Preclinical assessment of a propanol-amine derivative as a novel anti-persister molecule candidate for antibacterial co-therapy

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Energy and persistence conquer all things

- *Benjamin Franklin*

Abstract

The increasing rate of antibiotic resistance development, together with the limited number of novel antibacterial compounds, is causing a true public health crisis where treatment options for patients infected with multidrug-resistant bacteria are down to only a few last-line antibiotics. Greatly contributing to the difficult treatment of these bacterial infections is the presence of a small fraction of persister cells; phenotypical variants which are highly tolerant to antibiotic treatment. Persister cells are responsible for the recalcitrant nature of chronic infections, contribute significantly to the antibiotic tolerance of biofilms and evidence is mounting that persister cells may serve as a reservoir for the development of antibiotic resistance. Clearly, there is an urgent need for novel therapies that allow the treatment of multidrug-resistant infections while effectively clearing the antibiotic-tolerant persister fraction. Despite the increasing insights into the molecular mechanisms behind persistence, their apparent redundancy and species-specific mechanisms are hampering the rational development of targeted anti-persister therapies.

One possible way to circumvent this is the use of screenings specifically directed towards non-dividing cells, allowing the identification of novel anti-persister compounds from large chemical libraries. Previous research within the SPI group successfully used this top-down approach to identify SPI009, a propanol-amine derivative capable of decreasing the persister fraction of *Pseudomonas aeruginosa* in combination with the conventional antibiotic ofloxacin.

This doctoral research project focused on the further characterization of this novel compound and the determination of its mechanism of action. While originally identified in a screening against *P. aeruginosa*, extensive *in vitro* testing revealed the ability of SPI009 to directly kill persister cells of several clinically relevant pathogens, including the other ESKAPE pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*,

Acinetobacter baumannii and *Enterobacter* spp.), *Burkholderia cenocepacia* and the model organism *Escherichia coli*. Additionally, the observed antibiotic-independent effect of SPI009 allows successful combination with antibiotics from mechanistically distinct classes to further increase antibacterial activity. SPI009 showed capable of sensitizing bacteria to antibiotic treatment and, strikingly, restores antibiotic sensitivity even in (multidrug) resistant strains. The use of several *in vitro* set-ups revealed a potent anti-biofilm activity and ability of SPI009 to eradicate intracellular infections while the first *in vivo* tests demonstrated a significant increase in survival of *P. aeruginosa* infected *Caenorhabditis elegans* upon treatment with the combination therapy of SPI009 and ciprofloxacin. These characteristics greatly increase the clinical potential of our compound as SPI009-based combination therapies have the potential to be employed in a great variety of bacterial infections.

In a second part of this project, we aimed to unravel the mechanism of action of SPI009. This is not only essential for future clinical development but may also increase our knowledge about persister cells and ways to eradicate them, thereby contributing to the development of future anti-persister therapies. A combination of molecular-genetic and cellular approaches was used to demonstrate that SPI009 is capable of directly killing both persister and non-persister cells through extensive membrane damage. High levels of membrane damage result in cell lysis while intermediate permeabilization can significantly increase the sensitivity towards different antibiotics, confirming the potential of SPI009 as a candidate for antibacterial combination therapies.

Within the frame of this PhD, we also explored the anti-persister effects of Art-175 in *P. aeruginosa* and *A. baumannii*. Artilysin®s are comprised of a bacteriophage-produced endolysin coupled to a lipopolysaccharide-destabilizing peptide, allowing lysis of Gram-negative cells and, as such, possessing potential anti-persister activity. The demonstrated ability of Art-175 to quickly kill both normal and persister cells makes it an attractive alternative anti-persister therapy to treat bacterial infections in a highly specific manner.

