Repurposing as a tool to identify antibiotic potentiators with anti-biofilm activity

Repurposing as a tool to identify antibiotic potentiators with anti-biofilm activity

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Thesis submitted to obtain the degree of Doctor in Pharmaceutical Sciences

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LIST OF ABBREVIATIONS

AD	Artificial dermis
AME	Aminoglycoside modifying enzyme
AMP	Antimicrobial peptide
AMR	Antimicrobial resistance
Ara4N	Aminoarabinose
BAC	Bacterial artificial chromosome
Bcc	*Burkholderia cepacia* complex
BF	Biofilm
CA-MRSA	Community-associated methicillin resistant *Staphylococcus aureus*
CDC	Centers for disease control and prevention
CF	Cystic fibrosis
CFTR	Cystic fibrosis transmembrane conductance regulator
CFU	Colony forming unit
c-di-GMP	Cyclic diguanylate
CNS	Central nervous system
Cp	Plasma concentration
CTB	CellTiter Blue
DMSO	Dimethyl sulfoxide
DOS	Diversity-oriented synthesis
eDNA	Extracellular deoxyribonucleic acid
EPM	Extracellular polymeric matrix
ESBL	Extended spectrum beta-lactamase
ESKAPE	*Enterococcus faecium*, *Staphyolococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and Enterobacter species
FBS	Fetal bovine serum
FDA	Food and drug administration
FICI	Fractional inhibitory concentration index
GFP	Green fluorescent protein
HA-MRSA	Healthcare-associated methicillin-resistant *Staphylococcus aureus*

HGT	Horizontal gene transfer
Hla	α-hemolysin
HTS	High-throughput screening
ICA	Intercellular adhesion gene cluster
LOPAC	Library of pharmaceutically active compounds
LPS	Lipopolysaccharide
MFS	Major facilitator superfamily
MBL	Metallo beta-lactamase
MDR	Multidrug-resistant
MH	Mueller hinton
MIC	Minimal inhibitory concentration
MRSA	Methicillin-resistant *Staphylococcus aureus*
MTP	Microtiter plate
NIH	National institutes of health
NIHCC	National institutes of health clinical collection
NP	Natural products
NSAID	Non-steroidal anti-inflammatory drug
PBP	Penicillin binding protein
PEL	Pellicle
PIA	Polysaccharide intercellular adhesin
PMN	Polymorphonuclear neutrophil
PNAG	Poly-N-acetylglucosamine
PS	Physiological saline
PSL	Polysaccharide synthesis locus
PSM	Phenol soluble modulin
QS	Quorum sensing
RND	Resistance nodulation division
ROS	Reactive oxygen species
R&D	Research and development
SBDD	Structure-based drug design

SERM	Selective estrogen receptor modulator
SSRI	Selective serotonin reuptake inhibitor
TCS	Two-component system
VISA	Vancomycin-intermediate *Staphylococcus aureus*
VRSA	Vancomycin-resistant *Staphylococcus aureus*
YPD	Yeast pepton dextrose
3D	Three-dimensional

Chapter I:
Introduction

Today, antibiotics have become a victim of their own success [1]

1. Bacterial resistance: do bacteria win the battle?

1.1. Bacterial resistance: a global emerging threat

Throughout history, infectious diseases have caused pandemic disasters decimating complete civilizations. Even today, infections remain a major cause of disease worldwide and respiratory infections, HIV/AIDS, and malaria are the main causes of death in low-income countries [2, 3]. Also in high-income countries infections remain an important cause of morbidity and mortality. For instance, up to 10% of hospitalized patients will suffer from a nosocomial infection [4]. Mainly elderly, patients with underlying diseases (like cystic fibrosis), or patients undergoing medical treatments and surgery (like immunosuppression, chemotherapy, transplantations, or implantation of foreign bodies) are vulnerable to infections by opportunistic pathogens.

The discovery of penicillin was a milestone in the history of medical science [1, 5, 6]. Many more antibiotics were developed during the golden era of antimicrobial drug discovery, and they revolutionized medicine in many respects [1, 5, 6]. Antibiotics made incurable diseases like tuberculosis and pneumonia treatable [1]. Also, antibiotics enabled the development of modern medicine because they have successfully treated or prevented infections in patients who are receiving immunosuppressing cancer chemotherapy or who have had organ transplants or joint replacements, among others [7, 8]. Perioperative antimicrobial prophylaxis has contributed to a decrease in the incidence of surgical site infections [8, 9]. However, bacteria develop resistance against antibacterial agents, sometimes shortly after their introduction to the market (Figure 1) [5].

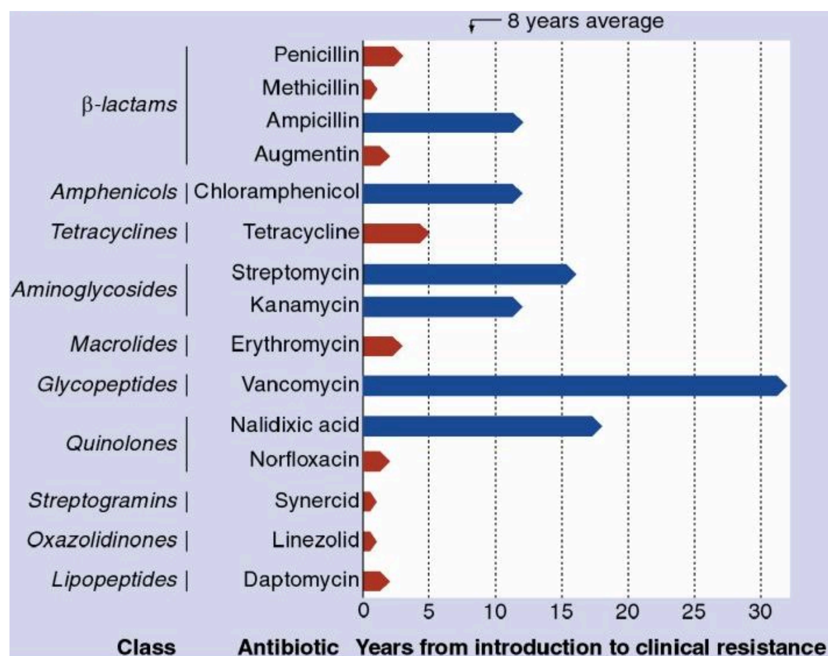

Figure 1. Resistance development against antibiotics happens within 8 years on average. Bars mark the number of years between introduction to the market and the first report of clinical resistance (red: resistance occurred in less than 8 years, blue: resistance development took longer than the average 8 years) [10].

Mis- and overuse of antibiotics, both in humans and animals [11], global trade, and migration contributed to the spread of resistant micro-organisms [12]. The prevalence of multidrug-resistant (MDR) pathogens is increasing and infections that are untreatable with last resort antibiotics have been reported [13]. The World Health Organization recognized antimicrobial resistance (AMR) as one of the top three threats to human health [14]. Yearly, 50,000 people die in the USA and Europe from infections caused by resistant pathogens, and this number is estimated to be over 700,000 deaths globally [15]. If we do not take action to tackle AMR now, by 2050 10 million people are estimated to die every year from drug-resistant infections which are more deaths than caused by cancer [16].

In addition, predictive macroeconomic models have estimated that the world will produce around 8 trillion USD less yearly by 2050 when AMR is not addressed [15]. This huge number is based only on the effects of lost economic output resulting from increased rates of death and illness [15]. It does not take into account the rise in healthcare costs associated with drug-resistant infections, or the problems that will arise in other medical areas. Indeed, AMR also threatens the safety and efficacy of common surgeries, like hip replacements or caesarian sections, and of immunosuppressing chemotherapy, among others [8, 15, 16].

Most nosocomial infections are caused by a small group of pathogens, referred to as the ESKAPE bugs (i.e. *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and Enterobacter species) [17]. The prevalence of these ESKAPE pathogens in our hospitals is increasing and they effectively “escape” the effects of antibacterial drugs [17, 18]. The Centers for Disease Control and Prevention (CDC) assessed the threats of AMR based on several factors, including clinical and economic impact, incidence and 10-year projection of incidence, or the availability of effective antibiotics. Based on these assessments, CDC classified the threat level by important pathogens as urgent, serious, and concerning (Table 1) [7]. The rise of AMR and the lack of novel antibiotics might lead us towards a ‘post-antibiotic era’ [19].

Table 1. CDC assessment of antibacterial resistance threats [7].

Urgent threats	*Clostridium difficile*
	Carbapenem-resistant Enterobacteriaceae
	Drug-resistant *Neisseria gonorrhoeae*
Serious threats	Multidrug-resistant *Acinetobacter*
	Drug-resistant *Campylobacter*
	Fluconazole-resistant *Candida*
	ESBLs producing Enterobacteriaceae
	Vancomycin-resistant Enterococci
	Multidrug-resistant *Pseudomonas aeruginosa*
	Drug-resistant nontyphoidal *Salmonella*
	Drug-resistant *Salmonella* Typhimurium
	Drug-resistant *Shigella*
	Methicillin-resistant *Staphylococcus aureus*
	Drug-resistant *Streptococcus pneumoniae*
	Drug-resistant tuberculosis
Concerning threats	Vancomycin-resistant *Staphylococcus aureus*
	Erythromycin-resistant Group A *Streptococcus*
	Clindamycin-resistant Group B *Streptococcus*

1.2. Intrinsic, acquired, and adaptive resistance

Intrinsic, acquired and adaptive resistance are three main resistance types that can be distinguished in bacteria [20]. Intrinsic resistance originates from innate properties, applies to all strains of a species and limits the activity of all antibiotics of a certain class [5, 11, 20]. For instance, differences in cell wall structure between Gram-negative and Gram-positive bacteria contribute to distinct susceptibilities towards antibiotics. Gram-negative bacteria possess a thin peptidoglycan cell wall surrounded by a poorly permeable outer membrane containing lipopolysaccharides. This outer membrane is responsible for the intrinsic resistantance towards glycopeptide antibiotics, among others [11, 21]. In contrast, Gram-positive bacteria possess thick layers of peptidoglycan and teichoic acids threading through, but lack the outer membrane [21].

Acquired resistance usually occurs when susceptible bacteria obtain new genetic material with resistance genes (e.g. plasmids and transposons) from resistant micro-organisms. The transfer of new genetic material might take place by conjugation, transformation or transduction, and can result in the expression of efflux pumps or antibiotic degrading enzymes, or in a bypass of the antibacterial target [5, 11]. Plasmids might contain genes that cause resistance towards important classes of antibiotics, but also genes encoding virulence determinants [11]. In addition, acquired resistance might also arise after spontaneous mutations in the chromosomes of susceptible strains conferring resistance to structurally similar compounds [11].

Finally, adaptive resistance is a reversible and temporary phenomenon enabling bacteria to survive antibacterial treatment. It is observed in several bacterial species upon exposure to nonlethal or consecutive incremental amounts of antibiotics, or in other stress conditions [20, 22]. When the trigger is removed from the environment, bacteria quickly return to the non-resistant phenotype. Adaptive resistance originates from changes in protein expression, for instance, a downregulation of porins or an upregulation of efflux pumps [20, 22]. Adaptive resistance is not transferred vertically as is the case in innate and acquired resistance [20, 22].

Horizontal gene transfer (HGT) and the increased expression of genes encoding efflux pumps, resulted in the emergence of MDR pathogens. HGT leads to the acquisition and accumulation of genes originating from resistance plasmids or transposons causing resistance towards several classes of antibiotics. A single resistance plasmid can contain several resistances genes and once acquired by a bacterium, resistance plasmids are stably maintained and transferred efficiently to other cells [23, 24]. Some main resistance mechanisms towards important antibacterial drug classes will be summarized in the subsequent paragraphs based on examples in three bacterial species: *Staphylococcus aureus*, representing Gram-positive micro-organisms, and *Pseudomonas aeruginosa* and *Burkholderia cenocepacia*, representing Gram-negative micro-organisms.

1.3. Resistance in *Staphylococcus aureus*

S. aureus is a Gram-positive coccus that colonizes the human skin and nose as a commensal. However, *S. aureus* is also responsible for a plethora of infections, ranging from skin and soft tissue infections to life threatening endocarditis, pneumonia, and sepsis [25]. Over time, *S. aureus* developed resistance towards several antibiotics, making treatment challenging.

1.3.1. Beta-lactam resistance

Resistance towards penicillin was observed in 1942, almost simultaneously with its introduction in the early 1940s [26]. Certain *S. aureus* strains produced penicillinases that hydrolyze the β-lactam ring in the penicillin molecule [11]. Twenty years later, 80% of *S. aureus* strains were resistant towards penicillin [26]. Methicillin and oxacillin are penicillinase-resistant, however, resistance occurred shortly after their introduction due to acquisition of the *mec*A resistance gene. This gene is located on the mobile genetic element SCC*mec,* and encodes an alternative penicillin-binding protein, PBP2a [5, 11]. This PBP2a has a similar activity as the original PBP, but shows lower affinity for β-lactam antibiotics, resulting in resistance towards all β-lactam antibiotics [27]. Methicillin resistant *S. aureus* (MRSA) infections occurred mainly in healthcare environments (healthcare associated MRSA, HA-MRSA) affecting older patients with comorbid conditions, often resulting in pneumonia and bacteremia [28]. More recently, MRSA infections in a healthy younger population unrelated to the healthcare environment (community associated MRSA, CA-MRSA) emerged [28]. In contrast to HA-MRSA, clinical syndromes are mainly skin and soft tissue infections, but infections with CA-MRSA might also result in severe sepsis. In addition, CA-MRSA often carries genes for the Panton-Valentine leukocidin, explaining the increased

virulence [28]. Both HA-MRSA and CA-MRSA carry the SCC*mec* containing the *mec*A gene, although the SCC*mec* in HA-MRSA strains is larger [28].

1.3.2. Vancomycin resistance

Vancomycin is the drug of choice to treat MRSA infections [29]. However, an MRSA isolate with reduced susceptibility towards vancomycin (vancomycin intermediate-resistant *S. aureus*, VISA) was for the first time reported in 1997 [30]. The reduced susceptibility of VISA is mainly caused by a thickened cell wall that sequesters vancomycin and limits its action [5, 31]. In 2002, a vancomycin resistant *S. aureus* (VRSA, MIC>256µg/ml) was isolated in a diabetes patient's infected chronic foot ulcer [5]. The VRSA possessed the *van*A resistance gene, probably acquired from the vancomycin resistant *Enterococcus faecalis* that co-infected the wound [5]. Acquisition and expression of *van*A changes the vancomycin binding site D-ala D-ala into D-ala D-lactate, reducing the affinity for vancomycin a 1000 times [5].

1.3.3. Resistance to other antibacterial agents

MRSA possesses several efflux pumps belonging to the major facilitator superfamily (MFS), e.g. TetK, expelling tetracyclines, and NorA, expelling fluoroquinolones and several disinfectants [25, 32]. In addition, resistance towards fluoroquinolones is also mediated by mutations in *grl*A/*grl*B and *gyr*A/*gyr*B, resulting in a lower affinity for their targets topoisomerase IV and DNA gyrase [27]. Resistance towards aminoglycosides originates mainly in the activity of aminoglycoside modifying enzymes (AME). These enzymes inactivate the aminoglycoside molecule by acetylation, adenylation or phosphorylation, preventing binding of the aminoglycosides to their target, the 30S ribosomal subunit [11, 33]. Against last resort antibiotics, like linezolid, resistance is described as well and originates from mutations in the 23S rRNA gene [34, 35].

1.4. Resistance in *Pseudomonas aeruginosa*

P. aeruginosa is a Gram-negative micro-organism ubiquitous in nature (e.g. soil, water, plants) and persisting in hospital settings [36]. Although *P. aeruginosa* is rarely part of the normal human microbiome, hospitalized patients are frequently (50%) colonized [36]. *P. aeruginosa* is an important causative agent of nosocomial infections [37]. Treatment of these infections is challenging because *P. aeruginosa* possesses several intrinsic and acquired resistance mechanisms [5, 37, 38].

1.4.1. Resistance towards β-lactams

The expression of AmpC, a chromosomally encoded serine β-lactamase, is induced upon the exposure to ampicillin and narrow-spectrum cephalosporins. This results in the deactivation of these β-lactams [39-41]. Although ureidopenicillins (e.g., piperacillin) and extended-spectrum cephalosporins (e.g., ceftazidime) are hydrolyzed by AmpC too, they are weak AmpC inducers and thus effective in treating *P. aeruginosa* infections. However, an increased AmpC production (derepressed production of AmpC), due to mutations in regulatory genes, renders these ureidopenicillins and extended-spectrum cephalosporins ineffective. In this

case, treatment with carbapenems is an option as they are not hydrolyzed by AmpC [36, 40, 41]. Several other β-lactamases have been identified in *P. aeruginosa* isolates, among them four carbenicillin hydrolyzing β-lactamases (PSE-1, PSE-4, CARB -3, and CARB-4), class A extended spectrum β-lactamases (ESBL, hydrolyzing carboxypenicillins, ureidopenicillins, extended-spectrum cephalosporins and aztreonam), classical and extended-spectrum oxacillinases and carbapenemases or metallo β-lactamases (MBLs) [37, 42].

In addition, also the activity of efflux pumps belonging to the resistance nodulation division (RND) family contributes to β-lactam resistance. RND efflux pumps are three-component systems consisting out of an energy dependent pump in the cytoplasmic membrane, an outer membrane protein and a linker protein in the periplasmic space [5, 37, 43]. MexAB-OprM, MexCD-OprJ, MexEF-OprN, and MexXY-OprM are clinically important RND efflux pumps in *P. aeruginosa* [37]. Especially MexAB-OprM contributes to the resistance towards β-lactams, only imipenem is not expelled [11, 37, 40]. Resistance towards imipenem is due to a downregulation of the outer membrane protein OprD [37].

1.4.2. Resistance towards fluoroquinolones

Fluoroquinolones are substrates for MexAB-OprM, MexCD-OprJ, MexEF-OprN, and MexXY-OprM [37]. Mutations in their regulatory genes (e.g. *nalB* for MexAB-OprM) causes efflux pump overexpression resulting in MDR phenotypes [37, 44]. Secondly, structural changes in the fluoroquinolone target enzymes DNA gyrase and topoisomerase IV result in lower binding affinity for fluoroquinolones [37]. These alterations originate in point mutations in quinolone-resistance determining regions of *gyrA/gyrB* or *parC/parE* [37, 45, 46].

1.4.3. Resistance towards polymyxin

In susceptible Gram-negative organisms, the cationic polymyxins and other antimicrobial peptides (AMPs) bind the negatively charged lipopolysaccharides (LPS) located in the outer membrane. This results in membrane destabilization and permeabilization, leading to self-promoted uptake [47, 48]. Next, they disrupt the cytoplasmic membrane and interfere with several intracellular processes [47]. *P. aeruginosa* is not inherently resistant towards polymyxin as the positively charged 4-amino-4-deoxy-L-arabinose (aminoarabinose, Ara4N) that is responsible for polymyxin resistance in *Burkholderia* species (see below) is not constitutively present in *P. aeruginosa* LPS. Presence of Ara4N reduces the net negative charge and subsequently the binding affinity of AMPs and polymyxin. However, several *P. aeruginosa* strain have mutations in the *pmr*A/*pmr*B and *pho*P/*pho*Q two-component systems (TCS) (as well as in other TCS) resulting in their activation and leading to upregulation of the genes responsible for the synthesis and transport of Ara4N to the LPS [49].

1.4.4. Resistance towards aminoglycosides

Impermeability due to a decreased uptake (by changes in LPS) [40, 50-53], and the activity of AMEs [52] are major aminoglycoside resistance mechanisms. *P. aeruginosa* can carry up to five different AMEs resulting in broad-spectrum aminoglycoside resistance [50]. Increased

efflux (by upregulation of MexXY) is a relatively rare resistance mechanism [37]. In 2003, another aminoglycoside resistance mechanism was reported in a clinical isolate from a Japanese patient [37]. This aminoglycoside-resistant *P. aeruginosa* clinical isolate possessed a 16S rRNA methylase (RmtA) responsible for 16S rRNA target modification resulting in high-level resistance to various aminoglycosides, including amikacin, tobramycin and gentamicin [37].

1.5. Resistance in *Burkholderia cenocepacia*

B. cenocepacia is a Gram-negative opportunistic pathogen grouped together with 19 other phenotypically similar species in the *Burkholderia cepacia* complex (*Bcc*) [54]. *B. cenocepacia* possesses a large genome (over 8Mbp in the case of *B. cenocepacia* J2315) compared to other Gram-negative bacteria and is known for its versatility (e.g., *Bcc* cause onion rot but also promote plant growth, or degrade pollutants in soil and groundwater) [55, 56]. Moreover, their original habitat, the rhizosphere soil, contains plenty other micro-organisms producing antibiotics, generating a selective pressure resulting in a species with innate resistance to a great part of our antibiotic drug arsenal (including β-lactams, aminoglycosides, and fluoroquinolones) [55-58]. The extreme resistance is shown by the ability of *Bcc* species to use penicillin as a carbon source and to survive in chlorhexidine solutions [47]. *B. cenocepacia* possesses Ara4N as a constitutive part of the lipid A and inner core of the LPS and is thus resistant towards polymyxin B and antimicrobial peptides, antibiotics that are considered to be the last-resort treatment of MDR Gram-negative bacteria [49]. In addition, many other mechanisms conferring resistance towards polymyxins and AMPs are described, including the efflux pump NorM [49]. Similarly, aminoglycoside self-promoted uptake is disturbed in *B. cenocepacia*, explaining their resistance [59]. Other resistance mechanisms, like β-lactamases and efflux pumps expelling aminoglycosides, chloramphenicol, fluoroquinolones, and tetracyclines are present in *B. cenocepacia* as well [60, 61].

United we stand, divided we fall [62]

2. Biofilms: what, where and why?

Since Van Leeuwenhoek, Koch and Pasteur described their observations, centuries of research followed unraveling prokaryotic life. Now, more than ever before we know how bacteria behave, cause infections and persist. The term 'biofilms' was first introduced by Costerton et al. in 1978 [63], and since then the awareness grew that this mode of bacterial growth contributes to the inability to cure certain infections, as one of their characteristics is a reduced susceptibility towards antimicrobial agents.

2.1. Biofilms: some important characteristics

Although bacteria are single-celled organisms, they can organize themselves in complex sessile multicellular consortia, known as biofilms [64, 65]. Biofilms are formed everywhere: in industrial settings, in natural environments, and in the human body [66]. Bacterial cells residing in biofilms are attached to each other or to a surface and they are embedded in a matrix of extracellular polymeric substances [65]. The biofilm cells are phenotypically different from their planktonic counterparts because growth rate and gene transcription is altered [65].

2.2. Biofilm formation

Biofilm formation is a complex developmental process [67]. Adhesion, proliferation, and detachment are the three mayor steps in biofilm formation [68]. The process is dynamic and starts with planktonic bacteria that attach reversibly to a surface (Figure 2). Pili, flagella, receptors, or other adhesive surface appendages make contact with biotic or abiotic surfaces [69]. Adhesion is followed by the secretion of extracellular polymeric substances resulting in an irreversible attachment [69]. Subsequently, cells proliferate resulting in the formation of micro-colonies. The biofilm grows and cells differentiate, resulting in a mature biofilm with multilayered cell clusters [65, 69]. Quorum sensing (QS), or cell-to-cell signaling, plays an important role in the development of the biofilm [65]. Finally, cells disperse from the biofilm and form biofilms elsewhere [70]. This dispersion happens passively by external forces like hydrodynamic shear stress [67] or actively initiated by the bacteria themselves. This active detachment is under the control of several sensory systems, e.g. QS [68] and c-di-GMP [67] and involves surfactant-like molecules [68].

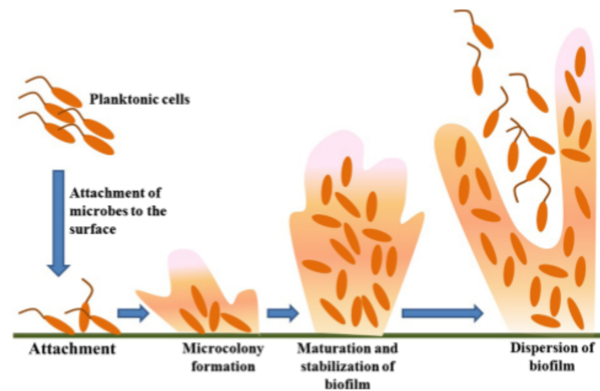

Figure 2. Simplified representation of the different steps in biofilm formation. After attachment of planktonic cells to a surface, they produce an extracellular polymeric matrix (EPM) and form micro-colonies, resulting in a mature biofilm. Finally, cells disperse and the process can start again [70].

2.3. Biofilm structure and organization

A biofilm is built up out of microbial cells and their self-produced extracellular matrix, holding the cells together and accounting for approx. 90% of the biofilm biomass [67]. The matrix contains proteins, exopolysaccharides and extracellular DNA (eDNA) [71] and provides the cells with water and nutrients. The composition of the matrix is species dependent, e.g. the matrix of *P. aeruginosa* biofilms might contain the exopolysaccharides alginate, Pel, and Psl [72], while the matrix of *S. aureus* strains possessing the ica-operon is composed of polysaccharide intercellular adhesin (PIA), which is a polymer of N-acetylglucosamine (PNAG) [73, 74], and teichoic acids [68]. Moreover, major differences in exopolysaccharide composition are observed within a bacterial species as well. For instance, mucoid strains of *P. aeruginosa,* as encountered in lungs of cystic fibrosis (CF) patients, produce alginate while other *P. aeruginosa* strains do not produce alginate [75]. Besides being strain-dependent, the matrix composition is also determined by environmental conditions like medium, substrate, and the age of the biofilm [73, 75]. Young *P. aeruginosa* biofilms were dispersed upon DNase treatment, suggesting eDNA is an important matrix component. In contrast, no dispersion was induced in mature biofilms, as other extracellular polymeric substances formed the biofilm matrix [75]. The shape of the biofilms is dependent on several conditions, for instance, if glucose is available as a carbon source, mushroom-shaped biofilms will be formed in flow-chambers, while flat biofilms are formed if citrate is the only available carbon source [75]. Cell clusters within the biofilm structure can grow to considerable sizes (up to a few millimeters) and water channels provide transport of metabolic waste products and nutrients [76]. These water channels are maintained by rhamnolipids in *P. aeruginosa* biofilms [75]. Biofilms shield cells from the activity of chemical disinfection or antimicrobial therapy, and from host immune defenses like phagocytosis and opsonisation [69, 71]. This contributes to their recalcitrant nature.

2.4. Recalcitrance of biofilms

Biofilms show a reduced susceptibility to antimicrobial agents compared to planktonic cells. Both resistance and tolerance mechanisms contribute to this recalcitrance [65, 77]. Resistant bacteria grow in the presence of antibiotics, and the mechanisms enabling them to do so are

described above. In contrast, tolerant bacteria survive a transient exposure to otherwise bactericidal antibiotics because they grow slowly and have a reduced metabolism [78]. Some relevant examples of resistance, tolerance and other mechanisms contributing to the recalcitrance of biofilms will be given in the following paragraphs.

2.4.1. Reduced growth rate and metabolic activity

Bacteria residing in the deeper biofilm areas have a reduced growth rate and metabolic activity due to oxygen and nutritional limitations and the accumulation of toxic waste products. Moreover, beside nutrient limitations, a general stress response is responsible for slow growth in cells residing in biofilms [79]. For instance, the expression of sigma factor *rpo*S was increased in biofilms [80]. RpoS mediates the expression of many genes involved in stress and starvation processes (e.g. RpoS controls the regulation of 10% of the *Escherichia coli* genome) [81]. Although it was thought that RpoS was only expressed during stationary phase, it is recognized now that RpoS expression is induced at high cell densities, thus also in biofilms. RpoS mRNA was identified in sputum of cystic fibrosis patients chronically infected with *P. aeruginosa* [79].

The slow-growing cell subpopulations have an increased tolerance to antibiotics that target processes active in dividing or metabolically active bacteria [65, 67, 69, 75]. For instance, inactive subpopulations are tolerant to ciprofloxacin, tetracycline, or tobramycin, while active subpopulations are sensitive [65]. However, this decreased susceptibility of inactive subpopulations does not apply to all antibiotics: colistin kills the slow growing subpopulation, while the metabolically active subpopulation survives by inducing the expression of MDR efflux pumps and by modifying the LPS with aminoarabinose [75, 82].

2.4.2. A specific phenotype

The gene expression between planktonic and biofilm cells is altered which partially contributes to the difference in resistance observed. Various genes are expressed only in biofilms, e.g. *ndvB* gene in *P. aeruginosa* PA14 strain [75]. In these biofilms, *ndvB* gene products produce periplasmic glucans that sequester tobramycin [75]. Another gene, *tolA*, was upregulated in *P. aeruginosa* biofilms compared to planktonic cells [83]. TolA changes the LPS structure resulting in a decreased binding affinity for aminoglycosides and other polycations [83].

Several studies report increased activity of efflux pumps in biofilms [65, 84]. E.g., PA1874-1877, an efflux pump in *P. aeruginosa*, was more expressed in biofilms than in planktonic cells, conferring biofilm resistance towards several fluoroquinolones and aminoglycosides [65]. Also, as biofilms consist out of several phenotypic subpopulations, the expression of efflux pumps is dependent on the location in the biofilm, for instance, a higher expression of MexAB-OprM in cells populating the substratum was observed in *P. aeruginosa* biofilms [65, 85].

2.4.3. Reduced antibiotic penetration: sequestration and deactivation

In general, diffusion of most antibiotics through the biofilm is not hampered [76, 86], however, positively charged antibiotics like aminoglycosides poorly penetrate the biofilm as they are sequestered by the EPM [86, 87]. Addition of alginate lyase and DNase to mucoid *P. aeruginosa* biofilms increases the diffusion of these antibiotics through the biofilm [75, 82]. In contrast, the inability of ampicillin to diffuse through a *Klebsiella pneumoniae* Kp1 biofilm is not due to sequestration, but to the activity of β-lactamases [80]. Also in *P. aeruginosa* biofilms, β-lactamases accumulate within the biofilm resulting in β-lactam neutralization while penetrating the biofilm [70, 82, 88].

2.4.4. Horizontal gene transfer and mutators

Horizontal gene transfer within the biofilm happens more frequently than in planktonic cells, [89] since biofilm bacteria typically reside closer to each other [82]. The efficient transmission of antimicrobial-resistance genes between bacteria of the same species or between different species can make the biofilm community more resistant and virulent [67, 69, 70]. Also, *P. aeruginosa* biofilm cells are characterized by an increased mutability compared to planktonic cells, for example, a hypermutable subpopulation ('mutators') is present in the lungs of CF patients [82]. Mutators, deficient in the DNA mismatch repair system, exhibit high frequencies of point mutations, and hence develop rapidly antimicrobial resistance [34, 90].

2.4.5. Persister cells

Persisters are a small subpopulation of drug-tolerant dormant cells. They are not mutants as re-inoculation of persister cells gives rise to a population with both persisters and non-persisters. Persisters are present both in biofilms and planktonic cultures, but those in biofilms are problematic as the matrix protects them from host immune defenses [77]. When an antibiotic therapy is stopped, the remaining persister cells act as a reservoir and are responsible for repopulating the biofilm (Figure 3) [67, 89, 91]. More and more it is accepted that persister cells are an important cause of recalcitrance in biofilm infections [91].

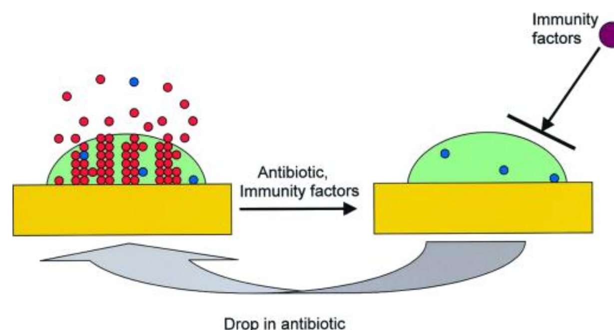

Figure 3. Representation of the killing of non-persisters (red dots) in the biofilm and in the planktonic culture. However, host immune factors and antibiotics are unable to kill surviving persisters (blue dots) in the biofilm, while planktonic persisters are efficiently removed [77].

2.5. Biofilm infections

Commensal biofilms are encountered in dental plaque and in the intestine [92], but biofilms are also estimated to be involved in 80% of all bacterial and fungal infections [70]. Biofilm-related infections are a serious clinical problem as they are often very difficult to eradicate [65, 93]. Biofilms might arise on medical indwelling devices, such as endotracheal tubes, vascular or urinary catheters, prosthetic cardiac valves, implants and prosthetic joints. In addition, biofilms are formed in CF patients' lungs, and in chronic wounds, and they are involved in infections like otitis media, chronic sinusitis and vaginosis [93].

The bacteria used in the experiments in this dissertation are important pathogens involved in respiratory tract infections in CF patients and in chronically infected wounds. For this reason I will focus on these two clinical conditions in the following paragraphs.

2.5.1. Biofilm infections in cystic fibrosis patients

2.5.1.1. Cystic fibrosis

CF is the most common lethal autosomal recessive genetic disorder in the Caucasian population, affecting one in 2500 [94]. Although life expectancy has increased drastically, many CF patients still die in young adulthood [95]. CF originates in mutations in the cystic fibrosis transmembrane conductance regulator gene (*CFTR*), coding for a chloride channel [96]. A defect in chloride secretion in the respiratory epithelium results in the production of viscous dehydrated mucus. Mucociliary clearance is impaired in removing this thick mucus layer, which subsequently forms an ideal environment for bacterial colonization and infection [96].

2.5.1.2. Bacteria causing lung infections in CF patients

The lower respiratory tract of young CF patients is colonized with *S. aureus* and *Haemophilus influenzae* [97]. However, from later infancy on *P. aeruginosa* predominates [97]. Other micro-organisms that might be involved in lung infection are *Bcc* species and *Stenotrophomonas maltophilia*, among others (Figure 4) [97].

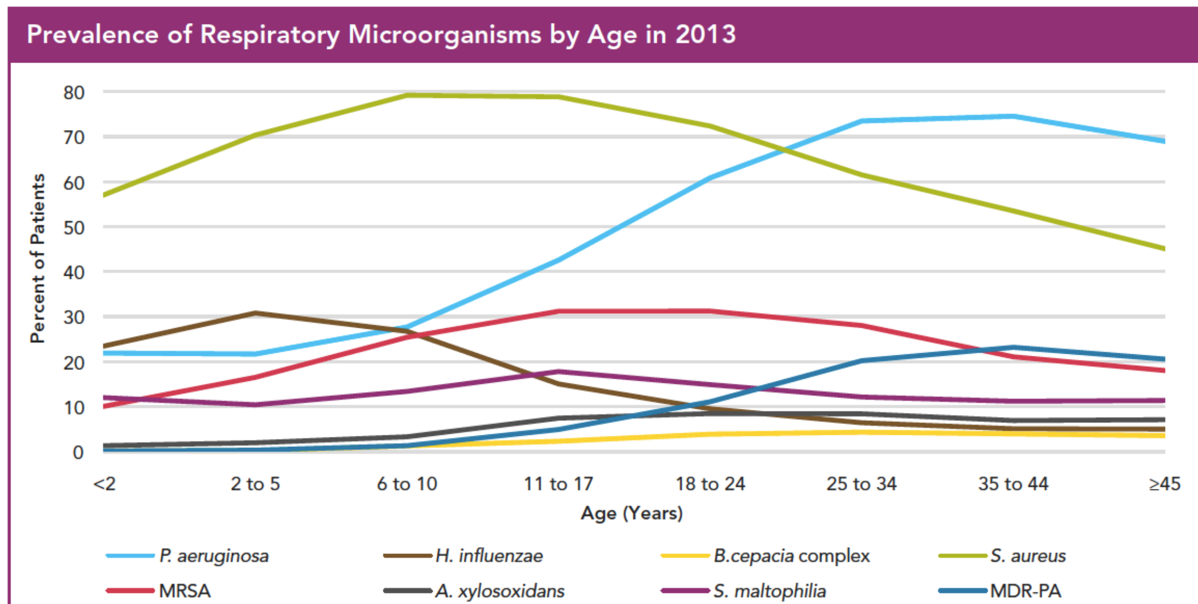

Figure 4. The distribution and evolution of bacterial species colonizing the lung of CF patients in function of their age [98].

Chronic lung infection with *P. aeruginosa* and subsequent severe lung damage is the main cause of morbidity and mortality in CF patients [87, 95]. Initially, CF patients become infected with non-mucoid *P. aeruginosa*. However, the CF lung environment stimulates a switch towards a mucoid phenotype [99]. This switch occurs after a mutation in the anti-sigma factor *mucA* [100]. In contrast to the non-mucoid phenotype, the mucoid phenotype overproduces alginate. Alginate activates a strong immune response that is harmful for the surrounding lung tissue [101]. Moreover, alginate causes an increased resistance towards antibiotics and the activity of host immune factors as this mucoid matrix protects the micro-colonies [67, 99].

Also *Bcc* species form biofilms in the lungs of CF patients [58]. Up to 8% of CF patients are infected with *Bcc* [102], of which *B. cenocepacia* and *B. multivorans* are the most prevalent (85-97% of all *Bcc* infections) [103]. *Bcc* infection can result in the cepacia syndrome, which is an invasive and often fatal pneumonia with acute deterioration of the lung function and septicemia [56, 87].

2.5.1.3. Antimicrobial treatments in CF patients

The treatment of pulmonary infections in CF patients has improved greatly due to the introduction of inhaled antibiotics (tobramycin, amikacin, colistin, aztreonam, and levofloxacin) [95, 104]. Advantages of inhalation therapy are the relatively quick and simple administration and a targeted drug delivery [104]. This targeted delivery allows high intrapulmonary drug concentrations, while systemic toxicity is limited [104].

Preventing the establishment of chronic *P. aeruginosa* infection is the focus of current therapy; patients are isolated from those with a chronic infection, and an aggressive eradication therapy is initiated when colonization is identified [82]. This therapy consists out

of nebulized tobramycin as monotherapy or oral ciprofloxacin in combination with nebulized colistin for 3 weeks to 3 months [82]. Additionally, long-term daily nebulization of DNase helps to prevent infection with *P. aeruginosa*, reducing the need for antibiotics and enhancing the lung function [82, 105]. This approach has changed the epidemiology of chronic *P. aeruginosa* lung infections in CF patients: chronic lung infections with *P. aeruginosa* that were common in children, now mainly occur in older patients [82]. Chronically infected patients require a life-long antibiotic treatment to suppress the infection [82], which consists out of at least one nebulized antibiotic: tobramycin, colistin, or aztreonam [104]. Inhaled tobramycin has the most impact in improving the lung function, as well as in reducing the bacterial load and the number of exacerbations [104]. In addition, this chronic suppressive therapy is supplemented every 3 months with other antipseudomonal antibiotics during 2 weeks [82]. CF patients facing exacerbations of *P. aeruginosa* infection are treated with two anti-pseudomonal antibiotics with distinct mode of action: a β-lactam (ceftazidime, cefepime, piperacilline/tazobactam, meropenem, or aztreonam [106]) combined with an aminoglycoside or ciprofloxacin [107]. Effective chronic suppressive therapies are lacking in case of *Bcc* infections [58] and no standardized regimens to treat *Bcc* have been developed [108].

In addition, different antibiotic adjuvants are currently investigated. These adjuvants might improve the efficacy of antibiotic treatments by increasing the susceptibility of the bacteria/biofilms towards the antibiotics or the host immune system, or they might cause a reduction in bacterial virulence [95]. For example, the alginate oligosaccharide OligoG increases the activity of macrolides, tetracyclines and β-lactams towards formed biofilms of *P. aeruginosa* and *Bcc* and is currently investigated to treat CF patients in a clinical trial phase II [95]. Garlic extracts (allicine and ajoene) have anti-biofilm properties as well [95] and interfere with QS [109]. Garlic-treated biofilms of *P. aeruginosa* were more susceptible towards tobramycin than the control and this was also proven in a mice pulmonary infection model. However, a clinical trial in 26 CF patients could not demonstrate a significant difference compared to placebo [95]. Gallium formulated with gentamicin in liposomes increased the activity of gentamicin against *P. aeruginosa* CF clinical isolates *in vitro* [110] and gallium nitrate was active towards *P. aeruginosa* biofilms *in vitro* and in animal models [111]. The latter is currently in phase II clinical trial for CF patients [112].

2.5.2. Chronic wounds

Chronic wounds, including pressure sores, venous ulcers, and diabetic foot ulcers, are a major cause of morbidity and 1-2% of the population in developed countries will face a chronic wound in their lifetime [113, 114]. The incidence is increasing with increasing life expectancy, and an increase in life style diseases such as diabetes, obesity, and vascular diseases. Many factors contribute to the emergence and persistence of chronic wounds, including patient’s factors like an uncontrolled immune response, but also the microbial load and type of bacterial species determines if a wound becomes (chronically) infected [115].

2.5.2.1. The wound healing process, biofilms, and the uncontrolled immune response

The normal wound healing processes, i.e. coagulation, inflammation, cell proliferation and re-epithelialization, are disturbed in chronic wounds [113, 116]. Although the role of biofilms is

not completely understood, it is assumed that biofilms arising after wound colonization are responsible for a delay in healing and development into a chronic state as they induce chronic inflammation [76, 117, 118]. This hypothesis is supported by the fact that biofilms were present in only 6% of acute wound specimens, compared to 90% in specimens of chronic wounds [76, 119]. In normal wound healing, bacteria and debris are removed in the inflammatory phase and this prevents infection [120]. In chronic wounds, however, the inflammatory phase is prolonged because polymorphonuclear neutrophils (PMNs) that migrated to the tissue around the wound to kill the invading bacteria, are ineffective in clearing the biofilm cells [76]. PMNs release toxic compounds, for instance reactive oxygen species (ROS) and elastase, that contribute to severe surrounding tissue damage and non-healing of the wound [76]. Beside toxic compounds released by immune cells, also bacteria release toxins enabling them to invade deeper into the tissue, contributing to the host damage [121].

2.5.2.2. Four situations depending on the bacterial load present in wounds

The term contamination is used to describe the condition in which non-replicating bacteria are temporarily present in wounds, while the term colonization refers to growing bacteria in wounds. In both conditions, there is no host damage and wound healing is not delayed [113]. In contrast, higher numbers of bacteria prevent wounds from healing. The transition between colonization and non-healing is thought to take place when over 10^5 colony forming units/gram tissue are present [122]. Local infection or critical colonization is the condition between colonization and infection. In this situation, bacteria do cause a delay in wound healing [113]. This situation is often observed in elderly patients with an impaired immune response [121], or when bacteria form biofilms [123]. Critical colonization is characterized by non-healing wounds that clinically not look infected [120]. Critical colonization can finally result in an infection with host injury, clinical signs like redness, pain, and warmth, or septicemia [123]. However, these classic signals are not always displayed in chronic wounds. Foul odor, wound breakdown and discoloration of granulation tissue are other signs pointing towards infection [122].

