figure 1 E-G). In contrast to *M. tuberculosis*, surprisingly no effect was observeable against the other tested closely related mycobacterial species (Table 1). Cytotoxicity was checked against cell lines derived from different human tissues to further characterise KSKs. Among others, kidney and liver cells were tested as well as the monocytic cell line THP-1. KSK-104 and KSK-106 showed no cytotoxic effects at the highest tested concentration of 100 µM on any cell line. Additionally, the molecules have been tested against various nosocomial bacteria, such as *Staphylococcus aureus* Mu50, *Acinetobacter baumannii* ATCC BAA-1605 and *Pseudomonas aeruginosa* ATCC 27853. None of the nosocomial bacteria responded to treatment with KSKs (data not shown). Taken together, these results suggest that the effect of KSKs is based on a mechanism and/or target that is specific for *M. tuberculosis*.

Structure-activity relationship was investigated by testing different derivatives of KSK-104 and KSK-106 in microbroth dilution assays (Figure 2). These experiments demonstrated that region A should consist of a phenyl moiety which is ideally substituted in *para*-position with either a phenyl (KSK-104) or a penthyl ether (KSK-106). Region B needs to contain two alkoxyamides which are not further substituted and region C, however, provides best efficiency against *M. tuberculosis* when the benzyl moiety is unsubstituted as well. However, KSK-104 and KSK-106 remain the most actives molecules that have been found during our experiments.

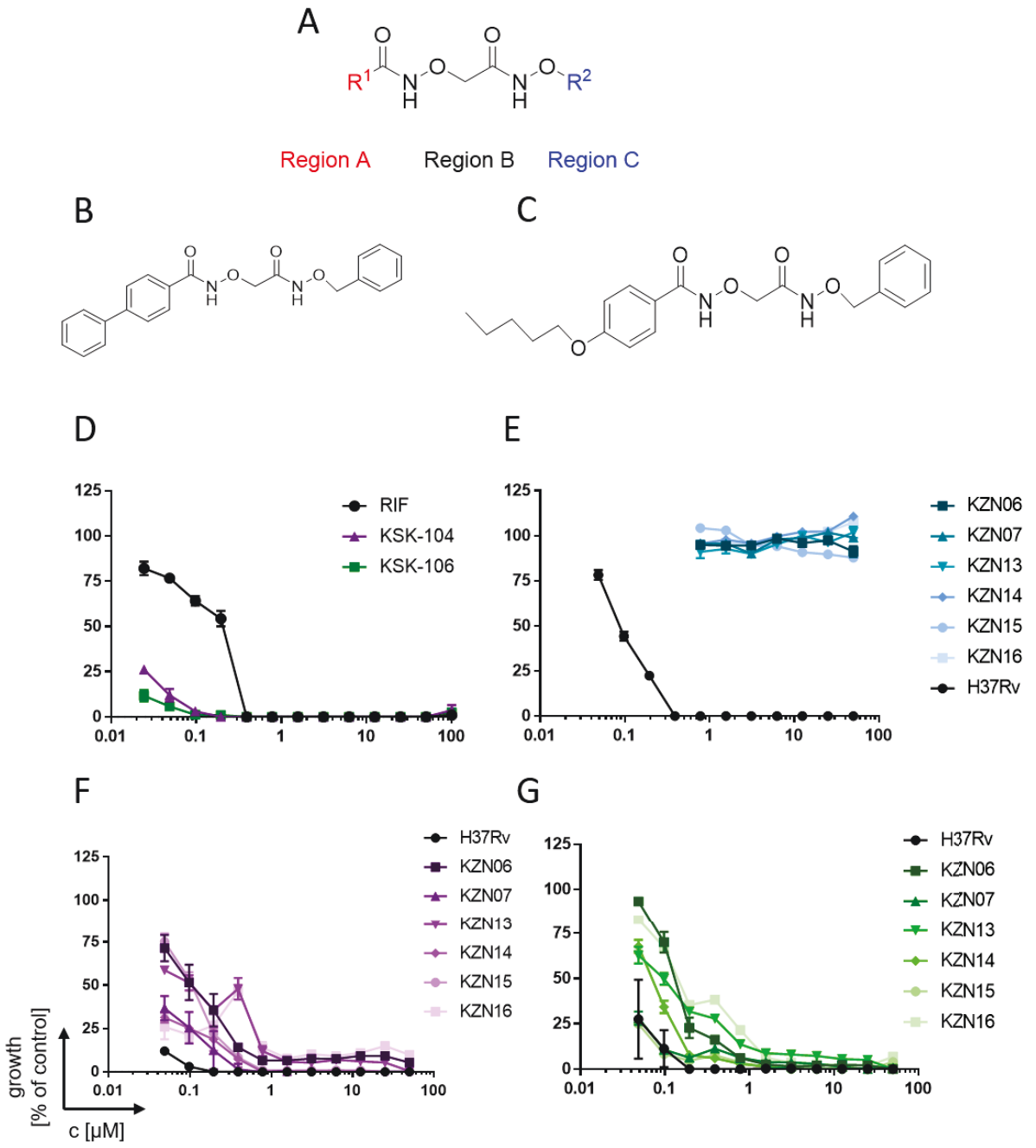


Figure 1 – Structures of KSK molecules and antibacterial activity *against M. tuberculosis*.

The basic structure of alkoxyamide-based molecules (A) and the structures of anti-TB compounds KSK-104 (B) and KSK-106 (C). Both molecules are known to be precursors of HDAC-inhibitors and are based on an alkoxyamide structure. (D-G) Dose-response curves of KSK compounds against *M. tuberculosis* strains. Anti-TB activity against the laboratory strain H37Rv is shown in (D). KSK-104 (purple triangle) and KSK-106 (green square) are compared

to rifampicin (RIF, black circle) which is less effective than KSKs. Additionally, anti-TB activity was evaluated against *M. tuberculosis* XDR strains obtained from KwaZulu-Natal, South Africa. While rifampicin does not show any activity against XDR strains (E), KSK-104 (F) and KSK-106 (G) are active against XDR strains in a micromolar scale.

Table 1 – Minimal inhibitory concentration of KSKs and RIF against various *Mycobacterium* species. KSKs and rifampicin (RIF) have been tested in microbroth dilution assays against various mycobacteria. All concentrations are given in µM.

strain	KSK-104	KSK-106	RIF
M. tuberculosis H37Rv	0.048	0.096	0.39
M. tuberculosis mc^{2}6230	3.125	0.78	0.097
M. tuberculosis CDC1551	0.78	0.78	1.56
M. tuberculosis Erdman	0.19	0.19	0.097
M. tuberculosis KZN06	1.56	1.56	> 100
M. tuberculosis KZN07	1.56	1.56	> 100
M. tuberculosis KZN13	3.125	1.56	> 100
M. tuberculosis KZN14	1.56	1.56	> 100
M. tuberculosis KZN15	1.56	1.56	> 100
M. tuberculosis KZN16	3.125	1.56	> 100
M. abscessus (CF001s)	> 100	> 100	> 100
M. marinum DSM 44344	> 100	> 100	0.39
M. smegmatis mc²155	> 100	> 100	25
M. bovis BCG Pasteur	> 100	> 100	3.125
M. bovis AF2122/97	> 100	> 100	> 100