Overall, this PhD project contributes to the development of novel anti-persister therapies through the exploration of the previously described Art-175 as a potential anti-persister strategy and the detailed and extensive characterization and determination of the mechanism of action of the novel anti-persister molecule SPI009. While there are still some hurdles to overcome in the further clinical development of SPI009, this novel compound shows good potential and may serve as a scaffold for the development of future anti-persister therapies.

Samenvatting

De toenemende snelheid van resistentieontwikkeling en het beperkte aantal nieuwe antibacteriële middelen in de ontwikkelingspipeline hebben de mogelijke behandelingen voor patiënten geïnfecteerd met multidrugresistente bacteriën herleid tot een beperkt aantal *last resort* antibiotica. Daarnaast draagt de aanwezigheid van een kleine fractie persistorcellen, fenotypische varianten die in staat zijn antibioticumbehandeling te overleven, sterk bij tot de moeilijke behandeling van deze infecties. Persistorcellen zijn verantwoordelijk voor de recalcitrante aard van chronische infecties, dragen in sterke mate bij tot de antibioticumtolerantie van biofilmen en blijken de ontwikkeling van antibioticumresistentie te bevorderen. Er is dus een duidelijke nood aan nieuwe therapieën die multidrugresistente infecties aanpakken en bovendien in staat zijn de antibioticumtolerante persistorfractie te elimineren. Ondanks een toenemende kennis over de mechanismen achter persistorvorming, wordt de ontwikkeling van doelgerichte therapieën sterk bemoeilijkt door een grote redundantie en het voorkomen van species-specifieke mechanismen.

Een mogelijke manier om deze problemen te omzeilen is het toepassen van grootschalige screenings, specifiek gericht op niet-delende cellen. Voorafgaand onderzoek binnen de SPI-groep paste deze *top-down approach* succesvol toe, wat leidde tot de identificatie van SPI009. Dit propanolamine-afgeleid molecule veroorzaakt een significante verlaging van de persistorfractie van *Pseudomonas aeruginosa,* in combinatie met het conventionele antibioticum ofloxacine.

Dit doctoraatsproject focust op de verdere karakterisering van dit nieuwe molecule en de bepaling van zijn werkingsmechanisme. Hoewel initieel ontdekt in *P. aeruginosa*, blijkt SPI009 in staat persistorcellen af te doden van verschillende klinisch relevante pathogenen waaronder de ESKAPE-pathogenen (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *P. aeruginosa* en *Enterobacter* spp.), *Burkholderia cenocepacia* en het modelorganisme *Escherichia coli.* Het feit dat SPI009 op een antibioticum-

onafhankelijke manier blijkt te werken, laat een verdere versterking van het antibacteriële effect toe door combinatie met verschillende klassen antibiotica. SPI009 is bovendien in staat multidrugresistente bacteriën terug gevoelig te maken voor antibioticumbehandeling. Het gebruik van verschillende *in vitro* setups toont bovendien aan dat de component een sterke anti-biofilm activiteit bezit en dat de combinatietherapie met ciprofloxacine in staat is intracellulaire infecties te elimineren en de overleving van *P. aeruginosa* geïnfecteerde *Caenorhabditis elegans* nematoden sterk te verhogen. Deze experimenten leveren een duidelijk bewijs dat SPI009-gebaseerde combinatietherapieën mogelijk een grote variëteit aan bacteriële infecties kunnen behandelen, wat het klinische potentieel van de component sterk doet toenemen.

In een tweede deel van het project onderzoeken we het werkingsmechanisme van SPI009. Dit is essentieel voor de eventuele verdere ontwikkeling van de component en bevordert onze kennis over persistorcellen en de mogelijkheden tot uitroeiing ervan. Een combinatie van genetische en cellulaire technieken toont aan dat SPI009 zowel persistor als niet-persistorcellen direct kan afdoden via een sterke beschadiging van de bacteriële membraan. De ontdekking dat een uitgebreide membraanbeschadiging resulteert in cellysis, terwijl een mindere mate van beschadiging de cel gevoeliger maakt voor antibiotica behandeling, bevestigt het potentieel van SPI009 als kandidaat voor antibacteriële combinatietherapieën.