2.5.2.3. A diverse microbial flora in chronic wounds

In most cases, the microbiota in a wound is composed out of two to five different bacterial species [76]. Initially, skin commensals such as *S. aureus* and β-hemolytic streptococci are found in wounds [29]. For example, *S. aureus* was isolated from 43% of infected and 88% of clinically non-infected leg ulcers [122]. In a later stage, Gram-negative bacteria, especially coliforms, and anaerobes are encountered as well [29].

2.5.2.4. Antimicrobial treatment of chronic wounds

Treatment of chronic wounds is a multistep approach, starting with debridement in which necrotic tissue, senescent cells, bacteria, or foreign material are removed [69, 119]. There is sufficient evidence that frequent (weekly) debridement increases the healing rates of chronic wounds [119]. Debridement cannot remove the biofilm completely, but it forces the biofilm to

reattach and reform. During this time (2-3 days), the biofilm is more susceptible to antibiotics and host immune defenses [119].

Antibiotic therapy should be fine-tuned upon the identification of the causing pathogens, their antibiotic susceptibility and the patient's clinical response [124]. Wounds with clinical signs of infection are often treated with systemic antibiotics [125]. Topical antibiotics, like fusidic acid, mupirocin, polymyxin, or metronidazole are also used in low-grade infections [125-127]. However, clinical signs of infection are not always clearly present in chronic wounds. It is often difficult for clinicians to determine if the micro-organisms present are responsible for the non-healing and if subsequently antibiotic therapy is appropriate and justifiable [29]. Generally, clinically non-infected wounds should not be treated with systemic antimicrobial therapy [128]. Moreover, sufficient evidence for the effectiveness of antibiotics in treatment of chronic wounds is lacking [29, 122]. Lipsky et al. recommend a topical antiseptic for a short time in chronic wounds with no clear clinical signs of infection [125]. However, also the use of antiseptics in treatment of chronic wounds is a subject of debate [129]. Antiseptics are cytotoxic to cells involved in the wound healing process, although this cytotoxicity is mainly observed solely *in vitro* [129]. Moreover, the cytotoxicity depends on the concentration of the antiseptic [129] and it is not observed when applying ointments, gels, or dressings with a more sustained release, in contrast to solutions [125]. An example to illustrate the complexity is povidone iodine; *in vitro* data suggest cytotoxicity at 10%, while this is not observed clinically, and a 3% povidone iodine hydrogel has shown to contribute positively to wound healing [126]. In addition, iodine is active towards *S. aureus* and *P. aeruginosa* biofilms [126], justifying its use in chronic wound treatment. Moreover, antiseptics are preferred over topical antibiotics, as antibiotics are more prone to resistance development [125, 129]. In addition, the topically used antibiotics neomycin and bacitracin cause contact sensitization in 10% of patients [127, 129].

Beside debridement and subsequent topical or systemic antibacterial treatment, other measures like wound hydration are indispensable [130], together with tackling the underlying diseases that are the primary cause of chronic wounds [122].

It is perhaps ironic that the current challenges are in part the result of the past success [131]

3. From golden era to discovery void

Almost all antibiotic classes currently in use were discovered between the 1930s and 1980s (Figure 5). Consequently, this era is referred to as the golden era in antibacterial drug discovery. In 1967, the US Surgeon General declared "that we had essentially defeated infectious diseases and could close the book on them" [91, 132]. Pharmaceutical industry focused more on treatments for cancer and chronic diseases. However, due to the emergence of MDR bacteria, new antibiotics are urgently needed.

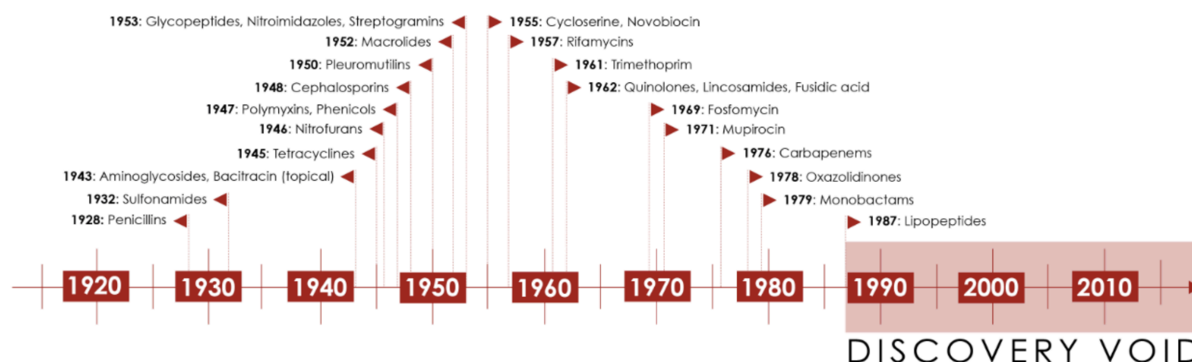

Figure 5. Time-line representing the discovery of new antibiotic classes. Most antibiotics were discovered between 1930 and 1980. In the following years, referred to as the discovery void, very few new antibiotics were discovered [133].

3.1. The golden era: 1930s to 1980s

The discovery of sulfonamides and penicillin in the 1930s was the start of a golden era that lasted more than 40 years [19, 132]. Aminoglycosides, and later tetracyclines, cephalosporins, macrolides, glycopeptides, quinolones, and carbapenems were developed [132, 134, 135]. Most of these drug classes were discovered by empirical screening of natural products [132, 136]. The basic chemotypes of these classes were used as scaffolds to develop new analogues using medicinal chemistry. This synthetic tailoring resulted in drugs with a broader spectrum, an increased potency, a better safety profile and/or a new possible route of administration [132, 136]. This is exemplified by the fact that from 12 antibacterial scaffolds over 200 antibacterial analogs were developed and approved, especially β-lactams, tetracyclines, quinolones, and macrolides [132]. Although these derivatives are likely to more rapidly lead to resistance by existing mechanisms [135], optimization of antibiotics was and is still important in antibacterial drugs discovery [137].

However, between 1970 and 1999, mupirocin was the only drug marketed with a new mode of action. All other antibiotic drugs developed in this period were modifications of existing drugs [19, 137].

3.2. A new millennium, a new hope?

Several initiatives were launched to stimulate the investigation for new antibiotics, e.g. the US Generating Antibiotic Incentives act, the 10x'20 act from the Infectious Diseases Society of

America, and the Innovative Medicines Initiative New Drugs for Bad Bugs, the Urgent Need, Antibiotic Action initiative and the Antibiotic Resistance (ReAct) in Europe [19, 135]. From 2000 on, twenty-six new antibacterial drugs and two new β-lactam/β-lactamase inhibitor combinations were approved by the Food and Drug Administration (FDA) [19, 138-143]. These new drugs are mainly derivatives of existing scaffolds, i.e. nine quinolones, three glycopeptides, a macrolide, four carbapenems, two cephalosporines, and a tetracycline [19]. Nevertheless, six of the newly approved drugs belong to five new classes, which is a considerable improvement over earlier years (Table 2) [19]. However, despite being new marketed classes of antibiotics, these antibiotics are based on old discoveries as the oxazolidinones, the lipopeptides, and pleuromutilines were patented already in 1978, 1987, and 1952, respectively [144, 145]. Moreover, none of these first-in-class drugs is active against Gram-negative micro-organisms [19].

Table 2. The first-in-class antibiotics launched since 2000. Based on information from [19]. C. = Clostridium, M. = Mycobacterium.

Year of approval	Name	Class	Spectrum	Mode of action
2000	linezolid	oxazolidinone	Gram-positive	protein synthesis inhibitor
2003	daptomycin	lipopeptide	Gram-positive	formation of cell membrane pores
2007	retapamulin	pleuromutilin	Gram-positive	protein synthesis inhibitor
2010	fidaxomicin	tiacumicin	*C. difficile*	binds RNA polymerase
2012	bedaquiline	diarylquinoline	*M. tuberculosis*	ATP synthase inhibitor

3.3. The low commercial potential of antibiotics

In 1990, research and development (R&D) for antibacterial drugs was operating in 18 big pharmaceutical companies [19]. By now, only AstraZeneca, Novartis [146], GSK, Sanofi-Aventis [19], and Merck [6] remain [147] and many R&D is done in small biotechnological companies. For instance, in 2005, 7 antibacterials were in clinical development in large pharmaceutical companies, while 12 in biotech companies [132]. One of the strategies used by small biotech companies is the purchase of niche products from big pharma that quit their development [132]. For instance, Abbott Laboratories discovered and patented tiacumicins in 1986. However, only 26 years later, the small pharmaceutical company Optimer Pharmaceuticals launched tiacumicin B or fidaxomicin (Dificid), a first-in-class drug to treat diarrhea caused by *C. difficile* [19]. Nevertheless, Optimer Pharmaceuticals had to close down their business as the revenue for Dificid was disappointing due to the availability of cheap generics [148]. Niche products, addressing only a small segment of the market, are not commercially interesting as no broad or empirical use is possible [132]. Only if diagnostic methods improve, these niche antibacterial drugs will become interesting for big pharma. Till then, pharmaceutical companies will focus on broad-spectrum agents [132, 147].

The exodus of pharmaceutical industry from antibacterial R&D two decades ago [149] was due to (i) scientific, (ii) economic and (iii) regulatory challenges [147].

First of all, the identification of new antibiotic classes is extremely difficult. Efforts to identify new antibiotics by screening large existing libraries of small molecules failed [133]. For instance, GSK performed 70 high-throughput screens between 1995 and 2001 but identified only in 7% of the screens lead compounds [147] of which none made it into clinical trials [135]. Screenings in other therapeutic areas have a success rate that is 10 times more in this phase [147]. A main obstacle is the bacterial impermeability [133].

Secondly, investment in R&D of new antibacterial agents is economically unattractive compared to other classes of drugs. Although costs of R&D have been rising last years for all classes of drugs, this is especially problematic for antibiotics as the return on investment is lower and the risk higher [15]. The return on investment in a traditional business model depends on the price of the new drug multiplied by the volume of sales, and both are low for new antibiotics for several reasons [147]. Antibiotics are mainly used in curative short-term therapies, while drugs used to treat chronic diseases are taken for a long period on a pill-a-day basis [132, 144]. Drugs in other therapeutic areas with similar short periods of treatment have been commercially lucrative thanks to high prices [15]. However, antibiotics are generally low in price because cheap generics prevail the antibiotic market and the price of new antibiotics is benchmarked against the prices of these generics [147]. In addition, new antibiotics will often be reserved for a small group of patients to prevent resistance development, limiting their use and subsequently the revenue [136, 147, 150]. Moreover, resistance will develop inevitably when the new drug is used, resulting in its devaluation [151]. However, resistance development is unpredictable, contributing to the economic uncertainty regarding return on investment of antibiotics over time [7]. These factors contribute to a limited (or even negative) and unpredictable return on investment and a new business model that de-links the volume of sales from the revenues is thus necessary [147, 151].

The regulatory requirements imposed by the FDA are a third barrier for development of new antibiotics as clinical trials have become more expensive and time consuming, while risk of failing increased [151]. A reformation in regulatory requirements is necessary, improving the feasibility and clinical relevance of clinical trials [151]. One of the ideas proposed is the Limited Population Antibacterial Drug regulatory approval pathway, in which trials focus on MDR pathogens [7, 151]. Drugs approved after such trials will receive a narrow indication label [7, 151]. These trials will be smaller and thus less expensive and shorter in time [7, 151]. Several initiatives were recently launched to attract the pharmaceutical industry back to antibiotic development and to fill the pipeline again. However, it is questionable if these initiatives are sufficient and if this eventually will result in the discovery of a sufficient amount of new antibiotics. The pharmaceutical industry is currently not able to meet the needs necessary to fight AMR, so it is important that academia remain dedicated into AMR research and development of new treatment strategies.

Natural products, an unsurpassed source of evolved chemical diversity [152]

4. Platforms used for antibiotic drug discovery

Most of the antibiotics currently in use were discovered by empirical whole-cell screening of natural products (NP). However, the systematic screening of the same pool of NP resulted in re-identifying similar classes of already known antibiotics [153]. This caused a switch towards a target-based approach and the application of new techniques based on genomics, high-throughput screening (HTS), structure based drug design (SBDD), and combinatorial chemistry [153].

4.1. Phenotypic whole-cell screening of natural products

NP are secondary metabolites produced by bacteria, fungi, and plants displaying antibacterial activity to protect themselves [132]. Particularly soil actinomycetes provided many NP that were a tremendous source for new antibiotics [132, 136]. Over 23,000 NP are characterized and they are complex and diverse chemical compounds with several stereo-specific carbon centers and sizes ranging from small (100 Da) to large molecules (> 1000 Da) [153].

NP were screened in a low-throughput manner, a method that did not change for several decades [144, 153]. Soil samples were collected all over the world, from which bacteria and fungi were isolated [153]. NP were extracted from culture broths and their effect on growth inhibition of test organisms was analyzed in whole-cell screens [145, 153]. A pharmaceutical company like Eli Lilly was able to test 35,000 strains annually applying this approach [153]. NP that exhibited antibacterial activity were included in subsequent assays. Toxicity tests in animals were conducted to evaluate the selectivity [145] and medicinal chemistry changed features of active NP resulting in antibiotics with more drug-like properties and less toxicity [132, 154]. Also, the mode of action was determined, although this was often not elucidated until years after the introduction to the market.

Gradually, a more target-based approach gained interest over empirical screenings [145]. For example, it was appreciated that, among others, the cell wall synthesis machinery had proven to be a successful and selective target. For this reason, Merck used another endpoint than growth inhibition in their phenotypic screens, namely the formation of spheroplasts [145, 155]. This screen resulted in the discovery of fosfomycin and thienamycin [145, 155].

However, interest in NP screening decreased as no new classes of antibiotics were discovered anymore. In 30 years, only one new class of antibiotics was discovered, represented by daptomycin [153]. Similar NP are abundant and these are consequently rediscovered when screening fermentation broths. These NP mask the presence of other (low-abundant) antibiotics [154], making it increasingly difficult to discover new classes [144]. An example of this is streptomycin, which is produced by approx. 1% of all actinomycetes, whereas daptomycin is produced by only 0.000001% of all actinomycetes [153, 154]. In order to overcome this background noise, ‘dereplication’ methods were introduced to separate known compounds from new compounds in NP extracts [145]. Anyway, in the 1990s, the platform

was largely abandoned, as alternative promising methods had become available to discover new antibacterial drugs [132, 156].

4.2. Genomics

In 1995, the first whole genome sequence of a bacterial pathogen (*H. influenzae)* became available and this was the start of the genomic era for antibacterial drug discovery [144]. Bacterial genomes were enormous sources of information, among others, revealing new targets for antibiotic drug discovery [132].

4.2.1. Target discovery

Genomes of relevant pathogenic bacteria were analyzed and compared using bioinformatics for conserved genes that were absent or do not share sequence similarity in humans [136], leading to the identification of 150-350 (number depends on the span of the bacterial spectrum) potential targets [137].

However, there are several reasons why a single target-based screening turned out not to be suitable for antibacterial drug development. First of all, whether a gene is essential or not can strongly differ among bacterial species, even between strains [136]. This might be due to additional analogous enzymes or the activity of alternative biochemical routes [156]. E.g. methionyl tRNA synthetase was thought to be a valid target as it showed a high sequence homology between several pathogens, among them *S. pneumoniae*. However, 30% of the clinical isolates of *S. pneumoniae* were not susceptible to potent inhibitors of methionyl tRNA synthetase as they possessed an unrelated methionyl tRNA synthetase [136]. Furthermore, essential genes *in vitro* might become non-essential during disease [157] as nutrients are readily available in the human body and might provide reaction products making the inhibited enzyme not necessary for survival [137]. In addition, the target has to be ‘druggable’, which means that it should possess binding sites for drug-like molecules and that binding of such a molecule results in a biological effect [145]. It is accepted now that many of the promising targets are ‘undruggable’ [157].

4.2.2. Finding an inhibitor for the selected target: HTS, SBDD, and synthetic compounds

After finding a potentially suitable target, inhibitors for this target can be identified using HTS of large chemical libraries [135-137]. An alternative to HTS is SBDD, an *in silico* method where ligands are designed based on the crystallographic structure of the drug target [135]. Although inhibitors with activity against the purified targets were identified in HTS or designed in SBDD*,* they often failed to have antibacterial activity due to inefficient intracellular penetration [135-137, 145, 154].

Between 1995 and 2001, GSK screened a synthetic compound library (consisting of 500,000 compounds) against 67 isolated targets from *S. pneumoniae*. The hit rate was low: leads were identified against 5 targets only, and none of them made it to clinical trials [135]. The major cause for failure in the GSK screening programs, and in HTS in other pharmaceutical companies, was the low molecular diversity in their library [145]. Indeed, the early combinatorial compound collections turned out to be worthless in antibacterial drug discovery

[158]. Traditional combinatorial chemistry creates from few chemical scaffolds many unique molecules, assembled in 'combi-chem' libraries [153, 158]. These compounds are stereochemical diverse and distinct in the appendages around the scaffold, but miss the so called 'skeletal diversity' [158]. The combi-chem libraries were used in combination with historical chemical product libraries [154] that were developed for screening programs in other therapeutic areas [136]. These libraries were not solely developed against enzymes, but also against G-protein coupled receptors or ion channels. These targets show little similarity with the bacterial targets, and consequently, the library compounds showed low affinity for the bacterial enzymes [145], adding to the low hit rate. Furthermore, most antibacterial drugs possess different characteristics compared to non-antibacterial drugs, for instance, they do not meet Lipinsky's rule of 5, they are larger, more complex and less lipophilic (Figure 6) [136, 154, 159]. Compounds that meet Lipinksy's rule of 5 tend to lack the potential to penetrate bacterial cells [135].

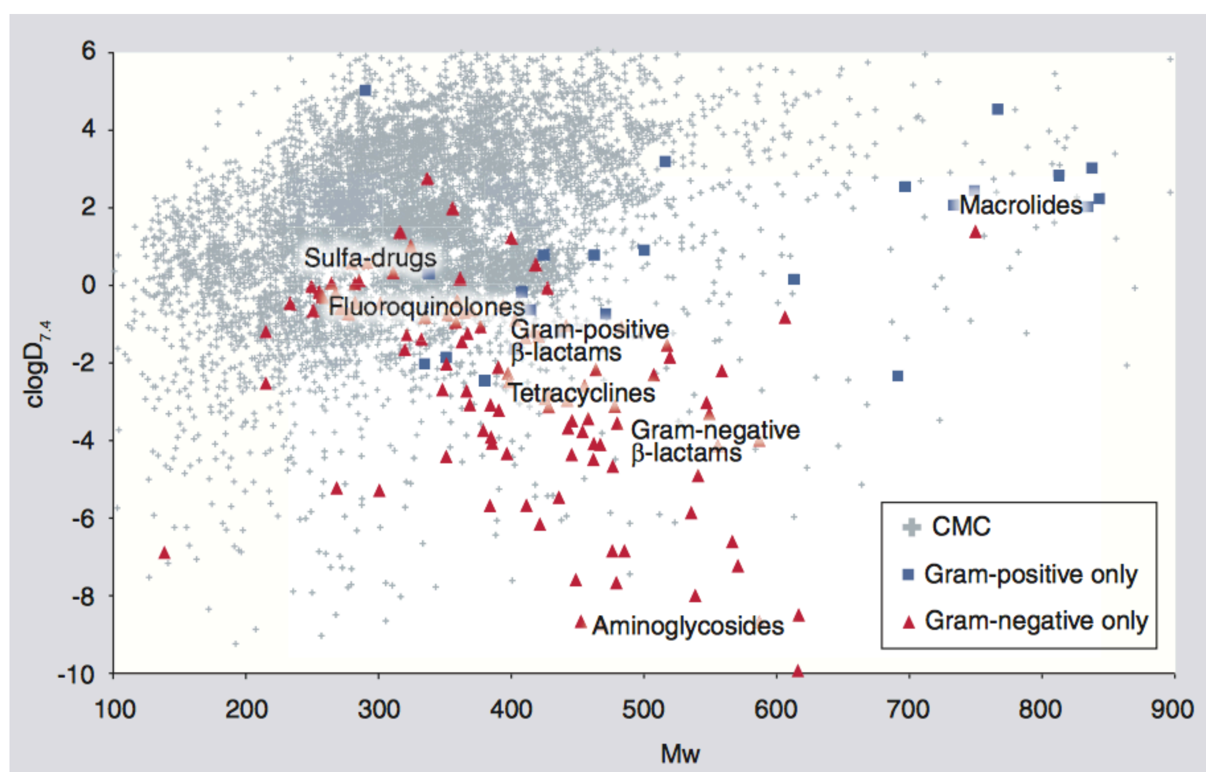

Figure 6. Antimicrobial drugs differ both in size (molecular weight, Mw) and lipophilicity (ClogD) from other drugs (CMC: 4623 compounds from the commercially available Comprehensive Medicinal Chemistry database) [137].

4.3. The post-genomic era

It is clear that the lack of success in the 1990s was not due to lack of innovation [145], but to the selection of a single target and its insufficient validation, the suboptimal *in vitro* screening process and screening huge random synthetic libraries with limited quality, complexity, and diversity [135, 137, 145, 159]. These important pitfalls from the genomic era have been comprehensively evaluated and helped to improve the current drug discovery methods. Genomics provided meaningful knowledge about important pathogens, as well as enabling

technologies, now applied in more sophisticated whole-cell screens [157]. Also, these techniques facilitate target identification and subsequently lead optimization [146, 160]. Moreover, there is again increased interest in NP, a proven source for antibiotic drug discovery [132, 154], now further explored by applying genome derived methods. Also the necessity of developing libraries focused on antibacterial research is recognized [137, 144, 161]. In addition, the use of AMPs as such or as lead molecules is currently further investigated, and several AMPs are in clinical trials. The naturally occuring AMPs are cationic amphipatic molecules with diverse structures (α-helical peptides, disulfide bound β-sheet peptides, loop structured peptides, or extended peptides) and sizes (ranging from 12 to 100 aminoacids) [162]. Hence, AMP represent a unique and large area of chemical space.

4.3.1. Revival of whole-cell screens

4.3.1.1. Classical empiric whole-cell screens

Recently conducted high-throughput whole-cells screens resulted in interesting hits, leads, and even marketed new drugs. The automation, miniaturization and techniques available for subsequent target identification, made whole-cell screenings attractive again [160].

Andries et al. screened a commercially available library against the rapidly-growing non-pathogenic surrogate *Mycobacterium smegmatis* [144, 160], and found compounds active against *Mycobacterium tuberculosis*, including a series of diarylquinolones that, after chemical optimization, led to the development of bedaquiline [160]. The mode of action was elucidated by whole-genome sequencing of cells that became resistant upon bedaquiline exposure [160]. Bedaquiline is the first antibacterial drug marketed that was discovered applying HTS [144].

A disadvantage of empirical whole-cell screenings for growth inhibition is the oft-recurring identification of compounds that disrupt the bacterial membrane in a non-specific way (detergent activity, alkylation, energy poisoning) [135, 145]. This is often an indication of cytotoxicity towards mammalian cells and hence these hits are useless [135].

4.3.1.2. Target-based whole-cell screens

Many hits identified in target-based cell-free screenings were useless as they lacked antibacterial activity. Now, target-based and whole-cell screenings are combined and this has resulted in the discovery of promising compounds [137, 157, 163, 164]. For instance, the NP platensimycin was discovered after screening 250,000 NP extracts in an 'agar diffusion two-plate differential sensitivity assay' [137, 163, 165]. In such screenings, the sensitivity of a target depleted mutant for the library compounds is compared to the sensitivity of the wild type strain [137].

Another method is the construction of reporter cells or biosensors [137]. A promoter that is selectively and strongly induced upon the inhibition or depletion of a selected target is fused with a reporter (e.g. *luxCDABE* operon of *Photorhabdus luminescens*) [159]. An inhibitor of the target will cause an up-regulation of the responsive promoter and hence also the reporter, resulting in the generation and emission of, in the case of the *luxCDABE* operon,

bioluminescence [159]. Reporter assays have been described for several bacteria, including *Bacillus subtilis* [135] and *P. aeruginosa* [159].

4.3.1.3. Whole-cell screens in the presence of mammalian cells

High content screening uses cells, e.g. macrophages, that are intracellularly infected with a pathogen, e.g. *Mycobacterium* [157]. This resembles more the *in vivo* situation, so hits found in these screenings are expected to demonstrate higher *in vivo* activity [157]. An additional asset in this approach is that cytotoxic compounds are immediately eliminated [157]. A phenotypic screen in which 57,000 compounds were tested against a GFP-labeled *Mycobacterium tuberculosis* H37Rv in Raw264.7 macrophages is described that resulted in several groups of active molecules [160, 166].

4.3.1.4. In vivo screens

Prontosil was discovered in 1932 by screening dyes in mice, and although it is impossible to screen the current libraries in mice, *in vivo* screening has several advantages over *in vitro* screening [144]. *In vitro* screenings might miss several promising compounds [144]. Some drugs require activation (also prontosil) by e.g. gut bacteria, and some bacterial targets are only expressed during infections [144]. A good alternative for mice is screening in the nematode *Caenorhabditis elegans* [144]. Furthermore, when screening in *C. elegans*, toxic compounds can be identified, and compounds with properties that would prevent them from being druggable (e.g. high protein binding), would not be identified as hits while they would bind to a target in *in vitro* screens [144]. More recently, other screening platforms for *Mycobacterium* species are described, in which the screening is performed in zebrafish larvae and amoeba [160]. However, the feasibility and asset of such platforms still has to be confirmed by screening large libraries [160].

4.3.2. Revival of NP discovery: back to the future

There are new NP waiting to be discovered, but it is thought that they are produced at an even lower rate as is the case for daptomycin, so in theory tens of millions of actinomycetes would have to be tested on an annual base. Off course, this is impossible applying the methods used decades ago, which allows testing only tens of thousands actinomyces annually [154]. However, Cubist Pharmaceuticals developed a high-throughput miniaturized fermentation and screening, enabling them to screen 10^7 actinomycetes annually [154].

Also, new sources can be invoked, e.g. marine actinomycetes [154], yielding new NP and thus potential new antibacterial drug scaffolds [132]. Furthermore, it is estimated that 99% of all bacteria on earth do not grow under laboratory conditions [144]. These uncultured bacteria are an untapped source of new NP and novel techniques to grow these are being developed [137, 144, 167]. An innovative approach to grow uncultured bacteria in a high-throughput manner is their *in situ* cultivation using the isolation chip or 'iChip' (Figure 7) [144, 168]. Ling et al. used this device to isolate and grow 10,000 uncultured bacteria, and tested subsequently the antimicrobial activity of their NP extracts against *S. aureus* NCTC8325-4. This resulted in the identification of teixobactin, a depsipeptide representing the first member of a new class of antibiotics [169]. An alternative for trying to culture uncultured bacteria is analyzing soil for

their DNA. Based on metagenomics, several companies were able to produce antibiotics from soil DNA (Figure 8) [144].

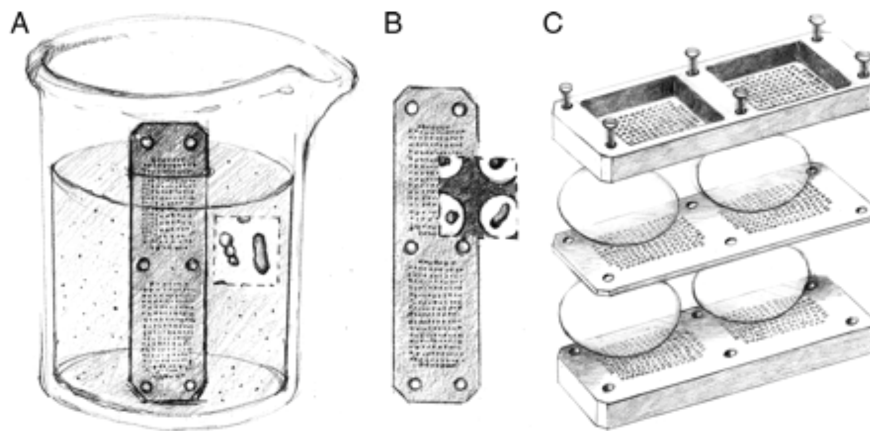

Figure 7. Representation of the iChip device. The iChip consists of hundreds diffusion chambers (wholes are 1 mm in diameter). A. The iChip is dipped in a diluted soil sample containing agar. B. Each whole captures a single cell C. Semi-permeable membranes prevent cell migration but allow diffusion of nutrients and growth factors, when the device is put back into the soil [168].

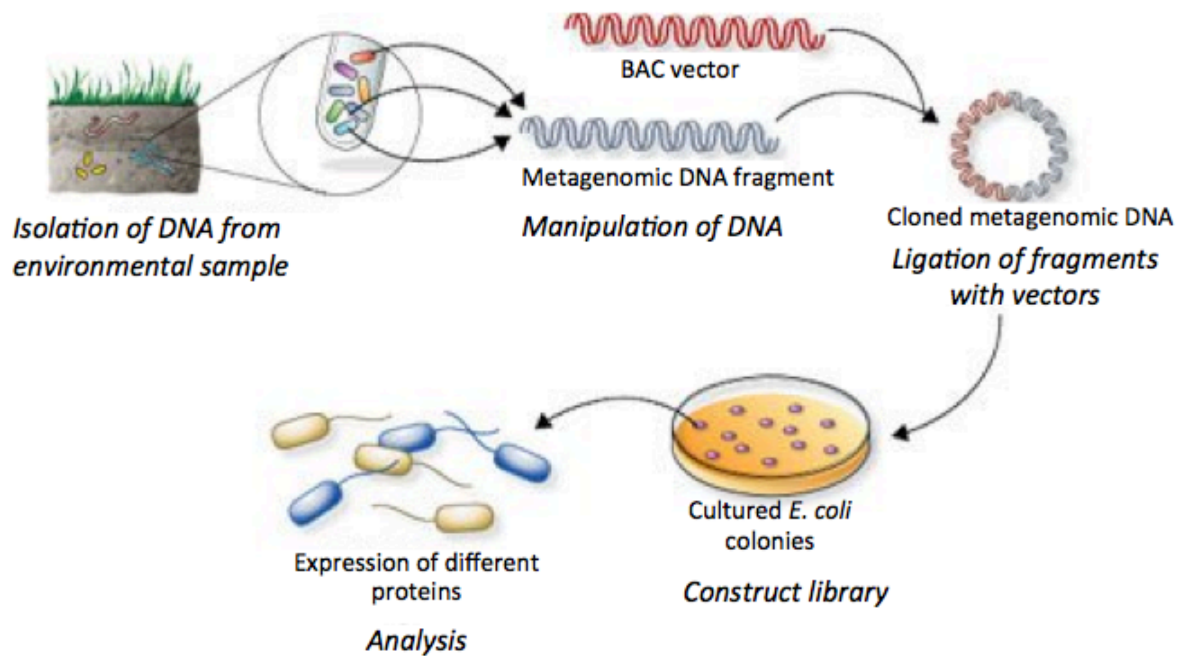

Figure 8. Simplified illustration of the metagenomic process applied to identify DNA of uncultivable bacteria in soil and producing NP from this isolated DNA, without necessitating to culture the uncultivable bacteria [170]. DNA isolated from soil samples is ligated in a Bacterial Artificial Chromosome (BAC). BAC is used as a vector and as it is based on F-plasmid DNA, it is passed through to the daughtercells when transformed into *E. coli*. Each colony of *E. coli* contains BAC with a different soil DNA fragment, resulting in a metagenomic library [171].

In addition, it is known now that the genomes of bacteria and yeast possess several gene clusters coding for enzymes producing secondary metabolites that are not expressed under conventional laboratory conditions. These cryptic pathways can be activated by several genetic methods, like overexpression of positive regulators, or by adapting the fermentation conditions [153]. As it is estimated that less than 10% of NP are produced by actinomycetes in fermentation broths, the other 90% cryptic gene clusters provide a source of new NP in known species [153, 154]. However, both the metagenomics approach with uncultured bacteria, as mining cryptic pathways, are very time-consuming and for this reason generally abandoned [144].

Mutational biosynthesis, precursor-directed biosynthesis, and combinatorial biosynthesis are molecular methods in which NP intermediates, precursors, or metabolic pathways are adapted resulting in new analogs of NP [153], while 'diverted total synthesis', 'function-oriented synthesis', and 'biology-oriented synthesis' are some examples of chemical approaches to synthesize analogues of NP [172].

It is clear that it is more difficult than sixty years ago, when new classes of antibiotics were well accessible as screening soil bacteria was enough. However, we have many tools now to find new antibiotics in sources that were thought empty before [154] or, like Baltz says: 'We are in the early stages of a Renaissance in antibiotic discovery for actinomycetes'[154].

Nevertheless, NP might have severe limitations concerning stability, toxicity, pharmacokinetic, and their production, making the development into marketed drugs sometimes very difficult [137]. Thus beside NP research, other strategies should be followed as well.

4.3.3. The position of synthetic compounds in the post-genomic era

Beside the HTS of pure NP [152], NP extracts or fermentation broths [163], there is definitely a future for synthetic libraries and SBDD as well.

4.3.3.1. Chemical space and Diversity-Oriented Synthesis

Classic combinatorial chemistry generated libraries of insufficient skeleton-diversity, and thus covered only a concise part of the chemical space (Figure 9) [158]. However, the greater the chemical space occupied by a library, the greater the chance to find a compound that has affinity for a biological target [158]. Obviously, it is impossible to cover the entire chemical space in a single library, and the question remains as to what is the biologically relevant chemical space that has to be addressed in a library. It is unknown whether we should look in the region where NP and known drugs reside or whether completely new drugs can be found in 'un-tapped areas of chemical space' [158].

Diversity-Oriented Synthesis (DOS) of compound libraries leads to smaller libraries than with combinatorial chemistry, but the compounds are more complex, stereochemically more diverse, and most importantly, the compounds differ greatly in their core structure [158]. The purpose of DOS is to design more suitable small molecule libraries that cover a considerable

part of the chemical space, including known bioactive areas of chemical space as well as unknown and unexplored regions [158].

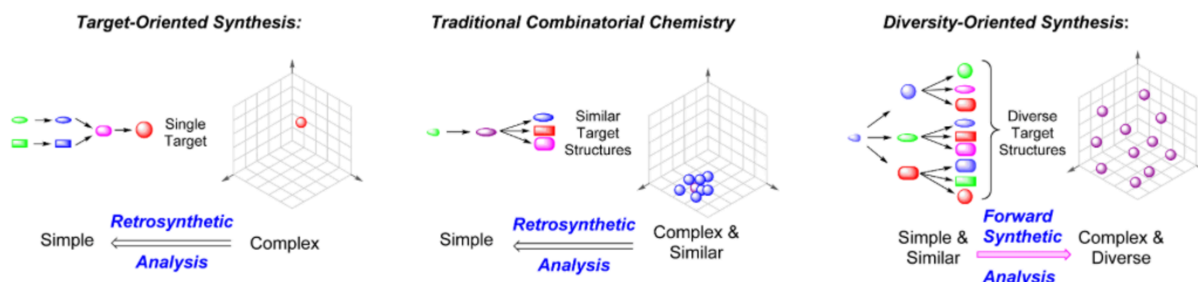

Figure 9. Comparison between target-oriented synthesis, traditional combinatorial chemistry and DOS. The chemical space occupied by DOS is more diffuse compared to traditional combinatorial chemistry [173].

4.3.3.2. Establishment of rules for intracellular penetration to build new libraries

It is clear that both more diverse and biological relevant compound libraries have to be designed. Antibiotics possess distinctive physicochemical properties compared to other drugs and these characteristics should be taken into account when designing new drugs. So far, general 'rules' for good antibiotic penetration into cells, especially into Gram-negative organisms are lacking, as the factors that determine uptake and efflux are only partially understood and each barrier is different [137, 147]. However, some features or rules of penetrations have been proposed [144, 145] and these can be used in guiding the development of synthetic compound libraries specific for antibacterial screenings and SBDD [144, 147].

The rules for penetration should be established based on characteristics of compounds that do penetrate into bacteria, however, the number of compounds that do so is limited [144]. Some deductions were made already that can be implemented when new libraries are made [144]. Good permeability is expected for fairly hydrophilic compounds smaller than 600 Da, as these compounds are able to be transported through porins [137, 145]. Also zwitterionic compounds are more likely to penetrate Gram-negative bacteria [137] and anions are poor efflux pump substrates [144]. In contrast, hydrophobic cations will penetrate poorly as they are preferred substrates for MDR efflux pumps [144]. Interestingly, it has been observed that synthetic compounds containing fluorine and boron, which are not often encountered in NP, are favorable for penetration [144]. The successful synthetic fluoroquinolones confirm this. Considering these characteristics in screenings for and design of new antibacterial compounds proved to be a valuable approach, as exemplified by the activity of Anacor Pharmaceuticals. This company used their boron chemistry platform in antibacterial drug discovery [144, 145, 174]. They found that ABX, a first-in-class compound that inhibits bacterial leucyl tRNA synthetase, showed both *in vitro* and *in vivo* activity towards several Gram-negative pathogens [145]. A related compound, AN3365, of which the structure is not disclosed yet, successfully passed clinical phase I trials [145, 174].

Remember two benefits from failure. First, if you do fail, you learn what isn't working and second, the failure provides you the possibility to try a new approach. Roger Von Oech [131]

5. Repurposing: old drugs, new hope?

5.1. A shortcut to finding 'new' drugs

Drug repurposing (or repositioning) is a term used to describe the use of drugs (either drug candidates, abandoned drugs, approved drugs, or withdrawn drugs) to treat a disease for which they were initially not developed for [175, 176]. The term drug repositioning was introduced in 2004 by Ashburn and Thor [177], and the number of publications using this and similar terms increased drastically in recent years [175]. However, the idea is not new and several drugs have been repurposed earlier. One of the most famous examples is sildenafil, which was in development for angina pectoris but was eventually marketed for erectile dysfunction, a side effect that appeared in the clinical trials [178, 179]. Indeed, in the past, repurposing was mainly based on serendipitous clinical observations [178], while in the last decade, small repurposing startup companies were founded and units were created in big pharmaceutical companies focusing on drug repurposing [179].

The main advantages of drug repurposing over *de novo* drug development are reduced time and costs in the R&D process, as knowledge concerning safety and pharmacology are available for the repurposing candidates [180]. However, clinical trials are required to prove efficacy for the new indication [181]. In addition, if higher doses of the repurposing candidate are required for the new indication and these fall outside the approved therapeutic window, additional phase I clinical trials have to be performed [181].

Drug libraries suitable for laboratory-based HTS were developed and are commercially available (e.g. John Hopkins Clinical Compound Collection, the NIH Chemical Genomics Center Pharmaceutical Collection, NIH Clinical Collection (NIHCC) [181], the Prestwick Chemical Library [182], the Library of Pharmaceutically Active Compounds (LOPAC) [160, 183], and the Screen-Well FDA-approved drug library V2 [184]). For example, the Prestwick Chemical Library consists out of 1280 approved off-patent drugs and has a high chemical and pharmacological heterogeneity [182].

5.2. Repurposing in the field of infectious diseases

5.2.1. Non-antibiotic approved drugs with antibacterial activity

For lack of a better name, drugs in use for non-bacteriological indications but with antibacterial activity are in literature often called 'non-antibiotic drugs' or 'non-antibiotics' [185]. These compounds might possess a direct antibacterial activity and/or enhance the activity of existing antibiotics by increasing the susceptibility of the bacteria towards the antibiotics, e.g. by controlling efflux pumps. In addition, they might also affect the pathogenicity of bacteria [186] or act by interference with the host resulting in an improved pathogen clearance [184, 187].

Several drug classes (e.g. antihistamines, local anesthetics, antihypertensive drugs, tranquilizers, anti-inflammatory drugs) are known to possess antibacterial activity, although they were not developed to treat bacterial infections [14, 186, 188]. The antimicrobial

properties of some of these compounds are known for decades [179, 186, 189]. For instance, a study dating from 1977 describes the antibacterial activity of tetracaine against *P. aeruginosa* [188]. Increased cell wall permeability and interference with the cell membrane, resulting in leakage of intracellular compounds followed by cell lysis was postulated as the mode of action [188]. Similarly, for several decades, studies have attributed bacteriostatic and bactericidal activities to the antifungal imidazoles. Sud and Feingold described in 1982 the distinct mode of action of miconazole and ketoconazole towards *S. aureus* [190]. In addition, several recent screening programs revealed promising repurposing candidates, as described below.

5.2.2. Screening repurposing libraries reveals new drug candidates

5.2.2.1. Screening for antibacterial activity

Harbut et al. screened the LOPAC library against *M. tuberculosis* in nutrient-deprivation conditions with the aim to identify bactericidal compounds [183]. Auranofin, clinically used to treat rheumatoid arthritis was identified in this screen and activity towards several other Gram-positive pathogenic bacteria was observed [160, 183]. The target of auranofin was identified as the bacterial thioredoxin reductase [183]. Interestingly, this screening resulted not only in the discovery of a promising repurposing drug candidate, but also in the identification of a valuable target essential in many Gram-positive bacteria [160].

In a screening campaign using the NIHCC 1&2, Younis and colleagues identified 24 non-antibiotic drugs, among them ebselen and 5-fluoro-2'-deoxyuridine [189]. These two compounds killed MRSA and VRSA at clinically achievable concentrations and the *in vitro* activity was confirmed *in vivo* in a mouse model for septicemic MRSA-infection [189]. The libraries used in the study of Younis et al. were also used in the present dissertation. These libraries contain 727 compounds that all passed human safety assessment and many of them are FDA approved drugs.

5.2.2.2. Screening for anti-virulence activity

The use of anti-virulence drugs as alternative for novel antibiotics is a new strategy to meet the demand for new therapies [180, 191, 192]. Anti-virulence drugs 'disarm' bacteria by inhibiting the production of disease-causing virulence factors rather than inhibiting bacterial growth or killing bacteria [192]. Anti-virulence drugs can be used both in prophylaxis or as helper compound of antibiotics in combination therapy during infection [192].

The Prestwick Chemical Library was screened against a *P. aeruginosa* biosensor for pyoverdine inhibitors [191]. Pyoverdine is an important virulence factor, and might thus be a valuable target [191]. Flucytocine, an antimycotic drug, was found to reduce the pyoverdine production at low iron levels, without affecting growth [191]. The effect was confirmed in a mouse model for pulmonary infection, where it protected mice from death [191]. The same research group performed a screen with the same library for QS-inhibitors in *P. aeruginosa* and identified the anthelmintic drug niclosamide as a potent QS inhibitor, protecting *Galleria mellonella* larvae from death after challenge with *P. aeruginosa* [180]. Another research group performed an *in silico* screen and found that raloxifene, a selective estrogen receptor

modulator, binds PhzB2, which is involved in pyocyanin production [193]. This resulted in a decreased pyocyanin production *in vitro* and an attenuation in *P. aeruginosa* virulence in infected *C. elegans* [193].