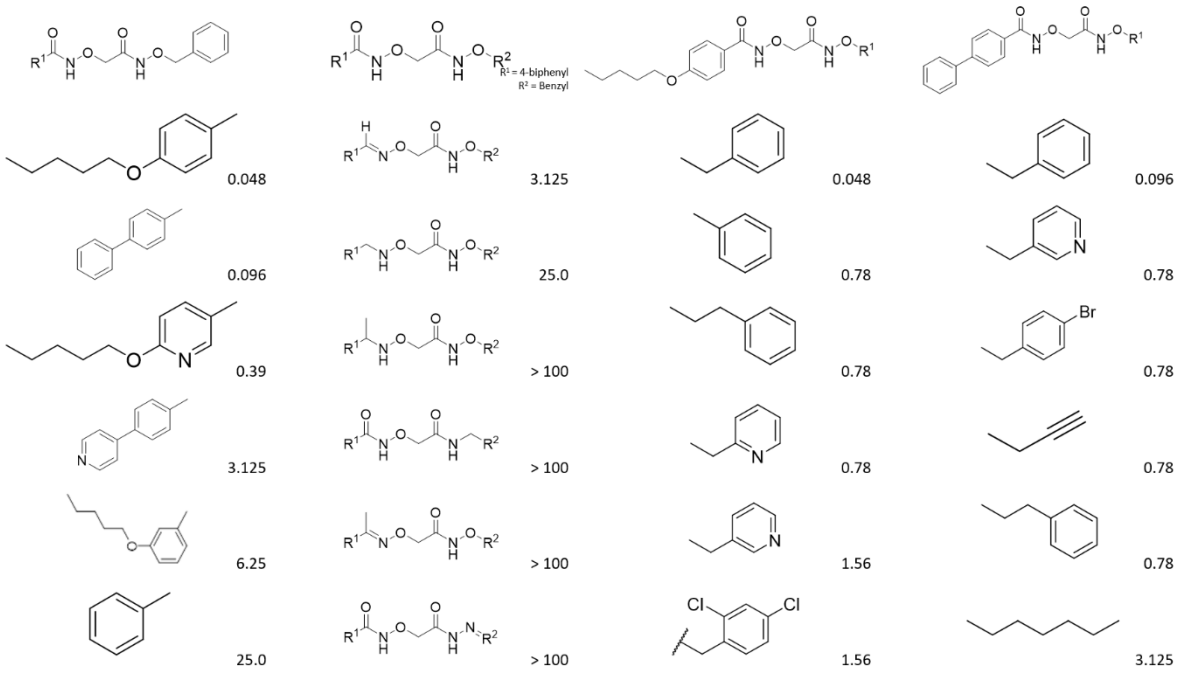


Figure 2 – Structure activity relationship data. Derivatives of KSK-104 and KSK-106 have been tested for activity against *M. tuberculosis* H37Rv. Although diverse substitutions and modifications were tried, no derivative was more active than the original KSK-104 and KSK-106 molecules *in vitro*.

KSKs have a bactericidal effect against *M. tuberculosis* H37Rv.

After we have shown that KSKs posses anti-TB activity, we were interested in further characteristics of these effects of the molecules. Therefore, we monitored the anti-TB effects of both molecules over 35 days performing a time-killing kinetic. We found that both, KSK-104 and KSK-106, exhibited a bactericidal effect within the first 10 days of incubation, resulting in ca. 2.5-log reduction in viability. No further reduction in viability occurred after day 10 (Figure 3 A). However, the viable cell number remained static and no resumption of growth was observed in monotherapy in contrast to the tested clinical drugs (Figure 3 B-F). Since the treatment of tuberculosis is always a combination therapy, we tested KSKs in combination with different first-line antibiotics and the recently conditionally approved drugs delamanid and bedaquiline. Both compounds showed an additive effect in combination with isoniazid, rifampicin, ethambutol and delamanid, with reduction of cell viability down to the

detection limit of 10^2 CFU/mL. Furthermore, particularly combination with KSK106 efficiently suppressed to resurgence of any surviving bacteria even after prolonged incubation periods (Figure 3 B-E). In contrast to the other tested antibiotics, bedaquiline substantially dampened the bactericidal effect of KSK compounds, indicative of negative drug-drug interference (Figure 3 F).

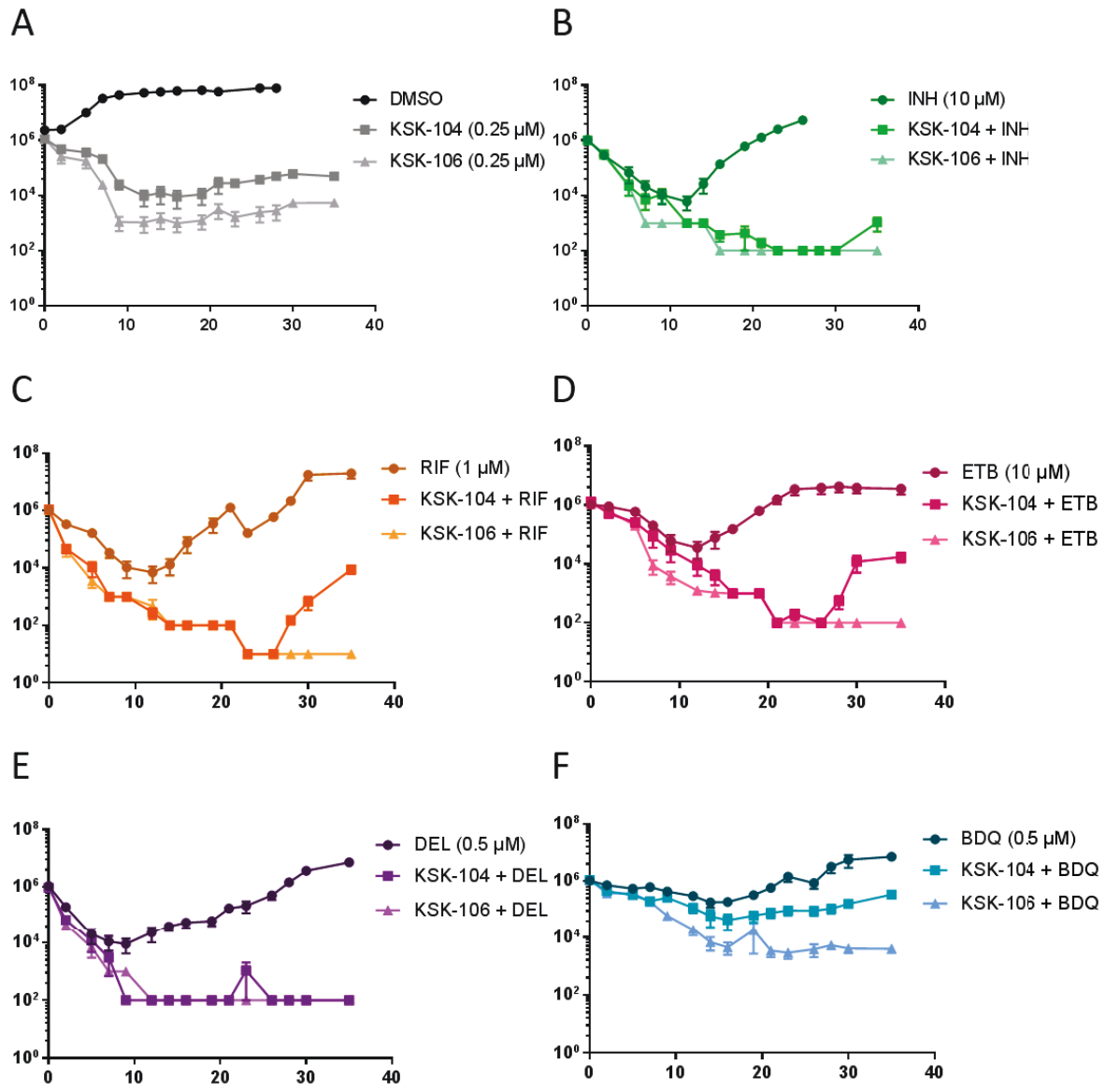


Figure 3 – *In vitro* killing kinetic with *M. tuberculosis* H37Rv.

M. tuberculosis H37Rv was treated with the indicated concentrations of first-line drugs in monotherapy and in combination with KSK-104 or KSK-106, respectively. KSK-104 (grey square) and KSK-106 (grey triangle) alone (A) are showing a bactericidal effect. Combination with different first-line drugs (B - F) led to additive effects regarding the killing efficiency and

delayed resurgence of bacteria surviving the initial treatment. Antibiotics alone (circle) showed resurgence of bacteria, which is delayed by several days when KSK-104 was added (squares) and completely prevented when KSK-106 was added to the culture (triangles). X-axis depicts time in days, Y-axis is showing CFU per mL. Experiments have been performed in triplicates.The limit of detection was 10 CFU/ml in C and 100 CFU/ml in A, B, D-F.

KSKs are killing *M. tuberculosis* after internalisation into THP-1-derived macrophages. *M. tuberculosis* is known to survive within macrophages and escaping the immune response mechanisms of the infected host. Therefore, we investigated whether KSKs are able to kill *M. tuberculosis* also after internalisation by macrophages. After 5 days, fluorescence microscopy was used to visualise the internalised bacteria within the THP-1-derived macrophages using an *M. tuberculosis* reporter strain constitutively expressing mCherry (Figure 4 A). As expected, untreated solvent control (DMSO) showed a strong fluorescence signal within the macrophages, whereas the sterile control showed no signal. Rifampicin (RIF) and streptomycin (STREP) were used as established anti-TB chemotherapeutics to compare the efficiency of KSKs. KSK-104 and KSK-106 both resulted in residual fluorescence samples lower than the RIF and STREP controls (Figure 4 A), indicative of a strong intracellular killing capacity. For further evidence and quantification, CFU plating was performed (Figure 4 B). Rifampicin and streptomycin reduced the intracellular bacterial burden (expressed as CFU/well) by approx. 50-fold. In contrast, both KSK compounds were more potent in this assay albeit used at lower concentration (0.5 µM vs. 3 µM rifampicin or 20 µM streptomycin), resulting in approx. 100-fold (KSK-104) and 1000-fold (KSK-106) reduction of the intracellular bacterial burden.These results reveal that KSKs are able to penetrate relevant human target cells and to inhibit *M. tuberculosis* growth within macrophages with higher efficiency than first-line drugs.