In een ander luik van dit onderzoeksproject wordt het anti-persistor effect van Art-175 geëvalueerd in zowel *P. aeruginosa* als *A. baumannii*. Art-175 is een zogenaamd Artilysine®, bestaande uit een door bacteriofagen geproduceerd endolysine gekoppeld aan een membraandestabiliserend peptide. De mogelijkheid van Art-175 om door middel van cellysis zowel persistor als niet-persistorcellen snel af te doden maakt het tot een aantrekkelijke kandidaat voor de ontwikkeling van zeer specifieke anti-persistortherapieën.

Het werk in deze thesis draagt bij tot de ontwikkeling van nieuwe anti-persistortherapieën. Dit enerzijds door de evaluatie van het anti-persistor effect van het reeds gekende Art-175 en anderzijds via de gedetailleerde karakterisering en bepaling van het werkingsmechanisme van het nieuwe anti-persistor molecule SPI009. Ondanks de hindernissen die overwonnen zullen moeten worden in de verdere klinische ontwikkeling van SPI009, biedt deze component interessante mogelijkheden in de behandeling van persistorcellen en als startpunt voor de ontwikkeling van nieuwe therapieën.

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List of abbreviations

2-AA	2’-amino acetophenon
ALT	Antibiotic lock therapy
AMK	Amikacin
AMP	Antimicrobial peptide
ATM	Aztreonam
ATP	Adenosine triphosphate
a.u.	Arbitrary units
BC	BODIPY™ TR Cadaverine
bp	Basepair
CAZ	Ceftazidime
CaF	Carboxyfluorescein
CF	Cystic fibrosis
CFE	Colony forming estimate
CFTR	Cystic fibrosis transmembrane conductance regulator
CFU	Colony forming unit
CIP	Ciprofloxacin
Cpm	Counts per minute
CSLM	Confocal Laser Scanning Microscopy
CST	Colistin
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethylsulfoxide
DOPE	1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine
DOPG	1,2-dioleoyl-*sn*-glycero-3-phospho-(1'-rac-glycerol)
ECDC	European Centre for Disease Prevention and Control
EDTA	Ethylenediaminetetraacetic acid
EPS	Extracellular polymeric substance
ESBL	Extended-spectrum β-lactamase
EUCAST	European committee on antimicrobial susceptibility testing
FICI	Fractional inhibitory concentration index
FM® 4-64	*N*-(3-Triethylammoniumpropyl)-4-(6-(4-(Diethylamino) Phenyl) Hexatrienyl) Pyridinium Dibromide
FU	Fluorescence units

HSL	Homoserine lactone
Hz	Hertz
ICU	Intensive care unit
IPM	Imipenem
LDH	Lactate dehydrogenase
LPS	Lipopolysaccharide
MBC	Minimal bactericidal concentration
MBL	Metallo-β-lactamase
MD	Multi-drug resistant
MDC	Minimal dormicidal concentration
MEL	Melittin
MEM	Meropenem
MIC	Minimum inhibitory concentration
MOI	Multiplicity of infection
MOPS	3-(*N*-morpholino)propanesulfonic acid
MQ	MilliQ
mRNA	Messenger RNA
MRSA	Methicillin-resistant *Staphylococcus aureus*
MRSE	Methicillin-resistant *Staphylococcus epidermis*
MSC	Minimal stationary cidal concentrations
ND	Not determined
NGM	Nematode growth medium
NPN	1-*N*-phenylnaphthylamine
OD	Optical Density
OFX	Ofloxacin
PBS	Phosphate buffered saline
PI	Propidium iodide
PIP	Piperacillin
PD	Pharmacodynamic
PK	Pharmacokinetic
PMB	Polymyxin B
PMF	Proton motive force
(p)ppGpp	guanosine pentaphosphate or tetraphosphate
PQS	*Pseudomonas* quinolone signal
qPCR	Quantitative PCR
QS	Quorum sensing
RIF	Rifampicin
RNAseq	RNA sequencing
RND	Resistance-nodulation-division
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
Rpm	Rotations per minute
rRNA	Ribosomal RNA
SAR	Structure-activity relationship