The NSAID diflunisal was identified to bind the phosphoryl-binding pocket of the transcription factor AgrA after *in silico* screening of the National Cancer Institute Library (90,000 compounds) [192]. AgrA is involved in the expression of several important *S. aureus* virulence factors, among them α-hemolysin (hla) and phenol soluble modulin type α (PSM) [192]. Incubation of MRSA in the presence of diflunisal resulted in a decreased *hla* expression by a factor 1,000 and a significant reduction of *psm α* expression [192]. Because the virulence factor PSM contributes to bone destruction during an *S. aureus* osteomyelitis infection, the protective effect of diflunisal towards PSM-induced osteoblast cell death was investigated [194]. Diflunisal potently inhibited killing by *S. aureus* of murine and human osteoblasts, without affecting the growth of *S. aureus*. In addition, a foam loaded with diflunisal was developed and the ability to inhibit pathogen-induced bone destruction was evaluated in mice with *S. aureus* osteomyelitis infection. Diflunisal did not cause a decrease in bacterial burden in the infected femurs, however, it reduced the bone destruction with 36% [194].

5.2.2.3. Screening in the presence of antibiotics to identify potentiators

The combination of antibiotics with non-antibiotic drugs as potentiators is a potentially valuable approach to overcome the problem of antibacterial drug resistance [195-197]. Several non-antibiotic drugs are described for additive or synergistic interactions with common antibiotics, restoring antimicrobial activity [187]. For instance, phenothiazines acted synergistically with aminoglycosides and macrolides against *Burkholderia pseudomallei* presumably by interaction with BpeAB-OprB and AmrAB-OprB efflux pumps [187]. A screening with 1059 previously approved drugs performed by Ejim and colleagues against *P. aeruginosa* PAO1, *E. coli* BW25113 and *S. aureus* ATCC 29213 in the presence of minocycline resulted in the identification of 6, 41, and 35 hits, respectively. These hits were non-antibiotic drugs that synergized with minocycline although they have never been used clinically to treat bacterial infections [198]. Disulfiram was one of the hits against *S. aureus*: alone, disulfiram has only weak antibacterial activity but it improved the activity of minocycline in a synergistic way against several MRSA strains, including MRSA USA300. Loperamide was a hit for *P. aeruginosa*: it has no antibacterial activity when used alone, however, it showed synergistic interaction with minocycline. The synergistic interaction was confirmed against several clinical strains of *P. aeruginosa*, and against *Salmonella enterica* Typhimurium. The efficacy of this combination was evaluated in a mouse model of infectious colitis caused by *S. enterica* Typhimurium. Treatment with the compounds alone had no impact on the infection, while the combination treatment reduced the microbial load in the cecum significantly [198].

5.2.2.4. Screening for compounds with activity towards the host

The Screen-Well FDA-approved drug library V2 (780 compounds) was screened against murine macrophages infected with *Yersinia pestis* CO92 [184]. This resulted in the identification of three hits (trifluoperazine, doxapram and amoxapine) that were able to

increase the survival of mice in a murine model of pneumonic plague [184]. Those compounds exhibited no bacteriostatic or bactericidal activity, nor did they affect bacterial virulence (no influence on type III secretion system or protease activity), leading to the conclusion that trifluoperazine, doxapram and amoxapine target host cell pathways resulting in a decreased pathogenesis [184].

In addition, 'inverse genomic signature' is a new *in silico* approach to rapidly identify antimicrobial treatments acting on host cells [199]. In brief, transcriptome profiles of human cells after treatment with an approved drug (available in public databases) are compared to profiles of cells after infection (or any disease state). Drugs causing an 'inverse similarity' in gene expression can further be examined as potential repurposing candidates in *in vitro* and *in vivo* experiments [199].

Chapter II:
Objectives

Biofilms are consortia of bacterial and/or fungal cells embedded in an extracellular matrix [200]. Bacteria can form biofilms in patient tissues, on body surfaces and on implanted medical devices [201]. The cells within these biofilms are phenotypically different from their planktonic counterparts [200] and they are more resistant and tolerant towards antibacterial treatments. Treating biofilm-related infections is challenging because it is difficult, or often impossible, to eradicate them with antibiotic therapy alone [201, 202]. In addition, the biofilm matrix protects the cells from host immune defenses [201] making biofilms reservoirs of persisting infections [70].

Biofilms are a considerable problem in health care settings and it is estimated that 65-80% of infections is biofilm-related [200, 201]. Pathogens like *S. aureus* and *P. aeruginosa* are leading causes of biofilm infections, and also *B. cenocepacia* is thought to form biofilms in the lungs of CF patients [201, 203]. The main aim of this dissertation was investigating the use of approved and off-patent drugs as potentiators of antibiotics against biofilms of these three important pathogens.

The first goal was the optimization of a quantification method based on the dye Cell Titer Blue (CTB). CTB is a non-fluorescent dye that is reduced by metabolically active cells into a fluorescent reaction product. The amount of fluorescence generated is related to the number of viable cells present. An existing protocol for CTB staining can be used in a high throughput way for quantification of the effect against biofilms of *B. cenocepacia* and *S. aureus*, but the signals generated by *P. aeruginosa* biofilms are too low and too variable. For this reason, we aimed to develop an alternative protocol using CTB, which enabled us using CTB for the rapid quantification of the effect of the treatments against *P. aeruginosa* biofilms as well (chapter III paper I).

Secondly, we aimed to identify drugs that enhanced the activity of antibiotics against biofilms. In order to identify these potentiators, we screened a library of approved and off-patent drugs in combination with tobramycin, against biofilms of *P. aeruginosa* and *B. cenocepacia*, and in combination with vancomycin against biofilms of *S. aureus* (chapter III paper II and III). Mature biofilms were formed in 96-well MTPs, which is a general *in vitro* biofilm model system allowing HTS. The biofilms were treated for 24 h with the combination antibiotic/library compound, and the effect was evaluated with CTB. For *S. aureus* and *B. cenocepacia*, the CTB method as described by Peeters et al. [200] was used, while for the screening against *P. aeruginosa* we used the new protocol described in paper I. Promising repurposing candidates were selected out of the hits resulting from these screening campaigns. Next, the activity of these selected hits was evaluated in lower concentrations and in combination with other antibiotics in the same *in vitro* biofilm model system. The effect was quantified by plate counts instead of CTB.

The final goal of this dissertation was to confirm the *in vitro* effect of our selected combinations in models that mimic the *in vivo* situation more closely. Efficacy of a treatment in these model systems is a better predictor for the therapeutic value of the combinations. The models used in these experiments were both *in vitro* models (a chronic wound biofilm model

and a three-dimensional organotypic cell culture model) and *in vivo* models (*Galleria mellonella* and a mouse lung infection model). In contrast to the screening against *S. aureus* and *B. cenocepacia*, only a very limited number of hits were identified in the screening against *P. aeruginosa*, and the activity of none of these hits was evalutated in other model systems. Hence, the results of this screening were not included in the Chapter III but the results of this screening are briefly summarized in Chapter IV.

Chapter III:
Experimental work

Paper I:
Optimization of resazurin-based viability staining for quantification of microbial biofilms.

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ABSTRACT

The resazurin-based viability staining is often used to quantify viable biofilm cells grown in microtiter plates (MTP). The non-fluorescent resazurin is reduced by metabolically active cells to resorufin which is fluorescent. The amount of fluorescence generated is related to the number of viable cells present. Unfortunately, the linear range of the method is restricted and the lower limit of quantification is approximately 10^6 colony forming units (CFU) per biofilm.

The goal of the present study was to optimize this method to broaden its applicability. We added fresh growth medium and resazurin to mature *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Burkholderia cenocepacia* and *Candida albicans* biofilms formed in MTP. Subsequently, the increase in resazurin-based fluorescence was followed over time and we determined the time needed to reach a specific value of fluorescence as well as the time to reach the maximum fluorescence. These time points correlate with the number of viable cells that were initially present and results were compared to plate counts.

Using these alternative read-outs, we were able to extend the linear range from 10^6–10^8 to 10^3–10^8 CFU per biofilm, meaning that lower numbers of viable cells can be measured and the effect of anti-biofilm treatments can be quantified more accurately. Moreover, this approach is less expensive and less laborious than conventional plating techniques.

INTRODUCTION

Conventionally used antibiotics are active against planktonic cells that cause acute infection, but often fail to completely eradicate biofilms leading to persistent infections [66, 111]. Biofilms are cell communities embedded in a highly hydrated self-produced extracellular matrix and are attached to biotic or abiotic surfaces. They show inherent tolerance and higher resistance to antibiotic treatment than their planktonic counterparts [80, 82, 111]. Therefore, there is an urgent need to develop novel anti-biofilm agents and to do so, good model systems and quantification methods are indispensable.

Several direct and indirect methods are used to quantify biofilms in the static microtiter plate (MTP) model system [64], each with its own advantages and disadvantages. Plating is a common quantification method that allows the determination of the number of culturable cells. Selective conditions can be used and isolates are available for further research. Furthermore, absolute cell numbers are obtained, and both very low and very high numbers of micro-organisms can be accurately quantified [204]. However, plating requires detachment and dispersal of the biofilm cells to obtain a homogenous cell suspension and scraping, vortexing and/or sonication is used for this purpose [205]. The number of cells will be underestimated if the dispersing is not done accurately. Moreover, cells will not grow and form visible colonies if they are in a state of starvation or under stress [206, 207]. In addition, since plating is time-consuming and labor-intensive, it is not suitable for HTS. The number of microorganisms can also be estimated indirectly using several staining methods, for example with fluorescein diacetate, tetrazolium salts or resazurin [200]. Resazurin (7-hydroxy-3H-

phenoxazin-3 one 10-oxide), also referred to as CellTiter-Blue (CTB) or AlamarBlue, is often preferred over other stains as it is easy to use, stable and non-toxic. Metabolically active cells reduce the blue and non-fluorescent resazurin to the pink fluorescent reaction product resorufin. This reduction is proportional to the number of metabolically active cells present. The fluorescence generated can be measured using plate readers [205, 208, 209]. Quantification with resazurin is fast, inexpensive and suitable for high-throughput estimation of the number of metabolically active cells. However, the results have to be interpreted carefully since only relative values are obtained and the resazurin method as conventionally used has a lower limit of quantification of approximately 10^6 colony forming units (CFU) per biofilm [200]. The present study evaluates an alternative approach of using resazurin to quantify the number of viable cells in bacterial and fungal biofilms.

MATERIAL & METHODS

Strains and culture conditions

Staphylococcus aureus Mu50, *Pseudomonas aeruginosa* PAO1 and *Burkholderia cenocepacia* LMG 16656 were cultured on Mueller Hinton agar plates (MH, Lab M, Lancashire, UK) and *Candida albicans* SC5314 on yeast-peptone-dextrose agar plates (YPD, BD, Franklin Lakes, NJ). From these pure cultures, overnight suspensions were made by inoculating 40 ml MH broth for the bacteria and 40 ml YPD broth for *C. albicans* with a loopful of microorganisms. All strains were grown aerobically at 37 °C.

Antibiotics and antiseptics

The following antibiotics and antiseptics were used: tobramycin (TOB, TCI, Tokyo, Japan), ciprofloxacin (CIP, Sigma, Bornem, Belgium), vancomycin hydrochloride hydrate (VAN, Sigma), amphotericin B (AMF, Sigma), miconazol (MICO, Certa, Waregem, Belgium), chlorhexidin digluconate (CHX, Fagron, Waregem, Belgium), cedium (CED, 1mg/ml benzalkonium chloride, NaEDTA, Na_2HPO_4 $2H_2O$, NaH_2PO_4 $2H_2O$, NaCl, Qualiphar, Bornem, Belgium), clindamycin (CLIN, Certa) and ozonated olive oil (O_3, Labo Althea, Kapelle Op Den Bos, Belgium). All compounds were dissolved in physiological saline (PS, 0.9% (w/v) NaCl), except for CHX which was dissolved in water of standard hardness (WSH) and MICO which was dissolved in dimethylsulfoxide (DMSO, Sigma); and subsequently diluted in PS. Where necessary, solutions were filter sterilized (0.22 µm, Whatman, Dassel, Germany) prior to use. Treatment with ozonated olive oil was performed as described in [210].

Biofilm formation in 96-well microtiter plates

Overnight suspensions were adjusted with MH broth for the bacteria and YPD broth for *C. albicans* to an optical density (595 nm) of 0.05 for *B. cenocepacia*, 0.2 for *P. aeruginosa* and *S. aureus* and 0.5 for *C. albicans*. These optical densities correspond to approximately 5×10^7 cells/ml. A hundred microliters of the diluted cell suspensions were transferred to the wells of a polystyrene round-bottomed 96-well microtiter plate (MTP, SPL Lifescience, Korea) and then incubated at 37 °C. Ten wells filled with sterile medium served as blank control. Medium was removed after 4 h and biofilms were rinsed with PS to remove non-

adhered cells. Fresh medium was subsequently added to the wells and plates were further incubated for 20 h.

Set-up of calibration curves

To investigate the relation between the number of metabolically active cells in the biofilm and the resazurin-based fluorescence signal obtained, biofilms were formed as described above. After 24 h of biofilm formation, medium was removed, biofilms were rinsed and 100 µl fresh PS was added to the wells. The plates were vortexed (5 min) and sonicated (5 min) (Branson 3510; Branson Ultrasonics Corp., Danbury, CT) and the entire content of the wells was collected. This process was repeated twice in order to harvest all biofilm cells. Serial 10-fold dilutions of this suspension were made in PS and 50 µl of each dilution was added to the wells of a round-bottomed sterile 96-well MTP together with 50 µl double concentrated MH medium (5 wells per dilution). Subsequently, 20 µl CellTiter-Blue was added to the wells and the fluorescence (λ_{ex} 535 nm/λ_{em} 590 nm) was measured every 5 min for 20 h using a multilabel microtiter plate reader (Envision; Perkin–Elmer LAS, Waltham, MA). Plates were incubated in the dark at 37 °C between the measurements and fluorescence measured in the blank control was used to correct for background signal. Simultaneously, the number of CFU per biofilm in each dilution was quantified using conventional plating techniques. The time to reach an absolute fluorescence value of 100,000 and the time to reach the maximum fluorescence was determined for each dilution. These time points and the number of CFU present in every dilution were then used to set up the calibration curves. The experiments were performed in triplicate for each strain.

C. albicans is usually grown in media containing relatively high concentrations of glucose because this fermentable carbohydrate enhances fungal growth. However, our preliminary results showed that the use of this medium leads to high background levels of fluorescence, indicating that the medium influences the measurement (Figure S1). In order to avoid this, we decided to grow *C. albicans* biofilms in YPD, but carried out the resazurin assay in MH.

Biofilm treatment and quantification

Biofilms formed as described above were treated with 100 µl of various antibiotics and antiseptics for 24 h at 37 °C. Contact time for CHX however ranged from 1 to 30 min and reaction was stopped using Dey Engley neutralizing broth (DENB, Lab M, 7 g/l lecithin, 6 g/l sodium thiosulphate, 5 g/l tween 80, 5 g/l tryptone, 2.5 g/l sodium bisulphite, 1 g/l sodium thioglycollate, 0.02 g/l bromocresol purple, 2.5 g/l yeast extract). The supernatants were removed subsequently and the biofilms were rinsed with PS. Conventional plating and resazurin staining were then used to determine the number of culturable and viable biofilm cells, respectively (designed as $CFU_{plating}$ and CFU_{CTB}).

To determine $CFU_{plating}$, 100 µl PS was added to the wells containing the treated biofilms and the MTP was sonicated and vortexed twice (same procedure as described above). The detached cells were quantified by conventional plating. To determine CFU_{CTB}, 100 µl MH was added to the wells and the MTP was sonicated and vortexed twice. Subsequently, 20 µl resazurin was added and the MTP was incubated for 20 h at 37 °C. The fluorescence

generated by the reduction of resazurin was measured every 5 min and the time to reach the fluorescence signal of 100,000 and the maximum fluorescence signal were determined. The previous derived calibration curves were then used to calculate the CFU_{CTB}. Finally, $CFU_{plating}$ and CFU_{CTB} were compared for each condition. Statistical analysis was performed with SPSS software using independent sample t-tests. P values < 0.01 were considered to be statistically significantly different.

RESULTS AND DISCUSSION

Bacteria growing in biofilms cause persistent infections which are often not cleared with conventional antibiotic treatment alone. New anti-biofilm agents are urgently needed and to this end, good model systems and quantification methods are essential. The non-fluorescent dye resazurin is used to quantify metabolically active cells. In the conventionally used assay, resazurin is diluted in PS and added to biofilms formed and treated in MTP. The fluorescence generated by the reduction of resazurin to its fluorescent reaction product by metabolically active cells is typically measured after 30–120 min. However, the linear range is restricted and the lower limit of quantification is approximately 10^6 CFU per biofilm, which means that the assay cannot discriminate between cell numbers lower than 10^6 CFU per biofilm [200].

In the present study, we optimized this resazurin-based quantification method. Mature *S. aureus*, *P. aeruginosa*, *B. cenocepacia* and *C. albicans* biofilms were formed in MTP. Subsequently, these biofilms were disrupted, detached and collected. Serial dilutions were made and wells of a sterile MTP were filled with these diluted cell suspensions together with fresh MH growth medium and resazurin. The fluorescence generated by the reduction of resazurin was followed over time. The time to reach an absolute fluorescence value of 100,000 and the time to reach the maximum fluorescence were determined for every dilution. The time to reach these fluorescence signals was plotted against the number of CFU present in every dilution, obtained by conventional plating, leading to the calibration curves shown in Figure 1. The linear range increased from 10^6–10^8 CFU per biofilm as described in [200] to approximately 10^3–10^8 CFU per biofilm. This results in a substantially decreased lower limit of quantification, making screenings for anti-biofilm agents more precise. Moreover, by measuring the fluorescence over an extended time, high fluorescence signals were generated for all species tested. This is interesting especially for micro-organisms that reduce resazurin slowly, like *P. aeruginosa*.

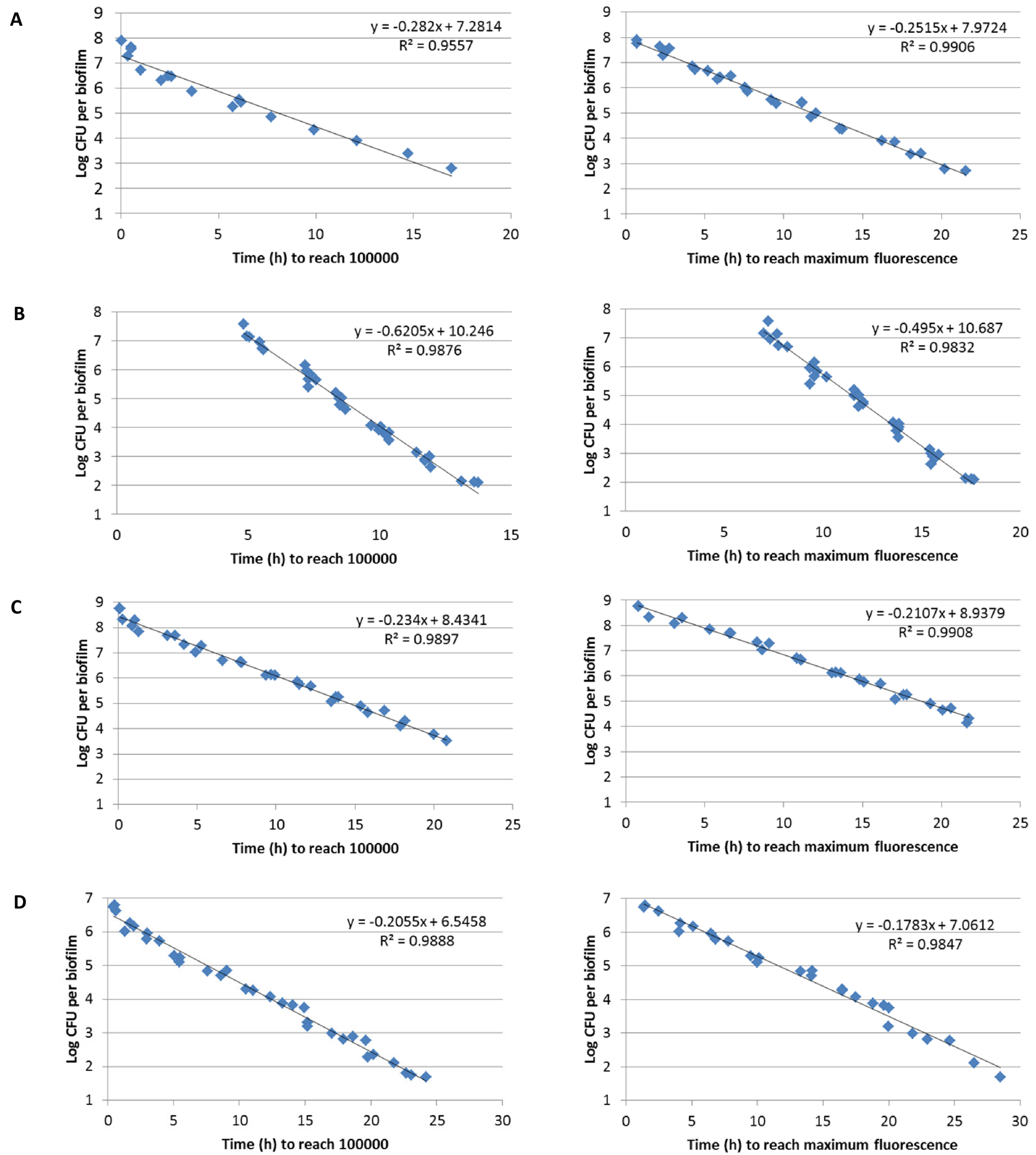

Figure 1. Calibration curves showing the relation between the time needed to reach an absolute fluorescence value of 100,000 (left) and maximum fluorescence value (right) and the number of CFU per biofilm determined by plating. A) *S. aureus*, B) *P. aeruginosa*, C) *B. cenocepacia*, D) *C. albicans*.

In order to validate our approach, mature biofilms of *S. aureus*, *P. aeruginosa, B. cenocepacia* and *C. albicans* were grown in MTP and treated with several anti-infective agents in various concentrations and/or contact times. Conventional plating was used to determine the $CFU_{plating}$, and resazurin staining was used to determine CFU_{CTB}. The $CFU_{plating}$ and CFU_{CTB} obtained were then compared for each treatment. As shown in Figure 2, the results obtained with both quantification methods were very similar and confirm that this method can be implemented in screenings to assess the number of viable cells.

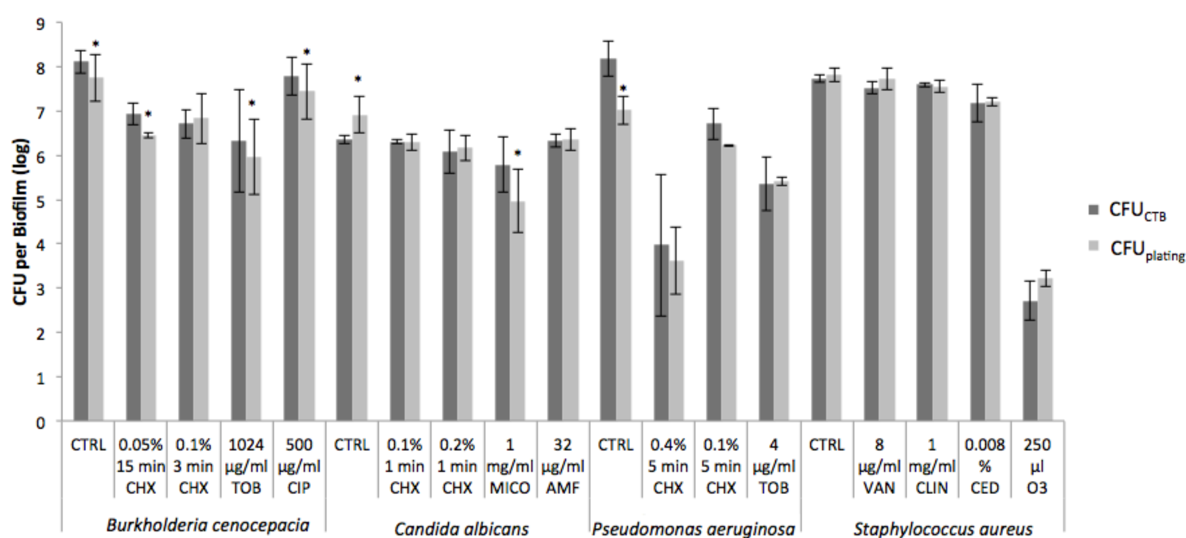

Figure 2. The number of CFU per biofilm as determined by plating ($CFU_{plating}$) and as calculated using the previously determined calibration curves (CFU_{CTB}). Error bars represent standard deviations. * Statistically significantly different ($p < 0.01$), ($n \geq 5$).

Both the time to reach a signal of 100,000 and the time to reach the maximum fluorescence were determined for every strain and treatment evaluated in this study. However, microorganisms that rapidly reduce resazurin reach the signal of 100,000 in a few minutes, consequently, the time to reach the maximum can be established more precisely and is therefore preferred. *S. aureus* rapidly reduces resazurin while the other organisms tested in the present study reduced resazurin more slowly. Hence, we calculated the CFU_{CTB} for *S. aureus* using the time needed to reach the maximum fluorescence, and for the other strains using the time needed to reach a fluorescence value of 100,000.

For some treatments, $CFU_{plating}$ and CFU_{CTB} are statistically significantly different (Figure 2). However, these differences are minor and not biologically significant as they do not exceed 1 log and will not influence the interpretation of the results obtained in screening assays.

CONCLUSION

The resazurin assay is a simple method to quantify the number of viable cells in biofilms formed in MTP. By optimizing this assay, we were able to decrease the lower limit of quantification from 10^6 to 10^3 CFU per biofilm, meaning that substantially lower numbers of viable cells can be measured and the effect of anti-biofilm treatments can be quantified more accurately. Moreover, the assay described is faster and less labor intensive than conventional plating.

ACKNOWLEDGEMENTS

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SUPPLEMENTARY DATA

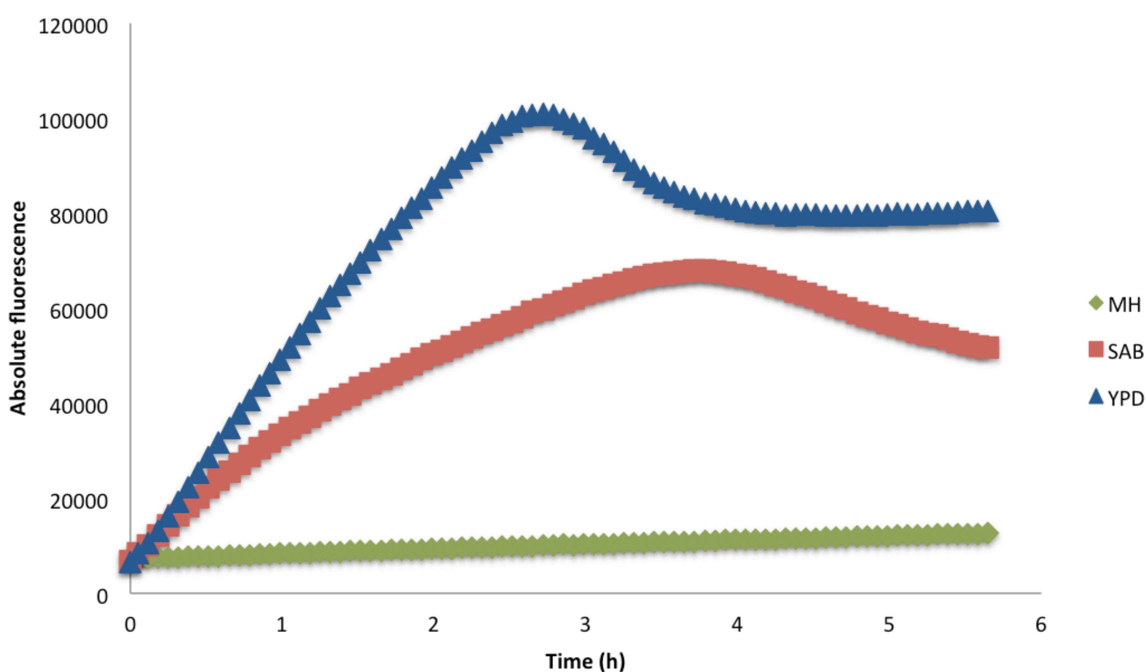

Figure S1. The use of Sabouraud broth (SAB) and Yeast Pepton Dextrose broth (YPD) leads to high background levels of fluorescence, indicating that these growth media influence the measurement, in contrast to Mueller Hinton broth (MH).

Paper II:
Screening a repurposing library for potentiators of antibiotics against *Staphylococcus aureus* biofilms

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ABSTRACT

Staphylococcus aureus biofilms are involved in a wide range of infections that are extremely difficult to cure with conventional antibiotic therapy.

We aimed to identify potentiators of antibiotics against mature biofilms of *S. aureus* Mu50, a methicillin-resistant and vancomycin-intermediate strain. Over 700 off-patent drugs from a repurposing library were screened in combination with vancomycin in an MTP-based biofilm model system. This led to the identification of 25 hit compounds, including four phenothiazines among which thioridazine was the most potent. Their activity was evaluated in combination with other antibiotics against both planktonic as biofilm grown *S. aureus* cells. The most promising combinations were subsequently tested in an *in vitro* chronic wound biofilm infection model.

Although no synergistic activity was observed against planktonic cells, thioridazine potentiated the activity of tobramycin, linezolid and flucloxacillin against *S. aureus* biofilm cells. However, this effect was only observed in a general 96-well MTP biofilm model and not in a chronic wound model of biofilm infection.

We identified several drug compounds which potentiated the activity of suboptimal concentrations vancomycin against biofilms formed in an MTP-based biofilm model. A selected hit compound failed to potentiate the activity of three antibiotics tested in a model that mimics specific aspects of wound biofilms. This study provides a platform for discovering and evaluating potentiators against bacterial biofilms and highlights the necessity of using relevant *in vitro* biofilm model systems.

INTRODUCTION

Staphylococcus aureus is a notorious opportunistic pathogen that is involved in a wide range of infections, including chronic wounds, endocarditis, and infections associated with indwelling medical devices [202]. Treatment of these infections is challenging due to the frequent occurrence of antimicrobial resistant strains such as methicillin-resistant *S. aureus* (MRSA) [211]. In addition, *S. aureus* often reside within biofilms at the infection site [202]. Biofilms are cell communities embedded in a self-produced extracellular matrix in which the bacterial cells are protected from the host's immune system and the activity of antimicrobial agents [202]. Biofilm-related infections often fail to respond to antibiotic therapy, leading to chronic infections for which more effective treatments are urgently needed [202].

An approach to fill the antibiotic pipeline is screening repurposing libraries [212]. In drug repurposing or repositioning, drugs are used to treat diseases they were initially not developed for [176]. This approach has several advantages over *de novo* drug development, e.g. long term toxicity and metabolic properties are already known [176]. This reduces the risk, time and costs to bring the repurposed drug to the market [213]. Although several non-antibiotic drugs have antimicrobial activity, [186] none of these are currently used in antibacterial

therapy since their bactericidal activity is often only observed at very high concentrations making them clinically less useful [189]. However, the combination of antibiotics with non-antibiotics as potentiators ('helper' compounds) could result in a greater antimicrobial effect and less toxicity when lower concentrations of both can be used. For this reason, the search for antibacterial potentiators has recently gained interest [187].

Many whole-cell screenings are conducted using general growth medium and measuring inhibition of bacterial growth as the endpoint [214]. However, growth conditions and endpoints used to measure antibacterial activity are important parameters and different hits can be identified depending on whether compounds are screened for activity against planktonic or biofilm cells [214, 215]. Investigating alternative endpoints, like the ability to inhibit biofilm formation or quorum sensing, can lead to interesting results [180, 216]. However, few screenings are performed against biofilms [217, 218] and few studies evaluated off-patent drugs for their possible potentiator activity towards antibiotics that could be used for topical treatment of biofilm-related infections (including chronic wound infections) [219]. In addition, few studies have investigated the activity of their hit compounds under conditions simulating the host environment. In the present study, we performed a repurposing screen in the presence of vancomycin against *S. aureus* biofilms, followed by validation of a selected hit in an *in vitro* chronic wound biofilm model.

MATERIAL & METHODS

Strains and culture conditions

The methicillin-resistant and vancomycin-intermediate strain *S. aureus* Mu50 was used in this study. Pure cultures were maintained on tryptic soy agar (TSA, Lab M, Lancashire, UK) plates. Overnight broth cultures (ON culture) were obtained by inoculating Mueller Hinton broth (MH broth, Lab M) with several colonies and incubated for 18 h with shaking. All cultures were kept aerobically at 37°C.

Drugs and antibiotics used

The National Institutes of Health Clinical Collection 1 and 2 (NIHCC 1&2, Evotec, San Francisco, USA) are repurposing libraries containing respectively 446 and 281 components at a concentration of 10 mM, dissolved in 100% DMSO and stored at -20°C in 96-well microtiter plates (MTPs). Ciprofloxacin (CIP) was obtained from Fluka (Buchs, Swiss), tobramycin (TOB) from TCI Europe (Antwerp, Belgium), rifampicin (RIF), gentamicin (GEN), vancomycin hydrochloride hydrate (VAN), linezolid (LNZ), tetracycline hydrochloride (TET), fusidic acid sodium salt (FA), flucloxacillin sodium (FLU), fluphenazine dihydrochloride (FLUPH), perphenazine (PERPH), trifluoperazine dihydrochloride (TRI) and thioridazine hydrochloride (TZ) were purchased from Sigma Aldrich (Bornem, Belgium). Solutions were made in physiological saline (PS, 0.9% w/v NaCl) and ointments in 70% white vaseline (Fagron, Waregem, Belgium) and 30% liquid paraffin (Fagron). Perphenazine was first dissolved in DMSO (Sigma Aldrich) and subsequently diluted in PS. For the MIC and checkerboard tests, drugs were dissolved in

ultrapure Milli-Q water (MQ, Millipore, MA, USA). All solutions were made fresh on the day of the experiment and filter sterilized (0.22 µm, Whatman, Germany).

Screening of the NIHCC 1&2 against mature biofilms of *S. aureus* Mu50 grown in 96-well MTPs

Biofilms were formed as previously described [220]. In brief, round-bottomed 96-well MTPs were filled with 100 µl of a diluted overnight bacterial suspension (OD_{595} 0.2, approx. $5x10^7$ CFU/ml) and incubated for 4 h at 37°C. Subsequently, wells were rinsed with PS to remove all planktonic cells and 100 µl fresh MH broth was added. Plates were incubated for an additional 20 h at 37°C. The 24 h old biofilms were rinsed with 100 µl PS. Subsequently, 49 µl PS, 1 µl of compound (final concentration: 100 µM), and 50 µl of a 80 µg/ml vancomycin solution was added (final concentration: 40 µg/ml) in case of combination treatment. In case of experiments carried out in the absence of vancomycin, 1 µl compound was added to 99 µl PS. All experiments were carried out at least in triplicate. A blank, a growth control and a control for vancomycin treatment alone were included on every plate. After 24 h incubation at 37°C, the supernatant was removed and biofilms were rinsed with 100 µl PS. The number of metabolically active cells was determined by resazurin staining (Cell Titer Blue, CTB, Promega, Leiden, The Netherlands) using a multilabel MTP-reader (λ_{ex} 535 nm/λ_{em} 590 nm, Envision, Perkin Elmer LAS, Waltham, MA) as previously described [200]. The blank corrected fluorescence signals generated by *S. aureus* biofilms treated with the combination of component and vancomycin were compared to the fluorescence signal generated after treatment with vancomycin alone. Successful hits were defined as combinations yielding a decrease in fluorescence signal of at least 90% compared to fluorescence generated by biofilms treated with vancomycin alone.

Determination of the MIC and FIC

MICs of thioridazine, fluphenazine, perphenazine and trifluoperazine against *S. aureus* Mu50 were determined in triplicate using flat-bottom 96-well MTP (TPP, Trasadingen, Switzerland) as described earlier [221]. The inoculum was standardized to approx. $5x10^5$ CFU/ml. The plates were incubated at 37°C for 24 h, and the optical density at 590 nm was measured using a multilabel MTP-reader (Envision). The MIC is the lowest concentration that generates a signal that is not significantly different from that of un-inoculated growth medium.

Interaction between thioridazine (50-0.78 µM), fluphenazine (100-1.56 µM), perphenazine (100-1.56 µM), or trifluoperazine (50-0.78 µM), and tobramycin (1024-4 µg/ml), gentamicin (512-2 µg/ml), vancomycin (32-0.125 µg/ml), ciprofloxacin (256-1 µg/ml), linezolid (32-0.125 µg/ml), tetracycline (256-2 µg/ml), fusidic acid (1-0.004 µg/ml), or flucloxacillin (1024-4 µg/ml) was determined by checkerboard assays and the fractional inhibitory concentration index (FICI) was calculated to determine whether the interaction was synergistic (FICI $\leq$ 0.5) or indifferent (FICI > 0.5). In order to do so, solutions of the antipsychotic phenothiazines were added to flat 96-well MTPs in a concentration gradient in one direction, and the antibiotic solutions were added in a concentration gradient in the other direction. A similar inoculum as in the MIC tests was subsequently added and the absorbance was measured after 24 h incubation. The FICI was calculated as follows: MIC_{AB} /MIC_A +

$MIC_{BA}/MIC_{B,}$ where MIC_{AB} is the lowest concentration of the phenothiazine in the presence of the antibiotic where the absorbance does not differ from the absorbance of the uninoculated blank control, MIC_A is the lowest concentration of the phenothiazine that causes an absorbance that does not differ from that of the uninoculated blank control, MIC_{BA} is the lowest concentration of the antibiotic in the presence of a phenothiazine that causes an absorbance that does not differ from the absorbance of the blank control, while MIC_B is the lowest concentration of the antibiotics that causes an absorbance not different from that of the blank control.

Biofilm eradication tests in 96-well MTPs

The activity of the four phenothiazines against *S. aureus* biofilms was evaluated in a 96-well MTP biofilm model. Mature biofilms of *S. aureus* were grown as described above and subsequently treated with thioridazine, fluphenazine, perphenazine or trifluoroperazine (10, 50, and 100 µM). The potentiating activity of thioridazine on antibiotics was investigated in the same model. Biofilms were treated with thioridazine (100 µM) in combination with tobramycin (1024 µg/ml), gentamicin (1024 µg/ml), flucloxacillin (1024 µg/ml), vancomycin (40 µg/ml), linezolid (20 µg/ml), tetracycline (128 µg/ml), ciprofloxacin (250 µg/ml), fusidic acid (1000 µg/ml) and rifampicin (512 µg/ml). After 24 h, the treatment solutions were removed and biofilms were rinsed with 100 µl PS. Cells were collected from the wells by 2 cycles of shaking (5 min, 700 rpm, Titramax 1000) and sonicating (5 min, Branson 3570E-MT, Branson Ultrasonic Corporation, Banbury, USA). The content of 5 wells was pooled in tubes containing 9 ml PS and the number of colony forming units per biofilm (CFU/BF) was determined by plating serial 10-fold dilutions on TSA plates. Experiments were performed in triplicate on at least 3 different days ($n \geq 3$).

Validating the activity of tobramycin, linezolid, or flucloxacillin and thioridazine in an *in vitro* chronic wound biofilm model

Preparation of the artificial dermis (AD) and biofilm formation were performed as described by Brackman et al. [222]. Briefly, an upper layer of the dermis was prepared by dissolving sodium hyaluronate (Lifecore Biomedical, MN, US) in PS, adjusting the pH to 3.5 followed by chemically crosslinking hyaluronate with ethylene glycol diglycidyl ether (EX810, Sigma). Freeze-drying containers were filled with this solution, stored overnight at 4°C, subsequently strored at -80°C, and freeze dried. Then, a second layer was prepared, containing hyaluronic acid and collagen. This was poured on the first layer. After freeze-drying, the dermis were removed from the freeze-drying vial, irradiated with a UV lamp to cross link the collagen, followed by sterilization in an oven (110°C, 1 h) [222]. The dermis were put in the wells of a 24-well MTP and moistend with 1 ml bolton broth containing 50% (v/v) plasma (Sigma), and 5% freeze-thawed horse blood. Ten µl of a diluted overnight *S. aureus* culture (10^4 CFU) was added to the dermis. After 24 h of biofilm formation at 37°C, the infected dermis was rinsed and subsequently solutions (100 µl) containing tobramycin (2048 µg/ml), linezolid (400 µg/ml), or flucloxacillin (20.5 mg/ml) alone and with 100 µl thioridazin (100 µM) were added. Alternatively, the AD were covered with 250 mg ointment containing tobramycin (15 mg/g), alone or combined with 0.25 mg/g thioridazine for 24 h. The AD were kept at 37°C during treatment. After treatment, AD were placed into tubes containing 10 ml PS, the sessile

cells were removed from the AD by three cycles of vortexing (30 s) and sonication (30 s) and the number of CFU/dermis was determined by plating the resulting suspensions. Every condition was tested in triplicate.

Statistical analysis
Statistical analysis were performed using SPSS version 23 software and data were analyzed with Kruskal-Wallis test.

RESULTS AND DISCUSSION

Hits resulting from screening NIHCC 1&2 against *S. aureus* biofilms
Over 700 compounds from the NIHCC 1&2 repurposing libraries were screened for their ability to potentiate the antimicrobial activity of vancomycin against mature *S. aureus* biofilms formed in 96-well MTPs. The effect of the treatment was quantified using CTB, measuring the number of surviving metabolically active cells. Compounds that caused a decrease in fluorescence signal of ≥90% when combined with vancomycin compared to treatment with vancomycin alone (set to 100%) were retained for further analysis. Twenty-five compounds (3.4%) met these requirements. This hit rate is much higher compared to the 0.025% average hit rate usually seen in HTS [223] but comparable to screenings using similar repurposing libraries [189, 218]. Hits were classified according to their therapeutic indications and belonged to the following categories: anti-infective agents (n=6), antipsychotic drugs and antidepressants (n=5), antineoplastic and hormonal drugs (n=7), and a miscellaneous group (n=7) (Table 1).

Subsequently, *S. aureus* biofilms were treated with the identified hits in absence of vancomycin. Six compounds (fluphenazine, trifluoperazine, sertraline, tamoxifen, oxymetholone, and amiodarone) did not cause a significant reduction in fluorescence signal compared to the growth control (Table 1). Hence, these compounds have no effect anti-biofilm effect on their own measured by CTB staining. In contrast, the potentiating activity of the other hit compounds is at least partially due to their intrinsic anti-biofilm activity.