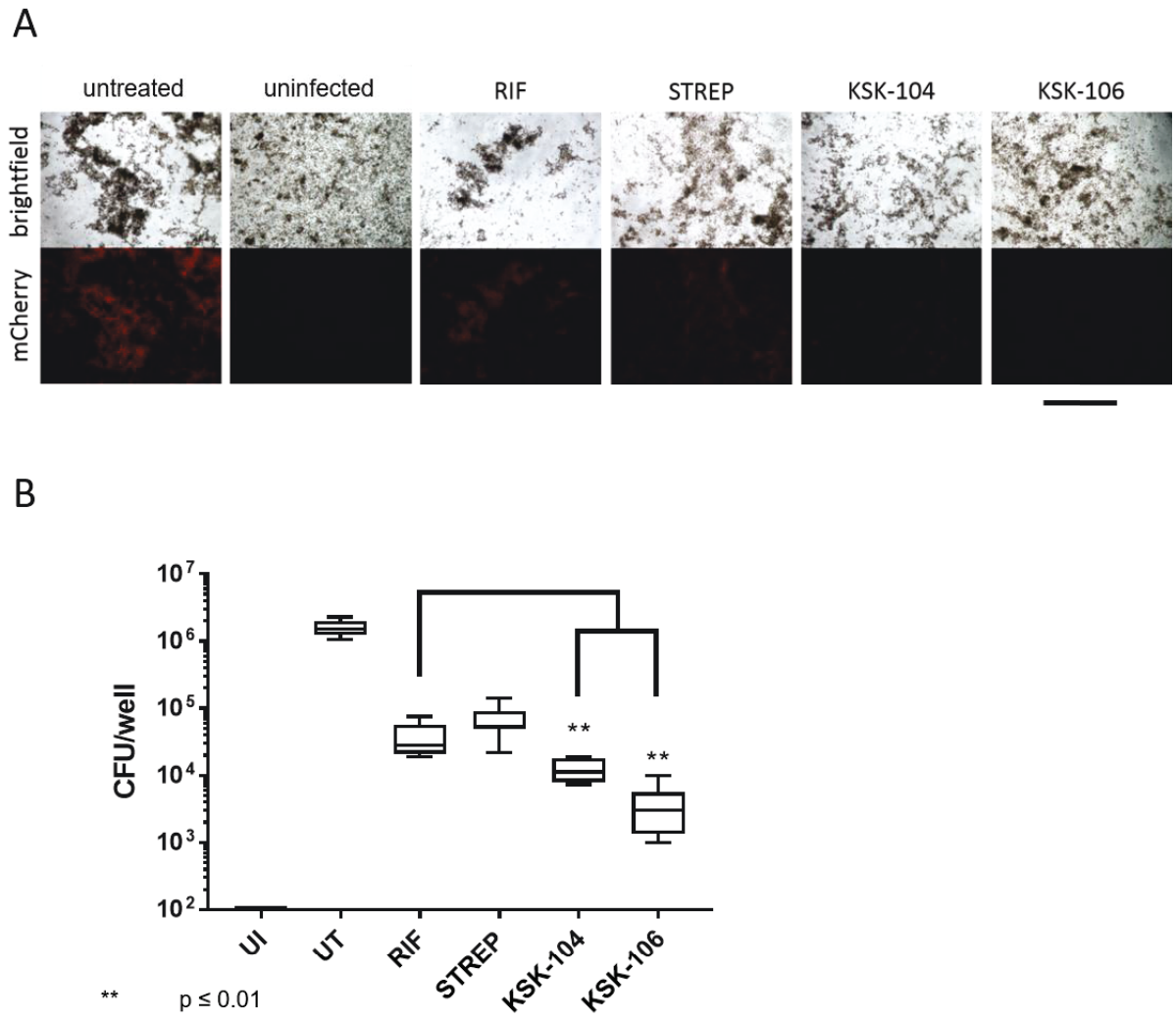


Figure 4 – Infection of THP-1-derived macrophages.

THP-1 cells were differentiated to macrophage-like cells and infected with a *M. tuberculosis* H37Rv reporter strain. (A) The upper line shows the brightfield pictures of macrophages, the lower line the fluorescence of the *M. tuberculosis* reporter strain expressing mCherry constitutively. Antibiotics were used in the following concentrations: rifampicin (RIF) 3 µM, streptomycin (STREP) 20 µM. KSK-104 and KSK-106 were used in concentrations of 0.5 µM. Magnification 40x, scale bar 50 µm (B) Box-plot of CFU counting after infection with *M. tuberculosis* H37Rv. Cells were treated with antibiotics and KSK concentrations as described above. UI = uninfected, UT = untreated (DMSO), RIF = rifampicin, STREP = streptomycin. Boxes are showing upper and lower quartile with median. Statistical

significance was calculated using Kolmogorov-Smirnov-test and student's t-test. All experiments have been performed in triplicates and repeated once with similar results.

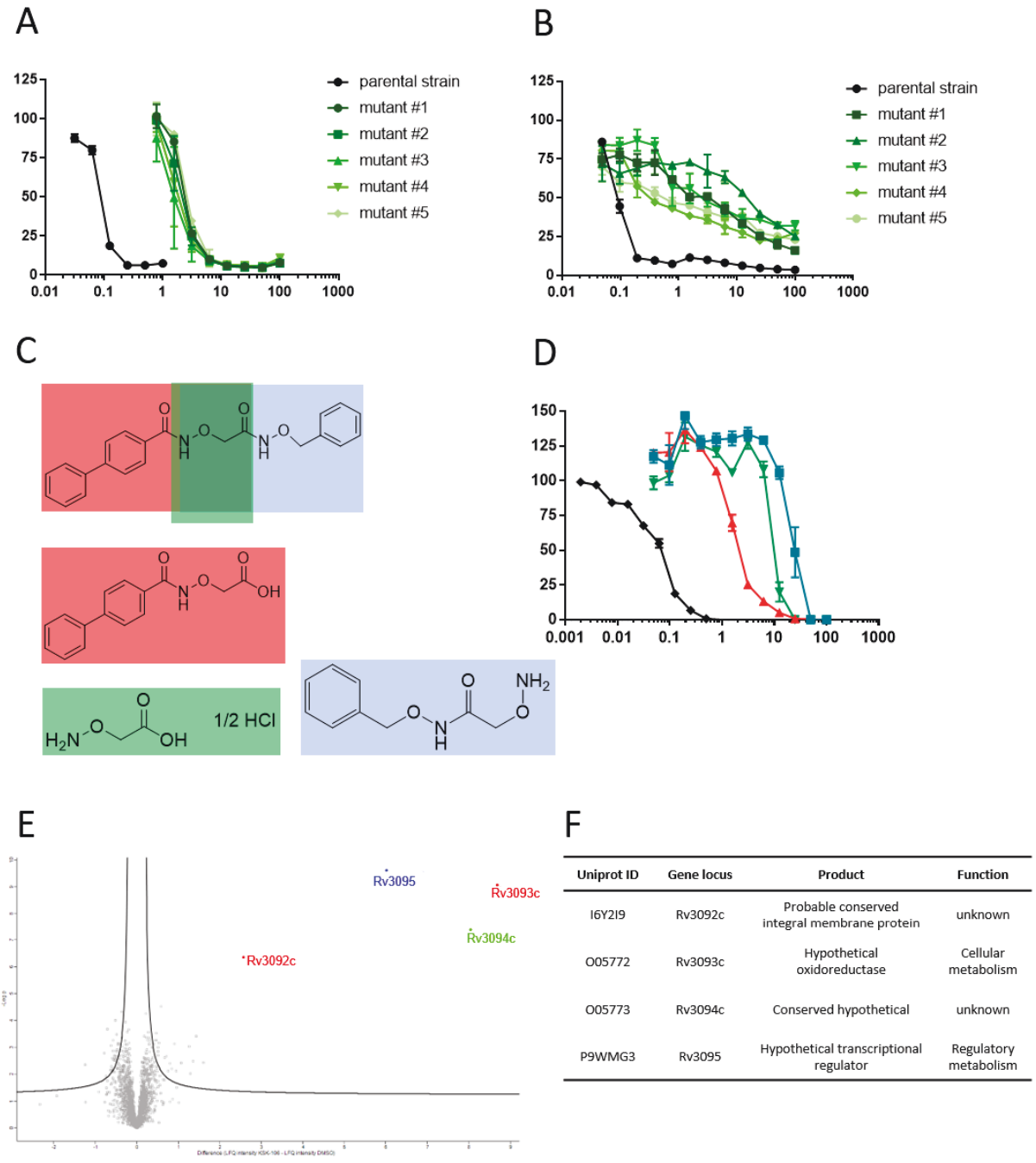
Amidohydrolases are linked to KSK activity.