SEM	Standard error of mean
SNP	Single nucleotide polymorphism
SPI009	1-((2,4-dichlorophenethyl)amino)-3-phenoxypropan-2-ol
SUV	Small unilamellar vesicles
TA	Toxin-antitoxin
TCA	Trichloroacetic acid
TIC	Ticarcillin
TSB	Tryptic soy broth
TZP	Piperacillin-tazobactam
VRE	Vancomycin-resistant *Enterococcus*
WHO	World Health Organization
WT	Wild type

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B.1 Overview of the genes identified in the 85 kb deletion region in *P. aeruginosa* SSF65-563

Aims and scope of the thesis

Decades of antibiotic misuse and oversubscription have resulted in a dramatic increase in antibiotic resistance, causing infections that were once easily cured to become a major threat to human health. However, even in the absence of antibiotic resistance some infections prove impossible to eradicate. This can be attributed to the presence of a small subfraction of antibiotic-tolerant persister cells, phenotypical variants capable of surviving treatment with high doses of antibiotics and reinitiating infection once antibiotic pressure drops. As such, persister cells are responsible for the recalcitrant nature of chronic infections, greatly contribute to the observed antibiotic tolerance in biofilms and have recently been suggested as a possible reservoir for the emergence of antibiotic resistance. Since their discovery in the 1940s, persister cells have been identified in most major pathogens, including the Gram-negative *Pseudomonas aeruginosa*, a major cause of nosocomial infections. As reviewed in **Chapter 1**, this pathogen demonstrates resistance to many conventional antibiotics through a combination of extensive intrinsic resistance and the ability to easily adopt additional resistance mechanisms. An overview is provided of different mechanisms underlying persister formation in *P. aeruginosa* and additionally, several strategies are presented for the treatment of chronic *P. aeruginosa* infections.

Strongly contributing to the chronic nature of bacterial infections and antibiotic tolerance of biofilms, persister cells form an attractive target for the development of novel antibacterial therapies. Indeed, the development of anti-persister molecules has the potential to significantly improve patient outcomes and, with the current antibiotic crisis in mind, provides an interesting alternative as an antibacterial strategy. **Chapter 2** provides an overview of the currently described anti-persister molecules, discusses possible novel targets and methods for the development of future anti-persister therapies and addresses the current obstacles in their further clinical development.

The general objective of this thesis is to contribute to the development of novel anti-persister therapies through the detailed characterization and determination of the mode of action of the propanol-amine derivative SPI009. This novel, small molecule anti-persister compound was previously discovered within our research group and was shown to significantly decrease the persister fraction of *Pseudomonas aeruginosa* in combination with a conventional antibiotic.More specifically, we aim to provide a detailed characterization of this novel anti-persister compound regarding its activity in different clinically relevant pathogens and in combination with mechanistically different antibiotics, using relevant preclinical model systems. **Chapter 3** discusses the identification of this novel compound and explores its activity in different *P. aeruginosa* clinical isolates. SPI009 is shown to directly kill persister cells in an antibiotic-independent manner and possess an advantageous secondary effect against actively dividing cells. Further characterization of the compound, as discussed in **Chapter 4**, shows a broad-spectrum activity against several clinically important pathogens. Furthermore, SPI009 demonstrates a potent anti-persister and anti-biofilm effect in several *in vitro* set-ups and the combination therapy of SPI009 proves capable of efficiently clearing pseudomonal infections in an *in vitro* and *in vivo* model.