Combining vancomycin with the antifungal imidazoles miconazole nitrate, oxiconazole nitrate or econazole nitrate resulted in a decrease in fluorescence signal of more than 90% compared to treatment with vancomycin alone. Interestingly, treatment with the imidazoles alone caused a significant decrease in fluorescence signal compared to the untreated control ($p< 0.05$) (Table 1). This indicates that these imidazoles substantially decrease the metabolic activity of *S. aureus* biofilms. For this reason, the effect of the combination cannot only be attributed to a direct potentiating effect. Activity of imidazoles against planktonic *S. aureus* has been known for decades [190]. Imidazoles cause a K^+ leakage indicating membrane damage [190] and their interaction with bacterial flavohemoglobin results in an increased level of intracellular reactive oxygen species (ROS) [224].

Sertraline, an antidepressant belonging to the selective serotonine reuptake inhibitors (SSRI), was identified as a potentiator in our screening. In absence of vancomycin, sertraline causes

no significant decrease in fluorescence signal meaning that it has no intrinsic activity against biofilms. Recently, Ayaz et al. showed that sertraline has both intrinsic and synergistic activity in combination with several antibiotics (among them moxifloxacin and levofloxacin) against planktonic *S. aureus* [225]. The authors suggest that efflux pump inhibition is the mode of action but this is not confirmed yet [225].

The phenothiazines trifluoperazine HCl, perphenazine, fluphenazine dihydrochloride and thioridazine HCl, were all identified as hits in the current screening. Antibacterial properties of the first marketed antipsychotic drug belonging to this class, chlorpromazine, were known since its release 60 years ago. However, at that time chlorpromazine was not an interesting antibiotic agent or lead compound due to its severe side effects and relatively low antibacterial activity [226]. Today, as phenothiazines with less side effects (e.g. thioridazine) are available, and the need for new antibiotic therapies is high, these drugs are being investigated as potential repurposing drug candidates. Toxicity is acceptable for short-term treatments and their *in vitro* activity, either alone or as potentiators of antibiotics, especially beta-lactams, is promising [227]. Moreover, the mode of action has been investigated in detail. Phenothiazines inhibit the activity of efflux pumps, alter the cell wall structure, and interfere with other cellular processes [32, 226-230]. Interestingly, due to increased concentrations intracellularly, phenothiazines enhance killing of phagocytosed MRSA at clinically achievable concentrations [211]. However, conflicting results are obtained in *in vivo* models [228, 230, 231] and activity against biofilm cells has not been described in literature. Being valuable and potential repurposing drug candidates, we selected these four phenothiazines (thioridazine, fluphenazine, perphenazine and trifluoperazine) to investigate their activity against *S. aureus* biofilms in more detail.

Table 1. Hits identified in a screen of the NIHCC 1&2 against biofilms of *S. aureus* are classified according to their therapeutic indication. Compounds were tested at a concentration of 100 µM in the presence of vancomycin (VAN, 40 µg/ml) or in absence of vancomycin. Data shown are average percentages with standard deviation. *Significant reduction in fluorescence compared to growth control, $p<0.05$. SSRI: selective serotonin reuptake inhibitor, SERM: selective estrogen receptor modulator, NSAID: non-steroidal anti-inflammatory drugs

COMPOUND	DESCRIPTION	Residual metabolic activity after treatment with compound and VAN compared to treatment with VAN alone ± SD	Residual metabolic activity after treatment with compound compared to untreated control ± SD
Anti-infectives			
Hexachlorophene	Desinfectant	0 ±0 *	8 ±10 *
Triclosan	Desinfectant	0 ±0 *	2 ±3 *
Miconazole	Antifungal drug	9 ±10 *	33 ±22 *
Econazole	Antifungal drug	5 ±5 *	24 ±24 *
Oxiconazole	Antifungal drug	0 ±0 *	10 ±10 *
Efavirenz	Antiviral drug	0 ±1 *	5 ±8 *
Antipsychotic drugs and antidepressants			
Fluphenazine	Antipsychotic	6 ±3 *	62 ±31
Perphenazine	Antipsychotic	9 ±3 *	48 ±24 *
Thioridazine	Antipsychotic	0 ±0 *	29 ±18 *
Trifluoperazine	Antipsychotic	0 ±0 *	67 ±36
Sertraline	Antidepressant	0 ±0 *	51 ±21
Antineoplastic and/or hormonal drugs			
Daunorubicin	Antineoplastic	0 ±0 *	18 ±16 *
Idarubicin	Antineoplastic	0 ±0 *	15 ±27 *
Toremifene	SERM	0 ±0 *	9 ±7 *
Tamoxifen	SERM	0 ±0 *	67 ±10
Clomifene citrate	SERM	0 ±0 *	13 ±1 *
Flutamide	Anti-androgen	2 ±1 *	24 ±14 *
Oxymetholone	Anabolic steroid	0 ±0 *	88 ±47
Miscellaneous group			
Amiodarone	Class III antiarrhythmic	6 ±9 *	68 ±6
Carvedilol	β blocker/α-1 blocker	8 ±6 *	41 ±6 *
Honokiol	Neolignal biphenol	0 ±0 *	0 ±0 *
Sodium loxoprofen	NSAID	3 ±1 *	42 ±29 *
MK886	Lipoxygenase inhibitor	5 ±2 *	39 ±25 *
5-Nonyloxytryptamine	5-HT_{1B} receptor agonist	0 ±0 *	37 ±15 *
Ethacrynic acid	Diuretic	5 ±5 *	1 ±1 *

Activity of phenothiazines against planktonic *S. aureus*

The MIC for planktonic cells was determined and thioridazine and trifluoperazine showed highest activity with MICs of 50 µM compared to 100 µM for fluphenazine and perphenazine. A possible interaction was evaluated in checkerboard assays measuring the growth inhibiting properties of varying concentrations of the phenothiazines and an antibiotic (vancomycin, tobramycin, ciprofloxacin, fusidic acid, gentamicin, tetracyclin or linezolid). FIC indices were > 0.5 for all combinations, indicating that there was no synergistic activity between phenothiazines and the antibiotics tested.

Activity of phenothiazines against *S. aureus* biofilm cells

We subsequently evaluated the biofilm eradicating effect of thioridazine, fluphenazine, trifluoperazine, and perphenazine in different concentrations (10-50-100 µM), using plate counts. Trifluoperazine and perphenazine did not result in a significant reduction in CFU/biofilm in contrast to thioridazine and fluphenazine (Figure 1). Thioridazine (50 µM or 100 µM) caused a significant reduction of approx. 1 log_{10} in CFU/biofilm compared to the untreated control. However, thioridazine has no biofilm eradicating effect at lower concentrations. Fluphenazine (100 µM) caused a significant reduction of 0.5 log_{10} while lower concentrations were inactive.

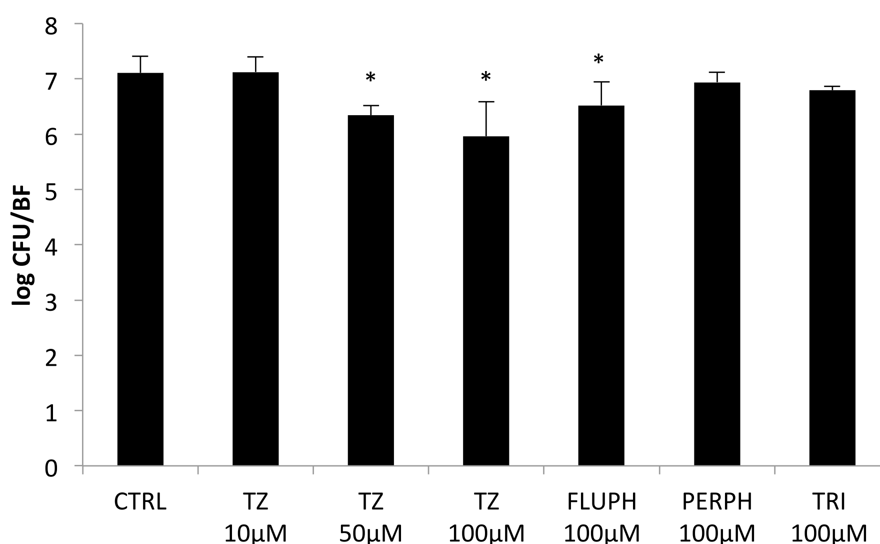

Figure 1. Treatment of mature *S. aureus* biofilms for 24 h with 100 µl PS (untreated control, CTRL), thioridazine (TZ, 10, 50, or 100 µM), fluphenazine (FLUPH, 100 µM), perphenazine (PERPH, 100 µM), or trifluoperazine (TRI, 100 µM). *Significantly different number of colony forming units/biofilm (CFU/BF) compared to CTRL ($n>3$; $p<0.05$, error bars indicate standard deviation)

Being the most potent compound, thioridazine was evaluated for its capacity to potentiate the activity of other antibiotics. Biofilms were treated with thioridazine (100 µM) in combination with vancomycin (40 µg/ml, MIC=8 µg/ml), ciprofloxacin (250 µg/ml, MIC=62.5 µg/ml), linezolid (20 µg/ml, MIC=2 µg/ml), fusidic acid (1000 µg/ml, MIC=2 µg/ml), tetracycline (128 µg/ml, MIC=64 µg/ml), tobramycin (1024 µg/ml, MIC=512 µg/ml), flucloxacillin (1024 µg/ml, MIC >512 µg/ml), gentamicin (1024 µg/ml, MIC=256 µg/ml) and rifampicin (512 µg/ml, MIC>512 µg/ml) and the effect was quantified using plate counts. Treatment with the

combination thioridazine and tobramycin resulted in a clear additional anti-biofilm effect, reducing the CFU/BF to approx. 5 $\log_{10}$ (Figure 2). Although similar results were observed for 50 µM thioridazine, lower concentrations (10 µM) failed to potentiate the activity of tobramycin. So far, it is unclear what the mechanism is behind the potentiating effect. Also treatment with thioridazin and linezolid or flucloxacillin resulted in a significant reduction in log CFU/BF compared to treatment with the respective antibiotics alone. Linezolid is a putative substrate for the LmrS efflux pump [232] so thioridazine might interfere with this multidrug efflux pump. Potentiating activity towards beta-lactams is described in planktonic cells and is due to alterations in the cell wall biosynthesis and a decrease in expression of *mecA* and *blaZ* [227]. Combined treatment with thioridazine and gentamicin, vancomycin, ciprofloxacin, tetracycline, rifampicin or fusidic acid did not result in a significant decrease in CFU/BF compared to treatment with the respective antibiotic alone (Figure 2). Although ciprofloxacin, tetracycline, and fusidic acid are substrates for NorA, NorB, TET38, and MdeA efflux pumps [27], no additional effect is observed when these antibiotics are combined with thioridazine. Finally, the failure of thioridazine to potentiate the activity of fusidic acid might be due to ion-ion interactions as we observed precipitation when both drugs are in contact.

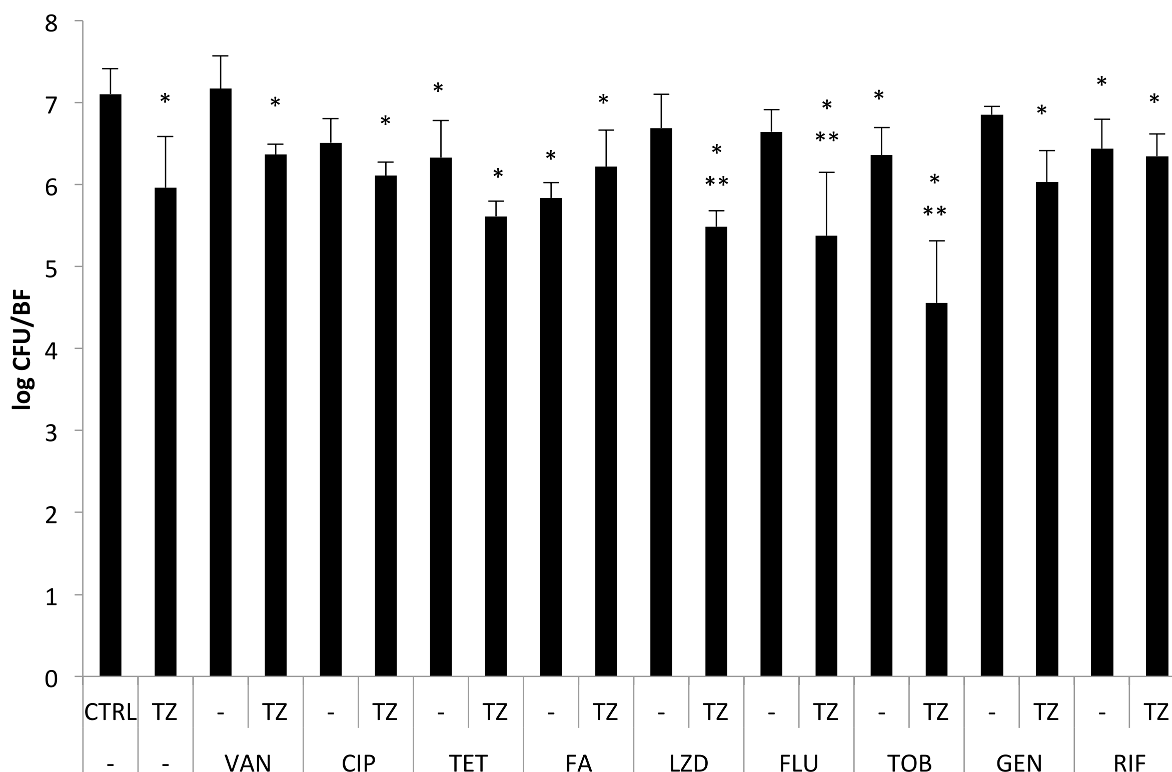

Figure 2. Treatment of mature *S. aureus* biofilms for 24 h with 100 µl PS (untreated control, CTRL), vancomycin (VAN, 40 µg/ml), ciprofloxacin (CIP, 250 µg/ml), linezolid (LNZ 20 µg/ml), fusidic acid (FA, 1000 µg/ml), tetracycline (TET, 128 µg/ml), tobramycin (TOB, 1024 µg/ml), flucloxacillin (FLU, 1024 µg/ml), gentamicin (GEN, 1024 µg/ml), and rifampicin (RIF, 512 µg/ml) alone or in combination with thioridazine (TZ, 100 µM). Antimicrobial activity was determined using plate counts. *Significantly different number of CFU/BF compared to CTRL ($p<0.05$), **Significantly different number of CFU/BF compared to treatment with the respective antibiotics alone ($p<0.05$). P-value TZ vs TOB and TZ was 0.055. Data shown are the average ($n\geq3$), error bars indicate standard deviation.

Activity against *S. aureus* biofilms grown in an *in vitro* chronic wound biofilm model
As thioridazine causes a significant additional reduction in the number of CFU/BF in combination with tobramycin, linezolid, or flucloxacillin, compared to treatment with the antibiotic alone in a 96-well MTP biofilm model system, the activity of these combinations was evaluated in an *in vitro* chronic wound biofilm model [222].

Biofilms of *S. aureus* formed in this model were treated with PS containing thioridazine and/or tobramycin, linezolid or flucloxacillin. Additionally, biofilms were treated with an ointment containing tobramycin and thioridazine. However, none of these treatments resulted in a decrease in the number of CFU (Figure 3), even though higher concentrations were applied. For example, we tested an ointment containing tobramycin concentrations 5 times higher compared to the ones present in the ophthalmic ointment Tobrex[R].

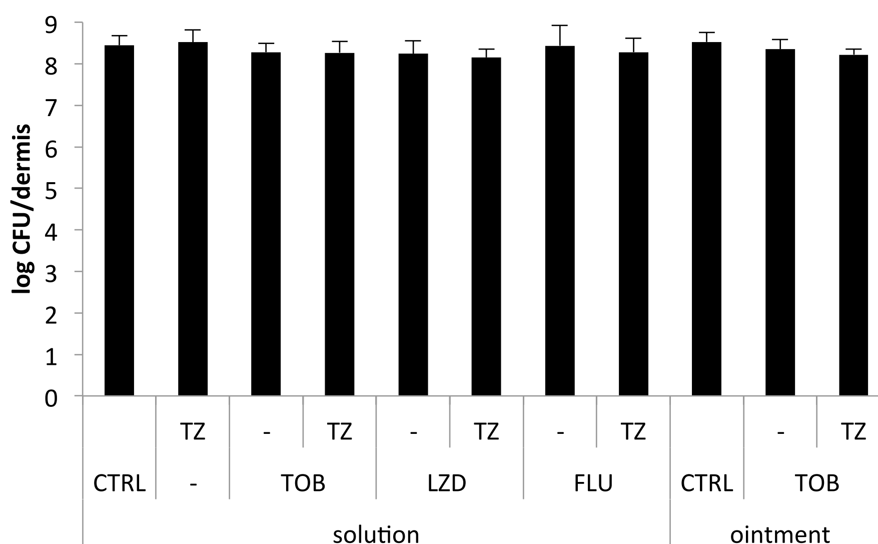

Figure 3. Evaluation of the activity of formulated solutions and ointments in the chronic wound biofilm model. Dermis were treated 24 h p.i. with 100 µl physiological saline (untreated control, CTRL), tobramycin (TOB, 2048 µg/ml), linezolid (LZD, 400 µg/ml), flucloxacillin (FLU, 20.5 mg/ml), alone or in combination with thioridazin (TZ, 100 µM), and with 250 mg ointment without compounds (CTRL) or including tobramycin (15 mg/g) alone or in combination with thioridazine (0.25 mg/g corresponding to 15 times the amount used in the solutions). Data shown are the average (n≥3), error bars indicate standard deviation.

The loss of antibiotic activity of the tested antibiotics is in accordance with the observation of Hill et al., who formed biofilms of *S. aureus* in a chronic wound biofilm constant depth film fermenter. These biofilms did not respond to treatment with high concentrations of flucloxacillin [233]. Also thioridazine was unable to cause a reduction in CFU/dermis, nor did it potentiate the activity of tobramycin, linezolid, or flucloxacllin. The high protein binding (99%) of thioridazine [234] might explain its deactivation in the chronic wound biofilm model since the medium used to moisten the artificial dermis contains 50% (v/v) plasma and 5% (v/v) horse blood. This hypothesis was evaluated in an experiment in which planktonic *S. aureus* Mu50 cells were treated with thioridazine (100 µM) in a plasma gradient. At 3% (v/v) plasma or less, thioridazine inhibits growth, whereas at 12% (v/v) plasma, *S. aureus* grows equally as the control without treatment. This means that thioridazine is deactivated at these

higher plasma concentrations (Figure S2). Loss of antibacterial activity of thioridazine due to protein binding was earlier postulated in *in vivo* tests [228, 230].

Other repurposing candidates in the NIHCC 1&2 library

Beside the antifungal imidazoles, sertraline and the phenothiazines described above, other anti-infectives, antineoplastic and hormonal drugs were identified. These drugs displayed an antimicrobial activity when used alone, except for tamoxifen which has no activity alone but does potentiate the activity of vancomycin (Table 1). However, these compounds are toxic and this limits their potential as repurposing candidate. Nevertheless, promising results were generated for chemotherapeutic drugs in other repurposing studies [189]. As the need is high for active compounds against biofilms, these drugs might find an application in catheter lock therapy and impregnation or coating of biomaterials [218].

Honokiol, MK-886 and 5-nonyloxytryptamine potentiated the activity of vancomycin and had antimicrobial activity on their own against biofilms of *S. aureus* Mu50. Honokiol has previous been described as being antimicrobial [235] and MK-886 and 5-nonyloxytryptamine were identified in another screen against planktonic MRSA [189]. Amiodarone and oxymetholone are potentiators of vancomycin and have no anti-biofilm effect on their own, in contrast to carvedilol and ethacrynic acid that cause a significant reduction in fluorescence signal compared to growth control when used alone. To our knowledge, these four drugs have not yet been identified as having antimicrobial activity or as antibacterial potentiators.

Several compounds were not withheld as hit in the present study although they were previously discovered as hits with antibacterial activity in other screening projects. Benzbromaron in combination with vancomycin reduced the fluorescence signal with 87% compared to treatment with vancomycin alone (Table S1). This drug was identified in a screening as interfering with the *S. aureus* quorum sensing system [236] and also shows anti *Candida albicans* biofilm activity [218]. In the presence of vancomycin, loperamide reduces the fluorescence signal with 88% compared to treatment with vancomycin alone (Table S1). Loperamide permeabilizes the outer cell membrane of Gram-negative bacteria and was identified in a screen in the presence of minocycline to be active against *Escherichia coli* and *Pseudomonas aeruginosa*, but not against *S. aureus* [198]. Although both benzbromaron and loperamide displayed activity in our screen, they were not withheld as hits due to the strict cut-offs applied in this study (residual activity of 10% or less compared to vancomycin alone).

In contrast, azelastine was not found to potentiate the activity of vancomycin in the present study, although it was previously identified as a potentiator of beta-lactams, macrolides, fluoroquinolones, aminoglycosides and tetracyclines against several Gram-positive bacteria (including *S. aureus*) [197]. Similarly, several NSAIDs [237], statins [238], and anesthetics [239], have been described to display antimicrobial activity but were not identified in the present screening, with the exception of loxoprofen. Several explanations can be given for this discrepancy. First of all, different strains are used in the different studies. Secondly, different concentrations are used among the studies. Thirdly, different media are used which can account for differences in the activity of these compounds (e.g. some statins need to be

activated by lactonases before they display antibacterial activity). Finally, most screening studies are targeting planktonic cells, while in the present study we specifically looked for potentiating activity against biofilm cells.

CONCLUSION

The aim of the present study was to identify compounds that increase the activity of antibiotics against established biofilms in a 96-well MTP model and subsequently investigate these combinations in a more relevant model system. Using this approach, we were able to identify 25 hits out of 727 off-patent drugs. Among these hits were four phenothiazines, of which thioridazine showed highest activity against both planktonic and biofilm cells. This compound was further evaluated in combination with several antibiotics in a 96-well MTP model. Combination with tobramycin, linezolid and flucloxacillin showed the highest activity. Evaluation in a recently described model for chronic wounds made it possible to estimate the value of these combinations in a model that mimics specific aspects of wound biofilms. Despite activity in the 96-well MTP biofilm model, no activity was observed in the chronic wound biofilm model. The present study provides a platform for discovering and evaluating potentiators against bacterial biofilms and highlights the necessity of using relevant *in vitro* biofilm model systems.

ACKNOWLEDGEMENTS

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SUPPLEMENTARY DATA

Table S1. The NIH Clinical Collection 1&2 were screened at a concentration of 100 µM in the presence of 40 µg/ml vancomycin for potentiators against biofilms of *S. aureus* Mu50. Hits were subsequently tested in absence of vancomycin. Effect was evaluated using CTB staining. The values shown in the left column represent the mean residual metabolic activity of the compound in the presence of vancomycin compared to treatment with vancomycin alone, and the standard deviation (SD). The values in the right column represent the mean residual metabolic activity of the compound compared to untreated control, and the standard deviation.

PUBCHEM CODE	COMPOUND NAME	MEAN (%)	SD	MEAN (%)	SD
CPD000449281	Nalbuphine	81	20		
CPD000449275	Raclopride	92	13		
CPD000449271	Zacopride	104	17		
CPD000449276	SKF 83566 D1 R agonist	101	18		
CPD000449316	3'-deoxydenosine	118	40		
CPD000449274	AM 404 N-arachidonoylaminofenol	67	17		
CPD000059053	Pilocarpine	122	37		
CPD000058291	Nifedipine	144	58		
CPD000042823	Flurbiprofen	74	12		
CPD000059136	Deferiprone	115	26		
CPD000058470	Loxapine	117	29		
CPD000326694	D-3-MEO-N-methylmorphinan	131	7		
CPD000449282	Duloxetine	79	13		
CPD000449320	Glycine	88	10		
CPD000449318	Benzene acetic acid	116	24		
CPD000058345	S-progesterone	117	7		
CPD000058961	Famotine	122	25		
CPD000449299	5-HTR-3 agonist	123	11		
CPD000466270	Pancuronium	162	21		
CPD000058175	Metronidazole	178	27		
CPD000449327	Benzeneacetic acid	114	6		
CPD000449323	Benzeneacetonitrile	110	18		
CPD000449328	Selegiline HCl	178	23		
CPD000449294	Zucapsaicin	111	33		
CPD000058513	Salbutamol	134	17		
CPD000057879	Vesamicol	129	48		
CPD000469289	Picrotin	144	26		
CPD000449268	Terazosin	161	44		
CPD000449319	Diphenylcyclopropenone	85	7		
CPD000449326	Thiazolidinecarboxylic acid	75	23		
CPD000466274	Mesoridazine	123	13		
CPD000449313	Pyridazinone difluoromethoxy	107	16		
CPD000466275	Phenothiazine	72	17		
CPD000449322	Cyclopentaquinolin	113	25		
CPD000058306	Clotrimazole	57	13		
CPD000058255	Loratadine	123	18		
CPD000058500	Phenelzine	76	7		
CPD000449311	Riluzole	134	23		
CPD000449312	Naltrindole	206	21		

CPD000449277	Nornicotine	134	12		
CPD000449269	Bifemelane	104	17		
CPD000449284	CGS antagonist	122	19		
CPD000449287	Cinanserin	70	13		
CPD000449272	Cisapride	68	32		
CPD000449273	Indatrline	16	14		
CPD000058520	Trazodone	45	10		
CPD000449301	Prazosin	64	7		
CPD000058525	Urapidil	83	27		
CPD000449278	Cotinine	73	12		
CPD000058313	Cycloserine	83	7		
CPD000466268	Fluvoxamine	70	8		
CPD000449270	Doxepin	82	9		
CPD000059133	Trifluoperazine HCl	0	0	72	23
CPD000058908	Hydroxy methylmorphinan	93	14		
CPD000449329	Ornithine	86	7		
CPD000148117	Maprotiline	72	33		
CPD000466272	Pizotyline	80	17		
CPD000059126	Beta-estradiol	135	13		
CPD000059046	Diacetyl diaminohexane	158	40		
CPD000058353	Diphengydramine	91	27		
CPD000449267	Galanthamine	115	15		
CPD000449296	Ifenprodil	157	26		
CPD000059171	Tetraethylthiuram	188	43		
CPD000449302	Piribedil	166	28		
CPD000058460	Ketoconazole	143	21		
CPD000058623	Tripelennamine	114	22		
CPD000449325	Pryazinecarboxamide	139	22		
CPD000059105	Amino tetrahydroacridine	290	152		
CPD000058319	Ethynylestradiol	161	54		
CPD000449317	Pyrimidinone arabinofu	218	46		
CPD000449324	L-glutaminic acid	147	31		
CPD000449305	Trifluoromethylphenylpiperazine	111	66		
CPD000449298	Pramipexole	230	55		
CPD000058189	Lidocaine	235	55		
CPD000449290	Indometacine	179	49		
CPD000058555	Tomelukast	78	50		
CPD000466269	Paroxetine	107	10		
CPD000449288	Epigallocatechin	83	26		
CPD000145728	Amino hydroxy benzoic	208	59		
CPD000449321	Oxiranecarboxylic acid	194	77		
CPD000338536	Cephalexine	85	1		
CPD000466390	Pidotimod	79	9		
CPD000466386	Ramipril	95	75		
CPD000469284	Fenpiverinium	31	5		
CPD000058610	Nortestosterone	54	2		
CPD000466384	Niztididne	69	41		
CPD000059047	Flucytosine	51	10		
CPD000048684	Oxcarbazepine	73	7		

CPD000466385	Troxipide	57	33		
CPD000466341	Actarit	39	8		
CPD000469183	Azelastine	54	9		
CPD000466388	Tocainide	96	31		
CPD000499525	Taxifolin	77	3		
CPD000466387	Levofloxacin	86	29		
CPD000469182	Cefatrizine	126	14		
CPD000466364	Idebenone	92	17		
CPD000466366	Levosulpiride	132	48		
CPD000238142	Pemoline	142	51		
CPD000466343	letrozole	131	68		
CPD000469184	Meropenem	140	17		
CPD000466339	Orlistat	139	41		
CPD000469179	Ondansetran	111	15		
CPD000059117	Levonorgestrel	244	92		
CPD000469197	Cetraxate	189	18		
CPD000149316	Alprazolam	161	73		
CPD000058464	Lamotignine	189	34		
CPD000059145	Crotamiton	185	19		
CPD000472526	Amfebutamone	166	31		
CPD000466340	Alfuzonsine	48	12		
CPD000449309	Amisulpride	95	6		
CPD000469292	Lofepramine	39	12		
CPD000466362	Perospirone	69	20		
CPD000059010	Docetaxel	76	1		
CPD000387107	Honokiol	0	0	0	0
CPD000469196	Tolterodine	70	9		
CPD000466363	Carmofur	58	9		
CPD000469181	Paroxetine	80	17		
CPD000466337	Olmesartan	89	5		
CPD000469593	Losartan	78	13		
CPD000466338	Temozolomide	136	38		
CPD000058528	Methyltestosterone	90	7		
CPD000469195	Tosufloxacin	67	14		
CPD000466361	Mecillinam	110	13		
CPD000469177	Atomoxetine	92	36		
CPD000466336	Artesunate	71	13		
CPD000058959	Itraconazole	91	27		
CPD000469193	Cefpodoxime	78	14		
CPD000058803	Buflomedil	105	45		
CPD000012114	Moclobemide	207	65		
CPD000466330	Halometasone	167	40		
CPD000466357	Triclabendazole	26	12		
CPD000466331	Rofecoxib	138	23		
CPD000471619	Bisoprolol	165	39		
CPD000466334	Ezetimibe	183	53		
CPD000469176	Tiagabine	268	42		
CPD000466355	Idarubicin	0	0	15	27
CPD000466360	Flubendazole	62	2		

CPD000466356	Tacrolimus	72	11		
CPD000469208	Valaciclovir	72	26		
CPD000466382	Clarithromycin	43	7		
CPD000466383	Aripiprazole	50	9		
CPD000471622	Trimebutine	102	14		
CPD000238198	Mestalone	81	17		
CPD000466370	Nisoldipine	20	1		
CPD000466371	Piceid	103	22		
CPD000149359	Secnidazole	119	17		
CPD000466369	Nifekalant	87	25		
CPD000466372	Nateglinide	76	21		
CPD000058691	Megestrol acetaat	93	11		
CPD000466374	Ormetoprim	188	34		
CPD000466377	Zileuton	89	28		
CPD000058350	Stavudine	102	18		
CPD000058918	Gabexate	98	44		
CPD000469293	Oxiconazole	0	0	10	10
CPD000469235	Kitasamycin	104	22		
CPD000466375	Famciclovir	131	16		
CPD000326828	Sotalol	109	35		
CPD000466373	Rufloxacin	75	13		
CPD000466389	Taxiflolin	160	87		
CPD000469211	Alosetron	66	23		
CPD000058423	Bupropion	98	6		
CPD000471621	Irsogladine	125	10		
CPD000466376	Acarbose	64	12		
CPD000469294	Benproperine	30	3		
CPD000466378	Fenprobamaat	82	1		
CPD000058926	Adamantan	73	11		
CPD000449280	Carvedilol	8	6	41	6
CPD000466379	Lomifylline	75	13		
CPD000466380	Pazufloxacin	50	12		
CPD000466381	Miglitol	60	7		
CPD000058373	Tranilast	60	4		
CPD000466345	Olanzapine	79	26		
CPD000449297	Nefazodone	27	6		
CPD000469185	Moxifloxacin	59	9		
CPD000469186	Nelfinavir	72	10		
CPD000469187	Pravastatin	129	25		
CPD000466344	Topotecan	97	24		
CPD000466303	Levetiracetam	80	14		
CPD000469142	Pramipexole	102	26		
CPD000466323	Risperidone	114	17		
CPD000469167	Pioglitazone	110	12		
CPD000469147	Cilastatin	124	23		
CPD000466348	Argatroban	155	53		
CPD000466327	Valdecoxib	101	10		
CPD000466346	Naftopidil	66	29		
CPD000156231	Nobiletin	151	91		

CPD000466304	Finasteride	82	9		
CPD000469145	Zolpidem	104	9		
CPD000048458	Viramune	98	15		
CPD000466325	Topiramate	81	14		
CPD000466350	Voriconazole	99	19		
CPD000469190	Fenoldopam	65	1		
CPD000471612	Rosiglitazone	71	6		
CPD000469191	Escitalopram	96	15		
CPD000058866	Zeranol	100	19		
CPD000466354	Latanoprost	53	11		
CPD000058576	Didanosine	114	23		
CPD000466298	Sertraline	0	0	51	21
CPD000466353	Calcipotriol	63	13		
CPD000466308	Epirubicin	32	7		
CPD000466329	Bicalutamide	99	10		
CPD000469192	benidipine	123	24		
CPD000466352	Amlexanox	59	12		
CPD000469148	Cerivastatin	74	8		
CPD000466309	Icariin	89	12		
CPD000466310	Methylandrosternediol	64	6		
CPD000466307	Triptolide	77	15		
CPD000469170	Rosiglitazone	83	23		
CPD000059106	Tegafur	93	12		
CPD000466392	Oligomycin	62	3		
CPD000469199	Benazepril	135	7		
CPD000058877	Oxymetholone	0	0	88	47
CPD000059060	Ipriflavone	101	10		
CPD000058286	Oxaprozin	127	8		
CPD000058510	Rolipram	119	14		
CPD000469200	Mosapride	138	3		
CPD000466391	Isoquercitrin	84	13		
CPD000058450	Flumazenil	96	18		
CPD000469164	Ozagrel	115	10		
CPD000466394	Hyperoside	87	8		
CPD000466322	Rifabutin	77	14		
CPD000469141	Esmolol	116	19		
CPD000466321	Tadalafil	89	10		
CPD000058957	Modafinil	108	7		
CPD000058570	Doxorubicin	12	1		
CPD000469209	Moxonidine	94	8		
CPD000058302	Nitrazepam	38	8		
CPD000387024	Pefloxacin	147	18		
CPD000469154	Venlafaxine	134	20		
CPD000469592	Pantoprazole	125	27		
CPD000469159	Fluticasone	89	5		
CPD000469161	Indinavir	118	36		
CPD000469160	Midazolam	121	6		
CPD000466319	Lamivudine	91	17		
CPD000469151	366-70-1	80	12		

CPD000469280	Esomeprazole	133	6		
CPD000059146	Sulfasalazine	158	22		
CPD000466313	Torasemide	128	17		
CPD000469156	Tropistrona HCl	139	11		
CPD000326795	Ranolazine	148	13		
CPD000058366	Nitrendipine	58	7		
CPD000469290	Saquinavir	46	1		
CPD000058970	Bifonazole	13	2		
CPD000469158	Sumatriptan	165	27		
CPD000466314	Exemestane	147	13		
CPD000466367	Nitazoxanide	126	35		
CPD000058398	Diazepam	69	16		
CPD000471623	Quetiapine	119	16		
CPD000112560	Rutin	125	16		
CPD000466317	Penciclovir	83	17		
CPD000466393	Calcitriol	208	44		
CPD000469140	Diphenoxylate	140	12		
CPD000449307	Felbamate	163	45		
CPD000058855	Droperidol	51	10		
CPD000035998	Pentoxifylline	71	6		
CPD000058461	Ketorolac	69	18		
CPD000466395	Ritonavir	65	4		
CPD000469210	Vinorelbine tartrate	60	6		
CPD000466335	Linezolid	43	3		
CPD000469203	Lomerizine	45	4		
CPD000466351	Efavirenz	0	1	5	8
CPD000466306	Irbesartan	72	6		
CPD000466305	Repaglinide	86	5		
CPD000238204	Ethylestrenol	58	19		
CPD000440694	Pterostilbene	29	12		
CPD000469144	Roxatidine	110	22		
CPD000471616	Dexbrompheniramine	79	9		
CPD000469168	Anagrelide	85	5		
CPD000471618	Tegaserod	101	21		
CPD000058475	Milrinone	97	26		
CPD000466315	Levocetirizine	136	16		
CPD000326936	Citalopram	85	26		
CPD000048468	Ticlopidine	92	13		
CPD000469165	Sodium loxoprofen	3	1	42	29
CPD000466316	Zafirlukast	98	9		
CPD000469152	Terbinafine	111	12		
CPD000466320	Isradipine	119	5		
CPD000466318	Valsartan	94	46		
CPD000449291	Piroxicam	43	6		
CPD000469282	Glycopyrrolate	161	11		
CPD000449286	Physostigmine	126	13		
CPD000058465	Lobeline	113	6		
CPD000058436	Doxylamine	90	24		
CPD000449266	Milnacipran	73	21		

CPD000449315	Fluoro pyrimidone	118	17		
CPD000466271	Chlorpheniramine	65	22		
CPD000466333	Dofetilide	46	17		
CPD000471620	Formoterol	93	19		
CPD000525252	Rizatriptan	94	7		
CPD000466332	Rifapentine	127	31		
CPD000469178	Loteprednol	91	9		
CPD000466359	Enalaprilat	81	16		
CPD000449292	Donepezil	113	15		
CPD000238177	Nimetazepan	69	12		
CPD000466365	Nicorandil	172	25		
CPD000466326	Telmisartan	206	43		
CPD000469143	Itopride	89	13		
CPD000466324	Rifaximin	87	6		
CPD000469188	Montelukast	71	27		
CPD000058253	Didezoxycytidine	87	2		
CPD000466276	Imidazol amine	78	6		
CPD000466280	Pyridobenzodiazepin	99	8		
CPD000466278	MK886	5	2	39	25
CPD000466277	Etomidate	105	7		
CPD000466281	Acetamide	73	10		
CPD000466283	Altanserin	60	7		
CPD000058420	Betaxolol	94	16		
CPD000466311	Indirubine	114	15		
CPD000466285	Azasetron	109	4		
CPD000466287	GR 89696	134	15		
CPD000058773	Delta-hydrocortisone	93	2		
CPD000058392	Diazoxide	85	9		
CPD000058612	Chloroadenosine	67	24		
CPD000058726	Ornidazole	112	16		
CPD000058572	Dimethyl phenylpiperzanine	97	12		
CPD000058507	Pirenperone	84	7		
CPD000059128	Mestramol	89	8		
CPD000059100	Aminoethyl pyridine	159	52		
CPD000059142	Benactyzine	65	21		
CPD000059158	Dichloroacetic acid	121	9		
CPD000059165	Bestatin	74	10		
CPD000469213	Toremifene	0	0	9	7
CPD000469214	Goserelin	73	4		
CPD000469212	Secoisolarciciresinol	63	2		
CPD000469217	Raltitrexed	75	16		
CPD000469229	Doxapram	93	4		
CPD000466294	Serotonin agonist	75	2		
CPD000112281	Brucine	75	6		
CPD000059115	Tryptoline	66	5		
CPD000058411	Fluphenazine	6	3	33	9
CPD000469233	Palonosetron	82	22		
CPD000058746	Naproxen	82	10		
CPD000058904	Mepivacaine	93	9		

CPD000058310	Benzbromaron	13	3
CPD000058300	Nimodipine	74	10
CPD000058701	Rolitetracycline	121	11
CPD000058715	Epirizole	104	17
CPD000058273	Azauridine	76	12
CPD000466922	Reichsteins	100	21
CPD000059086	Pyridinemethanol	124	16
CPD000449283	Haloperidol	60	15
CPD000449279	Stiripentol	73	6
CPD000449303	Fluperlapine	91	23
CPD000058660	Oxyphenomium	110	30
CPD000112358	Homoveratrylamine	145	43
CPD000058194	Tinidalzole	188	14
CPD000058741	Xanthinol	129	14
CPD000059111	Synephrine	159	33
CPD000058206	Resveratrol	80	17
CPD000059093	Malrol	100	27
CPD000059077	Aminoimidazole	114	9
CPD000059011	Enrofloxacin	80	20
CPD000058603	Dehydrocholic acid	89	49
CPD000058250	Cefaclor	114	14
CPD000059044	Benzylimidazole	84	23
CPD000469136	Duloxetine	32	7
CPD000469155	Vardenafil	146	15
CPD000469137	Ropivacaine	130	36
CPD000466301	Anastrozole	155	44
CPD000058462	Ketotifen	196	38
CPD000058769	Medroxy-progesterone	139	36
CPD000466919	Pinacidil	187	38
CPD000058266	Nitro-indazole	84	6
CPD000112269	Methoxytryptamine	89	24
CPD000059045	Phenothiazine	114	18
CPD000058553	Cladribine	79	14
CPD000469138	Granisetron HCl	90	9
CPD000466293	Rimcazole	108	27
CPD000466292	Nafadoride	84	13
CPD000058856	Desoximetasone	101	10
CPD000471617	Dexchlorpheniramine	154	18
CPD000466288	Guanidine	91	16
CPD000466290	SMR000466290	113	24
CPD000466284	AM251	147	38
CPD000466289	HTMT	106	6
CPD000466286	Benzo phenanthridine	68	28
CPD000466291	Methanesulfonamide	72	21
CPD000466279	H2-indol-2-on	71	12
CPD000466920	Beclosmethasone	120	31
CPD000058847	Omeprazole	69	9
CPD000469228	Dolastron	87	18
CPD000449310	Zolmitriptan	98	16

CPD000469223	Tremaulacin	52	12		
CPD000469227	Dactinomycin	33	8		
CPD000449308	Tramadol	77	2		
CPD000469226	Chlordiazeposide	106	24		
CPD000469225	Cefixime	129	35		
CPD000469224	Cefdinir	137	26		
CPD000469232	Lofexidine	147	41		
CPD000469221	Balsalazide	126	29		
CPD000469220	Olopatadine	55	8		
CPD000469287	Itavastatin	87	2		
CPD000058334	Cortisone	101	26		
CPD000058431	Cyproheptadine	72	14		
CPD000469230	Homoharringonine	65	7		
CPD000058318	Corticosterone	149	29		
CPD000471625	Vecuronium	94	16		
CPD000469219	Itibolone	119	31		
CPD000058212	Niacinamide	120	11		
CPD000059131	Nialamide	86	24		
CPD000469153	Vindesine	84	22		
CPD000058540	Vincristine	87	6		
CPD000466342	Lacidipine	71	37		
CPD000466347	Mirtazapine	33	14		
CPD000469285	Ampiroxicam	54	8		
CPD000466368	Glimepiride	67	25		
CPD000469198	Amlodipine	81	39		
CPD000469174	Rabeprazole	58	12		
CPD000058704	Clofazimine	26	10		
CPD000469166	Irinotecan	50	17		
CPD000058469	Lansoprazole	69	17		
CPD000149358	Desloratadine	70	19		
CPD000058772	Premarine	53	14		
CPD000058481	Mifepristaone	83	17		
CPD000112002	Etoposide	181	30		
CPD000238156	Sibutramine	115	28		
CPD000469632	Clobenpropit	76	21		
CPD000469231	Huperzine	102	28		
CPD000472527	Sibutramine HCl	108	13		
CPD000058410	Lorazepam	153	27		
CPD000469633	Azaspiro-decane-dione	128	29		
CPD000469631	Adenosine	167	16		
CPD000058296	Amiodarone	6	9	68	6
CPD000336944	Mevastatine	103	5		
CPD000469175	Imatinib	150	26		
CPD000468736	Melperone HCl	176	34		
CPD000469594	Parecoxib	181	40		
CPD000058504	Pergolide	185	14		
CPD000471626	Atracurium	65	27		
CPD000469218	Artemether	138	15		
CPD000058445	Ebselen	180	117		