The elucidation of the mode of resistance and mode of action might give a hint for further optimisation of KSKs to circumvent the emergence of resistance. Therefore, we isolated spontaneously resistant mutants against both KSKs. All resistant mutants we isolated against KSK-104 were showing high-level resistance (32-fold MIC shift, Figure 5 A) and occurred at a frequency of approx. 10^{-8}. For KSK-106, all isolated resistant mutants were showing a MIC shift of approx. 1024-fold, but had a much lower resistance rate which was < 10^{-10} (Figure 5 B). Consequently, we performed whole-genome sequencing and found several single nucleotide polymorphisms (SNPs, Figure S1). We found consistent SNPs in all five mutants of KSK-104 in the gene *Rv0552* which encodes a non-essential, conserved hypothetical protein with unknown function predicted to have amidohydrolase activity [15] and to act on carbon-nitrogen bonds but not on peptide bonds. Therefore, we used possible hydrolysis products of KSK-104 and found a shift in the MIC for the red hydrolysis products, revealing a pattern that is comparable to those of the resistant mutants we isolated (Figure 5 C+D). The KSK-106 resistant mutants showed different mutations. Interestingly, all but one mutant had a SNP in the *amiC* gene which also codes for a non-essential amidohydrolase. In contrast to KSK-104 resistant mutants, we not just found SNPs leading to an amino acid exchange, but we also found insertions that resulted in a frameshift and therefore yielding a non-functional protein.

Protein response of *M. tuberculosis* H37Rv to KSK-106 treatment.

M. tuberculosis H37Rv was treated with sublethal concentrations of KSK-106 (0.25x and 0.5x MIC) to gain insight into the stress response of the bacteria on proteomic level. The proteome analysis revealed that four specific proteins were significantly more abundant in treated cells compared to the DMSO control (Figure 5 E). All upregulated proteins seem to

be encoded in one operon that is controlled by the transcription factor *Rv3095* (uniport-ID: P9WMG3) that is also one of the upregulated proteins. The other significantly abundant proteins are *Rv3094c* (uniprot-ID: O05773), *Rv3093c* (uniprot-ID: O05772) and *Rv3092c* (uniprot-ID: I6Y2I9). Little is known about the *Rv3095*-operon (Figure 5 F). The transcription factor *Rv3095* is predicted to be an uncharacterised HTH-type transcriptional regulator of unknown function. The first gene in the potential operon, *Rv3094c*, is a conserved protein, predicted to have a FAD-binding site and Acyl-CoA dehydrogenase activity. *Rv3093c* is also a conserved protein of unknown function which might provide oxidoreductase activity. The last gene in the potential operon *Rv3092c* is also of unknown function. It was identified in the membrane fraction of *M. tuberculosis* and has properties of an integral membrane protein. All of these proteins have orthologues in other mycobacteria species such as *M. bovis*, *M. marinum* and *M. smegmatis*, but the genes are not arranged in an operon in the latter.



F

Uniprot ID	Gene locus	Product	Function
I6Y2I9	Rv3092c	Probable conserved integral membrane protein	unknown
O05772	Rv3093c	Hypothetical oxidoreductase	Cellular metabolism
O05773	Rv3094c	Conserved hypothetical	unknown
P9WMG3	Rv3095	Hypothetical transcriptional regulator	Regulatory metabolism

Figure 5 – Mode of action and resistance studies. Spontaneously resistant mutants were generated and MICs were tested in microbroth dilution assay for KSK-104 (A) and KSK-106 (B). The X-axis shows the concentration in µM and the Y-axis the growth in comparison to the DMSO growth control. Hydrolysis products of KSK-104 are shown in (C) and their MICs are given in (D). X- and Y-axis are used as in (A) and (B). Proteome analysis has been performed with KSK-106 sublethally treated cultures of *M. tuberculosis* H37Rv (0.25x and 0.5x MIC). The volcano plot depicts significantly increased proteins which are shown in colours and labelled.

For the plot, the results for KSK-106 in sum were compared with the DMSO control. The X-axis gives the abundance and the Y-axis the *p*-value for each protein. Information about the abundant proteins from (E) are shown in (F). The information was collected from the uniprot and mycobrowser database.

KSKs are causing cell wall stress in *M. tuberculosis*.

For further studies regarding the mechanism of action, we went on to check for certain stress markers in *M. tuberculosis*. The gene *iniB* (isoniazid-inducible gene B, *Rv0341*) is known to be upregulated when cell wall stress is elicited by isoniazid [16]. We checked via qPCR if *iniB* is upregulated when the cells are treated with sublethal concentrations of KSK-104 and KSK-106 (Figure 6 A). We found, in comparison to the rifampicin (RIF) control, that isoniazid (INH) is inducing the expression of *iniB* 3-fold. For KSK-104 and KSK-106 we also find upregulation of *iniB* expression by 3- and 2-fold, respectively. Although the results do not reach significant levels, this indicates that cell wall stress is linked to the mode of action of KSKs. Further experiments were performed to elucidate the mode of action. To check the integrity of the cell membrane, we performed a propidium iodide (PI) assay (Figure 6 B). PI is not able to pass the cell membrane unless the membrane is perforated. After passing the membrane, PI is intercalating into the DNA and fluorescence can be quantified. TritonX-100 was used as positive control and rifampicin (RIF) as negative control. For KSK-104 and KSK-106 we received the same result as for RIF and could not detect any fluorescence. Membrane perforation is therefore not the mode of action.

Since KSKs are having a specific activity against mycobacteria and have no effect on other bacteria, such as *S. aureus* or *P. aeruginosa*, we investigated a possible impact of KSKs on the composition of the mycolic acid methyl esters (MAME) in the mycobacterial cell wall. We isolated MAME from *M. tuberculosis* H37Rv cultures that have been treated with sublethal concentrations of KSKs, rifampicin, isoniazid or ethambutol. To analyse the composition of the mycolic acids, we performed thin layer chromatography (TLC, Figure 6 C). DMSO, rifampicin (RIF) and isoniazid (INH) were used as controls for comparison of the MAME

composition. For DMSO we found all three kinds of MAMEs (alpha, methoxy, keto) as well as for cells treated with RIF and ethambutol (EMB), which were not expected to have an impact on MAME synthesis. For INH, we isolated only small amounts of alpha- and methoxy-MAMEs as expected. KSK molecules had no impact on the MAME composition and, therefore, mycolic acid synthesis and transport can also be excluded as the target of KSKs. We went on testing whether KSK molecules are interfering with the ATP metabolism (Figure 6 D). The ionophore CCCP was used as positive control along with BDQ and Q203 [17]. For BDQ and Q203 we observed a strong ATP depletion after 24 h. For CCCP the effect was much weaker. In contrast to the negative control RIF, cells treated with KSK-104 and KSK-106 showed a similar response as CCCP treated cells. Thus, these results show that KSKs are able to decrease the ATP levels at a comparable level as CCCP does. Although the ATP depletion is not comparable to BDQ or Q203 [18], an indirect effect on the ATP generation is conceivable. For instance, a decrease of the proton motive force (PMF) by inhibition of the electron transport chain is feasible.