We next set out to unravel the mode of action of SPI009 as this can provide additional insights into the mechanisms behind persister formation and contribute to the development of future anti-persister therapies. **Chapter 5** covers the detailed analysis of the mechanism of action of SPI009 where both genetic and cellular approaches are employed to demonstrate the extensive membrane damaging activity of the compound. While most assays were performed in our model organism *P. aeruginosa*, microscopic analysis of *Staphylococcus aureus* confirmed SPI009-induced membrane damage in Gram-positive bacteria. In an attempt to identify genetic and phenotypic factors important in SPI009 activity, **Chapter 6** discusses the detailed phenotypic and sequence analysis of two clonal isolates showing a remarkable difference in SPI009 sensitivity.

Another interesting anti-persister therapy comprises the engineering of bacteriophage-produced lysins, capable of disrupting the bacterial membrane. Within the scope of this thesis, **Chapter 7** explores the anti-bacterial and anti-persister activity of Artilysin® Art-175 in the Gram-negative pathogen *Acinetobacter baumannii*.

Finally, **Chapter 8** provides a general discussion of the obtained results and future perspectives on the clinical development of SPI009 as a novel anti-persister therapy.

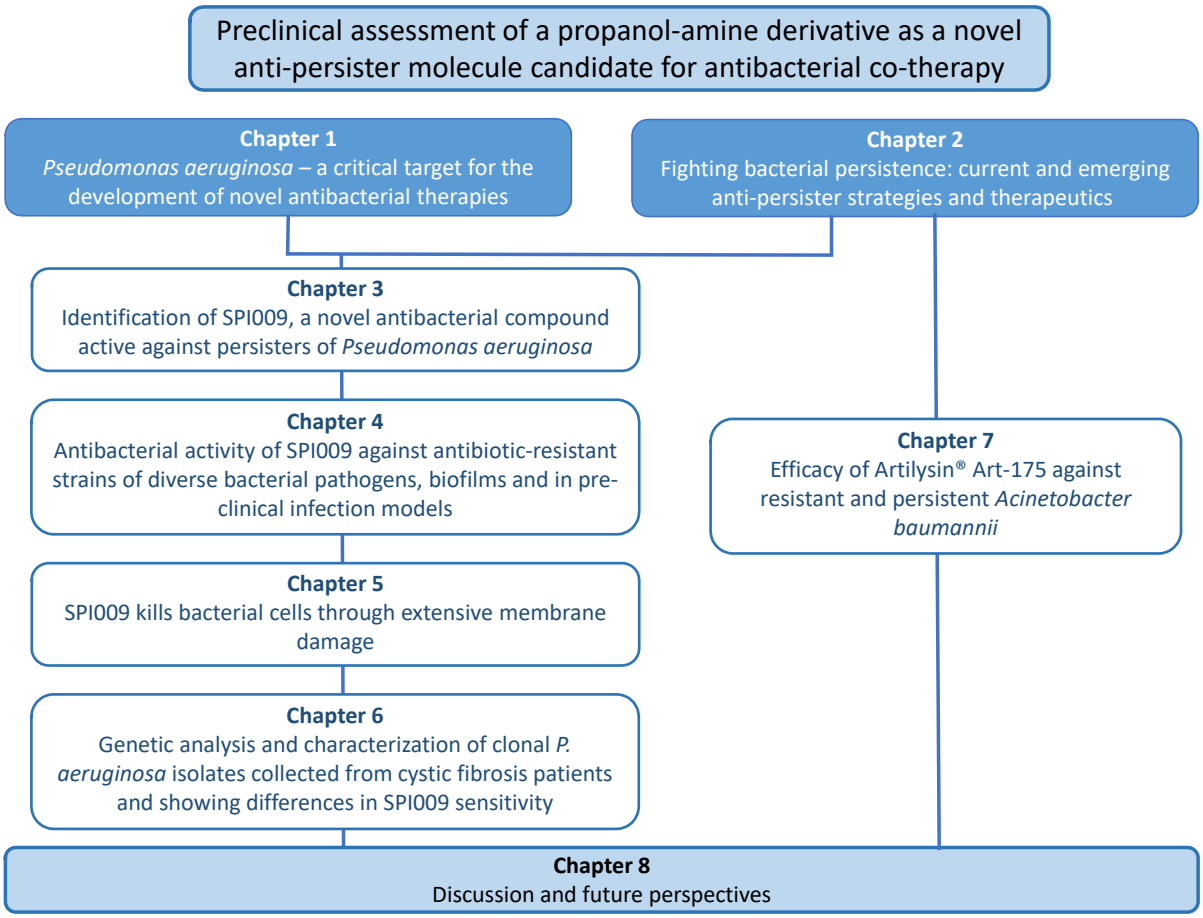


Figure I: Schematic outline of the thesis. Bright blue boxes represent literature chapters, while white boxes give an overview of the different experimental chapters.

PERSISTENCE

per·sis·tence (noun)/ pər ‘ sistəns

Firm or obstinate continuance in a course of action
in spite of difficulty or opposition

Chapter 1

Pseudomonas aeruginosa – a critical target for the development of novel antibacterial therapies

1.1 Introduction