CPD000468733	12066B	42	13		
CPD000469222	Teletromycine	140	17		
CPD000468732	CCPA	180	52		
CPD000468734	Methanone	57	11		
CPD000058878	Stanzolol	138	20		
CPD000238180	Zaleplon	152	12		
CPD000112594	Prostaglandin	98	25		
CPD000058344	Testosteron	206	21		
CPD000059075	Dehydroepiandrosterone	145	50		
CPD000466297	Sdm25n	195	62		
CPD000466299	Tiophene brome fluor	180	7		
CPD000466300	Nonyloxytryptamine	0	0	37	15
CPD000466295	Salmeterol	56	20		
CPD000466296	SB 205607	145	59		
CPD000326935	R-SCH 23390	89	14		
CPD000058230	Doxepin	94	12		
CPD000058382	Dipyridamole	81	8		
CPD000059151	Propofol	105	16		
CPD000058600	Ethacrynic acid	5	5	1	1
CPD000058187	Flutamide	2	1	24	14
CPD000058299	Fenofibrate	119	21		
CPD000058202	Furosemide	100	9		
CPD000038082	Fluorouracil	75	16		
CPD000471860	Folic acid	86	23		
CPD000653523	Hydrocortisone	74	6		
CPD000653536	Cortell	90	25		
CPD000058184	Ibuprofen	117	13		
CPD000040181	Ketoprofen	113	23		
CPD001906766	Minocycline	47	12		
CPD000058733	Miconazole	9	10	33	22
CPD000059134	Metyrapone	99	17		
CPD001317860	Pyrogluatmic acid	100	22		
CPD000058975	Nadolol	84	32		
CPD000058999	Disipal	86	32		
CPD000058192	Ofloxacin	98	16		
CPD000059120	Pindolol	84	10		
CPD000037139	Praziquantel	71	36		
CPD000059104	Phenylbutyric acid	117	22		
CPD000058326	Prednisolone	97	3		
CPD000058379	Phenergan	67	21		
CPD000058180	Perphenazine	9	3	29	8
CPD000718761	Prednisolone	101	10		
CPD000058506	Prilocaine	108	12		
CPD001227202	Prednisone	61	24		
CPD000059161	DL-penicillamine	80	23		
CPD000058579	Piperacilline	107	12		
CPD000857275	Quinidine	64	2		
CPD000653467	Ranitidine	79	19		
CPD001906767	Rifampicine	104	14		

CPD000058245	Retinoic acid	111	14		
CPD000471892	Spironolactone	90	32		
CPD000035999	Trimethoprim	90	8		
CPD000058219	Tyzine	83	21		
CPD000059176	Thyroxine	95	2		
CPD000058515	Trihexyphenidyl	137	38		
CPD000058403	Ursodeoxycholic	107	25		
CPD000059064	Dapsone	95	23		
CPD001370746	Symmertrel	109	15		
CPD000058849	Warfarin	113	8		
CPD000058394	Acetazolamide	109	28		
CPD000059083	Allopurinol	95	16		
CPD001906768	Atropine	79	5		
CPD000058264	Nalidixic acid	95	15		
CPD001567029	Triiodothyronine	63	2		
CPD000058284	Hydroflumethiazide	103	24		
CPD000058368	Annoyltin	56	25		
CPD000058613	Busulfan	80	23		
CPD000058269	Chlorzoxazone	82	17		
CPD000058429	Chlorothiazide	94	15		
CPD001370748	Cimetidine	83	14		
CPD000058433	Carisoprodol	80	35		
CPD000058364	Chlorpropamide	130	18		
CPD000058440	Bentyl	87	11		
CPD000312779	Chloroxine	42	5		
CPD000058723	Diflunisal	149	48		
CPD001370749	Econazole	5	5	24	24
CPD001370750	Ethionamide	173	62		
CPD000058719	Methocarbamol	199	19		
CPD000035778	Hydrochlorothiazide	181	40		
CPD001370751	Vistaril	70	10		
CPD000058356	Hexachlorophene	0	0	8	10
CPD000059082	Isoniazid	280	75		
CPD000058729	Duvadilan	249	9		
CPD000058267	Isoproterenol	242	50		
CPD000471847	Triclosan	0	0	2	3
CPD000058188	Mefenamic acid	86	10		
CPD000058832	Cantil	80	13		
CPD000058471	Metoclopramide	132	9		
CPD001370753	Methyldopa	96	44		
CPD000058271	Nitrofurantoin	16	9		
CPD000058486	nortriptyline	57	14		
CPD000058292	Naphazoline	54	5		
CPD000059024	Nicotinic	95	48		
CPD000058817	Norflex	92	28		
CPD001614498	Oxytetracycline	83	34		
CPD000718771	Procaine	87	14		
CPD000058714	Pyrimethamine	111	8		
CPD000058661	Pro-banthine	95	7		

CPD000058280	Probenecid	66	3		
CPD001906769	Pyridine 2 aldoxime	98	14		
CPD000058501	Primidone	82	6		
CPD000058275	Propylthiouracil	79	6		
CPD000036662	Pyrazinamide	95	20		
CPD000059079	Probesyl	80	12		
CPD000037657	Sulfisoaxole	75	15		
CPD000058223	Sulfamethoxazole	115	24		
CPD000058173	Sulfacetamide	90	20		
CPD000058991	Sulfinpyrzone	153	21		
CPD000326718	Sulindac	168	16		
CPD001906770	Tetracycline	114	27		
CPD000058537	Theophylline	127	30		
CPD000058363	Tolbutamide	106	7		
CPD000059118	Triamteren	70	14		
CPD000059081	Intropin	89	2		
CPD000058416	Amoxapine	91	6		
CPD000471872	Adenine	95	13		
CPD000036768	Atenolol	104	19		
CPD001491671	Tamoxifen	0	0	67	10
CPD000058418	Bumetanide	93	9		
CPD000058745	Clobetasol	136	10		
CPD000058254	Chlorpromazine	17	6		
CPD001491644	Cefazoline sodium	131	23		
CPD000059061	Captopril	126	2		
CPD000058372	Chlorambucil	98	15		
CPD000058809	Cefoxitin	116	27		
CPD000058321	Danazol	93	10		
CPD000058375	Diltiazem	133	11		
CPD001906774	Digoxin	154	16		
CPD000058346	17- beta estradiol	95	29		
CPD000058672	Edroponium	103	16		
CPD000058329	Fluocinolone acetonide	117	23		
CPD000042823	Flurbiprofen	141	35		
CPD000058455	Glipizide	119	11		
CPD000058393	Gemfibrozil	84	29		
CPD000058229	Glyburide	171	28		
CPD000058328	Hydrocortisone	163	41		
CPD000058829	Indapamide	140	19		
CPD001906775	Ipratropium bromide	109	19		
CPD000058388	Imipramine	111	13		
CPD000058463	Labetalol	116	21		
CPD000058466	Loperamide	12	7		
CPD000058833	Pro amatine	113	36		
CPD000653524	Medroxyprogesterone acetate	120	13		
CPD001906776	19-norethindrone acetate	77	3		
CPD000499579	19-norethindrone	96	23		
CPD000059074	Nicotine	102	13		
CPD001456372	Cardene	90	9		

CPD000058835	Nabmetone	165	14		
CPD000058490	Oxybutynin	119	36		
CPD000058605	Mestinon	132	61		
CPD001453705	Rythmol	133	31		
CPD001491654	Pfizerpen	87	15		
CPD000499581	Valproic acid	76	7		
CPD000058821	Procyclidine	75	18		
CPD000875264	Proxymetacaine	60	12		
CPD000058766	Naloxone	50	8		
CPD001906777	Spectinomycin	83	21		
CPD000058523	Tropicamide	62	14		
CPD000058290	Tolazamide	49	9		
CPD000058335	Triamcinolone acetonide	74	3		
CPD001456519	S-timolol	63	7		
CPD000058170	Thiabendazole	63	15		
CPD000058380	Thioridazine	0	0	35	16
CPD000058181	Altretamine	74	7		
CPD001491672	Phylloquinone	75	10		
CPD001491659	Eryped	84	4		
CPD000058422	Dibenzyline	24	8		
CPD000058693	Medrysone	132	27		
CPD000058524	Thalidomide	69	11		
CPD000857229	Aminolevulinec	37	1		
CPD001496929	Arbinoxamine	108	12		
CPD001496930	Demeclocycline	55	12		
CPD001496932	Westcort	81	18		
CPD000449328	Selegiline	100	16		
CPD000058840	6-2-ethoxy-1-naphthamido	86	23		
CPD000875314	Primaquine	42	11		
CPD001496934	Micropenin	117	10		
CPD001550033	Doxycycline	43	9		
CPD001233361	Beclomethasone	79	9		
CPD000058721	Cromolyn	82	16		
CPD000149600	Priscoline	88	5		
CPD000544948	Mercaptopurine	95	12		
CPD000427366	Azathioprine	67	12		
CPD000036735	Albendazole	97	10		
CPD000718755	Griseofulvin	101	3		
CPD000059006	Lincomycine	107	8		
CPD001496938	Methazolamide	111	18		
CPD001496939	Terbutaline	96	32		
CPD000471888	Mypirocin	76	2		
CPD000058331	Fluocinonide	113	4		
CPD000875233	Mefloquine HCl	21	4		
CPD001496941	Floxuridine	78	4		
CPD001563707	Mitoxantrone	55	8		
CPD001906784	Enalapril maleate	84	13		
CPD000058337	Budesonide	81	15		
CPD000466386	Ramipril	73	6		

CPD000718757	Depo medrol	85	15		
CPD000058383	Norepinephrine	61	7		
CPD001491664	Amcinonide	43	7		
CPD001317855	Clomid	0	0	13	1
CPD001819784	Phentolamine mono HCl	66	13		
CPD000058874	Fludarabine	73	14		
CPD000109709	Testosterone	80	19		
CPD000471891	Isotretinoin	68	4		
CPD000058376	Methimazole	66	2		
CPD000596519	Zonisamide	95	21		
CPD000058355	Brimondidine	64	4		
CPD000036734	Mebendazole	66	8		
CPD000058736	Meclizine	107	14		
CPD000058451	Flecainide	0	0		
CPD000146393	Dilantin	60	13		
CPD000059182	Miochol	56	5		
CPD000326766	Dantrolene sodium	26	2		
CPD001227192	Dexamethasone	75	20		
CPD000394012	Cogentin	66	11		
CPD000058324	Ganciclovir	63	13		
CPD000059219	Mesna	82	17		
CPD000058785	Meclomen	119	5		
CPD000471882	Fluconazole	146	18		
CPD001453712	Metaproterenol	177	46		
CPD000071170	Methoxsalen	133	41		
CPD000058224	Chloramphenicol	116	11		
CPD000499584	Tizanididne	191	4		
CPD001453706	Paroxetine	31	14		
CPD000550486	Mirtazapine	213	9		
CPD000010931	Etomidate	161	15		
CPD000499578	Moban	147	18		
CPD001453708	Fluvastatin	140	37		
CPD000058680	Urecholine	140	17		
CPD001496804	Cefuroxime	146	18		
CPD000718805	Cytoxan	153	15		
CPD000550478	Eszopiclone	66	7		
CPD000058802	Bendrofluazide	116	9		
CPD000058508	Raloxifen	52	37		
CPD000058351	Zidovudine	61	11		
CPD000058365	Clozapine	61	11		
CPD001317850	Amicillin	69	31		
CPD000058800	Acebutol	57	15		
CPD000058707	Amoxicilline	43	7		
CPD000857209	Epinephrine	61	27		
CPD000857239	Azacytidine	62	23		
CPD000058186	Buspar	42	8		
CPD000436311	Rimantadine	54	9		
CPD000059121	Podofilox	88	47		
CPD000058313	D-cycloserine	64	8		

CPD000059124	Cortisone	81	6		
CPD000058295	Clomipramine	47	14		
CPD001227191	Carbamazepine	58	36		
CPD000875213	Memantine	30	5		
CPD000036827	Desipramine	23	6		
CPD000326711	Mexiletine	60	22		
CPD000058438	Disopyramide	55	9		
CPD000673569	Stavudine	88	47		
CPD000097306	Doxazosin	36	19		
CPD000058963	Minoxidil	46	22		
CPD000059167	Propranolol	95	23		
CPD001496943	Ribavirin	106	43		
CPD000058309	Terazosin	158	25		
CPD000058635	Chlorthalidone	141	46		
CPD000058330	Methylprednisolone	136	60		
CPD001496977	Phenelzine	213	137		
CPD000058767	Naltrexone	162	15		
CPD000469282	Glycopyrrolate	145	20		
CPD000046147	Ethambutol	160	20		
CPD001453715	Cetirizine	128	17		
CPD000539527	Dicloxacillin	151	45		
CPD000718800	Meloxicam	143	32		
CPD001906781	Daunorubicin	0	0	18	16
CPD001906779	Rifapentine	152	49		
CPD000274084	Penicillin	98	5		
CPD000043336	Gatifloxacin	83	7		
CPD000550475	Clopidogrel	102	7		
CPD001551784	Cefotaxime	79	5		
CPD000466319	Lamivudine	90	7		
CPD001307702	Ondansetron	97	10		
CPD000339803	Betamethasone	89	11		
CPD000550473	Celecoxib	102	4		
CPD000058778	Aminomethylbenzenesulf	93	0		
CPD001906782	Thiothixene	75	2		
CPD000465669	Citalopram	89	14		
CPD000471864	Azithromycin	116	7		
CPD000673570	Lovastatin	73	9		
CPD000326785	Aminoglutetimide	103	7		
CPD000058452	Fluoxetine	11	5		
CPD001233272	Flunisolide	106	20		
CPD000058225	Acyclovir	93	10		
CPD000058443	Etodolac	71	9		
CPD000718785	Simvastatin	95	12		
CPD001227203	Rifabutin	88	12		
CPD001496951	Felodipine	76	19		
CPD000499582	Quinapril	96	15		
CPD000499573	Acitretin	50	13		
CPD000718798	Fexofenadine	83	9		
CPD001563899	Fluoromethadolone	108	30		

CPD000466298	Sertraline	8	3	51	21
CPD001566944	Carbidopa	111	15		

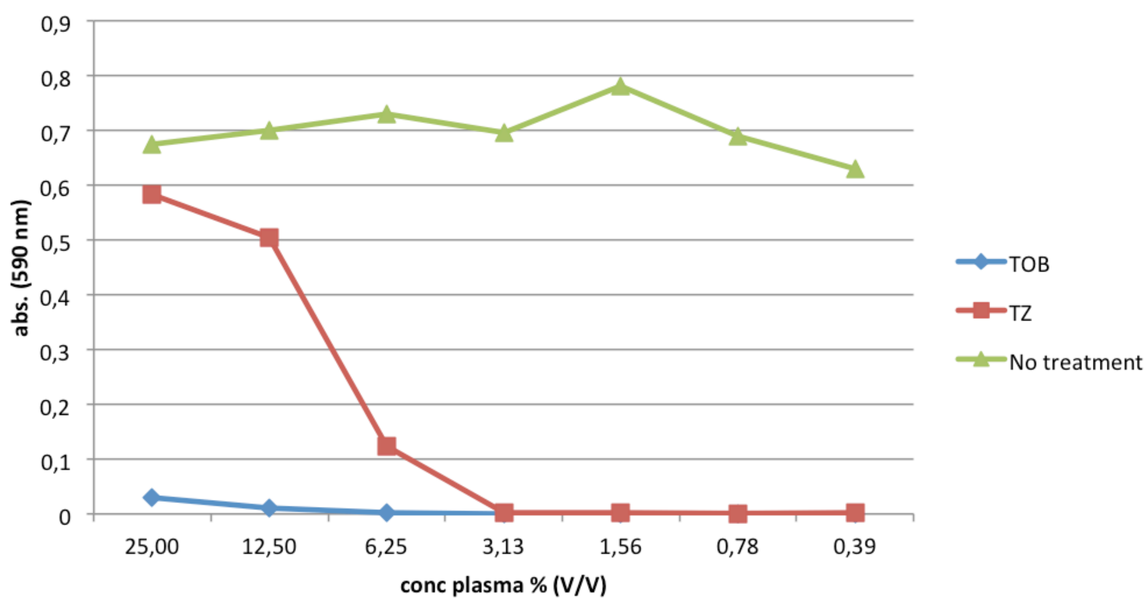

Figure S2. Activity of tobramycin (TOB) and thioridazine (TZ) or no treatment at different plasma gradients on the growth inhibition of *S. aureus* Mu50. Absorbance (abs.) is measured at 590 nm.

Paper III:
Evaluation of combination therapy for *Burkholderia cenocepacia* lung infection in different *in vitro* and *in vivo* models

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ABSTRACT

Burkholderia cenocepacia is an opportunistic pathogen responsible for life-threatening infections in cystic fibrosis patients. *B. cenocepacia* is extremely resistant towards antibiotics and therapy is complicated by its ability to form biofilms.

We investigated the efficacy of an alternative antimicrobial strategy for *B. cenocepacia* lung infections using *in vitro* and *in vivo* models. A screening of the NIH Clinical Collection 1&2 was performed against biofilms of *B. cenocepacia* formed in 96-well microtiter plates in the presence of tobramycin to identify repurposing candidates with potentiator activity. The efficacy of selected hits was evaluated in a three-dimensional (3D) organotypic human lung epithelial cell culture model. The *in vivo* effect was evaluated in the invertebrate *Galleria mellonella* and in a murine *B. cenocepacia* lung infection model.

The screening resulted in 60 hits that potentiated the activity of tobramycin against *B. cenocepacia* biofilms, including four imidazoles of which econazole and miconazole were selected for further investigation. However, a potentiator effect was not observed in the 3D organotypic human lung epithelial cell culture model. Combination treatment was also not able to increase survival of infected *G. mellonella*. Also in mice, there was no added value for the combination treatment.

Although potentiators of tobramycin with activity against biofilms of *B. cenocepacia* were identified in a repurposing screen, the *in vitro* activity could not be confirmed nor in a more sophisticated *in vitro* model, neither *in vivo*. This stresses the importance of validating hits resulting from *in vitro* studies in physiologically relevant model systems.

INTRODUCTION

Species belonging to the *Burkholderia cepacia* complex (*Bcc*) are opportunistic pathogens mainly known for causing chronic lung infections in cystic fibrosis (CF) patients and in patients with chronic granulomatous disease [240, 241]. *Burkholderia cenocepacia* belongs to the *Bcc* and accounts for approximately 45% of *Bcc* infections in CF patients in the United States [240, 241]. The complex interaction between *B. cenocepacia* and the CF lung is reflected by the various clinical outcomes and disease severity, from transient colonization to necrotizing pneumonia and sepsis resulting in early death. In addition, respiratory infections caused by *B. cenocepacia* are associated with lower survival following lung transplantation [242, 243]. Antimicrobial therapy against these infections frequently fails due to *B. cenocepacia's* resistance to many antibiotics and the induction of biofilm-specific tolerance mechanisms [203, 244, 245].

Compounds that improve the activity of antibiotics have been called helper compounds [185], potentiators [246], adjuvants [247], or resistance breakers [13]. The aim of the present study was to identify such compounds that reverse tolerance and/or resistance towards tobramycin by increasing the susceptibility of *B. cenocepacia* biofilms. To this end, we screened a

repurposing library containing compounds that passed human safety assessments and for which the toxicity and metabolic properties are already known [189]. Activity was evaluated in several *in vitro* and *in vivo* assays.

MATERIAL AND METHODS

Strains and culture conditions

B. cenocepacia LMG 16656 was maintained on tryptic soy agar plates (TSA, Lab M, Lancashire, UK). Overnight suspensions were obtained by inoculating Mueller Hinton broth (MH, Lab M) with several colonies and incubation with shaking for 18 h. A green fluorescent protein (GFP) producing *B. cenocepacia* K56-2 mutant was maintained on LB agar (Lab M) supplemented with 800 µg/mL trimethoprim (Ludeco, Brussels, Belgium) [248]. Overnight suspensions were made in LB broth supplemented with 800 µg/mL trimethoprim and inoculated for 23-24 h in a shaker (250 rpm). All cultures were kept aerobically at 37°C.

Library compounds and antimicrobial agents

The NIH Clinical Collection 1&2 (NIHCC 1&2, Evotec, San Francisco, CA) contain 727 compounds dissolved in 100% dimethyl sulfoxide (DMSO) at a concentration of 10 mM. Miconazole nitrate was obtained from Certa NV (Eigenbrakel, Belgium), meropenem from Astrazeneca (Zoetermeer, The Netherlands), econazole nitrate, ketoconazole, ciprofloxacin, and gentamicin sulfate from Sigma (Bornem, Belgium), oxiconazole nitrate from Santa Cruz Biotechnology (Heidelberg, Germany), and tobramycin from TCI chemicals (Tokyo, Japan). Azoles were dissolved in 100% DMSO (Sigma) and subsequently diluted in MilliQ water (Millipore, Billerica, MA) for MIC determination, in physiological saline (PS, 0.9% w/v NaCl) for the checkerboard assays, the tests with biofilms formed in 96-well MTPs, and the *G. mellonella* assay, or in GTSF-2 medium for the 3D organotypic human cell culture model [249]. Antibiotic stocks were made in MQ water or PS. All antibiotic solutions were filter sterilized (0.22 µm, Whatman, Dassel, Germany) and immediately used.

Library screen and identification of hits

B. cenocepacia LMG 16656 biofilms were formed in round-bottomed 96-well MTPs as described by Van den Driessche et al. [220] and subsequently treated with one µl library compound (final concentration: 100 µM), 49 µL PS and 50 µL of a 1024 µg/mL tobramycin solution (final concentration: 512 µg/mL). One µL of compound solution was added to 99 µL PS for the experiments carried out in absence of tobramycin. An untreated control, a control for tobramycin treatment alone and a blank were included on every plate. After 24 h, biofilms were rinsed with 100 µL PS and the effect of the treatment was evaluated using CellTiter-Blue staining (CTB, Promega, Leiden, The Netherlands). Fluorescence was measured using a multilabel MTP-reader (Envision, Perkin Elmer LAS, Waltham, MA) as described by Peeters et al. [200]. The blank corrected fluorescence signals generated by *B. cenocepacia* biofilms treated with the combination of component and tobramycin were compared to the fluorescence signals generated by biofilms that were treated with tobramycin alone. Hits were defined as compounds that caused a decrease in fluorescence signal of ≥ 90% in combination with tobramycin compared to the fluorescence signal generated by biofilms treated with

tobramycin alone. Hit compounds were also tested in absence of tobramycin and the fluorescence generated was compared to that from the untreated control.

Determination of the *in vitro* activity of econazole and miconazole against *B. cenocepacia*

To determine the MICs of miconazole and econazole, overnight *B. cenocepacia* LMG 16656 cell suspensions were adjusted to OD_{595} of 0.05 and diluted 1:50 in double concentrated MH. Subsequently, 100 µL cell suspension was added to flat-bottomed 96-well MTP (SPL Lifescience, Korea) containing 100 µL of 1/2 serial diluted imidazole solutions (concentration range 0-400 µM). OD_{590} was measured using a multilabel MTP reader (Envision) after 24 h. MICs were determined in triplicate on different days.

Biofilms were formed as described above and treated with 50 µL 200 µM econazole or miconazole (final concentration: 100 µM) and 50 µL 1024 µg/mL tobramycin (final concentration: 512 µg/mL). After 24 h, supernatants were removed and wells were rinsed. Cells were collected from the wells by 2 cycles of shaking (5 min, 700 rpm, Titramax 1000) and sonicating (5 min, Branson 3570E-MT, Branson Ultrasonic Corporation, Banbury, CT). The content of 5 wells was pooled in tubes containing 9 mL PS and the number of CFU per biofilm (CFU/BF) was determined by plating serial 10-fold dilutions on TSA plates. In addition, the potentiating activity of lower concentrations of econazole and miconazole (1 and 10 µM), as well as the combination of 100 µM econazole with meropenem (320 and 3200 µg/mL), ciprofloxacin (250 µg/mL) and gentamicin (512 and 2560 µg/mL) was evaluated in the same model system. Experiment were performed in triplicate on different days.

For checkerboard assays biofilms were treated with tobramycin (128 - 8192 µg/mL) and econazole (25 - 200 µM) or miconazole (25 - 200 µM). Potential synergistic activity of these treatments was assessed by calculation of the fractional inhibitory concentration index (FICI) based on metabolic activity determination using CTB. In detail, FICI was calculated as $FICI=(C_{TOB+IMI}/C_{TOB})+(C_{IMI+TOB}/C_{IMI})$, where $C_{TOB+IMI}$ is the concentration of tobramycin in the presence of the imidazole at which the fluorescence generated is equal to the fluorescence of the blank control, C_{TOB} is the concentration of tobramycin that causes a fluorescence equal to the fluorescence of the blank control, $C_{IMI+TOB}$ is the concentration of the imidazole that causes in the presence of tobramycin a fluorescence equal to that of the blank control and C_{IMI} is the concentration of the imidazole that causes a fluorescence signal equal to the fluorescence signal of the blank control. Interactions were scored as synergistic when FICI $\leq$ 0.5 [250].

Evaluation of the effect of econazole or miconazole and tobramycin in a 3D organotypic human cell culture model

A 3D human lung epithelial cell culture model was generated by growing A549 cells (ATCC CCL-185) on porous microcarrier beads in a low fluid shear bioreactor, as previously described [251, 252]. A549 cells were maintained in GTSF-2 medium supplemented with 2.5 mg/L insulin transferrin selenite (ITS, Sigma-Aldrich), 1.5 g/L sodium bicarbonate, and 10% heat-inactivated FBS (Life Technologies). All cultures were grown at 37°C under 5% CO_2 conditions.

Infection studies were performed using 3D cultures grown for 11 to 14 days, and at the time of infection cell culture medium without FBS and supplemented with 0.2% rhamnose was used. 3D cell cultures containing 2.5 x 10^5 cells in 250 µL per well were transferred to 48-well plates (SPL Life Sciences). An overnight culture of a GFP-producing *B. cenocepacia* K56-2 mutant was resuspended in cell culture medium and added to the 3D cells at a multiplicity of infection of ~15:1.

Treatments (156 µg/mL tobramycin and/or 10 µM econazole or miconazole) were added to the cell culture medium at the start of the infection. Plates were incubated at 37 °C under 5% CO_2 conditions for 1 h, rinsed with Hanks' Balanced Salt Solution (Life Technologies) where after fresh cell culture medium containing the treatment reagents was supplied. After 16 h additional incubation, the number of *B. cenocepacia* that associated with the 3D cells was determined. To this end, the content of each well was transferred to new wells to avoid inclusion of bacteria attached to the plastic surface. Cultures were rinsed twice with HBSS followed by the addition of 0.1% Triton X-100, vigorous mixing and plating. The experiments were performed in the absence and presence of 3D cells, under otherwise identical conditions, to assess the effect of tobramycin and/or imidazoles on the inhibition of bacterial association with an abiotic or biotic surface, respectively. Controls with no antimicrobials and uninfected 3D cell cultures were included in every assay. Assessment of the overall 3D cell culture model integrity and visualization of GFP-producing bacteria was performed using an EVOS FL Auto Imaging System (Life Technologies) at a magnification of 300x.

In addition, the 3D lung epithelial cell viability was evaluated based on a lactate dehydrogenase (LDH) assay. The LDH activity assay kit (Sigma) was used to measure the release of cytosolic LDH by 3D lung epithelial cells following exposure to *B. cenocepacia* K56-2. Medium from 3D aggregates infected with *B. cenocepacia* for 17 h was centrifuged for 15 minutes at 3700 rpm, where after the supernatants was used for LDH quantification following the manufacturer's instructions. A standard curve using NADH was included. As a positive control, 2.5 x 10^5 3D lung epithelial cells were lysed with 0.1% Triton-X100 in 250 µL (the same volume as for the test conditions). The data are presented as a percentage of LDH release from the positive control.

Evaluation of the potentiating effect in a *Galleria mellonella* infection assay

In vivo activity of tobramycin and econazole or miconazole was evaluated in the *G. mellonella* infection assay, as described by Brackman et al. [221]. *G. mellonella* larvae (Hengelsport de Poorter, Ghent, Belgium) were kept at 4°C in the dark on wooden flakes prior to use and divided in groups with similar weight distribution (300-450 mg, 450-550 mg or 550-650 mg). Equal amounts of larvae were taken from each group to compose new groups containing at least 12 larvae for the infected groups and 6 for the uninfected groups. Overnight cultures of *B. cenocepacia* LMG 16656 were centrifuged, resuspended in PS and adjusted to 10^7 CFU/mL. Larvae were injected with 10 µL cell suspension in the last left proleg and 10 µL of the treatment solution (512 µg/mL tobramycin, 50 µM econazole, 50 µM miconazole, or combinations) in the last right proleg using a syringe (BD ultrafine insulin

syringes, Becton Dickinson, Erembodegem, Belgium). Larvae were incubated at 37°C and survival was scored after 24, 48 and 72 h. Larvae were considered dead when no movement was observed in response to touch. To determine whether the treatment caused a reduction in the number of CFU/larvae 24 h post infection (*p.i.)*, larvae were homogenized (Polytron, Kinematica, Eschbach, Germany), serially diluted and plated on *Burkholderia cepacia* selective agar (Thermo Scientific, Erembodegem, Belgium).

Development of a formulation for inhalation containing tobramycin and miconazole

In contrast to tobramycin which is highly soluble in water, miconazole is very slighty soluble in water [253]. Therefore, it was decided to formulate the combination tobramycin-miconazole as a suspension for nebulization. In order to decrease the particle size of the suspended miconazole particles (i.e. down to desired particle size for deposit in the lungs), the wet bead milling technique was applied. The formulation was prepared by weighing 330 mg tobramycin and 15.4 mg or 61.6 mg miconazole into a 20 mL vial, followed by the addition of 1 mL Tween 80 solution (1 mg/mL) (Fagron, Waregem, Belgium), 4 mL PS, and 10 g zirconium oxide beads (diameter 0.5 mm, Netzsch Feinmahltechnik, Selb, Germany) as milling pearls. Subsequently, the vials were placed on a roller-mill (Peira, Beerse, Belgium) and grinding was performed at 150 rpm for 72 h. After milling, the microsuspension was separated from the grinding pearls by pipetting. The particle size distribution of miconazole was measured in triplicate by laser diffraction (Mastersizer S long bench, Malvern Instruments, Worcestershire, UK). The wet dispersion technique was applied using the 300RF lens (Malvern Instruments, Worcestershire, UK). The powders were dispersed in a solution of 0.2% Tween 80 in Miglyol 812 and subsequently vortexed and sonicated in order to eliminate agglomerates. In addition, biofilms formed in 96-well MTPs were treated with the formulation in a dilution of 1 in 129 (corresponding to 512 µg/mL tobramycin and 200 µM miconazole) to confirm the antibacterial activity. Experiments were performed six times independently and the effect was quantified by plate counts.

Mouse lung infection model

Animals were treated in accordance to the guidelines provided by the University of Antwerp and the European Directive for Laboratory Animal Care (Directive 2010/63/EU of the European Parliament). The laboratory Animal Ethics Committee of the University of Antwerp authorized and approved all animal experimentation in this study (file 2014-81). Female inbred BALB/c ByJRj mice of 8-9 weeks old (Janvier Labs, Le Genest-Saint-Isle, France) were kept under standard pathogen-free conditions with a constant temperature of 20-25°C, an average humidity of 50-60%, a 12 h dark-light cycle and food and drinking water ad libitum. Mice were rendered transiently leukopenic with cyclophosphamide (Endoxan, Baxter) at 150 mg/kg body weight (bw) given intraperitoneally 4 days prior to infection and 50 mg/kg bw administered both 2 days before infection and on the day of infection. To prepare the inoculum for intratracheal infection, a bacterial cryostock was thawed, diluted in LB broth (10^5 CFU/mL) and incubated for 24 h in a shaken incubator (25 rpm) at 37°C in a 14 mL vent cap sterile tube. Cells were harvested by centrifugation after 24 h and diluted in PBS to 2 x 10^7 CFU/mL. Before intratracheal challenge with *B. cenocepacia*, mice were anesthetized with isoflurane (Halocarbon, Norcross, GA). Next anesthetized mice were

inoculated intratracheally with *B. cenocepacia* (10^6 CFU/mouse) in 50 µL of PBS. Thereafter, infected mice were treated intranasally with 40 µl of the formulations described above, i.e. vehicle (0.02% Tween 80 dissolved in PS), tobramycin (66 mg/mL, corresponding to 120 mg/kg bw), miconazole (3.08 mg or 12.32 mg/mL corresponding to 5.6 and 22.4 mg/kg bw miconazole, respectively), and the combination, at 1 h, 24 h and 48 h *p.i.*. Mice were observed daily for functional behavior (fur quality, posture, state of activity and bw) and pneumonia symptoms (respiratory frequency). *B. cenocepacia* infected mice were sacrificed at day 3 *p.i.* by cervical dislocation. Lung, spleen and liver were excised, weighed and homogenized in 5 mL PBS and were used for the enumeration of CFU on LB agar or *Burkholderia cepacia* selective agar.

Statistics

Data are expressed as the mean ± standard error of the mean (SEM). SPSS version 23 software was used and data were analyzed using the Kruskal-Wallis test. P-values < 0.05 were considered statistically significant.

RESULTS

Library screen against *B. cenocepacia* biofilms in combination with tobramycin

Screening the NIHCC 1&2 against biofilms of *B. cenocepacia* formed in 96-well MTPs in the presence of tobramycin allowed us to identify 60 hits (8.2%). In the presence of tobramycin these hit compounds caused a decrease of ≥ 90% in fluorescence signal after CTB staining compared to treatment with tobramycin alone (Table S1). Hits were classified in four groups according to their therapeutic indication: anti-infective agents (n = 11), antipsychotics and antidepressants (n = 23), antineoplastic and/or hormonal drugs (n = 6), and a miscellaneous group (n = 20) (Table S2). Subsequently, *B. cenocepacia* biofilms were treated with the hit compounds alone; 19 compounds (i.e. miconazole, oxiconazole, ketoconazole, perphenazine, droperidol, thiothixene, clozapine, perospirone, tamoxifen, ethylestreol, montelukast, AM404, atomoxetine, nitrendipine, donepezil, procyclidine, tolterodine, and propranolol) did not cause a significant decrease in fluorescence. In contrast, 41 compounds caused a significant reduction in fluorescence compared to the untreated control suggesting their potentiating activity is at least partially due to an intrinsic activity against *B. cenocepacia* biofilms (Table S2).

Antifungal imidazoles are potentiators *in vitro*

Four antifungal imidazoles, i.e. econazole, miconazole, oxiconazole, and ketoconazole were identified as hits in the screening. Treatment of biofilms with econazole alone reduced the fluorescence significantly compared to the untreated control. Miconazole, oxiconazole, and ketoconazole did not cause a significant reduction in fluorescence compared to untreated control (Table S2).

Subsequently, *B. cenocepacia* biofilms were treated with 100 µM econazole or miconazole in combination with 512 µg/mL tobramycin and the antimicrobial effect was quantified by plate counts. Treatment with econazole or miconazole alone did not cause a reduction in the

number of CFU/BF. However, in combination with tobramycin, a significant reduction in the number of CFU/BF was observed, compared to treatment with tobramycin alone (Figure 1). Lower concentrations of econazole and miconazole were tested for potentiating activity towards tobramycin; there was no potentiator activity for 1 µM, while 10 µM econazole or miconazole were equally effective as 100 µM (Figure 1). The potentiator activity of econazole was subsequently investigated in combination with other antibiotics; 100 µM econazole caused a statistically significant reduction in CFU/BF in combination with 2560 µg/mL gentamicin, but not with 512 µg/mL gentamicin. Econazole did not potentiate the activity of meropenem or ciprofloxacin (Figure 1).

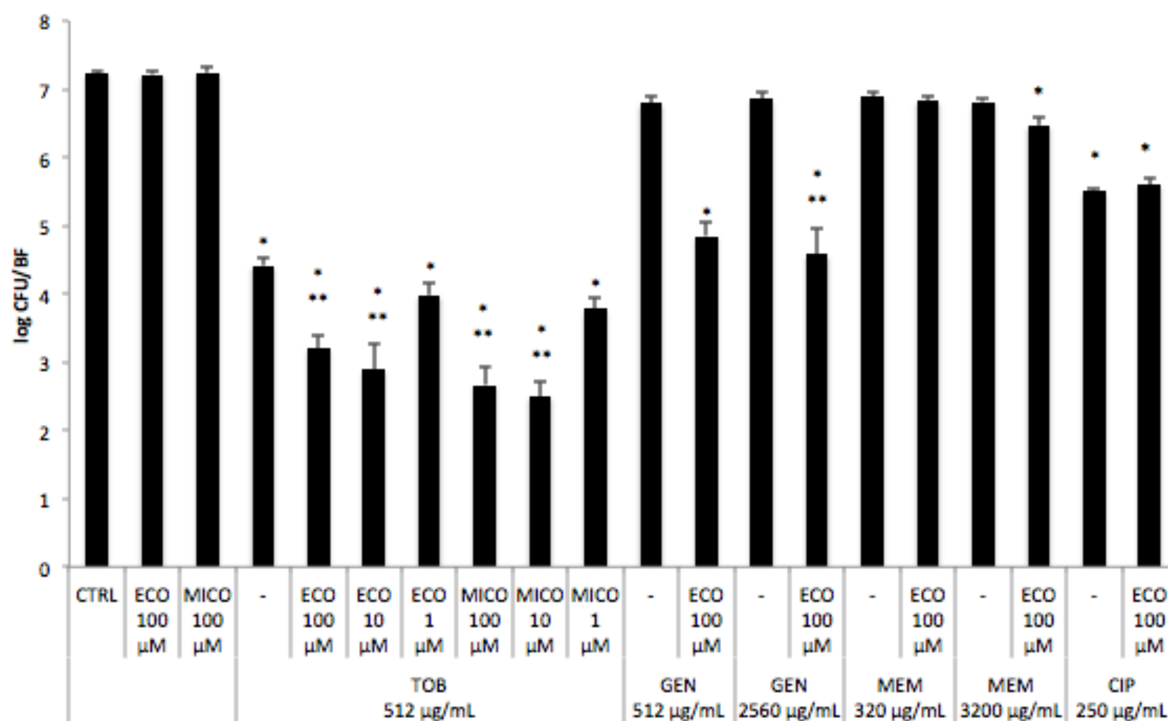

Figure 1. Number of CFU per biofilm after treating mature biofilms of *B. cenocepacia* LMG 16656 for 24 h with 100 µL PS (untreated control, CTRL), 1, 10, 100 µM econazole (ECO) or 1, 10, 100 µM miconazole (MICO), in combination with 512 µg/mL tobramycin (TOB), 512 and 2560 µg/mL gentamicin (GEN), 320 and 3200 µg/mL meropenem (MEM), or 250 µg/mL ciprofloxacin (CIP). Asterisks (*) indicate a significantly different number in log CFU/BF compared to untreated control, while double asterisks (**) indicate a significantly different number in log CFU/BF compared to treatment with the respective antibiotic alone (P-value < 0.05). (data shown are average; $n \geq 3$; error bars indicate SEM)

The MIC for econazole and miconazole against *B. cenocepacia* exceeded 200 µM. Subsequently, checkerboard assays were performed against biofilms of *B. cenocepacia* and showed that in the presence of the imidazoles, the concentration of tobramycin necessary to completely eradicate biofilms decreased substantially. As the imidazoles alone did not eradicate biofilms in the concentrations tested, and as higher concentrations could not be tested due to solubility issues, exact FICIs could not be calculated.

Evaluation of the activity of the combination treatment in a 3D organotypic cell culture model

B. cenocepacia K56-2 infected 3D lung epithelial cells without significantly affecting the 3D model integrity (Figure 2) and host cell viability (Figure S3) up till 17 h post infection. Specifically, based on the LDH release assay less than 10% cell death of infected cultures was observed, which was similar to that observed in the non-infected controls (Figure S3).

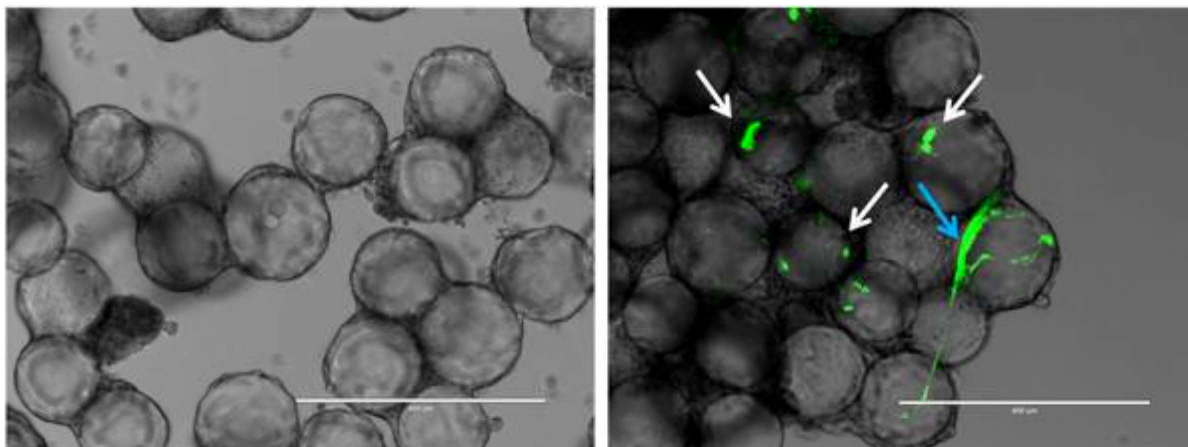

Figure 2. Left image showing an uninfected control where the microcarrier bead scaffolds are covered with A549 human lung epithelial cells, right image shows the 3D lung epithelial cells 17 h *p.i.*, green dots (white arrows) indicate intracellular growth of *B. cenocepacia* K56-2, while the large green structure (blue arrow) suggests that biofilm-like structures are formed. Magnification is 300x. Scale bar is 400 µm.