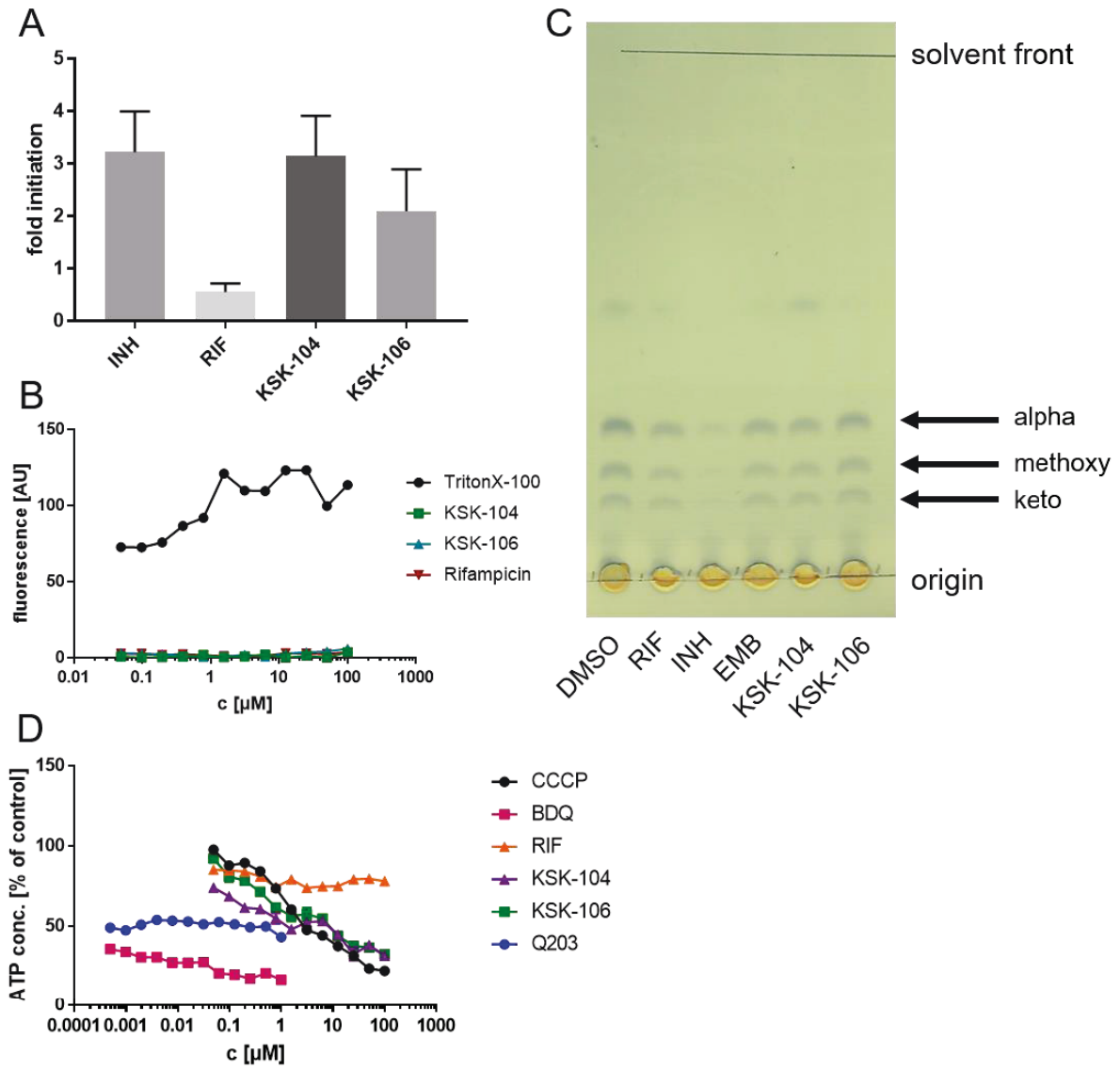


Figure 6 – Cell wall stress caused by KSK treatment. (A) qPCR was performed to measure the expression of *iniB*. The Y-axis shows the fold initiation in comparison to DMSO (=1). Experiments have been performed in triplicates. (B) Propidium iodide (PI) assay was performed using the indicated concentrations of KSKs, rifampicin (RIF) and TritonX-100 which was used as positive control. An increase in the fluorescence (Y-axis) is equal to membrane damage. (C) TLC performed with MAMEs isolated from *M. tuberculosis* H37Rv cultures that have been treated with sublethal concentrations of rifampicin (RIF), isoniazid (INH), ethambutol (EMB) and KSKs. Origin and solvent front are marked as well as the spots showing the three different types of MAMEs in *M. tuberculosis* H37Rv. (D) ATP depletion has

been quantified in cultures treated with the denoted concentrations of KSKs, CCCP, Q203 and bedaquiline (BDQ). CCCP, Q203 and BDQ were used as controls.

Discussion

In this study, we identified KSK-104 and KSK-106 as two promising molecules with high anti-TB activity. Both molecules can function as new chemotherapeutics against *M. tuberculosis* because of high potency to kill bacteria in nano-molar concentrations, high selectivity against *M. tuberculosis*, no detectable cytotoxicity and intracellular activity against bacteria within macrophages. Although we tested a great number of derivatives, we were not able to identify more active molecules than the original KSK-104 and KSK-106. Thus, it was possible to perform structure-activity relationship studies to understand the basic structure of KSK molecules. We found the two alkoxyamide groups of the molecules to be an essential determinant of the anti-TB activity. If one of the groups is missing, no effect against *M. tuberculosis* was detectable. Furthermore, region C is a variable region that can be modified and still provides acceptable results against *M. tuberculosis* in sub-millimolar range. For region A, a *para*-substitution is favourable, whereby all modifications yet made are decreasing the anti-TB potency of the molecules. The variability of region A-related *para*-substitutions and region C are notable opportunities to optimise the pharmacokinetic properties of the molecules after evaluation of *in vivo* efficiency.

Because of their high specificity against *M. tuberculosis*, a unique property of mycobacteria might be involved in either uptake, metabolism or target of the molecules. Spontaneously resistant mutants against both KSKs were isolated with rather low resistance rates (10^{-8} and $> 10^{-10}$, respectively), which gives the hint that most likely a whole cellular process might be involved as a target and not only one gene product. We identified SNPs in both sets of mutants which were located in amidohydrolases that are genes non-essential for *in vitro* growth [15,19]. Rv0552 and AmiC are both predicted to act on C-N-bonds, whereas only AmiC might be acting on peptide bonds. Proteome analysis, however, revealed that none of the amidohydrolases were more abundant when cells are treated with sublethal concentrations of KSK-106. It might be possible that the mutations found in the spontaneously resistant mutants are enabling the enzymes to act on intra-molecular C-N-bonds of KSK-104 and

KSK-106 [15], respectively. Thus, they might contribute to resistance against KSKs via hydrolysis. However, the SNPs we identified in *amiC* are leading to frameshifts and most likely to non-functional proteins. If we would expect hydrolysis of KSKs as a mechanism of resistance, a non-functional AmiC amidohydrolase would, therefore, be useless. Therefore, we rather think that KSKs are prodrugs, such as isoniazid[20], and the hydrolysis products are the active forms that provide anti-TB activity. It is conceivable that KSKs are entering the cells, are hydrolysed by Rv0552 and/or AmiC, respectively, whereafter the hydrolysis products provide the anti-TB activity. A point mutation in the amidohydrolases, however, is leading to lower affinity of the enzymes and, therefore, KSK prodrugs can not be hydrolysed to their active form. This theory is supported by the fact that no such hydrolases are found in humans or other nosocomial bacteria via BLAST search [21]. Therefore, KSKs are not hydrolysed in these organisms and the active form of the molecule is absent. This hypothesis might be proven by knockout and overexpression mutants of *Rv0552* and *amiC* in *M. tuberculosis* as well as nosocomial bacteria, such as *S. aureus*.

Regarding the mechanism of action, several targets could be ruled out. Although we found *iniB* as a marker for cell wall stress to be upregulated [16], we could not verify the formation of membrane pores or a change in the composition of MAMEs. Interestingly, KSKs do not have an additive effect in combination with BDQ as shown for other antibiotics. Recently, it was shown that BDQ is interfering with the antibacterial efficiency of isoniazid and moxifloxacin [22]. Nonetheless, we measured ATP concentrations in *M. tuberculosis* during our experiments and found a similar effect of KSKs as for the ionophore CCCP. Although the ATP depletion caused by BDQ was much higher than monitored for KSKs, we consider an indirect inhibition of ATP production as mechanism of action. BDQ is inhibiting the F_1F_0 ATP synthase [18], causing a strong and rapid ATP depletion. If the electron transport chain is inhibited, no PMF can be established to gain energy from ATP synthesis [23,24]. Furthermore, our hypothesis that KSKs are prodrugs complies with the electron transport chain as the target. One of the hydrolysis products we tested is a small hydroxamic acid which can bind metal ions [25,26],

such as HDAC inhibitors like suberanilohydroxamic acid that binds zinc ions [27]. It seems plausible that precursors of HDAC inhibitors are also potential chelators. In our view, the small hydroxamic acid which is the result of the hydrolysis of KSK-104 and KSK-106 by Rv0552 and AmiC, respectively, is binding metal ions from cytochromes in the electron transport chain [28]. This way the cytochromes do not work properly and no PMF can be established. Proteome analysis revealed that one specific group of proteins is abundant during sublethal treatment with KSK-106. To our best knowledge, this operon (referred to as *Rv3095*-operon) has yet not been mention as a response to antibiotic treatment. The function of each gene is yet unknown but data derived from homology give that *Rv3095* encodes for an HTH-motif transcription factor. It is known that this kind of transcription factors are found in eucaryotes and procaryotes [29,30] and are able to control expression of various cellular processes such as pathogenicity, antibiotic resistance and differentiation to name but a few [31-33]. It might be possible, however, that this operon is upregulated to compensate the deficiency in the electron transport chain since *Rv3094c*, for example, is a potential oxidoreductase with FAD-binding site. Cytochrome C, for instance, is also known to be an oxidoreductase containing heme [34] and *Rv3094c* might be able to takeover a part of the electron transport of a cytochrome. Of course, the *Rv3095*-operon needs further characterisation until this statement can be confirmed.