Pseudomonas aeruginosa is a rod-shaped, Gram-negative Gammaproteobacterium belonging to the order of the Pseudomonadales and characterized by its typical blue-green color.[1] *P. aeruginosa* genomes typically contain between 5.5 and 7 million base pairs (Table 1.1).[2] Recent genomic analyses report a core genome of about 4 400 genes, at least 6 600 accessory genes and an additional 30 000 or more rare genes that are unique to one or a limited number of isolates.[2–4] The presence of almost 300 genes encoding cytoplasmic membrane transport systems, the majority of which are involved in nutrient import, the highest proportion of regulatory genes and networks observed in known bacterial pathogens, its facultative anaerobe character, low nutritional demands and broad temperature range for growth allow *P. aeruginosa* to thrive in different niches and quickly adapt to hostile or changing environments.[5–7] Indeed, *P. aeruginosa* can be found in water and soil, plants, animal and human tissue but also on different surfaces in hospital environments.[1]

Table 1.1: Overview of genome size, % GC and number of CDS for selected *P. aeruginosa* strains.

Strain	Size	% GC	CDS[a]	GenBank	Reference
PAO1	6 264 404	66.6	5 584	AE004091.2	[6]
UCBPP-PA14	6 537 648	66.3	5 893	CP000438.1	[8]
PA7	6 588 339	66.4	6 286	CP000744.1	[9]
LESB58	6 601 757	66.3	5 927	FM209186.1	[10]
DK2	6 402 658	66.3	5 883	CP003149.1	[11]
PA1	6 498 072	66.4	5 910	CP004054.2	[4]
PACS2	6 492 423	66.3	5 903	AAQW01000001.1	[12]
M18	6 327 754	66.5	5 684	CP002496.1	[13]
9BR	6 801 503 and 48 358	66.1 and 58.7	6 154	AFXI00000001.1 and AFXI00000001.2	[14]
PA2192[b]	6 826 253	66.2	6513	NZ_AAKW00000000.1	[15]

[a]Coding DNA sequences; [b] Size and CDS were obtained from Genbank. Unless mentioned otherwise size, %GC and CDS are obtained from http://pseudomonas.com.