B. cenocepacia associated with the 3D lung epithelial cells both as dense clusters associated with single host cells and as structures that were spread out over multiple epithelial cells (Figure 2). Single bacteria associating with the 3D lung epithelial cells were also observed. 3D lung epithelial cells were exposed to 10, 50 and 100 µM of the imidazoles in the absence of *B. cenocepacia* for 17 h (Figure S4). The epithelial cells completely detached from their microcarrier bead scaffolds when exposed to 50 or 100 µM econazole or miconazole; this was not observed when cells were exposed to 10 µM of the imidazoles (Figure S4) and the latter concentration was chosen for further experiments. High concentrations of tobramycin (1000 µg/mL) did not affect the 3D lung model integrity (Figure S4). Adding 10 µM econazole or miconazole led to an additional reduction in *B. cenocepacia* biofilm formation in MTP in combination with 156 µg/mL tobramycin (Figure 3 and Figure S5), and this concentration was used to assess the number of viable and culturable bacteria associated with 3D cells. When the combination treatment was tested in this model, no potentiating effect of miconazole and econazole was observed, while the activity of tobramycin was similar as in the MTP (Figure 3).

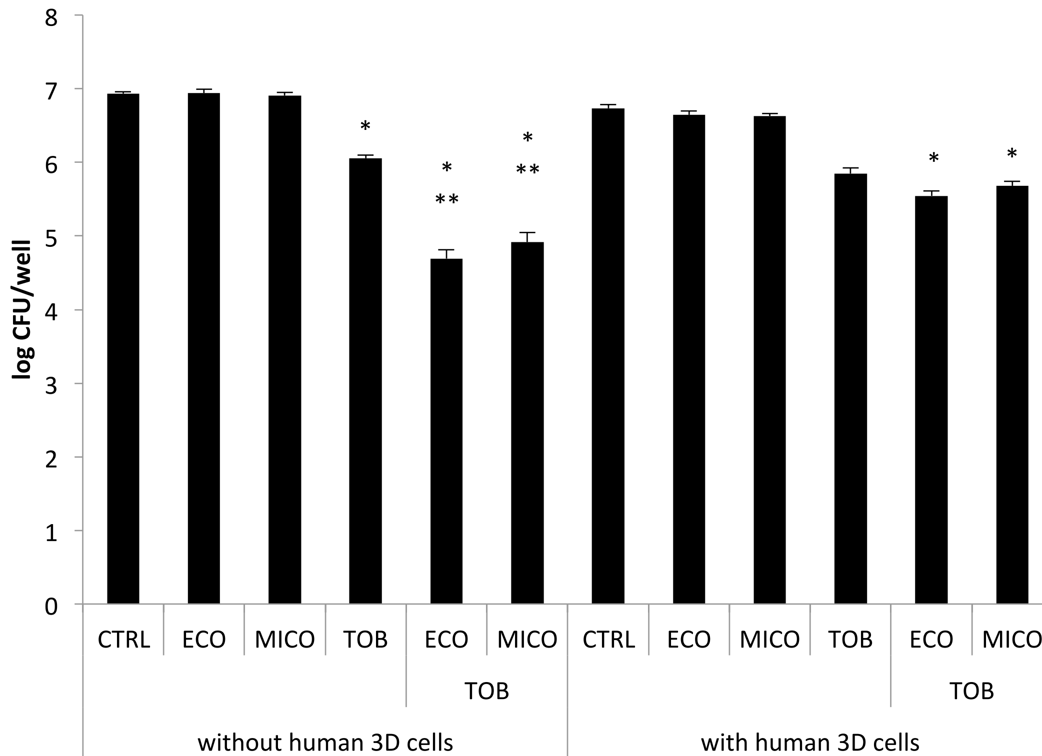

Figure 3. Effect of treatment with 10 µM econazole (ECO), 10 µM miconazole (MICO), 156 µg/mL tobramycin (TOB) or the combination on inhibition of biofilm formation of *B. cenocepacia* K56-2 with a plastic surface (left, number of biofilm cells per well) or the effect on the treatment on the number of bacterial cells associated with human 3D lung epithelial cells (right, number of host associated cells per well). Asterisks (*) indicate a significantly different number in CFU/well compared to no treatment, while double asterisks (**) indicate a significantly different number in CFU/well compared to treatment with tobramycin (P-value < 0.05). (data shown are average; n ≥ 3; error bars indicate SEM)

G. mellonella survival assay

G. mellonella larvae were injected with *B. cenocepacia* and treated with 10 µL vehicle (PS), 512 µg/mL tobramycin, 50 µM miconazole, 50 µM econazole, tobramycin combined with miconazole, or tobramycin combined with econazole. No differences were observed between the different treatments 24 h *p.i.*, which indicated that the treatment is not toxic at the concentrations applied. However, more than 50% of the larvae had died in the infected groups 48 h *p.i.*, regardless of the treatment which indicated that the treatment cannot protect the larvae against *B. cenocepacia* infection (Figure 4). These observations were confirmed by the fact that no differences in CFU/larvae were observed among the different groups 24 h *p.i.* (Figure S6).

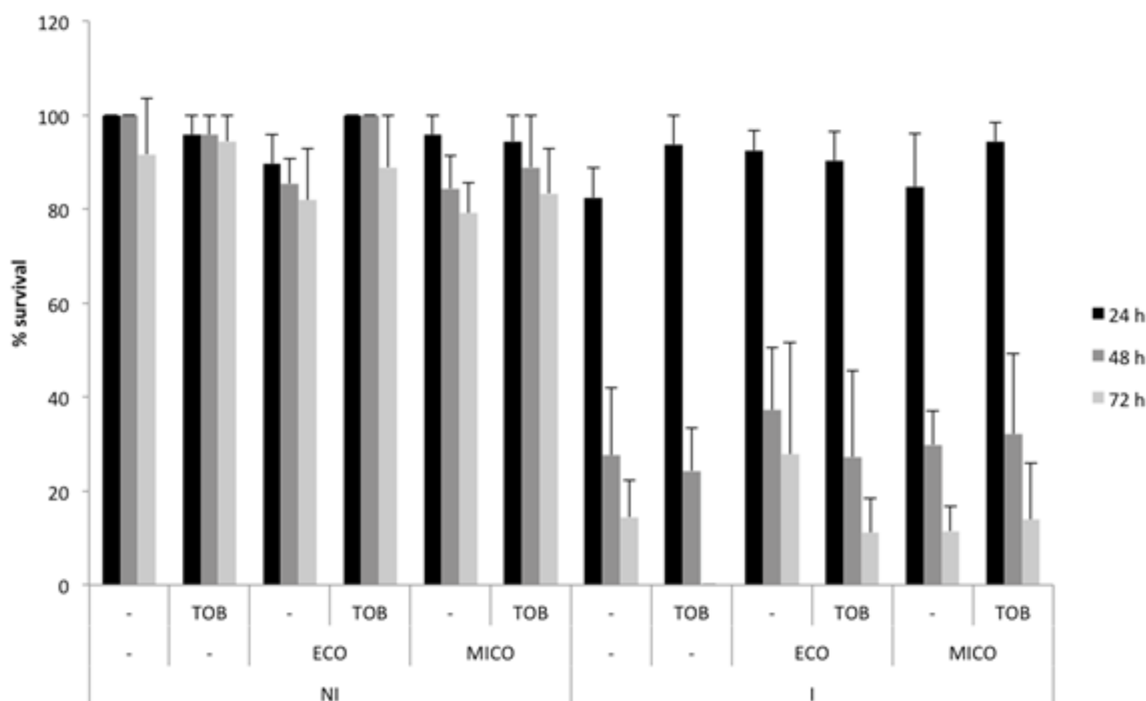

Figure 4. Percentage survival of *G. mellonella* in uninfected control groups (NI) and infected groups (I) 24, 48, and 72 h after administration of the different treatments (tobramycin (TOB), econazole (ECO), miconazole (MICO) or the combinations) and/or infection. (Data shown are average, n = 6, error bars indicate SEM)

Evaluation of the combination tobramycin and miconazole in a mouse lung infection model

The combination of tobramycin and miconazole was evaluated in a *B. cenocepacia* mouse lung infection model. To this purpose, a formulation for inhalation was developed in which the particle size was lower than 5 µm (i.e. d_{10} = 0.23 µm, d_{50} = 0.41 µm, and d_{90} = 3.68 µm), which is suitable for drug deposition in the lungs [254]. The formulation was found to be stable over 5 days (i.e. no shift in d_{50}-value) which covered the duration of the *in vivo* study. A similar tobramycin concentration (66 mg/mL) as the TOBI inhaler (Novartis, 60 mg/mL) was chosen for the treatment of *B. cenocepacia* infected mice, which is 129 times the concentration used in the MTP assay. The concentration of miconazole in the formulation was based on the ratio tobramycin/miconazole in the MTP experiments and the activity of the diluted formulation was confirmed against biofilms formed in 96-well MTPs (Figure S7). Treatment of infected mice with tobramycin resulted in prevention of dissemination to liver and spleen, and a reduction in lung burden of approx. 1 log (Figure 5). Mice treated with 22.4 mg/kg bodyweight miconazole suffered from side effects and were characterized by a hunchback posture, weak breathing and an overall bad fur condition. Also dissemination of bacteria to liver and spleen was observed in every miconazole treated mouse whereas only approx. half of the vehicle-treated mice (5/11) had dissemination of bacteria to liver and spleen (Figure 5). Therefore, a lower dose of miconazole (5.6 mg/kg bodyweight) was chosen for the evaluation of the combination therapy in mice. As shown in Figure 5, no significant difference in lung burden could be observed between mice treated with tobramycin and tobramycin combined with miconazole. Also mice treated with this lower dose of miconazole alone suffered from the same toxic effects as 22.4 mg/kg bodyweight miconazole.

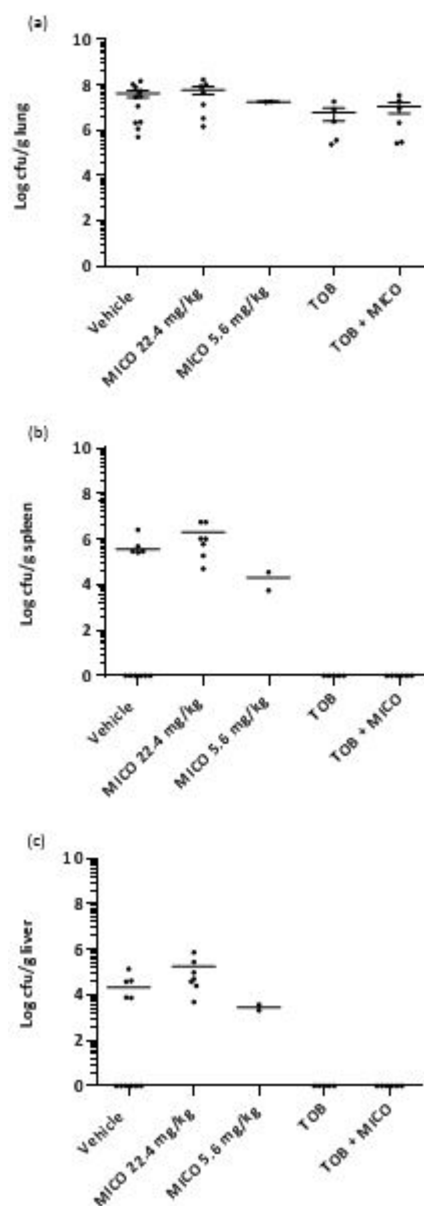

Figure 5. Colony counts in lung (a), spleen (b), and liver (c) in mice treated with: (i) vehicle, (ii) 22.4 mg/kg bodyweight miconazole (MICO), (iii) 5.6 mg/kg bodyweight miconazole, (iv) 120 mg/kg bodyweight tobramycin (TOB) and (v) 120 mg/kg bodyweight tobramycin combined with 5.6 mg/kg bodyweight miconazole (TOB + MICO). Error bars indicate SEM. 3 independent repeats of mice treated with vehicle ($n \leq 3$), 2 independent repeats of mice treated with miconazole 22.4 mg/kg ($n \leq 3$), 1 independent repeat of mice treated with miconazole 5.6 mg/kg ($n = 2$) and 1 independent repeat of mice treated with tobramycin + miconazole ($n = 6$).

DISCUSSION

Library screening identifies antifungal imidazoles as antibacterial potentiators
Screening two repurposing libraries against *B. cenocepacia* biofilms in the presence of tobramycin resulted in the identification of 60 hit compounds. Four antifungal imidazoles, i.e. econazole, miconazole, oxiconazole, and ketoconazole were among the hits identified. Other azoles present in the library were less or not active, e.g. the triazoles fluconazole, itraconazole, or voriconazole. This is in accordance with results from a structure-activity relationship study on several oxiconazole derivatives in which compounds with an imidazole moiety were shown to be more potent antibacterials against *Klebsiella pneumoniae, Escherichia coli* and *Staphylococcus aureus* compared to triazoles [255].

The MIC of econazole and miconazole against *B. cenocepacia* exceeded 200 µM, indicating that they potentiate tobramycin at concentrations that do not inhibit growth of planktonic cells when administered alone. This is in accordance with a study in which no growth inhibitory activity was observed for miconazole against planktonic cells of *Pseudomonas aeruginosa* and *E. coli*, while the combination of miconazole with polymyxin B resulted in a strong synergistic interaction [256].

The activity of imidazoles towards Gram-positive bacteria has been described before [190, 257-259]. Sud and Feingold investigated the bactericidal effect of miconazole and concluded that interference with the membrane of Gram-positive bacteria results in leakage of K^+ [190]. In addition, imidazoles bind *S. aureus* flavohemoglobin, a protein with NO dioxygenase activity, causing nitrosative and oxidative stress [224]. Miconazole also binds flavohemoglobin of *E. coli*, and *in vitro* data showed that the combination of a NO-donor, miconazole and polymyxin B nonapeptide were effective to treat four ESBL-producing *E. coli* isolates [260].

Activity of the combination in relevant model systems
Activity of tobramycin in combination with econazole or miconazole was tested in an *in vitro* 3D organotypic human cell culture model. 3D organotypic cell culture models are valuable research tools that mimic key aspects of the parental tissue and reduce the gap between *in vitro* cell culture models and physiological tissue [261, 262]. A 3D A549 lung epithelial cell culture model has previously been developed and validated, and was used previously to study the colonization of *P. aeruginosa* and *Francisella tularensis.* These studies demonstrated that the 3D lung epithelial cells generate more *in vivo*-like phenotypes compared to conventional monolayers [252, 263]. *B. cenocepacia* has been shown to both infect intracellular and form biofilms in the lungs of CF patients [203, 264, 265]. However, the biofilm formation capacity of *Burkholderia* species in CF lungs is still under debate [266]. Infection of the 3D lung epithelial model with *B. cenocepacia* K56-2 reflected aspects of the *in vivo* infection profile, as phenotypic characteristics that were indicative of both intracellular growth and biofilm formation were observed.

Tobramycin showed an increased inhibition on biofilm formation in MTP in the presence of econazole or miconazole, so the imidazoles tested both potentiate the biofilm-inhibiting and biofilm-eradicating activity of tobramycin, as described above. However, no potentiating effect could be observed in the 3D lung epithelial cell model (Figure 3).

The effect of the combination treatment was also evaluated in *G. mellonella*, which is a suitable model to test *Bcc* virulence [267]. *G. mellonella* was used before to estimate the activity of treatments towards *B. cenocepacia* infections, including novel agents with potentiator activity [221, 268]. Neither treatment with tobramycin, miconazole, econazole, nor the combination could protect the larvae against *B. cenocepacia* infection (Figure 4).

Finally, the combination of tobramycin and miconazole was evaluated in a *B. cenocepacia* mouse lung infection model. As 22.4 mg/kg bodyweight miconazole was toxic, a lower dose of miconazole (5.6 mg/kg bodyweight) was chosen for the evaluation of the combination therapy in mice. This dose is far below LD50 values for mice reported in literature, which range from 80 mg/kg for intravenous administration, to 220 mg/kg for intraperitoneal administration, and even up to 519 mg/kg upon oral administration [268]. However, also this lower dose appeared to be toxic for the mice used in the present study and no significant difference in lung burden could be observed between mice treated with tobramycin, and those treated with tobramycin and miconazole.

Different activity in a conventional *in vitro* biofilm model system and other model systems of host-pathogen interactions

Based on *in vitro* data the promising combination of tobramycin with econazole or miconazole was evaluated in other model systems which all indicated that the combination did not improve the outcome upon infection with *B. cenocepacia* infection. Discrepancies in antimicrobial activity between *in vitro* and *in vivo* situations are often observed [269]. This may be because basic *in vitro* models for susceptibility testing lack host factors (e.g. cellular and humoral immunity of the host, the pathogen's level of expression of virulence determinants and protein binding). Also, pharmacokinetic parameters as penetration into the site of infection are not taken into account. In the present study azoles lost their potentiating activity when they were investigated in models including host factors and it is thus likely that these host factors play a role in their inactivation. Protein binding of miconazole (reported to be 90%) might contribute to a substantial decrease in biologically active free fraction and loss of activity in the three models [270]. Also, a differential transcriptomic and phenotypic profile in the presence of host cells might resulting in a reduced susceptibility to the combination treatment.

The screening described in the present study was performed in one of the most frequently used models for the evaluation of novel biofilm inhibitory/eradication compounds, i.e. the static MTP biofilm model system [64]. Although this system has multiple advantages, it lacks many of the micro-environmental factors that are encountered during the natural course of infection of *B. cenocepacia*, including host components. Therefore, more advanced *in vitro* and *in vivo* model systems should be considered that are more reflective of the host

environment and thus better predict the efficacy of the combination therapy. Hence, it seems appropriate for future screenings of antimicrobial compounds that conventional *in vitro* model systems are complemented with model systems that incorporate key host factors in an early stage.

ACKNOWLEDGEMENTS

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SUPPLEMENTARY DATA

Table S1. The NIH Clinical Collection 1&2 were screened at a concentration of 100 µM in the presence of 512 µg/ml tobramycin for potentiators against biofilms of *B. cenocepacia* LMG 16656. Hits were subsequently tested in absence of tobramycin. Effect was evaluated using CTB staining. The values shown in the left column represent the mean residual metabolic activity of the compound in the presence of tobramycin compared to treatment with tobramycin alone, and the standard deviation (SD). The values in the right column represent the mean residual metabolic activity of the compound compared to untreated control, and the standard deviation.

PUBCHEM CODE	COMPOUND NAME	MEAN (%)	SD	MEAN (%)	SD
CPD000449281	Nalbuphine	30	14		
CPD000449275	Raclopride	50	24		
CPD000449271	Zacopride	58	40		
CPD000449276	SKF 83566 D1 R agonist	29	33		
CPD000449316	3'-deoxydenosine	106	77		
CPD000449274	AM 404 N-arachidonoylaminofenol	7	9	99	10
CPD000059053	Pilocarpine	114	29		
CPD000058291	Rifedipine	55	29		
CPD000042823	Flurbiprofen	121	58		
CPD000059136	Deferiprone	106	15		
CPD000058470	Loxapine	2	5	43	6
CPD000326694	D-3-MEO-N-methylmorphinan	39	24		
CPD000449282	Duloxetine	4	6	20	2
CPD000449320	Glycine	217	97		
CPD000449318	Benzene acetic acid	53	12		
CPD000058345	S-progesterone	27	12		
CPD000058961	Famotine	73	22		
CPD000449299	5-HTR-3 agonist	55	20		
CPD000466270	Pancuronium	52	13		
CPD000058175	Metronidazole	61	29		
CPD000449327	Benzenaeacetic acid	67	25		
CPD000449323	Benzeneacetonitrile	23	4		
CPD000449328	Selegiline HCl	57	8		
CPD000449294	Zucapsaicin	21	9		
CPD000058513	Salbutamol	69	19		
CPD000057879	Vesamicol	44	18		
CPD000469289	Picrotin	77	28		
CPD000449268	Terazosin	64	17		
CPD000449319	Diphenylcyclopropenone	26	20		
CPD000449326	Thiazolidinecarboxylic acid	75	40		
CPD000466274	Mesoridazine	23	5		
CPD000449313	Pyridazinone difluoromethoxy	58	40		
CPD000466275	Phenothiazine	17	10		
CPD000449322	Cyclopentaquinolin	48	10		
CPD000058306	Clotrimazole	25	3		
CPD000058255	Loratadine	12	10		
CPD000058500	Phenelzine	71	44		
CPD000449311	Riluzole	49	15		
CPD000449312	Naltrindole	22	10		

CPD000449277	Nornicotine	98	21		
CPD000449269	Bifemelane	6	2	23	9
CPD000449284	CGS antagonist	64	30		
CPD000449287	Cinanserin	17	8		
CPD000449272	Cisapride	75	56		
CPD000449273	Indatrline	12	11		
CPD000058520	Trazodone	37	15		
CPD000449301	Prazosin	75	55		
CPD000058525	Urapidil	123	43		
CPD000449278	Cotinine	70	26		
CPD000058313	Cycloserine	92	73		
CPD000466268	Fluvoxamine	13	12		
CPD000449270	Doxepin	11	4		
CPD000059133	Trifluoperazine	3	4	20	7
CPD000058908	Hydroxy methylmorphinan	95	32		
CPD000449329	Ornithine	108	25		
CPD000148117	Maprotiline	12	11		
CPD000466272	Pizotifen	7	5	51	9
CPD000059126	Beta-estradiol	30	13		
CPD000059046	Diacetyl diaminohexane	85	7		
CPD000058353	Diphengydramine	41	8		
CPD000449267	Galanthamine	100	26		
CPD000449296	Ifenprodil	20	14		
CPD000059171	Tetraethylthiuram	20	8		
CPD000449302	Piribedil	59	23		
CPD000058460	Ketoconazole	6	6	73	9
CPD000058623	Tripelennamine	34	5		
CPD000449325	Pyrazinecarboxamide	90	29		
CPD000059105	Amino tetrahydroacridine	47	41		
CPD000058319	Ethynylestradiol	14	4		
CPD000449317	Pyrimidinone arabinofu	102	25		
CPD000449324	L glutaminic acid	63	15		
CPD000449305	1-(3-trifluoromethylphenyl)piperazine	26	17		
CPD000449298	Pramipexole	103	34		
CPD000058189	Lidocaine	121	87		
CPD000449290	Indometacine	197	62		
CPD000058555	Tomelukast	71	34		
CPD000466269	Paroxetine maleate	5	6	23	4
CPD000449288	Epigallocatechin	189	42		
CPD000145728	Amino hydroxy benzoic	120	53		
CPD000449321	Oxiranecarboxylic acid	84	31		
CPD000338536	Cephalexine	51	13		
CPD000466390	Pidotimod	75	10		
CPD000466386	Ramipril	108	13		
CPD000469284	Fenpiverinium	66	20		
CPD000058610	Nortestosterone	40	1		
CPD000466384	Niztididne	67	14		
CPD000059047	Flucytosine	81	13		
CPD000048684	Oxcarbazepine	67	39		

CPD000466385	Troxipide	60	4		
CPD000466341	Actarit	85	28		
CPD000469183	Azelastine	6	7	36	3
CPD000466388	Tocainide	98	19		
CPD000499525	Taxifoli	62	12		
CPD000466387	Levofloxacin	89	8		
CPD000469182	Cefatrizine	82	17		
CPD000466364	Idebenone	31	14		
CPD000466366	Levosulpiride	81	38		
CPD000238142	Pemoline	92	22		
CPD000466343	Letrozole	89	18		
CPD000469184	Meropenem	75	20		
CPD000466339	Orlistat	88	29		
CPD000469179	Ondansetran	58	17		
CPD000059117	Levonorgestrel	98	14		
CPD000469197	Cetraxate	96	8		
CPD000149316	Alprazolam	81	6		
CPD000058464	Lamotignine	102	26		
CPD000059145	Crotamiton	73	27		
CPD000472526	Amfebutamone	89	21		
CPD000466340	Alfuzonsine	49	10		
CPD000449309	Amisulpride	56	11		
CPD000469292	Lofepramine	11	11		
CPD000466362	Perospirone	7	4	49	10
CPD000059010	Docetaxel	25	18		
CPD000387107	Honokiol	5	4	8	3
CPD000469196	Tolterodine	8	6	62	8
CPD000466363	Carmofur	62	12		
CPD000469181	N-methyl paroxetine	2	2	31	20
CPD000466337	Olmesartan	84	47		
CPD000469593	Potassium losartan	66	24		
CPD000466338	Temozolomide	108	54		
CPD000058528	Methyltestosterone	55	43		
CPD000469195	Tosufloxacin	75	40		
CPD000466361	Mecillinam	62	12		
CPD000469177	Atomoxetine	5	6	47	7
CPD000466336	Artesunate	52	25		
CPD000058959	Itraconazole	66	11		
CPD000469193	Cefpodoxime	68	13		
CPD000058803	Buflomedil	56	17		
CPD000012114	Clore morpholin ehyl	71	16		
CPD000466330	Halometasone	51	24		
CPD000466357	Triclabendazole	43	15		
CPD000466331	Rofecoxib	27	5		
CPD000471619	Bisoprolol	69	10		
CPD000466334	Ezetimibe	43	18		
CPD000469176	Tiagabine	38	12		
CPD000466355	Idarubicin	11	8		
CPD000466360	Flubendazole	52	17		

CPD000466356	Tacrolimus	34	15		
CPD000469208	Valaciclovir	76	19		
CPD000466382	Clarithromycin	38	21		
CPD000466383	Aripiprazole	45	16		
CPD000471622	Trimebutine	44	10		
CPD000238198	Mestalone	47	4		
CPD000466370	Nisoldipine	62	19		
CPD000466371	Piceid	73	38		
CPD000149359	Methyl nitro imidazole	74	22		
CPD000466369	Nifekalant	88	14		
CPD000466372	Nateglinide	96	19		
CPD000058691	Megestrol acetaat	41	16		
CPD000466374	Ormetoprim	110	31		
CPD000466377	Zileuton	51	11		
CPD000058350	Stavudine	74	44		
CPD000058918	Gabexate	68	21		
CPD000469293	Oxiconazole	4	4	65	16
CPD000469235	Kitasamycin	42	13		
CPD000466375	Famciclovir	76	18		
CPD000326828	Sotalol	66	12		
CPD000466373	Rufloxacin	74	17		
CPD000466389	Taxiflolin	90	21		
CPD000469211	Alosetron	86	21		
CPD000469159	Fluticasone	59	25		
CPD000469161	Indinavir	68	31		
CPD000469160	Midazolam	38	21		
CPD000466319	Lamivudine	77	17		
CPD000469151	366-70-1	117	31		
CPD000469280	Esomeprazole	34	18		
CPD000059146	Sulfasalazine	64	15		
CPD000466313	Torasemide	70	18		
CPD000469156	Tropistrona HCl	37	15		
CPD000326795	Ranolazine	62	24		
CPD000058423	Bupropion	83	33		
CPD000471621	Irsogladine	53	27		
CPD000466376	Acarbose	79	13		
CPD000469294	Benproperine	2	4	25	8
CPD000466378	Fenprobamaat	71	27		
CPD000058926	Adamantan	28	31		
CPD000449280	Carvedilol	11	12		
CPD000466379	Lomifylline	64	23		
CPD000466380	Pazufloxacin	69	20		
CPD000466381	Miglitol	76	44		
CPD000058373	Tranilast	59	35		
CPD000466345	Olanzapine	16	19		
CPD000449297	Nefazodone HCl	10	14	47	22
CPD000469185	Moxifloxacin	49	28		
CPD000469186	Nelfinavir	52	18		
CPD000469187	Pravastatin	143	52		

CPD000466344	Topotecan	79	36		
CPD000466303	Levetiracetam	79	21		
CPD000469142	Pramipexole	63	23		
CPD000466323	Risperidone	22	10		
CPD000469167	Pioglitazone	73	31		
CPD000469147	Cilastatin	66	24		
CPD000466348	Argatroban	74	35		
CPD000466327	Valdecoxib	59	19		
CPD000466346	Naftopidil	5	6	24	5
CPD000156231	Nobiletin	29	24		
CPD000466304	Finasteride	76	34		
CPD000469145	Zolpidem	81	29		
CPD000048458	Viramune	117	66		
CPD000466325	Topiramate	123	41		
CPD000466350	Voriconazole	94	29		
CPD000469190	Fenoldopam	94	39		
CPD000471612	Rosiglitazone	51	17		
CPD000469191	Escitalopram	22	14		
CPD000058866	Zeranol	58	20		
CPD000466354	Latanoprost	17	13		
CPD000058576	Didanosine	90	18		
CPD000466298	Sertraline	5	7	2	4
CPD000466353	Calcipotriol	38	10		
CPD000466308	Epirubicin	35	7		
CPD000466329	Bicalutamide	41	21		
CPD000469192	Benidipine	56	9		
CPD000466352	Amlexanox	79	13		
CPD000469148	Cerivastatin	109	28		
CPD000466309	Icariin	101	19		
CPD000466310	Methylandrosternediol	109	16		
CPD000466307	Triptolide	103	20		
CPD000469170	Rosiglitazone	88	19		
CPD000059106	Tegafur	79	13		
CPD000466392	Oligomycin	15	7		
CPD000469199	Benazepril	93	16		
CPD000058877	Oxymetholone	41	15		
CPD000059060	Ipriflavone	75	16		
CPD000058286	Oxaprozin	74	16		
CPD000058510	Rolipram	92	31		
CPD000469200	Mosapride	75	28		
CPD000466391	Isoquercitrin	93	25		
CPD000058450	Flumazenil	107	10		
CPD000469164	Ozagrel	127	32		
CPD000466394	Hyperoside	123	26		
CPD000466322	Rifabutin	27	20		
CPD000469141	Esmolol	72	21		
CPD000466321	Tadalafil	22	14		
CPD000058957	Modafinil	31	20		
CPD000058570	Doxorubicin	18	16		

CPD000469209	Moxonidine	74	58		
CPD000058302	Nitrazepam	48	40		
CPD000387024	Pefloxacin	105	88		
CPD000469154	Venlafaxine	77	44		
CPD000469592	Pantoprazole	49	50		
CPD000058366	Nitrendipine	8	4	73	10
CPD000469290	Saquinavir	11	7		
CPD000058970	Bifonazole	12	10		
CPD000469158	Sumatriptan	47	16		
CPD000466314	Exemestane	31	5		
CPD000466367	Nitazoxanide	6	4	16	13
CPD000058398	Diazepam	25	15		
CPD000471623	Quetiapine	25	13		
CPD000112560	Rutin	63	18		
CPD000466317	Penciclovir	60	11		
CPD000466393	Calcitriol	18	11		
CPD000469140	Diphenoxylate	76	39		
CPD000449307	Felbamate	73	26		
CPD000058855	Droperidol	9	7	62	10
CPD000035998	Pentoxifylline	79	17		
CPD000058461	Ketorolac	45	21		
CPD000466395	Ritonavir	32	7		
CPD000469210	Vinorelbine tartrate	5	3	22	9
CPD000466335	Linezolid	56	41		
CPD000469203	Lomerizine	38	11		
CPD000466351	Efavirenz	2	2	20	8
CPD000466306	Irbesartan	55	34		
CPD000466305	Repaglinide	64	14		
CPD000238204	Ethylestrenol	6	7	46	9
CPD000440694	Pterostilbene	5	9	30	10
CPD000469144	Roxatidine	88	37		
CPD000471616	Dexbrompheniramine	37	62		
CPD000469168	Anagrelide	47	32		
CPD000471618	Tegaserod	4	5	10	4
CPD000058475	Milrinone	78	21		
CPD000466315	Levocetirizine	83	16		
CPD000326936	Citalopram	27	11		
CPD000048468	Ticlopidine	18	9		
CPD000469165	Sodium loxoprofen	92	18		
CPD000466316	Zafirlukast	23	13		
CPD000469152	Terbinafine	45	26		
CPD000466320	Isradipine	23	18		
CPD000466318	Valsartan	91	17		
CPD000449291	Piroxicam	76	23		
CPD000469282	Glycopyrrolate	94	19		
CPD000449286	Physostigmine	62	20		
CPD000058465	Lobeline	40	30		
CPD000058436	Doxylamine	52	10		
CPD000449266	Milnacipran	75	26		

CPD000449315	Fluoro pyrimidone	80	24		
CPD000466271	Chlorpheniramine	24	10		
CPD000466333	Dofetilide	110	38		
CPD000471620	Formoterol	71	9		
CPD000525252	Rizatriptan	95	20		
CPD000466332	Rifapentine	10	6	35	8
CPD000469178	Loteprednol	97	21		
CPD000466359	Enalaprilat	107	15		
CPD000449292	Donepezil	5	4	57	5
CPD000238177	Nimetazepan	79	15		
CPD000466365	Nicorandil	156	39		
CPD000466326	Telmisartan	61	7		
CPD000469143	Itopride	50	21		
CPD000466324	Rifaximin	13	15		
CPD000469188	Montelukast	8	5	85	21
CPD000058253	Didezoxycytidine	45	8		
CPD000466276	Imidazol amine	51	27		
CPD000466280	Pyridobenzodiazepin	45	40		
CPD000466278	Indole propanoic acid	12	7		
CPD000466277	Imidazol carboxylic	37	11		
CPD000466281	Acetamide	66	49		
CPD000466283	Altanserin	42	10		
CPD000058420	Betaxolol	67	31		
CPD000466311	Indirubine	78	32		
CPD000466285	Azasetron	52	9		
CPD000466287	Gr 89696	54	15		
CPD000058773	Delta hydrocortisone	83	13		
CPD000058392	Diazoxide	65	14		
CPD000058612	Chloroadenosine	80	25		
CPD000058726	Ornidazole	91	21		
CPD000058572	Dimethyl phenylpiperzai	108	26		
CPD000058507	Pienperone	29	8		
CPD000059128	Mestramol	68	26		
CPD000059100	Aminoethyl pyridine	87	27		
CPD000059142	Benactyzine	125	14		
CPD000059158	Dichloroacetic acid	97	18		
CPD000059165	Bestatin	75	16		
CPD000469213	Toreminfene	19	1		
CPD000469214	Goserelin	38	13		
CPD000469212	Secoisolarciciresinol	66	11		
CPD000469217	Raltitrexed	90	25		
CPD000469229	Doxapram	79	25		
CPD000466294	Serotinen agonist	48	4		
CPD000112281	Brucine	68	8		
CPD000059115	Tryptoline	37	8		
CPD000058411	Fluphenazine	5	4	26	8
CPD000469233	Palonosetron	18	5		
CPD000058746	Naproxen	96	20		
CPD000058904	Mepivacaine	105	14		

CPD000058310	Dibromohydroxybenz	29	31		
CPD000058300	Nimodipine	21	5		
CPD000058701	Rolitetracycline	60	8		
CPD000058715	Epirizole	63	9		
CPD000058273	Azauridine	72	8		
CPD000466922	Reichsteins	53	17		
CPD000059086	Pyridinemethanol	93	12		
CPD000449283	Haloperidol	6	1	20	3
CPD000449279	Stiripentol	62	14		
CPD000449303	Fluperlapine	41	10		
CPD000058660	Oxyphenomium	57	10		
CPD000112358	Homoveratrylamine	90	10		
CPD000058194	Tinidalzole	104	27		
CPD000058741	Xanthinol	121	32		
CPD000059111	Synephrine	116	36		
CPD000058206	Resveratrol	56	10		
CPD000059093	Malrol	71	10		
CPD000059077	Aminoimidazole	52	6		
CPD000059011	Enrofloxacin	61	12		
CPD000058603	Dehydrocholic acid	72	23		
CPD000058250	Cefaclor	88	14		
CPD000059044	Benzylimidazole	90	20		
CPD000469136	Duloxetine hcl	6	2	10	4
CPD000469155	Vardenafil	90	15		
CPD000469137	Ropivacaine	84	14		
CPD000466301	Anastrozole	97	15		
CPD000058462	Ketotifen	70	8		
CPD000058769	Medroxy progesterone	94	14		
CPD000466919	Pinacidil	124	10		
CPD000058266	Nitro indazole	66	17		
CPD000112269	Methoxytryptamine	67	17		
CPD000059045	Phenothiazine	18	4		
CPD000058553	Cladribine	81	11		
CPD000469138	Granisetron HCl	45	8		
CPD000466293	Rimcazole	13	10		
CPD000466292	Nafadoride	11	6		
CPD000058856	Desoximetasone	62	18		
CPD000471617	Dexchlorpheniramine	58	13		
CPD000466288	Guanidine	104	17		
CPD000466290	Cpd000466290	18	7		
CPD000466284	Cpd000466284	93	25		
CPD000466289	HTMT	98	35		
CPD000466286	Benzo phenanthridine	53	27		
CPD000466291	Methanesulfonamide	66	15		
CPD000466279	2H-indol-2-one	66	9		
CPD000466920	Beclosmethasone	64	17		
CPD000058847	Omeprazole	56	17		
CPD000469228	Dolastron	49	16		
CPD000449310	Zolmitriptan	68	23		

CPD000469223	Tremaulacin	72	33
CPD000469227	Dactinomycine	42	12
CPD000449308	Tramadol	69	24
CPD000469226	Chlordiazeposide	66	25
CPD000469225	Cefixime	101	20
CPD000469224	Cefdinir	95	22
CPD000469232	Lofexidine	67	24
CPD000469221	Balsalazide	84	10
CPD000469220	Olopatadine	27	20
CPD000469287	Itavastatin	57	31
CPD000058334	Cortisone	61	35
CPD000058431	Cyproheptadine	28	27
CPD000469230	Homoharringonine	46	10
CPD000058318	Corticosterone	65	39
CPD000471625	Vecuronium	44	28
CPD000469219	Itibolone	58	37
CPD000058212	Niacinamide	102	46
CPD000059131	Nialamide	100	46
CPD000469153	Vindesine	45	9
CPD000058540	Vincristine	68	21
CPD000466342	Lacidipine	90	18
CPD000466347	Mirtazapine	58	10
CPD000469285	Ampiroxicam	78	4
CPD000466368	Glimepiride	85	23
CPD000469198	Amlodipine	12	2
CPD000469174	Rabeprazole	51	13
CPD000058704	Clofazimine	87	14
CPD000469166	Irinotecan	76	17
CPD000058469	Lansoprazole	71	13
CPD000149358	Chloro piperidin ylideen	24	9
CPD000058772	Premarine	96	30
CPD000058481	Mifepristaone	111	23
CPD000112002	Etoposide	79	6
CPD000238156	Sibutramine	81	16
CPD000469632	Clobenpropit	64	15
CPD000469231	Huperzine	81	9
CPD000472527	Sibutraminde	79	16
CPD000058410	Lorazepam	71	25
CPD000469633	Azaspiro decane dione	72	12
CPD000469631	Adenosine	82	13
CPD000058296	Amiodarone	84	11
CPD000336944	Mevastatine	80	8
CPD000469175	Imatinib	45	10
CPD000468736	Methylperon	65	12
CPD000469594	Parecoxib	82	7
CPD000058504	Pergolide	106	8
CPD000471626	Atracurium	70	15
CPD000469218	Artemether	95	15
CPD000058445	Ebselen	64	18

CPD000468733	12066b	14	10		
CPD000469222	Teletromycine	72	10		
CPD000468732	CCPA	97	24		
CPD000468734	Methanone	83	22		
CPD000058878	Stanzolol	99	14		
CPD000238180	Zaleplon	73	9		
CPD000112594	Prostaglandin	87	6		
CPD000058344	Testosteron	78	18		
CPD000059075	Dehydroepiandrosterone	79	6		
CPD000466297	Sdm25n	34	5		
CPD000466299	Dup 697	112	17		
CPD000466300	Nonyloxytryptamine	14	6		
CPD000466295	Salmeterol	37	6		
CPD000466296	Sb	67	14		
CPD000326935	R sch 23390	71	25		
CPD000058230	Doxepin	2	1	39	7
CPD000058382	Dipyridamole	36	12		
CPD000059151	Propofol	38	5		
CPD000058600	Ethacrynic acid	20	13		
CPD000058187	Flutamide	6	2	36	6
CPD000058299	Fenofibrate	50	35		
CPD000058202	Furosemide	33	9		
CPD000038082	Fluorouracil	33	14		
CPD000471860	Folic acid	50	16		
CPD000653523	Hydrocortisone	42	12		
CPD000653536	Cortell	45	5		
CPD000058184	Ibuprofen	55	20		
CPD000040181	Ketoprofen	52	13		
CPD001906766	Minocycline	32	3		
CPD000058733	Miconazole	1	1	49	11
CPD000059134	Metyrapone	50	16		
CPD001317860	Pyrogluatmic acid	82	35		
CPD000058975	Nadolol	68	25		
CPD000058999	Disipal	17	15		
CPD000058192	Ofloxacin	87	47		
CPD000059120	Pindolol	60	27		
CPD000037139	Praziquantel	40	21		
CPD000059104	Phenylbutyric acid	87	57		
CPD000058326	Prednisolone	61	19		
CPD000058379	Promethazine	4	8	30	10
CPD000058180	Perphenazine	3	3	46	8
CPD000718761	Prednisolone	58	20		
CPD000058506	Prilocaine	104	19		
CPD001227202	Prednisone	65	27		
CPD000059161	Dl penicillamine	88	28		
CPD000058579	Piperacilline	76	25		
CPD000857275	Quinidine	28	5		
CPD000653467	Ranitidine	47	19		
CPD001906767	Rifampicine	17	3		

CPD000058245	Trans retinoic	23	11		
CPD000471892	Spironolactone	34	13		
CPD000035999	Trimethoprim	90	25		
CPD000058219	Tyzine	91	13		
CPD000059176	Thyroxine	56	13		
CPD000058515	Trihexyphenidyl	20	9		
CPD000058403	Ursodeoxycholic	97	23		
CPD000059064	Dapsone	105	26		
CPD001370746	Symmertrel	58	15		
CPD000058849	Warfarin	74	7		
CPD000058394	Acetazolamide	78	21		
CPD000059083	Allopurinol	67	11		
CPD001906768	Atropine	76	17		
CPD000058264	Nalicixic acid	57	21		
CPD001567029	Triiodothyronine	21	6		
CPD000058284	Hydroflumethiazide	79	18		
CPD000058368	Amitryptiline	0	0	22	4
CPD000058613	Busulfan	71	22		
CPD000058269	Chlorzoxazone	84	19		
CPD000058429	Chlorothiazide	91	27		
CPD001370748	Cimetidine	107	30		
CPD000058433	Carisoprodol	103	42		
CPD000058364	Chlorpropamide	104	24		
CPD000058440	Bentyl	24	14		
CPD000312779	Chloroxine	5	6	44	11
CPD000058723	Diflunisal	63	15		
CPD001370749	Econazole	1	1	21	5
CPD001370750	Ethionamide	39	24		
CPD000058719	Methocarbamol	68	22		
CPD000035778	Hydrochlorothiazide	70	22		
CPD001370751	Vistaril	25	3		
CPD000058356	Hexachlorophene	0	1	5	6
CPD000059082	Isoniazid	73	26		
CPD000058729	Duvadilan	111	47		
CPD000058267	Isoproterenol	89	30		
CPD000471847	Triclosan	3	3	1	1
CPD000058188	Mefenamic acid	60	32		
CPD000058832	Cantil	72	13		
CPD000058471	Metoclopramide	57	23		
CPD001370753	Methyldopa	55	22		
CPD000058271	Nitrofurantoin	52	24		
CPD000058486	Nortriptyline	2	3	21	3
CPD000058292	Naphazoline	28	6		
CPD000059024	Nicotinic	50	20		
CPD000058817	Norflex	16	10		
CPD001614498	Oxytetracycline	39	13		
CPD000718771	Procaine	97	38		
CPD000058714	Pyrimethamine	54	20		
CPD000058661	Pro banthine	39	11		