In summary we identified two promising molecules as lead structures for anti-TB chemotherapeutics. The molecules meet all the requirements one would have for an efficient antibiotic. KSKs are able to kill *M. tuberculosis* in nano-molar concentrations *in vitro* and also after internalisation within macrophages, whereby no detectable cytotoxicity against various human cell lines was found. Moreover, the molecules have a very low resistance rate (10^{-8} and $> 10^{-10}$) and the mechanism of resistance is elucidated as well. This gives the opportunity to further optimise the structure of the molecules, whereas extensive structure-activity studies have already been thoroughly performed. In addition to that, a new mode of action was identified as the electron transport chain is the most likely target, for what reason

also XDR-TB strains are susceptible to KSKs. Further studies are, of course, necessary to find the exact target of KSKs and elucidate the role of the *Rv3095*-operon in this regard.

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Supplementary Material.

Alkoxyamid-based molecules interfere with energy metabolism of *Mycobacterium tuberculosis* to kill bacteria at nanomolar concentrations

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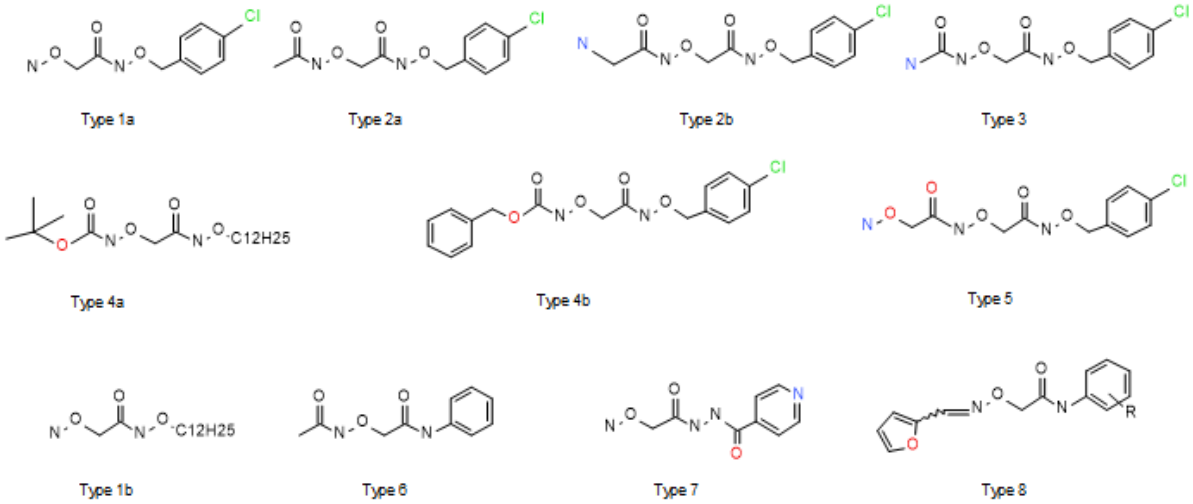

Figure S1. Published α-aminooxyhydroxamic acid derivatives with known antimycobacterial activity. Types 1-7 have been described in [11], type 8 in [15-16] .

Table S1 – SNPs of spontaneous resistant mutants. SNPs of KSK-104 (A) and KSK-106 (B) resistant mutants.

A

strain	mutations	
mutant #1	Rv0522: E131Q Rv0552: H67R	ppsE: C377R Rv3368c: G50S
mutant #2	Rv0522: E131Q Rv0552: H67R	ppsE: C377R Rv3368c: G50S
mutant #3	Rv0552: A229D	ppsA: W1294*
mutant #4	Rv0522: E131Q Rv0552: H67R	ppsE: C377R Rv3368c: G50S
mutant #5	Rv0522: E131Q Rv0552: H67R	ppsE: C377R Rv3368c: G50S

B

strain	mutations	
mutant #1	amiC: +ccgg in aa 347/473	ppsA: W1294*
mutant #2	amiC: +t in aa 100/473	ppsE: A465S Rv0818: L97P
mutant #3	amiC: P185T	ppsA: W1294* Rv0818: T99A
mutant #4		ppsA: W1294*
mutant #5	amiC: E129*	

7. Interleukin-26 activates macrophages and facilitates killing of *Mycobacterium tuberculosis*

In revision (minor revisions) in Scientific Reports

Impact Factor: 4.525 (2018)

Overall contribution to the paper: 25%

- Determination of minimal inhibitory concentrations (MICs) against *M. tuberculosis*
- Infection of THP-1 derived macrophage-like cells with *M. tuberculosis*
- Sample preparation for Scanning Electron Microscopy (SEM) of *M. tuberculosis*

8. Mining marine shell wastes for polyelectrolyte chitosan anti-biofoulants: Fabrication of high-performance economic and ecofriendly anti-biofouling coatings

Published in: Carbohydrate Polymers
Impact Factor: 6.044 (2018)
DOI: https://doi.org/10.1016/j.carbpol.2017.05.059
Overall contribution to the paper: 25%

- Contributed to manuscript drafting
- Determination of minimal inhibitory concentrations (MICs) against nosocomial pathogens
- Determination of MICs against biofilms
- Establishment of an anti-adhesion assay for chitosan-derived anti-biofoulants

9. Further publications

In addition, further minor contributions have been made to the following peer-reviewed publications:

Kamdem R. S. T., Pascal W., Rehberg N., **van Geelen L.**, Höfert S. P., Knedel T. O., Janiak C., Sureechatchaiyan P., Kassack M. U., Lin W., Kalscheuer R., Liu Z., Proksch P., Metabolites from the endophytic fungus *Cylindrocarpon sp.* isolated from tropical plant *Sapium ellipticum*. Fitoterapia. 128, 175-179, (2018).
DOI: 10.1016/j.fitote.2018.05.020

Rehberg N., Omeje E., Ebada S.S., **van Geelen L.**, Liu Z., Sureechatchayan P., Kassack M.U., Ioerger T.R., Proksch P., Kalscheuer R., 3-O-Methyl-Alkylgallates Inhibit Fatty Acid Desaturation in *Mycobacterium tuberculosis*. Antimicrob Agents Chemother. 23; 63(9), (2019).
DOI: 10.1128/AAC.00136-19

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

Meier D., Hernandez M.V., **van Geelen L.**, Muharini R., Proksch P., Bandow J.E., Kalscheuer R., The plant-derived chalcone Xanthoangelol targets the membrane of Gram-positive bacteria. Bioorg Med Chem. (2019).
DOI: 10.1016/j.bmc.2019.115151

10. Discussion and Perspectives

During this work, we demonstrated that compounds from different sources provide antimicrobial activity against various clinically important microorganisms. The focus was on bacteria, especially on pathogens that are classified as ESKAPE pathogens, the source of life-threatening nosocomial infections, and on *M. tuberculosis*, the causative agent of TB. Access to a large library of natural products and synthetic molecules allowed different approaches to tackle the rising problem of AMR.

As described in chapter 5, two natural products have been analysed that were isolated from the marine sponge *Dysidea granulosa*. This brominated phenoxyphenols, 4,6-dibromo-2-(2',4'-dibromophenoxy)phenol (2-bromo-PP) and 3,4,6-tribromo-2-(2',4'-dibromophenoxy)phenol (3-bromo-PP), kill both Gram-positive and Gram-negative bacteria. Broad-spectrum activity has been shown against the clinical important group of ESKAPE pathogens, including *E. faecalis*, *E. faecium*, *E. cloacae*, *E. coli*, *A. baumannii*, *K. pneumonia*, *P. aeruginosa* and MRSA. For MRSA and *P. aeruginosa*, in particular, it has been demonstrated that not just planktonic cells are killed by phenoxyphenols, but also dormant and biofilm-incorporated cells that are particularly hard to treat. Although a wide therapeutical window has been found for the treatment of planktonic cells, 2-bromo-PP and 3-bromo-PP need further optimisation regarding the anti-biofilm activity. Therefore, for further experimental purposes, it would be beneficial to synthesise derivatives to optimise the antibacterial potential. Regarding medicinal chemical optimisation, it is important not to add large functional groups to the molecules as the biofilm activity might be linked to the low molecular weight of the molecules, such as hordenine. The target identification is still pending for phenoxyphenols and tagged molecules with fluorescent properties, such as coumarin or cyanine, could give further inside in the uptake mechanism and localisation within the bacterial cell. Spontaneously resistant mutants had consistent mutations in a *tetR* transcriptional regulator and in the putative promotor region of *mtlA*, a EIIBC component of the mannitol-specific PTS. The gene *scrA*, the sucrose-specific EIIBC component of the PTS, is located in close genomic proximity to *tetR*. This might be related to a possible uptake mechanism of phenoxyphenols as EIIBC components have kinase-like properties. Therefore, possible phosphorylation of 2-bromo-PP and 3-bromo-PP might play a role in uptake via the bacterial PTS. Phosphorylated phenoxyphenol derivatives might verify this hypothesis in combination with knockout mutants and could demonstrate whether phosphorylation is also connected to the bacteriocidal activity of phenoxyphenols. A saturated transposon library will further reveal genes that are linked to the mechanism of action and point at possible drug targets to optimise the structure regarding anti-biofilm activity.