CPD000058280	Probenecid	100	96		
CPD001906769	Pyridine 2 aldoxime	117	39		
CPD000058501	Primidone	59	38		
CPD000058275	Propylthiouracil	48	11		
CPD000036662	Pyrazinamide	72	20		
CPD000059079	Probesyl	81	54		
CPD000037657	Sulfisoaxole	76	48		
CPD000058223	Sulfamethoxaole	113	61		
CPD000058173	Sulfacetamide	153	78		
CPD000058991	Sulfinpyrzone	118	45		
CPD000326718	Sulindac	121	52		
CPD001906770	Tetracycline	53	32		
CPD000058537	Theophylline	97	62		
CPD000058363	Tolbutamide	58	12		
CPD000059118	Triamteren	44	11		
CPD000059081	Intropin	65	31		
CPD000058416	Amoxapine	5	6	34	5
CPD000471872	Adenine	65	14		
CPD000036768	Atenolol	68	34		
CPD001491671	Tamoxifen	4	9	69	10
CPD000058418	Bumetanide	57	17		
CPD000058745	Clobetasol	44	9		
CPD000058254	Chlorpromazine	2	4	22	10
CPD001491644	Cefazoline sodium	75	18		
CPD000059061	Captopril	141	41		
CPD000058372	Chlorambucil	136	39		
CPD000058809	Cefoxitin	76	43		
CPD000058321	Danazol	96	32		
CPD000058375	Diltiazem	52	12		
CPD001906774	Digoxin	170	37		
CPD000058346	17- beta estradiol	54	27		
CPD000058672	Edroponium	86	15		
CPD000058329	Fluocinolone acetonide	67	14		
CPD000042823	Flurbiprofen	101	17		
CPD000058455	Glipizide	111	28		
CPD000058393	Gemfibrozil	127	45		
CPD000058229	Glyburide	99	21		
CPD000058328	Hydrocortisone	146	43		
CPD000058829	Indapamide	178	47		
CPD001906775	Ipratropium bromide	135	54		
CPD000058388	Imipramine	4	6	36	7
CPD000058463	Labetalol	117	32		
CPD000058466	Loperamide	2	4	36	6
CPD000058833	Pro amatine	103	34		
CPD000653524	Medroxyprogesterone acetate	18	12		
CPD001906776	19-norethindrone acetate	24	12		
CPD000499579	19-norethindrone	76	19		
CPD000059074	Nicotine	99	27		
CPD001456372	Cardene	27	11		

CPD000058835	Nabmetone	99	3		
CPD000058490	Oxybutynin	8	9	44	9
CPD000058605	Mestinon	99	16		
CPD001453705	Rythmol	12	4		
CPD001491654	Pfizerpen	68	26		
CPD000499581	Valproice acid	75	34		
CPD000058821	Procyclidine	6	7	48	20
CPD000875264	Proxymetacaine	43	17		
CPD000058766	Naloxone	66	34		
CPD001906777	Spectinomycin	52	18		
CPD000058523	Tropicamide	65	26		
CPD000058290	Tolazamide	94	58		
CPD000058335	Triamcinolone acetonide	49	12		
CPD001456519	S-timolol	67	17		
CPD000058170	Thiabendazole	63	18		
CPD000058380	Thioridazine	0	1	18	3
CPD000058181	Altretamine	60	21		
CPD001491672	Phylloquinone	84	15		
CPD001491659	Eryped	204	131		
CPD000058422	Dibenzyline	12	11		
CPD000058693	Medrysone	38	26		
CPD000058524	Thalidomide	80	67		
CPD000857229	Aminolevulinec	74	44		
CPD001496929	Carbinoxamine	51	45		
CPD001496930	Demeclocycline	57	29		
CPD001496932	Westcort	54	28		
CPD000449328	Selegiline	62	23		
CPD000058840	Nafcillin sodium	56	20		
CPD000875314	Primaquine	7	5	29	2
CPD001496934	Micropenin	94	15		
CPD001550033	Doxycycline	43	20		
CPD001233361	Beclomethasone	50	13		
CPD000058721	Cromolyn	94	11		
CPD000149600	Priscoline	43	16		
CPD000544948	Mercaptopurine	90	18		
CPD000427366	Azathioprine	98	40		
CPD000036735	Albendazole	88	25		
CPD000718755	Griseofulvin	69	26		
CPD000059006	Lincomycine	88	39		
CPD001496938	Methazolamide	107	43		
CPD001496939	Terbutaline	83	44		
CPD000471888	Mypirocin	122	70		
CPD000058331	Fluocinonide	64	10		
CPD000875233	Mefloquine hcl	4	4	11	8
CPD001496941	Floxuridine	79	14		
CPD001563707	Mitoxantrone	8	5	31	4
CPD001906784	Enalapril maleate	100	30		
CPD000058337	Budesonide	23	5		
CPD000466386	Ramipril	77	25		

CPD000718757	Depo medrol	63	29		
CPD000058383	Norepinephrine	92	22		
CPD001491664	Amcinonide	57	19		
CPD001317855	Clomid	13	12		
CPD001819784	Phentolamine HCl	53	9		
CPD000058874	Fludarabine	90	37		
CPD000109709	Testosterone	133	61		
CPD000471891	Isotretinoin	38	10		
CPD000058376	Methimazole	58	15		
CPD000596519	Zonisamide	67	15		
CPD000058355	Brimondidine	187	350		
CPD000036734	Mebendazole	52	16		
CPD000058736	Meclizine	29	13		
CPD000146393	Dilantin	55	22		
CPD000059182	Miochol	75	14		
CPD000326766	Dantrolene sodium	54	15		
CPD001227192	Dexamethasone	68	24		
CPD000394012	Benztropine mesylate	5	3	40	15
CPD000058324	Ganciclovir	71	28		
CPD000059219	Mesna	94	25		
CPD000058785	Meclomen	53	9		
CPD000471882	Fluconazole	82	11		
CPD001453712	Metaproterenol	88	15		
CPD000071170	Methoxsalen	83	36		
CPD000058224	Chloramphenicol	96	32		
CPD000499584	Tizanididne	75	16		
CPD001453706	Paroxetine hcl	6	8	10	7
CPD000550486	Mirtazapine	42	28		
CPD000010931	Etomidate	71	20		
CPD000499578	Moban	49	20		
CPD001453708	Fluvastatin	43	17		
CPD000058680	Urecholine	109	20		
CPD001496804	Cefuroxime	76	19		
CPD000718805	Cytoxan	125	13		
CPD000550478	Eszopiclone	209	365		
CPD000058802	Bendrofluazide	43	19		
CPD000058508	Raloxifen	13	4		
CPD000058351	Zidovudine	49	13		
CPD000058365	Clozapine	6	5	56	15
CPD001317850	Amicillin	62	7		
CPD000058800	Acebutol	59	20		
CPD000058707	Amoxicilline	46	17		
CPD000857209	Epinephrine	50	16		
CPD000857239	Azacytidine	51	10		
CPD000058186	Buspar	122	250		
CPD000436311	Rimantadine	29	10		
CPD000059121	Podofilox	60	14		
CPD000058313	D cycloserine	92	30		
CPD000059124	Cortisone	50	10		

CPD000058295	Clomipramine	5	4	22	6
CPD001227191	Carbamazepine	76	15		
CPD000875213	Memantine	35	10		
CPD000036827	Desipramine	6	4	28	7
CPD000326711	Mexiletine	62	19		
CPD000058438	Disopyramide	65	14		
CPD000673569	Stavudine	63	10		
CPD000097306	Doxazosin	21	14		
CPD000058963	Minoxidil	73	6		
CPD000059167	Propranolol	10	6	68	6
CPD001496943	Ribavirin	38	7		
CPD000058309	Terazosin	72	25		
CPD000058635	Chlorthalidone	54	38		
CPD000058330	Methylprednisolone	46	21		
CPD001496977	Phenelzine	67	13		
CPD000058767	Naltrexone	51	31		
CPD000469282	Glycopyrrolate	42	3		
CPD000046147	Ethambutol	109	28		
CPD001453715	Cetirizine	66	30		
CPD000539527	Dicloxacillin	67	13		
CPD000718800	Meloxicam	125	15		
CPD001906781	Daunorubicin	8	5	32	10
CPD001906779	Rifapentine	37	15		
CPD000274084	Penicillin	73	31		
CPD000043336	Gatifloxacin	94	38		
CPD000550475	Clopidogrel	56	20		
CPD001551784	Cefotaxime	109	71		
CPD000466319	Lamivudine	113	45		
CPD001307702	Ondansetron	66	12		
CPD000339803	Betamethasone	83	30		
CPD000550473	Celecoxib	95	54		
CPD000058778	Aminomethylbenzenesulf	90	19		
CPD001906782	Thiothixene	5	6	75	18
CPD000465669	Citalopram	44	15		
CPD000471864	Azithromycin	63	24		
CPD000673570	Lovastatin	85	28		
CPD000326785	Aminoglutetimide	129	59		
CPD000058452	Fluoxetine	1	1	17	9
CPD001233272	Flunisolide	73	18		
CPD000058225	Acyclovir	61	21		
CPD000058443	Etodolac	48	8		
CPD000718785	Simvastatin	70	13		
CPD001227203	Rifabutin	16	8		
CPD001496951	Felodipine	15	7		
CPD000499582	Quinapril	69	13		
CPD000499573	Acitretin	18	3		
CPD000718798	Fexofenadine	64	17		
CPD001563899	Fluoromethadolone	89	18		
CPD000466298	Sertraline	1	2	17	5

CPD001566944	Carbidopa	81	55

Table S2. Overview of the hits identified after screening the NIH Clinical Collection 1&2 against biofilms of *B. cenocepacia*. Hits are classified according to their therapeutic indication. Compounds were tested at a concentration of 100 µM in the presence or absence of tobramycin (512 µg/ml). The values shown in the left column represent the mean residual metabolic activity of the compound in the presence of tobramycin compared to treatment with tobramycin alone, and the standard deviation (SD). The values in the right column represent the mean residual metabolic activity of the compound compared to untreated contol, and the standard deviation. X means that the compounds did not cause a significant difference in fluorescence signal compared to the signal of untreated biofilms. SNRI: serotonin-norephinephrine reuptake inhibtor, SSRI: serotonin reputake inhibitor, TCA: tricyclic antidepressant, LTRA: leukotriene receptor antagonist.

#	PUBCHEM	COMPOUND	DESCRIPTION (function or structure)	MEAN (%)	SD	MEAN (%)	SD	
Anti-infective agents								
1	CPD000058356	Hexachlorophene	Desinfectant	0	1	5	6	
2	CPD000471847	Triclosan	Desinfectant	3	3	1	1	
3	CPD000058733	Miconazole nitrate	Antifungal drug	1	1	48	11	x
4	CPD001370749	Econazole nitrate	Antifungal drug	1	1	21	5	
5	CPD000469293	Oxiconazole nitrate	Antifungal drug	4	4	65	16	x
6	CPD000058460	Ketoconazole	Antifungal drug	6	6	73	9	x
7	CPD000466351	Efavirenz	Antiviral drug	2	2	20	8	
8	CPD000466367	Nitazoxanide	Anti-protozoal drug	6	4	16	13	
9	CPD000875314	Primaquine phosphate	Anti-protozoal drug	7	5	29	2	
10	CPD000875233	Mefloquine HCl	Anti-protozoal drug	4	4	11	8	
11	CPD000312779	Chloroxine	Anti-microbial drug	5	6	44	11	
Antipsychotics and antidepressants								
12	CPD000058411	Fluphenazine HCl	Phenothiazine	5	4	26	8	
13	CPD000058180	Perphenazine	Phenothiazine	3	3	46	8	x
14	CPD000058380	Thioridazine HCl	Phenothiazine	0	1	18	3	
15	CPD000059133	Trifluoperazine HCl	Phenothiazine	3	4	20	7	
16	CPD000058254	Chlorpromazine HCl	Phenothiazine	2	4	22	10	
17	CPD000058855	Droperidol	Butyrophenone	9	7	62	10	x
18	CPD000449283	Haloperidol HCl	Butyrophenone	6	1	20	3	
19	CPD001906782	Thiothixene	Thioxanthene	5	6	75	18	x
20	CPD000058470	Loxapine succinate	Dibenzoxazepine	2	5	43	6	
21	CPD000058365	Clozapine	Dibenzoxazepine	6	5	56	15	x
22	CPD000466362	Perospirone HCl	Azopirones	7	4	49	10	x
23	CPD000449282	Duloxetine	SNRI	4	6	20	2	
	CPD000469136	Duloxetine HCL	SNRI	6	2	10	4	
24	CPD000466298	Sertraline HCl	SSRI	3	5	10	9	
25	CPD000469181	N-methylparoxetine	SSRI	2	2	31	20	
	CPD001453706	Paroxetine HCl	SSRI	6	8	10	7	
	CPD000466269	Paroxetine maleate	SSRI	5	6	23	4	
26	CPD000058452	Fluoxetine HCl	TCA	1	1	17	9	
27	CPD000058295	Clomipramine HCl	TCA	5	4	22	6	
28	CPD000058388	Imipramine HCl	TCA	4	6	36	7	
29	CPD000036827	Desipramine HCl	TCA	6	4	28	7	
30	CPD000058486	Nortriptyline HCl	TCA	2	3	21	3	
31	CPD000058368	Amitriptyline HCl	TCA	0	0	22	4	
32	CPD000058416	Amoxapine	TCA	5	6	34	5	
33	CPD000058230	Doxepin HCl	TCA	2	1	39	7	
34	CPD000449297	Nefazodone HCl	other	10	14	47	22	

Anticancer therapy and/or hormonal therapy								
35	CPD001906781	Daunorubicin	Anthracycline	8	5	32	10	
36	CPD000469210	Vinorelbine	Antimitotic	5	3	22	9	
37	CPD001563707	Mitoxantrone	Topoisomerase inhibitor	8	5	31	4	
38	CPD001491671	Tamoxifen	SERM	4	9	69	10	x
39	CPD000238204	Ethylestrenol	Anabol steroid	6	7	46	9	x
40	CPD000058187	Flutamide	Antiandrogene	6	2	36	6	
Miscellaneous group								
41	CPD000469188	Montelukast sodium	LTRA	8	5	85	21	x
42	CPD000058379	Promethazine HCl	Histamine antagonist	4	8	30	10	
43	CPD000469183	Azelastine HCl	Histamine antagonist	6	7	36	3	
44	CPD000449274	AM404	Metabolite paracetamol	7	9	99	10	x
45	CPD000466272	Pizotifen meleate	Serotonine antagonist	7	5	51	9	x
46	CPD000387107	Honokiol	Neolignal biphenol	5	4	8	3	
47	CPD000469177	Atomoxetine HCl	SNRI	5	6	8	3	x
48	CPD000469294	Benproperine	Cough agent	2	4	25	8	
49	CPD000058366	Nitrendipine	Ca channel blocker	8	4	73	10	x
50	CPD000440694	Pterostilbene	Stilbenoid	5	9	30	10	
51	CPD000471618	Tegaserod maleate	5-HT 4 agonist	4	5	10	4	
52	CPD000449269	Bifemelane HCl	Choligeric drug	6	2	23	9	
53	CPD000449292	Donepezil HCl	Acetylcholinesterase inhibitor	5	4	57	5	x
54	CPD000058466	Loperamide HCl	Opioid receptor agonist	2	4	36	7	
55	CPD000058490	Oxybutynin chloride	Anticholinergic	8	9	44	9	
56	CPD000058821	Procyclidine HCl	Anticholinergic	6	7	48	20	x
57	CPD000394012	Benztropine mesylate	Anticholinergic, benzatropine	5	3	40	15	
58	CPD000469196	Tolterodine tartrate	Antimuscarinic	8	6	62	8	x
59	CPD000466346	Naftodipil	α1-adrenergic R antagonist	5	6	24	5	
60	CPD000059167	Propranolol HCl	Beta blocker	10	6	68	6	x

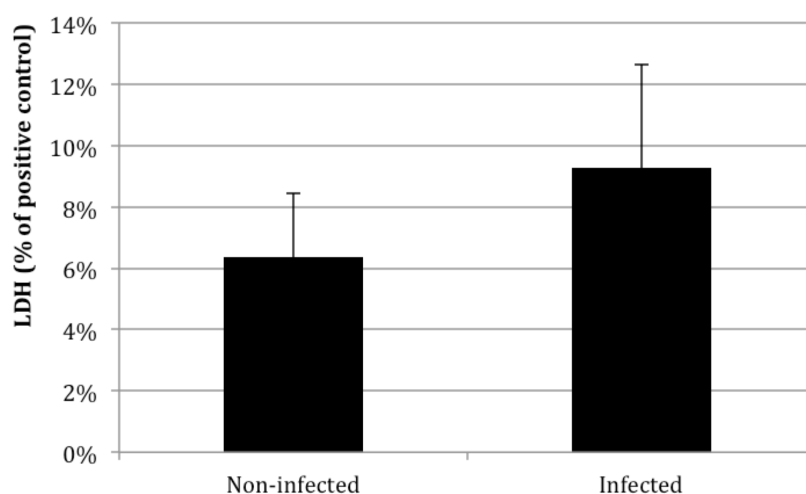

Figure S3. Lactate dehydrogenase (LDH) release assay of 3D A549 lung epithelial cells that were non-infected and infected with *B. cenocepacia* K56-2 for 17h. LDH release is presented as a percentage of a positive control (3D lung epithelial cells lysed with Triton-X100). Data shown are average, error bars indicate standard deviation.

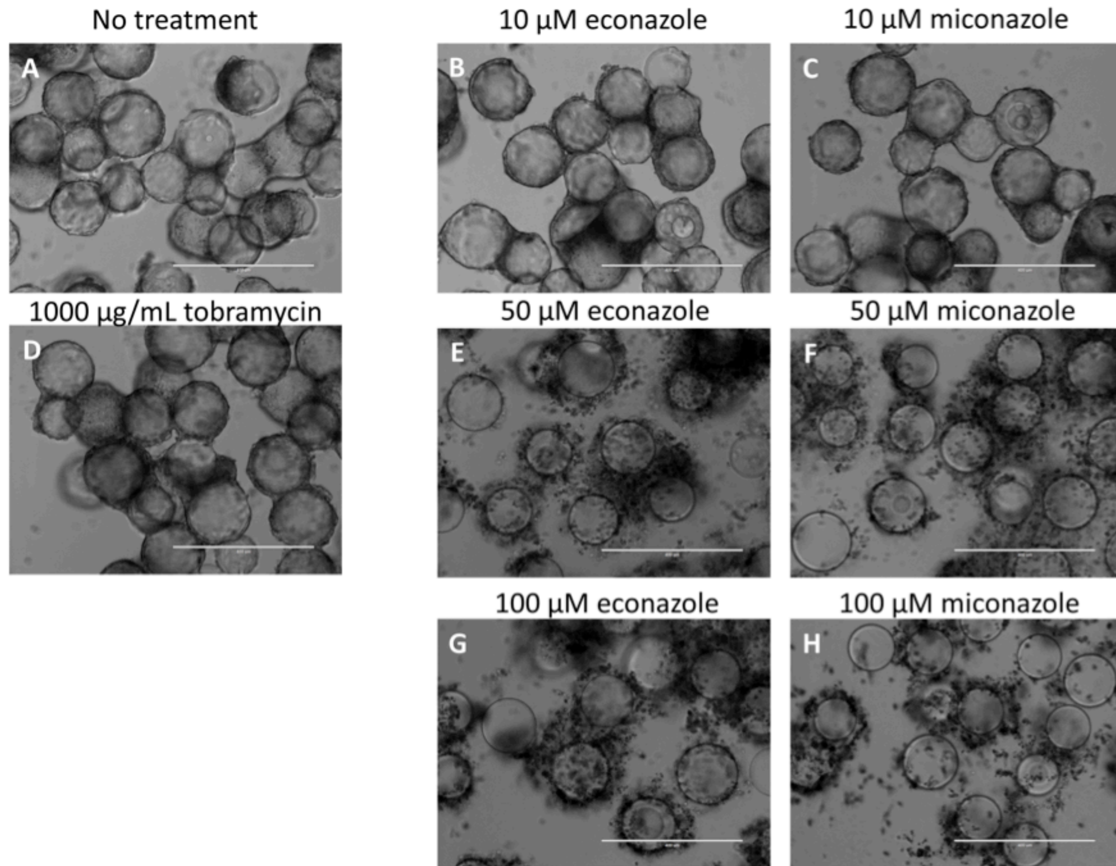

Figure S4. Exposure of 3D A549 lung epithelial cells for 17h to 10, 50, 100 µM econazole (B, E, G); 10, 50, 100 µM miconazole (C, F, H) or 1000 µg/mL tobramycin (D). A control condition where no antimicrobial agents were added is also included (A). High concentrations of the imidazoles resulted in detachment of host cells from microcarrier beads. Magnification is 300x. Scale bar is 400 µm.

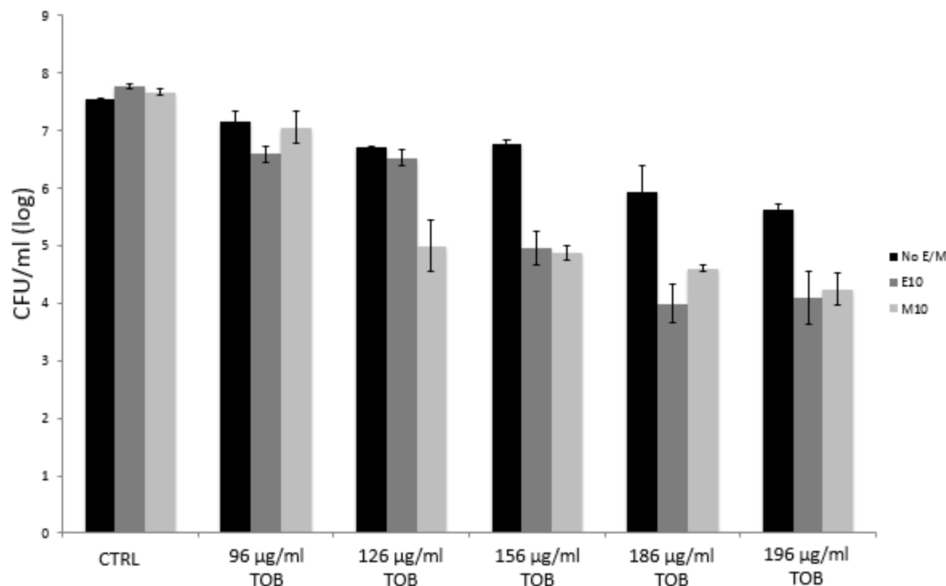

Figure S5. Preliminary data indicating that 10 µM econazole (E) or miconazole (M) in combination with ≥ 156 µg/mL tobramycin causes an additional reduction in *B. cenocepacia* biofilm formation in MTP compared to treatment with tobramycin alone, n=1, error bars indicate standard error of the mean on the technical replicates.

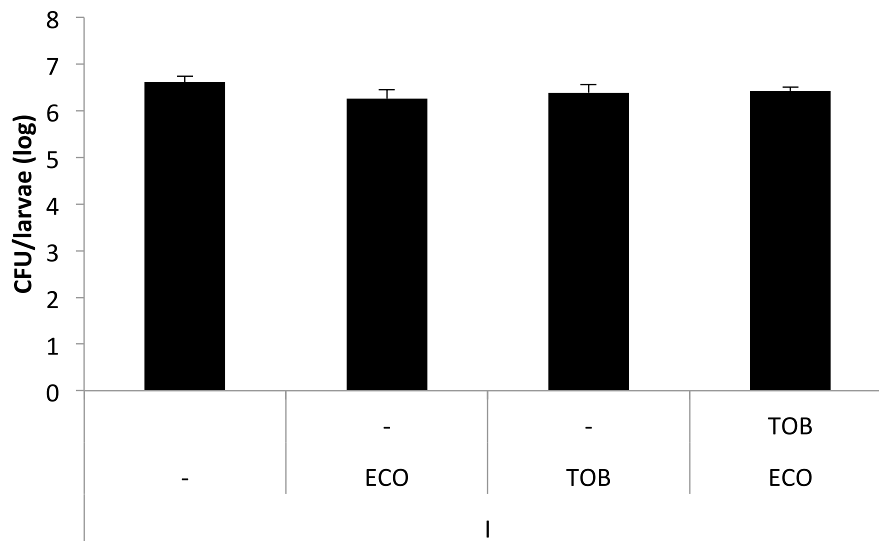

Figure S6. *G. mellonella* was infected with *B. cenocepacia* LMG 16656 and treated with tobramycin (TOB), econazole (ECO), or the combination. CFU/larvae was determined 24 h *p.i.* and treatment. Lavae were homogenized and plated on selective *Burkholderia* medium. Data shown are average, n≥3, error bars indicate standard error of the mean.

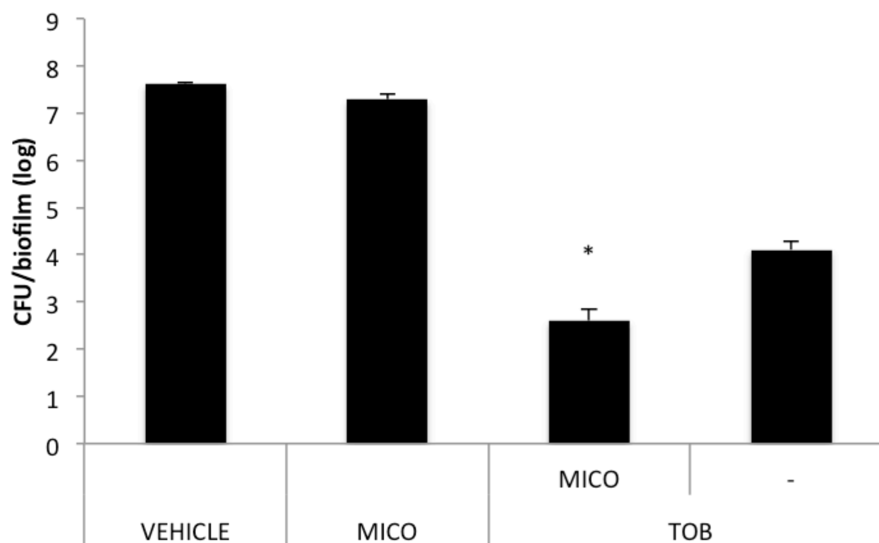

Figure S7. In order to evaluate the antimicrobial activity of a formulation for inhalation prior to use in the mouse lung infection model, biofilms of *B. cenocepacia* LMG 16656 were formed in 96-well MTPs and subsequently treated with the diluted (129x) formulation. This diluted formulation corresponds to vehicle (tween80 dissolved in PS), 512 µg/mL tobramycin (TOB), 200 µM miconazole (MICO) or the combination of tobramycin and miconazole. The asterisk indicates a significantly different number in log CFU/BF compared to treatment with tobramycin alone (P value < 0.05). Data shown are average, n≥3, error bars indicate standard error of the mean.

Chapter IV: Broader international context, relevance, and future perspectives

1. The continuous need for new antibacterial drugs

Antibiotics are critical for our health as we need them to treat and prevent the spread of infectious diseases [9, 16, 271]. However, the use of antibiotics results in a selective pressure towards resistant bacteria [16]. Between 1930 and 1980, AMR was tackled by the introduction of new classes of antibiotics, but this is where the shoe pinches now. Although new antibiotic classes active against Gram-positive pathogens were introduced more recently, the last time a new class of antibiotics active against Gram-negative bacteria has been introduced to the market was in the 1960's [13, 144] and new antibiotics active against both Gram-positive and Gram-negative bacteria are urgently needed.

The screening of NP in empirical whole-cell screens resulted in the discovery of many important antibacterial classes in the fifties and sixties of the 20th century [135]. The antibiotics developed in this 'golden era' and their derivatives are still indispensable today [135]. Nevertheless, this once so successful approach was abandoned as the NP source failed to provide new antibiotics [154]. All hope was put on new techniques introduced in the nineties, i.e. genomics, HTS, SBDD, and combinatorial chemistry [153]. Huge libraries were screened in target-based assays, but not a single lead compound was developed into a marketed antibacterial compound so far [132, 135]. There are multiple reasons for this failure, for instance, screening against isolated targets instead of whole cells [160], and screening libraries that did not cover a broad chemical space [135, 144]. More recent screenings met these flaws and implemented the techniques in whole-cell assays [160]. For instance, whole cells of *B. cenocepacia* K56-2 were screened with the Canadian Chemical Biology Network Compound Collection. This collection covers an ample chemical space as it is built up out of several different libraries: 16,000 synthetic small molecules from the Custom Library, 9989 synthetic small molecules from the DIVERSet Library, 1120 off-patent small molecules from the Prestwick Chemical Library, 361 NP from the BIOMOL Natural Products Library, 1214 NP from the Spectrum Collection and 690 synthesized lactams, lactones and piperidines from the Dennis Hal Compounds library, in total 30,259 compounds [272].

2. Drug repurposing and repurposing libraries

However, the development of new antibacterial drugs is extremely time-consuming, expensive and risky [189]. Moreover, the return on investment is relatively low for antibacterial therapies [147]. The use of existing drugs for the treatment of diseases for which they were not initially developed, is called drug repurposing [177]. Because toxicity and metabolic profiles of these drugs are very well characterized by clinical use, the costs and risks to develop and market these drugs is reduced [177]. Repurposing libraries are built out of approved drugs or drugs tested in clinical trials and are commercially available to enable HTS for the identification of repurposing candidates for a selected target/disease [182]. The screening of libraries containing drugs approved for non-bacteriological indications offers an alternative approach in antibiotic drug discovery [184]. Screening of these libraries resulted in

the identification of several interesting repurposing candidates with confirmed *in vivo* effect to treat bacterial infections [183, 189, 198].

In this dissertation, the NIHCC 1&2 repurposing libraries were used, which consist out of 727 compounds that were tested in clinical trials and passed human safety assessment. Most of the compounds are approved and off-patent drugs currently in use [273]. The compounds are dissolved in dimethyl sulfoxide (DMSO) and distributed in 96-well MTPs [273]. However, is screening 727 compounds enough to represent the entire space of existing drugs? FDA registered over 100,000 drug products [178]. However, this huge number is misleading as several drug products are different formulations, brand names, salts or esters, from only one 'molecular entity'. Thus, this number can be reduced to 3936 approved molecular entities and 4935 not approved molecular entities but used experimentally in humans worldwide, thus 8969 drugs in total [178]. When drugs unsuitable for laboratory-based HTS are removed (e.g. molecules insoluble in DMSO or unstable molecules), 7631 'small molecule' drugs remain [178]. So, the NIHCC 1&2 represents approx. 10% of all compounds that can be tested for repurposing purposes. The number of drugs in the NIHCC 1&2 is equal to the Screen-Well FDA-approved drug library V2 [274], and only slightly lower than the Prestwick Chemical Library which contains 1280 drugs (85% FDA approved) [182, 272], and the John Hopkins Clinical Compound Collection which contains 1600 compounds [275]. The NPC Screening Resource (NIH Chemical Genomics Center Pharmaceutical Collection) is one of the most comprehensive libraries, with 2400 approved drugs [182, 276]. The different therapeutic groups present in the NIHCC 1 are shown in Figure 1. Although a substantial number of drugs are anti-infective drugs and drugs used to treat diseases related to the central nervous system (CNS), the library covers all main therapeutic groups and thus possesses a high pharmacological heterogeneity.

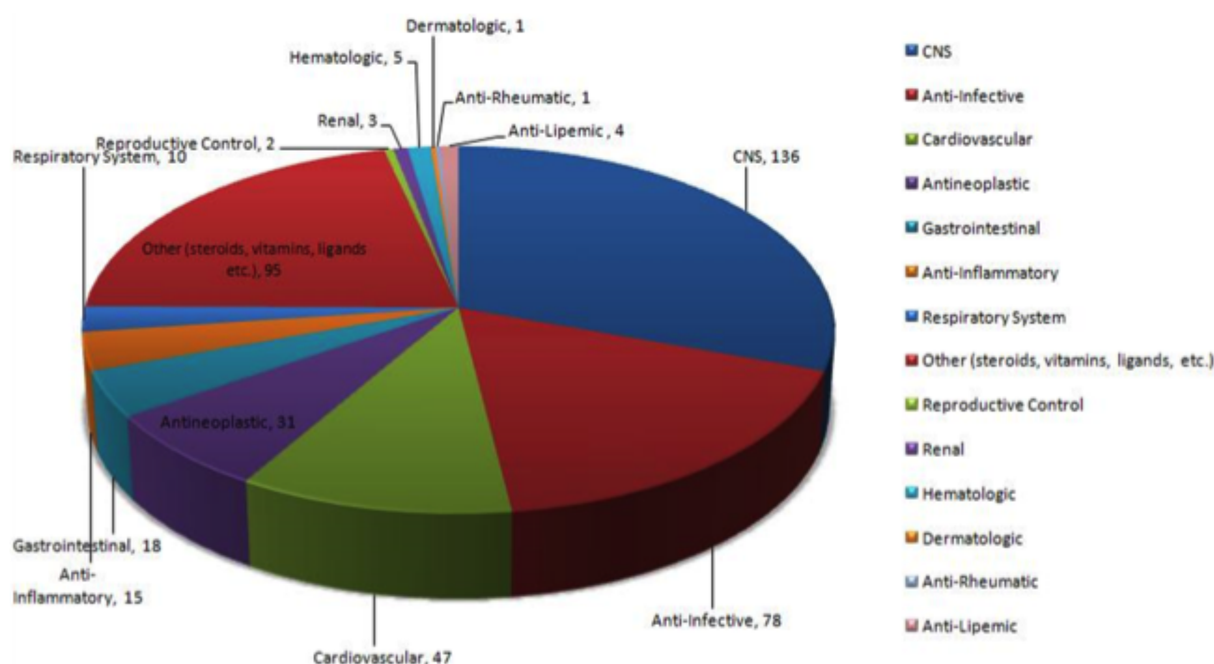

Figure 1. Pie chart representing the amount of drugs in each therapeutic group as contained in the NIHCC 1 [273].

In addition to the diversity of the library, also the quality is crucial in order to obtain reliable results upon screening, and we did encounter problems regarding the quality of the NIHCC 1&2. For example, a hit compound in the screening against *P. aeruginosa* biofilms was annotated with the code CPD000058451 in the documents provided by Evotec. This code corresponds to flecainide acetate in the Pubchem database. However, flecainide acetate, purchased from Sigma, had no activity towards *P. aeruginosa* biofilms in combination with tobramycin. Analysis of the mass spectrum revealed that the compound provided in the library was not flecainide but chlorhexidine. This stresses the importance of confirming hits with independently obtained pure compounds [277]. Luckily, this is quite easy to do for compounds present in repurposing libraries, as all tested compounds are well characterized and readily available for further research. The suppliers of the NIHCC 1&2 noted other quality problems as well and recognized they should have controlled all the vender supplied material before including the products in their library. The NIHCC 1&2 we purchased in 2013 is not available anymore but substituted by the NIH Clinical Collection which contains now 719 compounds in which incorrect compounds were replaced with the correct compounds and about 26 compounds were dropped for various reasons (personal communication).

3. The screening approach and the need for anti-biofilm therapies

Beside the selection of the library, the model in which the library screen is performed is equally important. Ideally, the model resembles the host-environment closely, and allows HTS. The screening against purified enzyme targets allows HTS but hits identified often failed to overcome the impermeability of bacterial cells [144]. Whole-cell screening is thus a more valuable approach and several repurposing screenings for new antibacterial therapies have been performed in whole-cell growth-based assays [183, 189, 278]. Generally, these screenings are performed against planktonic bacterial cells under optimal growth conditions in nutrient rich cell culture medium (e.g. Mueller Hinton or Luria Bertani broth).

For instance, Younis et al. screened the NIHCC 1&2 in a whole-cell assay against planktonic cells of various pathogens (*Enterococcus faecium* ATCC 700221, *S. aureus* USA300, *Klebsiella pneumoniae* ATCC BAA-1705, *Acinetobacter baumannii* ATCC BAA-1605, *P. aeruginosa* ATCC 15442, *Enterobacter cloacae* ATCC BAA-1143) [189], and Torres et al. screened the Prestwick Library against planktonic *S. aureus* TCH1516 [278]. Also Jacobs et al. screened the Prestwick Chemical Library for antimicrobial agents active against planktonic *E. coli* 8295 and the ESKAPE pathogens (*E. faecium* 824-05, *S. aureus* USA300, *K. pneumoniae* cKP1, *A. baumannii* 98-37-09, *P. aeruginosa* PAO1, and *E. cloacae* PMD1001) growing in Mueller Hinton broth [279]. In contrast to the screening performed by Younis et al. [189] or Torres et al. [278], in which the effect of the treatment was evaluated by measuring bacterial growth inhibition, Jacobs et al. developed an adenylate kinase assay which identified compounds that disrupt cellular integrity [279].

However, bacteria growing in nutrient rich laboratory media do not resemble the *in vivo* situation and it is thus important to screen existing libraries in alternative conditions [214, 280]. For example, Miller et al. performed a screening with the LOPAC library against *M. smegmatis* both in nutrient rich medium (Luria Bertani broth) and in carbon and nitrogen-limited medium and identified different hits in both conditions [214].

Also, bacteria can be non-growing and protected in biofilms *in vivo*. Biofilms contain micro-organisms organized in an EPM, acting as a reservoir that is responsible for persisting infections and showing increased resistance and tolerance towards antibiotic treatment [70]. Since they are estimated to be responsible for 80% of infections, it is important that new drugs are also effective against bacteria residing in biofilms [70].

Torres et al. selected 9 hit compounds from the initial screen against planktonic cells and tested their activity against biofilms of *S. aureus* [278]. Only three hit compounds were active: niclosamide, carmofur, and auranofin caused a reduction in CFU/BF of 1-2 log compared to untreated controls after 20 h [278]. Also Jacobs et al. evaluated the activity against biofilms of one hit compound identified in their primary screening against planktonic cells: the antihistamine drug terfenadine showed antimicrobial activity against planktonic *S. aureus* and its activity at 10xMIC was evaluated against biofilms formed by *S. aureus* UAMS1. This treatment resulted in a 2.7-fold increase in adenylate kinase release, corresponding to a 1.1-log reduction in biofilm cell viability [279].

Colquhoun and colleagues performed a screening of the complete Selleck library (853 approved drugs) against *A. baumannii* 98-37-09 growing in conventional laboratory medium (Mueller Hinton broth), but also in human serum, lung surfactant, and against biofilms [215]. They identified 90 compounds (73 antibiotics and 17 non-antibiotic drugs) with antimicrobial activity in at least one screening condition. Of the antibiotics identified, only four (rifampin, rifaximin, ciprofloxacin, and tetracycline) exhibited antibacterial activity in all conditions tested. From the 17 non-antibiotic drugs, four compounds (epirubicin, idarubicin, nebivolol and pyrimethamine) were identified as hits in the screening performed in MH, but they were not active in the other test conditions. Zink pyrithione was active both against cells in MH and against biofilms, while two other compounds, ciclopirox and ribavirin, were active in MH and lung surfactant. Doxorubicin was active against serum grown cells only, while eight drugs were active solely in the lung surfactant screen. The differences in screening conditions result in differences in bacterial physiology and this affects the organism's susceptibility: the screen resulted in the identification of several compounds showing activity in one or more of the alternative test conditions without activity against *A. baumannii* grown in conventional medium [215]. These compounds would thus have been missed if the screening was performed solely in conventional growth medium, although they may be promising anti-*Acinetobacter* drugs [215].

4. Combination therapy to treat biofilm infections

Looking for repurposing compounds active against established biofilms in monotherapy might result in very low hit rates as exemplified by the screening by Colquhoun (one hit) [215] and Torres (three hits) [278]. The eradication of mature biofilms using antibiotic monotherapy is very difficult and requires high concentrations and long treatments, which is often clinically impossible due to toxicity [111, 281]. The combination of drugs with different modes of action might improve the success of anti-biofilm therapies [90, 201, 282].

4.1. Combination of antibiotics to treat biofilm infections

Several combinations of antibiotics were shown to better kill biofilms than antibiotic monotherapy as illustrated by the following examples. Neither moxifloxacin nor daptomycin were able to kill *S. aureus* biofilms *in vitro*, while the combination of moxifloxacin or daptomycin with clarithromycin resulted in a significant reduction of biofilm survival [281]. Similarly, combining linezolid and daptomycin resulted in greater effect compared to treatment with the respective antibiotics alone [281]. The combinations rifampicin and vancomycin, and rifampicin and linezolid were superior to vancomycin or linezolid monotherapy in a rat model of foreign-body osteomyelitis [281]. An *in vitro* evaluation of the activity of eight antibiotics against *P. aeruginosa* biofilms revealed synergistic activity only between tobramycin and clarithromycin, while no anti-biofilm activity was observed for the single antibiotics [283]. This synergy between tobramycin and clarithromycin was not observed in planktonic cultures, and this might be explained by the fact that clarithromycin enhances the penetration of tobramycin by inhibiting the alginate synthesis, among others [283]. Another study investigated the combination of tobramycin with colistin and found that the combination was superior to monotherapy in killing biofilms of *P. aeruginosa in vitro* [90]. In a rat lung infection model, treatment with the combination resulted in reduced mortality rates and lower CFUs recovered from the lungs compared to monotherapy [90].

Clinically, combinations of antibiotics are used as well in an attempt to eradicate or control biofilms, for example in the treatment of prosthetic joint infections caused by *S. aureus* biofilms [281] or lung infections in CF patients caused by *P. aeruginosa* biofilms [90, 284]. The treatment in which nebulized tobramycin or colistin is combined with oral ciprofloxacin is successful to eradicate *P. aeruginosa* when the colonization is recent [284]. However, combination treatment fails to eradicate *P. aeruginosa* in chronically infected CF patients [284]. Also in the case of *S. aureus* biofilm infections, surgical removal of the nidus of infection is sometimes the only way to resolve the infection [202]. It is clear that conventional antimicrobial therapy, even when using combinations, often fails to eradicate infections caused by biofilms and that novel alternative strategies are urgently needed [285, 286].

4.2. Helper compounds to improve antibacterial activity

A strategy to restore or increase the antibacterial activity of antibiotics is the use of helper compounds [185], also called potentiators [246], adjuvants [247], or 'antibiotic resistance breakers' [13]. Reversal of antibiotic resistance by the helper compound can extend the

antimicrobial arsenal and increase the lifespan of existing antibiotics [187]. These helper compounds have no antimicrobial effect when administered alone, but they increase the activity of antibiotics in combination therapy [14].

One very well-known example of this strategy is the successful combination of β-lactam antibiotics with β-lactamase inhibitors [13, 14]. The addition of clavulanic acid to amoxicillin allowed the continuous use of amoxicillin against pathogens that are amoxicillin resistant due to activity of certain β-lactamases. Without this combination, the use of amoxicillin would be limited, while Augmentin (the marketed combination) was the best-selling antibiotic in 2001 [14].

Nevertheless, the number of successfully used combinations is limited as there has not been a broad exploitation of this concept in the past [13]. However, the identification of helper compounds is currently an important topic of research [247]. For instance, numerous researchers have been investigating compounds for their ability to block the QS system [287]. QS regulates several important bacterial virulence factors (e.g. rhamnolipids and proteases) and QS inhibitors might attenuate bacterial virulence resulting in a facilitated eradication by the host immune system [287]. In addition, QS inhibitors increase the susceptibility of bacterial biofilms towards antibiotics [287]. QS inhibitors have been identified in natural sources, e.g. iberin (horseradish), ajoene (garlic [287]), or hamamelitannin (witch hazel) [288, 289], but also synthetic compounds, like furanones [287], or off-patent non-antibiotic drugs, like the anthelmintic drug niclosamide [180] are described as QS inhibitors. Another example of a strategy that seems promising for restoring the activity of existing antibiotics is the use of helper compounds that inhibit efflux pumps [290]. Also here, existing drugs (i.e. reserpine, phenothiazines, calcium channel antagonists, SSRIs, and proton pump inhibitors) have been identified as efflux pump inhibitors [290]. Unfortunately, their use as helper compounds is often limited by the fact that they require concentrations too high to be clinically useful [290].

A so far under-investigated source of potentiators are drugs in use for non-bacteriological indications. For example, Ejim et al. screened 1057 previously approved drugs to identify potentiators of minocycline against planktonic *P. aeruginosa* PAO1, *E. coli* BW25113 and *S. aureus* ATCC 29213 [198]. This screening resulted in the identification of several hits, among them loperamide. Treatment with loperamide alone had no effect against *P. aeruginosa*, but combination with minocycline resulted in a synergistic interaction and similar effects were observed in other Gram-negative pathogens [198]. The *in vivo* efficacy was confirmed in a mouse model of infectious colitis caused by *Salmonella enterica* Typhimurium [198]. Treatment with loperamide or minocycline alone had no impact on the infection, while the combination caused a significant decrease in bacterial load in the cecum [198]. Loperamide is thus an attractive candidate to develop for clinical use in a combination therapy as it increases the susceptibility of microbes to an existing antimicrobial agent and thus enhancing the antibacterial activity

4.3. Helper compounds to improve anti-biofilm activity

The strategy of combining antibiotics with compounds that increase the susceptibility of biofilms has been investigated as well and several combinations with improved anti-biofilm activity have been described [95, 111]. For instance, DNase and alginate lyase enhance the penetration of aminoglycosides through *P. aeruginosa* biofilms resulting in a decreased biofilm growth and lower bacterial counts in CF sputum [286]. Similarly, the activity of antibiotics towards *E. coli* biofilms was improved in combination with a lipopeptide biosurfactant originating from *Bacillus licheniformis* [286]. Besides improving the penetration of antibiotics, also compounds that induce biofilm dispersion, inhibit efflux pumps or interfere with the QS system, might contribute to a better anti-biofilm activity of antibiotics [111, 286, 291]. For instance, garlic-treated *P. aeruginosa* biofilms were more susceptible towards tobramycin than control biofilms. This was also confirmed in a mouse pulmonary infection model, although a clinical trial in 26 CF patients failed to demonstrate a significant difference between patients receiving the garlic extract and placebo-treated patients [95, 109].

De Cremer et al. screened 1600 off-patent drugs for potentiator activity towards miconazole against biofilms of *Candida albicans* SC 5314. This resulted in the identifications of 8 hit compounds (hexachlorophene, pyrvinium pamoate, artesunate, broxyquinoline, dihydroartemisinin, gentian violet, bithionate disodium, and nitroxoline) [292]. Although several screenings of repurposing libraries against bacterial biofilms have been performed [215, 278], none of them screened against bacterial biofilms in the presence of an antibiotic for potentiators, like De Cremer et al. did against fungal biofilms. In this dissertation, we performed such a screening for the first time.

5. Screening a repurposing library for combination therapy against bacterial biofilms

The screenings in this dissertation were performed against three important pathogens: *S. aureus*, *P. aeruginosa,* and *B. cenocepacia*. The screening against *B. cenocepacia* and *P. aeruginosa* was performed in the presence of the antibiotic tobramycin. Tobramycin is commonly used to treat *P. aeruginosa* infections in CF patients, but it fails to eradicate *P. aeruginosa* in chronically infected patients (MIC *P. aeruginosa* PAO1: 1 µg/ml [293], concentration used: 2 µg/ml) [283]. *B. cenocepacia* is intrinsically resistant towards tobramycin (MIC *B. cenocepacia* LMG 16656: 256 µg/ml [294], concentration used: 512 µg/ml). The screening against *S. aureus* biofilms was performed in combination with vancomycin, the drug of choice to treat MRSA infections. *S. aureus* Mu50 is intermediate resistant towards vancomycin (MIC: 8 µg/ml [295], concentration used 40 µg/ml). The concentration at which library compounds were used was 100 µM.

Biofilms were formed in 96-well MTPs and treated after 24 h with the antibiotics and/or the library compounds (dissolved in physiological saline with 1% DMSO). The effect of the treatments after 24 h was evaluated with CTB staining. CTB is reduced by metabolically active cells into a fluorescent reaction product. Quantification of the fluorescence generated

gives an indication of the number of metabolically active cells present in the biofilm. This indirect quantification method is less labor intensive and time consuming than direct quantification by using plate counts [220]. For *B. cenocepacia* and *S. aureus*, the method as described by Peeters et al. was used [200]. In this method, CTB is diluted in physiological saline, added to the wells containing the treated biofilms, and the fluorescence signal generated is measured after 30 minutes incubation for *S. aureus*, and 1 h for *B. cenocepacia*. However, for *P. aeruginosa*, the fluorescence signals generated with this protocol were too low and variable. We optimized the protocol in order to overcome this problem and to allow us to use CTB for measurement of activity against *P. aeruginosa* biofilms as well. CTB was diluted in Mueller Hinton broth and the increase in fluorescence signal was followed over time till a threshold fluorescence signal of 100,000 was reached. The time necessary to reach this threshold correlates with the number of biofilm cells initially present [220].

Hits in our screening were compounds that, in the case of *S. aureus* and *B. cenocepacia,* caused a decrease of at least 90% in fluorescence compared to the fluorescence generated by biofilms that were treated with antibiotic alone. For *P. aeruginosa*, compounds were considered as hits if the amount of CFU/biofilm was lower than 10^3 after combination treatment. The hit rate resulting from the screenings performed for this dissertation was 8.2%, 1.7%, and 3.4% for potentiators of tobramycin or vancomycin against biofilms of *B. cenocepacia*, *P. aeruginosa*, and *S. aureus*, respectively. Although there are no data available for other screenings with a similar set up, we can compare this hit rate with that of other repurposing screens. A screening performed with the same library against planktonic *S. aureus* USA300 resulted in a hit rate of 3.3% (concentration used: 16 µM) [189], another repurposing screening in the presence of ½ MIC minocycline against planktonic *S. aureus* ATCC 29213, *P. aeruginosa* PAO1, and *E. coli* BW25113 resulted in hits rates of 15%, 4.5%, and 13% (concentration used: 10 µM) [198]. A screen of the Prestwick Chemical Library against planktonic *S. aureus* TCH1516 identified 104 compounds (hit rate: 8.1%, concentration used: 10 µM). A screen using the same library for compounds (tested at 20 µM) that inhibit biofilm formation of *C. albicans* SC5314 resulted in a hit rate of 3.25% [218]. Thus, the hit rate in our screening was comparable with these in other repurposing screenings. It should however be noted that we used higher concentrations compared to these other screenings, which might help explain the high hit rate in our screenings.

The hit compounds we identified could be divided in several groups based on their therapeutic indication: anti-infective agents, anti-psychotics and antidepressants, anticancer drugs and/or hormone related drugs, and a miscellaneous group.

5.1. Anti-infective agents

The NIHCC 1&2 library contained 47 antibiotics belonging to different classes. Almost all hits identified in the screening against *P. aeruginosa* were antibiotics: seven fluoroquinolones, rifampicin, and doxycycline. This result contrasted to the results from the screening against *S. aureus* and *B. cenocepacia*, where not a single antibiotic was a hit for *S. aureus*, and only chloroxine was a hit for *B. cenocepacia*. For instance, in the screening against *S. aureus* biofilms, linezolid (100 µM, corresponding to 34 µg/ml) was not identified as a hit compound

in combination with vancomycin, as it only caused a reduction of 50% in CTB signal. However, *S. aureus* Mu50 is a linezolid susceptible strain, as the MIC is only 2 µg/ml [296]. This illustrates the high resistance encountered in mature biofilms towards conventional antibiotics in contrast to planktonic cells and supports our strategy to look for potentiators among drugs with non-bacteriological indications.

Representatives of other antimicrobial drugs (antiseptics, antiviral, anthelmintic and antifungal drugs) present in the library were also identified as hits. Among them, four antifungal imidazole drugs, i.e. miconazole, ketoconazole, econazole and oxiconazole, showed a strong potentiating effect towards tobramycin against *B. cenocepacia* biofilms. Other azoles tested were not identified as hits. The antifungal imidazoles did not potentiate the activity of tobramycin against biofilms of *P. aeruginosa.* Against biofilms of *S. aureus*, ketoconazole was not effective, while the three other imidazoles mentioned above were identified as hit compounds. The activity observed against *S. aureus* probably originates in the intrinsic antibiotic activity of the antifungal imidazoles towards Gram-positive bacteria. The bactericidal effect of miconazole towards *S. aureus* is described by Sud and Feingold in which interference with the membrane results in leakage of K^+ [190]. In addition, imidazoles bind *S. aureus* flavohemoglobin, a protein with NO dioxygenase activity, causing nitrosative and oxidative stress [224, 258]. Miconazole also binds flavohemoglobin of *E. coli*, and *in vitro* data showed that the combination of a NO-donor, miconazole and polymyxine B nonapeptide (to increase the intracellular miconazole concentration) were effective to treat four ESBL-producing *E. coli* isolates [260]. However, to our knowledge, potentiating activity of antifungal imidazoles towards tobramycin against biofilms of *B. cenocepacia* had never been reported before.

5.2. Antipsychotics and antidepressants

The NIHCC 1&2 contains a large amount of drugs related to the central nervous system (Figure 1), and many of these drugs were amongst the hits identified in the screening against *B. cenocepacia* and *S. aureus*, but not against *P. aeruginosa*. Four phenothiazine antipsychotics and the antidepressant sertraline, a selective serotonin reuptake inhibitor (SSRI), were hits for *S. aureus*. These compounds were not identified as hit in the screening against planktonic *S. aureus* with the same library performed by Younis et al. [189], probably because they screened with lower concentrations (16 µM in contrast to 100 µM). A third of all hits identified against *B. cenocepacia* biofilms were antipsychotics and antidepressants, among them five antipsychotic phenothiazines. Although these drugs are not used to treat bacterial infections, their antimicrobial activity has been described extensively in literature [196, 225, 226, 297, 298]. Phenothiazines are known efflux pump inhibitors [32] and synergy with several antibiotics, especially β-lactams, has been observed *in vitro* [227, 297, 299, 300] and *in vivo* in *C. elegans* [231]. However, results obtained in mice are conflicting [228, 230] and no studies were found that describe any activity towards bacterial biofilms.

5.3. Anticancer and hormonal therapy

Several antineoplastic and hormonal drugs showed intrinsic activity towards established biofilms, or potentiator activity towards tobramycin or vancomycin against biofilms of *B. cenocepacia, P. aeruginosa, and S. aureus*. We did not pursue research on any of these hit compounds as, in our opinion, the compounds are too toxic to be repurposing candidates. However, other researchers did explore the potential of anticancer drugs identified in their screen with promising results. Younis et al. identified the anticancer drug 5-fluoro-2'-deoxyuridine as a hit against planktonic MRSA [189]. Activity of the compound was confirmed in a septicemic MRSA mice infection model with concentrations of 5-fluoro-2'-deoxyuridine much lower compared to concentrations therapeutically used for cancer treatment, and thus with reduced toxicity [189].

5.4. Miscellaneous group

Beside the antibiotics, the antiseptic hexachlorophene, and the anticancer drug bicalutamide, only one other drug was identified as a hit against biofilms of *P. aeruginosa*: L-pyroglutamic acid 7-amido-4-methylcoumarin. This compound is a strong potentiator of tobramycin, as the biofilms treated with this combination were not able to reach the threshold fluorescence signal of 100,000. The compound has also intrinsic activity towards *P. aeruginosa* biofilms. In contrast, the compound was not a hit in the screening against *B. cenocepacia* and *S. aureus*.

Treatment with the antihistaminics azelastine and promethazine, in combination with tobramycin, decreased the fluorescence signal generated by *B. cenocepacia* biofilms significantly compared to treatment with tobramycin alone. Promethazine has a tricyclic structure and belongs to the phenothiazine family, so the mode of action might be in line with that from the antipsychotic phenothiazines. Azelastine was not identified as a hit in the screen against *S. aureus* and has thus no potentiating activity towards vancomycin against *S. aureus* biofilms. In contrast, azelastine has been described to potentiate β-lactams, macrolides, fluoroquinolones, aminoglycosides and tetracyclines against several planktonic *S. aureus* strains *in vitro* [197].

Loperamide was identified as a hit in the screening against *S. aureus*, but not against *B. cenocepacia* and *P. aeruginosa*. Interestingly, loperamide was previously identified to potentiate the activity of minocycline against planktonic *P. aeruginosa* [198].

Other hit compounds identified in our screenings have been described for antibacterial properties in literature: honokiol [235, 301] which was a hit for *S. aureus* and *B. cenocepacia*, MK-886 and 5-nonyloxytryptamine [189] which were hits for *S. aureus*, and tegaserod [198], propranolol [302], and pterostilbene [303] which were hits in *B. cenocepacia.* Four hits for *S. aureus*, and 13 for *B. cenocepacia* have, to our knowledge, never been reported for having an antibacterial effect.

6. Validation of selected hits *in vitro*

We selected the antifungal imidazoles miconazole and econazole as promising repurposing candidates for *B. cenocepacia.* We rationalized that, if potent activity was observed in relevant *in vivo* models, the step to bring them to the market to treat another antimicrobial disease would be smaller, in comparison to drugs belonging to other therapeutic groups.

The phenothiazine antipsychotics trifluoperazine, fluphenazine, perphenazine and thioridazine were selected for further tests in *S. aureus*. Potent *in vitro* activity of phenothiazines was observed in several studies [32, 195, 227, 297, 304], but *in vivo* data were not consistent [228, 230, 231]. We selected these phenothiazine compounds because the activity towards biofilms had never been investigated in previous studies.

No further tests were performed with *P. aeruginosa* because the only hit that was not an antibiotic, antiseptic or anti-cancer drug, L-pyroglutamic acid 7-amido-4-methylcoumarin, turned out not to be an approved drug. Probably, the compound passed safety assessments in a clinical trial, but it was not clear to the provider Evotec why the compound was included in the library (personal communication).

The selected compounds were purchased and biofilms were treated with them to confirm the results from the screening by another quantification method, i.e. plate counts. Different concentrations were tested in combination with several antibiotics to identify other potentially promising combinations. The antifungal imidazoles miconazole and econazole did not have intrinsic activity against *B. cenocepacia* biofilms, however, they caused an additional decrease in CFU/BF in combination with tobramycin compared to treatment with tobramycin alone. This effect was not observed with ciprofloxacin or meropenem. Treatment with 100 µM trifluoperazine or perphenazine did not affect formed *S. aureus* biofilms, while treatment with 50 and 100 µM thioridazine or 100 µM fluphenazine caused a significant decrease in CFU/BF compared to no treatment. Treatment with thioridazine caused no potentiating activity towards vancomycin, so the effect seen in the screening was due to the intrinsic activity of thioridazine alone. However, there was a potentiating activity towards tobramycin, flucloxacillin and linezolid as these combinations caused a significant reduction in CFU/BF compared to treatment with antibiotics alone. Thus, overall we were able to confirm the results observed in the screening against biofilms and were able to validate the hits *in vitro*.

7. Validation of selected hits in other model systems

The biofilms used to screen for potentiators of antibiotics were formed on the bottom of 96-well MTPs, which is a closed biofilm model system that is often used [93]. However, this basic biofilm model differs a lot from the *in vivo* host environment [93]. It is thus necessary to confirm the potentiating activity of the selected hits in other, more relevant models as well. We evaluated the effect of miconazole and econazole with tobramycin in a three-dimensional (3D) organotypic human lung epithelial cell culture model, which is a more sophisticated *in*

vitro model that better mimics the *in vivo* situation [261]. We also used two *in vivo* models, i.e. the invertebrate *Galleria mellonella* infection assay and a mouse acute lung infection model. In addition, the potentiating activity of thioridazine towards tobramycin, flucloxacillin, and linezolid was evaluated in an *in vitro* model for chronic wound infections. However, in contrast to the activity observed in the general MTP model, the potentiating activity of our selected hit compounds could not be confirmed in any of these more sophisticated infection models. The reason for this failure is not clear, but probably host factors play an important role in deactivation of the compounds or in decreasing the susceptibility of the bacteria.

8. Recommendations for future screenings

8.1. Determination of the concentration to be used in the screening

The screenings in this dissertation were performed with 100 µM of each library compound. This concentration is higher than concentrations used in other screenings described in literature (i.e. 16 µM [189], 10 µM [198, 278], 20 µM [218] and 50 µM [279]). One hundred µM is relatively high and surpasses the clinically achievable levels for several drugs. For most drugs, the therapeutic plasma concentration (Cp) is well below 100 µM [305] and concentrations above the Cp might be toxic. For example, the Cp for miconazole is approx. 2.4 µM, which is well below the concentration used in our screening. Not surprisingly, we observed loss of integrity of the 3D organotypic human lung epithelial cells at 100 and 50 µM miconazole and econazole, indicating that the compounds are toxic at these concentrations. Similarly, severe toxicity was observed upon administration of miconazole to mice. Also in the case of thioridazine, 100 µM is well above the therapeutic Cp, which is between 0.02 and 0.54 µM. Indeed, drugs in use for non-bacteriological indications with antibacterial effect often possess this effect only at concentrations too high to use clinically without toxicity problems [189]. For this reason, it is reasonable to screen in lower concentrations. However, screening at higher concentrations like we did has advantages as well. It is likely to identify more hits with a potential in topical formulations or for catheter lock therapy. Secondly, analogues of these hits might be evaluated for activity at lower concentrations. Also, a second screen with the hits identified can be performed at lower concentrations and further tests continued only with those hits (if any) active at sub-micromolar concentrations.

8.2. Selection of the model system for screening

We have evaluated the effect of the antifungal imidazoles and the antipsychotic phenothiazines in more relevant model systems. Unfortunately, we could not confirm antibacterial activity in these model systems. The failure of identifying hits with activity in more relevant models might be explained by the fact that we performed the screening *in vitro*, against biofilms formed on plastic in closed systems without the presence of host factors that bacteria usually encounter *in vivo*. Indeed, problems with host-free antimicrobial discovery efforts have been reported before, because both pathogens and tested compounds might behave differently *in vitro* and in a host [306]. Although the compound's adsorption, distribution, metabolism, elimination, and toxicity are known for the compounds in our repurposing libraries, these factors are not taken into account in *in vitro* screenings [306]. In

order to meet some of these potential shortcomings, it might be valuable to perform future screenings in other model systems (Figure 2) that do include host factors and still enable HTS, e.g. in *C. elegans* [307-309] or in the presence of mammalian cells [184].

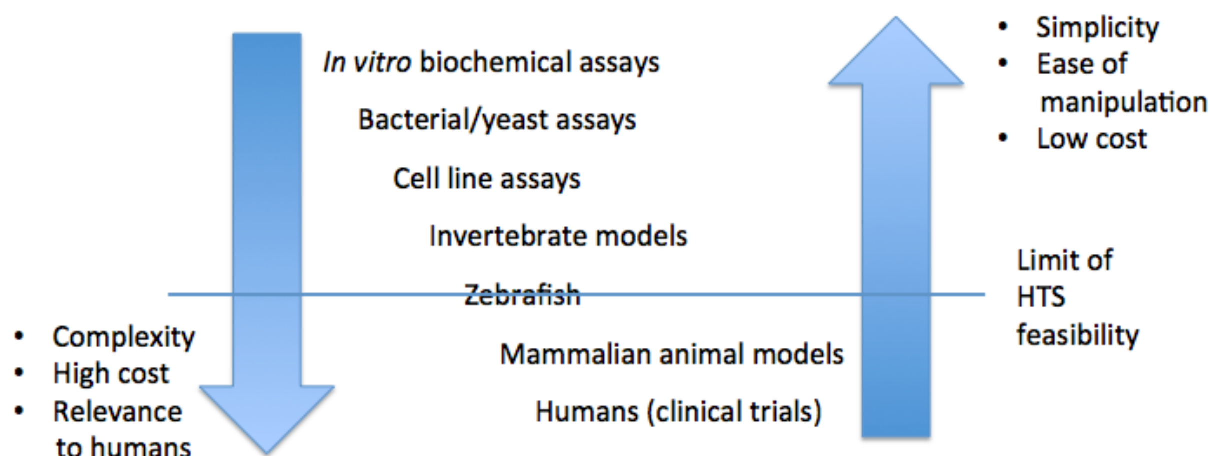

Figure 2. Different screening tools in drug discovery ordered according to their efficiency to perform HTS in one way and their relevance to humans in the other way [310].

For instance, Andersson et al. screened 780 FDA-approved drugs (Screen-Well-FDA-approved drug library V2) in an *in vitro* assay to asses RAW 264.7 murine macrophage viability following infection with *Y. pestis* CO92 [184]. Of the 94 non-antibiotic hits that prevented macrophage cytotoxicity during infections, 17 were tested in a murine model of pneumonic plague and trifluorperazine, doxapram, and amoxepine increased animal survival [184].

In addition, several screenings using *C. elegans* as a platform for antibacterial drug discovery have recently been described [311-313]. *C. elegans* is a free-living nematode and HTS is feasible because *C. elegans* is relatively inexpensive to maintain, it is small (1 mm), has a rapid generation time, and there are no ethical issues with its use [311, 314]. In contrast to traditional *in vitro* screenings, antimicrobial drug screening in *C. elegans* enables the evaluation of toxicity of the test compound to the host and *in vivo* efficacy simultaneously [309, 311, 312]. *C. elegans* can be infected and killed by a wide range of important human pathogens, like *P. aeruginosa*, *S. enterica*, *B. cenocepacia*, *E. faecalis*, or *S. aureus* [221, 309].

Rajamuthiah et al. evaluated the activity of 640 FDA-approved drugs (Biomol 4 Library) in an automated *C. elegans*-MRSA infection assay [311]. This screen resulted in the identification of the anthelmintic drug closantel which prolonged the survival of *C. elegans* upon infection with *S. aureus* MW2 BAA-1707 [311]. Moy et al. screened approx. 37.000 small molecules and NP in an automated *C. elegans* assay infected with *E. faecalis* MMH594 [312]. Twenty-eight compounds without known antimicrobial activity increased survival of *C. elegans* [312]. Six of these compounds are thought to be virulence inhibitors or host immune enhancers as no significant inhibition of microbial growth *in vitro* was observed [312]. Indeed, in contrast to traditional *in vitro* screenings, whole animal screenings allow the

identification of compounds that target bacterial virulence or enhance immune defenses, mechanisms that are only manifested when the host/pathogen relationship is intact [311, 312]. However, although *C. elegans* is a powerful tool in drug discovery, it has some shortcomings as well and confirmation of activity in mammals remains essential [314, 315]. Because *C. elegans,* and other model invertebrates like *Drosophila melanogaster*, lack important parts of the human immune system (e.g. leukocytes, complement pathway, and adaptive immunity) [306], the use of vertebrate models like *Danio rerio* (zebrafish) is currently under investigation. *D. rerio* is more similar to humans and screens with zebrafish embryos and larvae can be conducted in microtiter plates [315]. Nevertheless, the equipment cost is higher compared to the costs to maintain *C. elegans* and *D. melanogaster* [315]. Recently, an optimized method to allow HTS in zebrafish against *S. epidermidis* and *Mycobacterium marinum* [316] has been published, although such screenings have not yet been reported.

8.3. Compound prioritization process

Based on our experiences, some changes to the entire workflow would seem appropriate for future studies. While major changes to the primary screening do not seem necessary, the introduction of a second screening step could be valuable. In this second screening, lower concentrations of hit compounds could be tested, allowing a better selection of the most potent compounds to be included in further studies. Also, a secondary screening could have been performed with a broad selection of hits in more relevant *in vitro* infection models before starting *in vivo* studies. The antifungal imidazoles lost their potentiator effect in the 3D organotypic human lung epithelial cell culture model, and this was confirmed in two different *in vivo* models. It seems thus that the 3D model might be a valuable predictor for the *in vivo* activity. Thus, a secondary screen for the evaluation of the activity of a broad selection of hits in this model would possibly have allowed to eliminate hits that lost their potentiator activity *in vivo*.

8.4. Valorization

Since we were not able to confirm *in vivo* activity of the selected hit compounds, no immediate valorization resulted from this screening project. Nevertheless, several hits identified in our screening remain to be evaluated in more detail. However, even if further evaluation of our hit compounds would reveal potentiator activity in *in vivo* models, we are far from bringing these drugs to the market in the fight against resistant pathogens. Several issues will have to be addressed first, for instance, formulations for the combination therapy will have to be developed, drug-drug interactions will have to be evaluated, and the best drug ratios and dosing regimens will have to be determined for the combination therapies.

9. The future of drug repurposing for antibacterial treatments

Is drug repurposing a good idea to find new antibacterial therapies? For sure it is a good idea to investigate the activity of known drugs for their antibacterial activity. There are positive results observed for several repurposing candidates in mice infection models [184, 189, 198]. Even if these compounds will never make it to the market, they can reveal interesting information. For instance, the drugs can be used as lead compound for development of analogues with increased activity at lower concentrations or the targets they work on can be identified and other compounds that bind more selectively to them might be developed or screened for. Of course, in this scenario, the assets of reduced risk, time and costs no longer apply. It should be clear that the search for antibacterial drugs is long and many obstacles are on the way, and this is not different in a repurposing approach. All things considered, all possible approaches to find new antibacterial agents are important, and one of them is drug repositioning.

Chapter V:
Summary - Samenvatting

Biofilms are communities of bacterial cells embedded in an extracellular matrix and attached to a surface. Treatment of biofilm-related infections is challenging because the cells within the biofilm are more resistant and tolerant towards antibiotic treatment compared to planktonic cells. In addition, the biofilm matrix protects the cells from host immune defenses, contributing to the persistence of biofilm-related infections. Biofilms are a growing problem in health care settings, but currently there are no anti-biofilm treatments available. The aim of this dissertation was to find compounds that enhance the activity of existing antibiotics against biofilms of three important bacterial pathogens: *S. aureus*, *P. aeruginosa* and *B. cenocepacia*.

Compounds that enhance or restore the activity of antibiotics are called helper compounds or potentiators. In order to find these potentiators, we screened a library containing 727 off-patent drugs in the presence of tobramycin or vancomycin against biofilms of *P. aeruginosa*, *B. cenocepacia*, or *S. aureus*. The use of drugs to treat diseases different than the initial indication they were developed for, is called drug repurposing. Drug repurposing has several advantages over *de novo* drug development, including reduced development time and cost. Multiple drugs used for non-bacteriological indications are known to possess antibacterial activity or to synergize with antibiotics. Several screenings have been performed with off-patent drug libraries to evaluate (i) the antibacterial activity in monotherapy against planktonic cells, (ii) the antibacterial effect in monotherapy against biofilms, or (iii) the potentiator activity towards antibiotics against planktonic cells. However, a comprehensive screening with off-patent drug libraries to evaluate the potentiator activity towards antibiotics against bacterial biofilms has never been performed before.

Before we initiated the screening project, we adjusted the existing protocol of the CTB-based viability staining. The non-fluorescent dye CTB is reduced by metabolically active cells to resorufin which is fluorescent. The amount of fluorescence generated is related to the number of viable cells present. In the original protocol, CTB is diluted in physiological saline, added to the biofilms, and the fluorescence generated is measured after 30 min to 2 h incubation. In our optimized protocol, we diluted CTB in fresh growth medium. Subsequently, after adding it to the biofilms, the increase in CTB-based fluorescence was followed over time and we determined the time needed to reach a specific value of fluorescence as well as the time to reach the maximum fluorescence. These time points correlate with the number of viable cells that were initially present. Using these alternative read-outs, we were able to extend the linear range from 10^6–10^8 to 10^3–10^8 CFU per biofilm, meaning that lower numbers of viable cells can be measured and the effect of anti-biofilm treatments can be quantified more accurately.

Subsequently, we performed the screening against mature biofilms of *P. aeruginosa*, *B. cenocepacia*, or *S. aureus*. The biofilms were treated with 100 µM of the library compound in combination with 2 µg/ml tobramycin, 512 µg/ml tobramycin, or 40 µg/ml vancomycin, for *P. aeruginosa*, *B. cenocepacia*, or *S. aureus* respectively. After 24 h, the effect of the treatment was quantified using the optimized CTB protocol against the biofilms of *P. aeruginosa*, and the original CTB protocol against *B. cenocepacia* and *S. aureus*. The

screening resulted in the identification of 60 hits for *B. cenocepacia*, 25 hits for *S. aureus* and 12 hits for *P. aeruginosa*.

The antifungal imidazoles miconazole and econazole were among the hits identified against *B. cenocepacia*. We confirmed their potentiating effect towards tobramycin by plate counts: treatment with 10 - 100 µM miconazole or econazole did not cause a decrease in CFU/biofilm, however, in combination with tobramycin, the azoles caused a statistically significant decrease of 1.5 log CFU/biofilm compared to treatment with tobramycin alone. Nevertheless, this *in vitro* biofilm model system does not resemble the *in vivo* conditions. For this reason, we evaluated the effect of the combination in other *in vitro* and *in vivo* model systems as well. We used the three-D organotypic human cell culture model, *Galleria mellonella*, and a mouse lung infection model. Unfortunately, the potentiating effect of the imidazoles towards tobramycin could not be confirmed in any of these models.

The antipsychotic phenothiazines thioridazine, fluphenazine, perphenazine, and trifluoperazine were identified as hits against *S. aureus*. In contrast to fluphenazine, perphenazine, and trifluoperazine, thioridazine showed anti-biofilm activity in monotherapy: treatment with 100 - 50 µM thioridazine caused a significant reduction in CFU/biofilm compared to untreated biofilms. The potentiating effect of 100 µM thioridazine was evaluated against a broad panel of antibiotics. The combination of thioridazine with tobramycin, flucloxacillin, and linezolid resulted in an additional reduction in CFU/biofilm compared to treatment with the antibiotic alone. We used a chronic wound biofilm model to confirm the effect in a more relevant situation. However, despite the use of high concentration, biofilms formed in this model were not affected by treatment with thioridazine, the antibiotics, or the combination treatment.

To summarize, the screening of a repurposing library in combination with antibiotics against bacterial biofilms resulted in the identification of several potentiators. We could confirm the activity of several selected hits against biofilms formed in 96-well MTPs by plate counts. Nevertheless, the selected hits lost their potentiator activity when evaluated in more sophisticated models containing host factors.

Biofilms zijn opgebouwd uit bacteriën omgeven door een zelfgeproduceerde extracellulaire matrix. Zij vormen zich zowel op biotische als op abiotische oppervlakken. De behandeling van biofilm-gerelateerde infecties is een uitdaging. Cellen die zich in de biofilm bevinden vertonen immers een verhoogde resistentie en tolerantie in vergelijking met planktonische (vrij levende) cellen. Bovendien beschermt de matrix de bacteriën in de biofilm tegen het immuunsysteem van de gastheer, wat ook bijdraagt aan de persistentie van biofilm-gerelateerde infecties. Biofilms zijn een groeiend probleem, maar een specifieke behandeling tegen infecties ten gevolge van biofilms bestaat vooralsnog niet. De identificatie van componenten die de activiteit van bestaande antibiotica verhogen tegen biofilms was het doel van deze scriptie. Biofilms gevormd door drie belangrijke pathogenen, nl. *S. aureus*, *P. aeruginosa* en *B. cenocepacia* werden onderzocht.

Componenten die de activiteit van antibiotica verhogen worden ook wel ‘potentiators’ genoemd. Om deze potentiators te identificeren werd een bibliotheek van 727 componenten gecreend, waarvan de meerderheid *off patent*-geneesmiddelen zijn. Deze screening tegen *P. aeruginosa* en *B. cenocepacia* werd uitgevoerd in de aanwezigheid van het antibioticum tobramycine. In het geval van de screening tegen *S. aureus* was dit vancomycine. Het gebruik van geneesmiddelen voor de behandeling van andere ziekten dan deze waartegen de geneesmiddelen ontwikkeld werden, wordt benoemd met de term ‘drug repurposing’. Drug repurposing heeft verschillende voordelen in vergelijking met *de novo* ontwikkeling van geneesmiddelen, bijvoorbeeld een verlaagde ontwikkelingskost en een verkorte ontwikkelingstijd. Van verschillende geneesmiddelen die gebruikt worden om ziekten van niet-bacteriële oorsprong te behandelen, is geweten dat ze ook anti-bacteriële activiteit bezitten of de activiteit van antibiotica versterken. Meerdere screenings werden reeds uitgevoerd met bibliotheken bestaande uit geneesmiddelen gebruikt in allerhande indicaties. Deze screenings evalueerden ofwel (i) de antibacteriële activiteit van deze geneesmiddelen in monotherapie tegen planktonische cellen, ofwel (ii) de antibacteriële activiteit in monotherapie tegen biofilms, ofwel (iii) de potentiator activiteit tegenover antibiotica tegen planktonische cellen. Echter, een uitgebreide screening met deze bibliotheken om potentiators te identificeren met activiteit specifiek tegen bacteriële biofilms werd nog niet eerder uitgevoerd.

Vooraleer de screening werd aangevat, werd een bestaande quantificatiemethode geoptimaliseerd om het anti-biofilm effect van de behandelingen beter te evalueren. Deze quantificatiemethode is gebaseerd op een leefbaarheidskleuring, gebruik makend van CellTiter-Blue (CTB). CTB is een niet-fluorescente kleurstof die door metabool actieve cellen gereduceerd wordt, waardoor de fluorescente stof resorufin ontstaat. De hoeveelheid fluorescentie die gevormd wordt, hangt af van het aantal levende en metabool actieve cellen dat aanwezig is. In het originele protocol wordt CTB verdund in fysiologisch water, toegevoegd aan de biofilm. Vervolgens wordt de fluorescentie gemeten die ontstaat tijdens een 30 minuten tot 2 uur durende incubatie. In het aangepaste protocol wordt CTB verdund in vers groei medium. Daarna wordt het toegevoegd aan de biofilms en wordt de toename in CTB-gebaseerde fluorescentie gemeten gedurende een 18-tal uur. Vervolgens wordt de tijd bepaald die nodig was om een specifieke fluorescentie waarde (i.e. 100 000) of om de

maximum fluorescentiewaarde te bereiken. De tijd om deze waarden te bereiken is gerelateerd aan het aantal levende cellen dat initieel aanwezig was. Het lineair gebied van dit alternatief protocol is veel breder dan dat van het originele protocol (van 10^6–10^8 tot 10^3–10^8 kolonie-vormende-eenheden per biofilm (KVE/BF)). Hierdoor kunnen lagere hoeveelheden leefbare cellen gemeten worden en kan het anti-biofilm effect van de behandelingen accurater geëvalueerd worden.

Vervolgens werd de screening uitgevoerd tegen mature biofilms gevormd door *P. aeruginosa*, *B. cenocepacia*, en *S. aureus.* Deze biofilms werden behandeld met een component uit de bibliotheek in een concentratie van 100 µM, in combinatie met 2 µg/ml tobramycine in het geval van *P. aeruginosa*, van 512 µg/ml tobramycine in het geval van *B. cenocepacia*, of van 40 µg/ml vancomycine in het geval van *S. aureus.* Na 24 uur werd vervolgens het effect van de behandeling bepaald door middel van CTB kleuring. In het geval van *P. aeruginosa* biofilms werd het geoptimaliseerde protocol gebruikt, terwijl het originele protocol gebruikt werd voor de kwantificatie van het behandelingseffect tegen biofilms van *B. cenocepacia* en *S. aureus*. Uiteindelijk werden 60 hits geïdentificeerd in de screening tegen *B. cenocepacia,* 25 hits in de screening tegen biofilms van *S. aureus* en 12 hits in de screening tegen *P. aeruginosa.*

De imidazoles miconazole en econazole, die als antifungale geneesmiddelen gebruikt worden, werden geïdentificeerd als hit in de screening tegen *B. cenocepacia*. Dat deze imidazoles effectief de activiteit van tobramycine versterkten kon bevestigd worden met een andere kwantificatietechniek, namelijk uitplaten. De behandeling met 10 -100 µM miconazole of econazole leidde niet tot een daling in KVE/BF. In combinatie met tobramcyine veroorzaakten de imidazoles evenwel een statistisch significante daling van 1,5 log KVE/BF in vergelijking met de behandeling met tobramycine alleen. Echter, het *in vitro* biofilm model waarin de biofilms werden gevormd, bootst niet de omstandigheden na die *in vivo* aangetroffen worden. Daarom werd het effect van de combinatie tobramycine-imidazoles ook geëvalueerd in andere *in vitro* en *in vivo* modelsystemen. Hiervoor werd een 3D organotypisch humaan celcultuur model gebruikt, alsook larven van de grote wasmot *Galleria mellonella* en muizen waarin een longinfectie werd geïnduceerd. Helaas kon het potentiërend effect van de imidazoles in deze modellen niet aangetoond worden.

De fenothiazine antipsychotica thioridazine, fluphenazine, perphenazine en trifluoperazine werden als hits geïdentificeerd in de screening tegen *S. aureus*. In tegenstelling tot fluphenazine, perphenazine en trifluoperazine vertoonde thioridazine anti-biofilm activiteit op zichzelf: behandeling met 100 en 50 µM thioridazine resulteerde in een significante daling in KVE/BF in vergelijking met het aantal KVE/BF in onbehandelde biofilms. Het potentiërend effect van 100 µM thioridazine werd vervolgens geëvalueerd in combinatie met verscheidene antibiotica. Toevoegen van thioridazine aan een behandeling met tobramycine, flucloxacilline of linezolid zorgde voor een extra afdoding in vergelijking met behandeling met de respectievelijke antibiotica alleen. Ook hier werd vervolgens de activiteit van de combinaties getest in een model dat een beter idee geeft van activiteit in een relevante klinische situatie. Hiervoor werd gebruik gemaakt van een biofilm model dat een chronische wonde simuleert.

Ondanks het feit dat hoge concentraties gebruikt werden, waren de in dit model gevormde biofilms niet gevoelig aan de behandeling met de componenten alleen of in combinatie.
Samengevat resulteerde de screening van een herpositionering bibliotheek in aanwezigheid van antibiotica in de identificatie van verschillende potentiators. De activiteit van enkele geselecteerde hits tegen biofilms gevormd in 96-well MTPs konden bevestigd worden door middel van uitplatingen. In meer gesofisticeerde modellen verloren deze geselecteerde componenten echter hun potentiator activiteit.

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Curriculum vitae

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° 8 January 1989, Aalst, Belgium

PROFESSIONAL EXPERIENCE

2012-present PhD student at the laboratory of pharmaceutical microbiology, Ghent University

After optimizing a quantification method for bacterial viability I screened over 700 drug compounds against bacterial biofilms formed by several important bacterial pathogens. I am familiar working with bacterial cultures and testing efficacy of antibiotics both in *in vitro* and *in vivo* model systems. Beside these technical and analytical skills I developed my scientific writing and presentation skills. During my PhD, I coached five master students individually and supervised practical courses. In addition, I developed a passion for problem solving and worked in a dynamic team.

2011-2012 Trainee in a local pharmacy (Burst) and in the hospital Sint-Elisabeth (Zottegem)

2010-2011 Erasmus scientific research internship Universidad Complutense (Madrid)

EDUCATION

2007-2012 Master of Science in pharmaceutical care (Ghent University)

2001-2007 Latijn-wetenschappen-wiskunde (Dames Van Maria, Aalst)

ACTIVITIES AND INTERESTS

I have a passion for history that I express in reading historical novels and travelling around the world to visit historical places and learn to know other cultures. No better way to communicate in local languages, so for many years I took language classes. I like to hike, walk and run. I am secretary of the Marnixring Corneel Heymans in Gent and member of the general meeting of the Neutraal Ziekenfonds Vlaanderen.

LANGUAGE SKILLS

Native Dutch
Fluent English
Conversational French - Spanish - German

Appendix

CONFERENCES

2015 ICAAC, posterpresentation (San Diego)
2015 Knowledge for Growth, posterpresentation (Gent)
2014 ICAAC, posterpresentation (Washington D.C.)
2014 Knowledge for Growth, posterpresentation (Gent)
2014 Belgian society of microbiology, oral presentation (Brussel)
2013 ESGB Eurobiofilm, posterpresentation (Gent)
2013 Knowledge for Growth, posterpresentation (Gent)

PUBLICATIONS

- **Van den Driessche F.**, Rigole P., Brackman G., Coenye T., Optimization of resazurin-based viability staining for quantification of microbial biofilms, *Journal of microbiological methods*, 2014/98, 31-34
- De Cremer K., De Brucker K., Staes I., Peeters A., **Van den Driessche F.**, Coenye T., Cammue B., Thevissen K., Stimulation of superoxide production increases fungicidal action of miconazole against *Candida* biofilms, *Scientific reports,* 2016*, 6, art.nr. 27463*
- **Van den Driessche F**., Rigole P., Swimberghe R., Brackman G., Coenye T., Screening a repurposing library for potentiators of antibiotics against *Staphylococcus aureus* biofilms, International Journal of Antimicrobial Agents, submitted
- **Van den Driessche F**., Vanhoutte B., Brackman G., Crabbé A., Capoen D., Rigole P., Vercruysse J., Verstraete G., Vervaet C., Cos P., Coenye T., Evaluation of combination therapy for *Burkholderia cenocepacia* lung infection in different *in vitro* and *in vivo* models, Plos One, submitted
- Richter K., **Van den Driessche F**., Coenye T., Innovative approaches to treat *Staphylococcus aureus* biofilm-related infections, Essays in Biochemistry, submitted

TRAININGS

2015 TechTransfer skills training (Gent)
2014 Doctoral schools advanced academic English writing skills (Gent)
2014 Summerschool on bacterial biofilms (Leuven)
2013 Doctoral schools communication skills (Gent)