Eradication of hard-to-treat subpopulations of pathogenic bacteria can be achieved via antibiotic therapy but most clinical antibiotics are not effective against persisters or biofilms. Only small molecules, such as brominated phenoxyphenols, are able to penetrate the EPS matrix of biofilms. Another strategy to fight the resurgence of infection due to biofilms is the prevention of biofilm formation itself as presented in chapter 8. Coating with chitosan-derivatives has been used to prevent the initial adherence of bacteria to a polystyrene surface. As a consequence, microcolonies were not formed and biofilm formation was abolished. The great advantage of chitosan-derivatives is the low selective pressure on bacteria. Chitosan and all tested derivatives do not provide antibacterial activity, thus the emergence of resistance is very unlikely. Clinical use of chitosan-derivatives could result in lower rates of biofilm formation on the surface of catheters or joints, for instance, when the plastic or metal surfaces are coated with chitosan-derived polymers. Other than this, persisters and already formed biofilms remain particularly difficult-to-treat subpopulations. Quorum quenching molecules, such as quercetin, are suitable molecules and provide anti-biofilm activity as long as the biofilm is not yet formed [196]. Application after biofilm formation is, therefore, useless and the dispersal of the biofilm comes into focus. Dispersin is a promising candidate to degrade the biofilm matrix of MRSA and allow antibiotics to reach the biofilm-embedded bacteria [197]. Peptide 1018 is a small peptide that is interfering with the stress-induced (p)ppGpp signalling during biofilm formation. It prevents biofilm formation of clinically important pathogens *P. aeruginosa*, A. baumannii, *E. coli*, *K. pneumonia* and *S. aureus* in rather low concentrations [225]. Persisters are the other subpopulation exhibiting high antimicrobial tolerance. Clinically used antibiotics are largely inactive against persisters because most of the cellular metabolism is downregulated. New strategies, such as metabolite-enabled killing, need to be optimised. The metabolism of persisters is restarted with high doses of metabolites, and antibiotics are able to provide antimicrobial activity again [226]. Other than this, new drug targets need to be evaluated employing gDNA libraries or transposon mutagenesis.

In chapter 6, synthetic precursors of histone deacetylase (HDAC) inhibitors were tested against Mtb. The alkoxyamide-based molecules KSK-104 and KSK-106 exhibit anti-TB activity in nanomolar concentrations and are, more importantly, also highly active against XDR-TB strains. A wide variety of derivatives has been tested, and the essential structure for anti-TB activity has been revealed. Besides the low resistance rate of Mtb against KSKs, no detectable cytotoxicity and the specific anti-TB activity are notable advantages of these molecules. Since the target has not been validated yet, further experiments are necessary. To date, we hypothesise that KSKs are interfering with the electron transport chain (ETC). Therefore, the ATP concentration in the cells is depleted. Hydrolysis of KSKs by Rv0552 and/or AmiC results in a molecule with a hydroxamic acid moiety. Hydroxamic acids are known to be chelators of

metal ions, e.g. zinc ions of HDACs [227]. As KSKs are precursors of HDAC inhibitors a similar mode of action seems plausible although Mtb has no histones or HDACs. Therefore, we postulate that KSKs chelate iron ions from essential Fe-S-clusters from the cytochromes of the ETC and inhibit formation of the proton motive force (PMF). This would explain the different patterns of ATP depletion of BDQ, Q203 and KSKs. In addition, this model would also explain the rather low resistance rates of Mtb against KSKs because no protein is targeted but ion-clusters of the ETC. Despite this postulated mode of action, further derivatives of KSKs would enable us to investigate whether there are interaction partners of KSKs or their hydrolysis products, respectively. In this regard, KSK derivatives containing an alkyne moiety and still providing anti-TB activity are in the focus of interest as they might be able to bind a specific target and are still suitable for pulldown experiments. To circumvent the development of resistance against the postulated KSK-prodrug, a hydroxamic acid-derivative that provides high anti-TB activity and does not need intracellular activation is of particular interest. A further step to promote the elucidation of the mode of action of KSKs are pulldown experiments with whole-cell protein lysates. Since biotin is a rather large molecule and a biotinylated KSK-derivative did not provide anti-TB activity, which might be linked to either the missing phenyl-ring in region A, diffusion issues or both, KSK-derivatives with an alkyne moiety for click-chemistry would be more suitable. This molecule should provide anti-TB activity in a similar concentration range as compared to the corresponding non-alkyne variant and could be used to employ protein pulldowns to reveal binding of KSK-derived molecules to a protein target that is yet unknown. Furthermore, fluorophore-labelled KSK-derivatives could contribute to the elucidation of the mode of action. Fluorescence microscopy could be employed to identify a potential co-localisation of KSKs and GFP-tagged constituents of the mycobacterial ETC. Additionally, a thermofluor assay would reveal a potential protein target of KSKs and could, finally, verify the interaction of Rv0552 and AmiC with KSKs [228]. Genetic approaches using a gDNA or saturated transposon library in Mtb could also provide further information about the mode of action. The heterologous overexpression of Mtb genes in recombinant *M. bovis* could provide further insight into KSK-related mechanisms, such as uptake, activation of prodrugs or resistance when *M. bovis* is rendered susceptible. The upregulation of proteins appertaining to the *Rv3095*-operon as stress-response to sub-inhibitory treatment with KSK-106 is another interesting matter. To date, the function of the proteins of this operon has not been evaluated. Predictions were made upon sequence comparison yielding a transcriptional regulator (Rv3095), an acyl-CoA dehydrogenase with FAD-binding site (Rv3094c), an oxidoreductase (Rv3093c) and an integral membrane protein (Rv3092c). We hypothesise that Rv3095 is, as predicted, the transcriptional regulator of the *Rv3095*-operon including all of the mentioned genes. The information provided by the UniProt and Mycobrowser databases is rather scarce but allows speculation about a probable function. The sequence similarities of Rv3094c reveal

properties that are comparable to NADH-dehydrogenases. Furthermore, Rv3093c has hypothetically properties of an oxidoreductase that point toward this protein being a constituent of the ETC or other processes related to energy metabolism. It seems to be very likely that the upregulation of an operon is a specific stress response to compensate for the effects of an antibiotic [229]. Assuming that our hypothesis is correct and KSKs are indeed targeting the ETC, the *Rv3095*-operon might very likely be related to the energy metabolism as well. NADH-dehydrogenases contribute to the formation of the PMF, but do not contain metal ions, such as Fe(II) or Fe(III), and are therefore not affected by KSKs. The upregulation of the *Rv3095*-operon might be a compensation mechanism of Mtb against PMF and ATP depletion. The possible NADH-dehydrogenase Rv3094c might be able to transport electrons via the mycobacterial electron carrier menaquinone and translocate H^+ ions to buildup the PMF. In this hypothesis, Rv3093c, the hypothetic oxidoreductase, catalyses the recycling of menaquinone by oxidation. The regulatory mechanism of Rv3095 can be deciphered by gene deletion and/or overexpression of the predicted transcription factor *Rv3095* followed by (RT-)qPCR analyses. The exact function of this operon, however, needs to be evaluated carefully. Further techniques to characterise this operon are gene deletion and overexpression mutants along with protein isolation to determine the reaction in an adequate assay, such as a colourimetric assay to monitor the enzymatic activity of Rv3094c and Rv3093c, respectively. Brugger *et al.*, for instance, provided a protocol to quantify the oxidoreductase activity with different redox dyes [230] and Jin *et al.* employed an assay to monitor NADH-dehydrogenase activity [231]. Using these assays, it would be possible to check whether the postulated activity of the proteins can be verified. The function of Rv3092c, however, remains elusive because the sequence information provides no other data than sequence similarities to integral transmembrane proteins. It might, therefore, play a role as a transporter or receptor. Interference with energy metabolism has been in the focus for drug development against Mtb for the past decade [232]. In 2013, the imidazopyridine Q203 (telacebec) was discovered and is in clinical trials to date. As inhibitor of the ETC, it interferes with the cytochrome *bcc* complex of Mtb and inhibits the formation of the PMF [233]. Although the mechanism of interference of KSKs regarding PMF formation is different, the outcome is the same. In contrast to that, bedaquiline, a diarylquinoline, targets the hydrophobic regions of subunit c and ε of the F_1F_0 ATP synthase and directly inhibits ATP synthesis [222]. Furthermore, Kuroso and Crick highlighted in 2009 that MenA is a promising drug target. MenA is an essential enzyme for the synthesis of menaquinone, the electron carrier in the ETC of Mtb. As other bacteria utilise a combination of ubiquinone and menaquinone for electron transport, mycobacteria use exclusively menaquinone making its synthesis a drug target unique to mycobacteria [234, 235]. Other than this, lantibiotics form pores in the mycobacterial cell membrane resulting in a dissipation of the membrane potential and loss of the PMF [236]. In addition to that, the

phenothiazine thioridazine is predicted to target the ETC through inhibition of the NADH-dehydrogenase NDH-2. Furthermore, thioridazine is able to inhibit efflux pumps that are a central point of intrinsic drug resistance of Mtb. The dependency of efflux pumps on the PMF or ATP hydrolysis is therefore directly linked to the energy metabolism rendering it an even more promising drug target [237]. However, Lee *et al.* recently demonstrated that energy metabolism inhibitors are interfering with conventional anti-TB drugs, especially isoniazid and moxifloxacin [238]. Both antibiotics are linked to an increase in intracellular ATP levels. Depletion of these ATP levels due to combination therapy with e.g. bedaquiline decreases their anti-TB potency. Although we demonstrated an additive effect of KSKs in combination with classical first-line drugs such as isoniazid, Lee *et al.* demonstrated that the combined effects of anti-TB drugs need to be carefully monitored and evaluated.

Natural compounds isolated from various sources like sponges, fungi or endophytes as well as synthetic compound libraries are rich sources of antimicrobial compounds. Proteins naturally produced in the body, such as the cytokine interleukin-26 (IL-26), are able to provide antimicrobial activity as it has been demonstrated by Meller *et al.* [239]. The effect depends on membrane pore formation and was observed for Gram-positive and Gram-negative ESKAPE pathogens. In chapter 7, the anti-TB potential of IL-26 was evaluated, and “bleb” formation was verified employing Scanning Electron Microscopy (SEM) pointing out that membrane disruption is the mode of action against Mtb as well.

The sources of new antimicrobial compounds are rich as described in chapter 5. Especially soil bacteria have great potential to reveal their abilities to produce yet unknown molecules to combat microorganisms. Since soil bacteria are often hard to cultivate, one of the biggest challenges is their growth under laboratory conditions as pure cultures. That’s why Chaudhary *et al.* developed a new bioreactor that is able to mimic the natural environment of soil bacteria to allow cultivation under controlled laboratory conditions [240]. This method allows the isolation and enrichment of prior uncultivatable bacteria and the production of new antimicrobial molecules by challenging the bacteria or fungi with other microorganisms.
Another method to tackle the problem of AMR is drug repositioning also known as drug repurposing. Established drugs are screened for antimicrobial potency. The great advantage of this method is a large library of different drugs and approval as drug by the responsible administrations. The National Institutes of Health (NIH), for instance, offers a compound collection of approx. 2,400 FDA-approved drugs for screening for drug repurposing. Drug repurposing requires a systematic review of drugs and is based on networking, text mining and systematic acquisition of data [241]. Many drugs have been found to provide antibacterial activity due to drug repurposing. Oxyclozanide is used to treat helminthiasis but was found to

provide activity against MRSA by membrane disruption [242]. Another example is pentetic acid usually administered to treat hypocalcemia. During a screening it was found to provide activity against *P. aeruginosa* and reduce biofilm formation, which is an important virulence factor for bacteria [243].

Another option to fight bacterial infection is the use of monoclonal antibodies which has become increasingly promising in the past years. Humanised monoclonal antibodies, such as bezlotoxumab, passed clinical trials and have been approved by the FDA and the EU. The humanised monoclonal antibody bezlotoxumab is directed against the *Clostridium difficile* toxin B. The antibody binds toxin B and consequently inactivates it. Although bezlotoxumab is not suitable for antimicrobial therapy, it is able to prevent resurgence of *C. difficile* infections [244, 245]. Other than bezlotoxumab, monoclonal antibodies can also target bacterial surface proteins like SpA of *S. aureus* or polysaccharides like alginate produced by *P. aeruginosa*, which is of particular interest because it is associated with biofilm formation and resurgence of bacterial infections [246, 247].

Another vivid field of research to combat AMR is phage therapy. Phages are non-living biological particles that consist of DNA or RNA and a protein capsule. Their reproduction is completely depended on a bacterial host which is an important characteristic regarding the safety. They undergo either a lysogenic cycle and integrate into the bacterial genome or a lytic cycle and use the bacterial resources to reproduce themselves before they lyse the bacterial cell and reinitiate a new infection cycle. The infection range of bacteriophages is divers. Some phages are limited to a certain strain while others have a wide range of bacterial hosts. Animal experiments were performed and showed limited success. Murine sepsis caused by the Gram-negative pathogens *A. baumannii* and *P. aeruginosa* was treated with intraperitoneal injection of phages and cured a significant proportion of mice [248]. Therapy with bacteriophages of diabetic foot ulcers that were infected with MRSA led to recovery of all patients in a study [249]. The clinical important hard-to-treat bacterial subpopulation, especially biofilms, can also be targeted by phage therapy *in vitro* which is a great advantage in comparison to conventional antibiotics. Furthermore, bacteriophage-derived lytic proteins are bioengineered and provide anti-biofilm activity *in vitro* [250]. Phage therapy, however, is facing the same problems as antibiotics and is often limited to topical use only. Bacteriophages are restricted in their therapeutical range as antibiotics and resistances occur during therapy [251]. The effect of bacteriophages depends on the recognition of receptors on the bacterial cell surfaces. Alteration or loss of certain receptors confers resistance to bacteriophages. Additionally, bacteria are able to integrate phage DNA into their genome utilising the CRISPR/Cas9 system which enables bacteria to protect themselves against bacteriophages.

During this thesis, we identified potent molecules to tackle the problem of AMR. Research and development are, of course, important strategies to overcome the worldwide problem of rising AMR. Nevertheless, other factors need to change to prevent progression of the AMR problem. The use of antibiotics needs to be controlled more rigidly. Antibiotic or antibiotic-containing cremes and solutions can be bought in many countries without any prescription [252]. This leads to unnecessary and more importantly to uncontrolled use of antibiotics. This is a major driving factor of AMR, in particular in agriculture as mentioned in chapter 1.1.5. At the same time, the accessibility of antibiotics must be assured. Urgently needed new antibiotics have to be delivered to the countries and hospitals where they are needed most. In some countries, antibiotics are not available because of political or economic interests. A study by Kallberg *et al.* demonstrated that clinical important antibiotics are only available in Europe, North America and Central Asia, but not in Latin America or sub-Saharan Africa [253]. Along with increased accessibility, information has to be provided to instruct patients in the proper use of antibiotics. Misuse and false prescription are often factors that are contributing to emergence of resistance among pathogenic bacteria. An additional aspect to tackle the AMR crisis is patient monitoring and systematic screening. While the Benelux and Scandinavian countries are screening all patients before hospitalisation for multidrug-resistant pathogens, such as MRSA, the widely held practice in Germany is screening of patients only over 60 years during hospitalisation. Spread of HA-MRSA could be kept at bay if all patients would be screened with a nose- and/or throat-smear test. A positive test result would consequently lead to isolation of the patient to prevent further spread of bacteria. The effects of this policy are evident because MRSA incidence rates particularly in the Benelux and Scandinavian countries are significantly lower than in the rest of the EU [148].

Im summary, the AMR crisis has become a global threat. The number of multidrug-resistant pathogens is increasing consistently and needs to be monitored. Prevention and control of antibiotic misuse are vital, and systematic screening of hospitalised patients is an aim that is possible in a rather short period of time. To ensure constant advancement in antimicrobial drug discovery, research and development of new antimicrobial strategies and new drug targets have to be sponsored and promoted to be one step ahead of newly arising AMRs.

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I declare that I have not used sources or means without declaration in the text. All the passages taken from other works in the wording or in the meaning have been clearly indicated with sources. This thesis has not been used in the same or similar version to achieve an academic grading or is being published elsewhere.

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Lasse van Geelen