1.2 *P. aeruginosa* pathogenicity

The *P. aeruginosa* genome encodes various virulence factors and many regulatory mechanisms to adapt to hostile environments, reflecting its extensive pathogenicity. *P. aeruginosa* is capable of producing different types of virulence factors, depending on the stage of infection, most of which are regulated by one of the three quorum sensing (QS) systems, Las, Rhl or PQS (*Pseudhhomonas* Quinolone Signal).[16] During acute infections surface-associated virulence factors such as flagella, pili and lipopolysaccharide(s) (LPS) are

produced which contribute to the pathogen's main goals of invasion, dissemination and extensive tissue damage. Secreted virulence factors at this stage include exotoxin A and phospholipase C, causing severe tissue damage, and proteases such as LasA, LasB, protease IV and alkaline protease, breaking down tissue and immune system components.[5,17] In a second stage of infection, *P. aeruginosa* is mainly focused on optimal survival within the host, resulting in the production of virulence factors such as siderophores (pyoverdin and pyochelin) and alginate, the latter being involved in the transition to a mucoid phenotype and biofilm formation.[17,18]

1.2.1 *P. aeruginosa* as a common source of nosocomial infections

Its large and flexible genome, combined with the ability to survive in and adapt to changing environments results in *P. aeruginosa* being one of the most critical pathogens in today's medicine.[19] As an opportunistic pathogen, *P. aeruginosa* causes life-threatening infections in immunocompromised patients such as cancer and AIDS patients, but also in burn victims,[20] causing it to be the 4th most common cause of healthcare-associated or nosocomial infections in Europe (8.9%).[21,22] Recent data from the European Centre for Disease Prevention and Control (ECDC) identified *P. aeruginosa* as the most common cause of intensive care unit (ICU)-acquired pneumonia (18.8%) with other infections sites including surgical sites, the urinary tract and the human bloodstream.[21,23] While generally being acknowledged as a nosocomial pathogen, *P. aeruginosa* is also responsible for several community-acquired infections such as microbial keratitis[24], acute otitis externa[25] and skin infections such as folliculitis and 'green nail syndrome'.[26]

1.2.2 *P. aeruginosa* in cystic fibrosis patients

Despite its important role in several hospital-acquired and community-acquired infections, *P. aeruginosa* is probably best known for causing life-threatening chronic lung infections in cystic fibrosis (CF) patients. CF is caused by an autosomal recessive genetic defect of the 'cystic fibrosis transmembrane conductance regulator' (CFTR) gene, causing impaired chloride and sodium ion transport in slime-producing epithelial cells and resulting in thick and sticky mucus in the lungs, intestinal tract, reproductive system and secretory glands. This thick mucus layer decreases the antimicrobial activity of the human immune system and disturbs the function of the epithelial cilia, impairing mucociliary clearance of bacteria.[27,28] Despite the

fact that *CFTR* mutations cause several serious symptoms, 80-95% of all patients will eventually succumb to the consequences of chronic bacterial infection.[28,29] While the overall prevalence of *P. aeruginosa* has been reported to decrease over the past years, it still remains the dominant species in adult CF patients.[30]

1.3 Antibiotic resistance

Research from the ECDC showed that 14% of *P. aeruginosa* clinical isolates exhibit resistance against at least three classes of antibiotics, labelling them as being multi-drug resistant (MDR),[22] and even the occurrence of pan-drug resistant strains has been reported.[31–33] The presence of practically all known enzymatic and mutational mechanisms of bacterial resistance greatly contribute to the increasingly difficult treatment of *P. aeruginosa* and render it a phenomenon of antibacterial resistance.[34] Table 1.2 provides an overview of the different resistance mechanisms present in *P. aeruginosa,* which will be further discussed in the following paragraphs.

Table 1.2: Overview of the antibiotic resistance mechanisms present in *P. aeruginosa*

	Stable	Inheritable	Inducible by environment	Mechanisms	Examples of resistance determinants
Intrinsic	+	+	-	Low membrane permeability	OprF, OprD, OprB
				Multidrug efflux pumps	MexAB-OprM, MexCD-OprJ, MexEF-OprN, MexXY-OprM
				β-lactamases	AmpC, PoxB
Acquired	+	+	-	Horizontal gene transfer	β-lactamases, MBL, ESBL, 16S rRNA methylase, aminoglycoside modifying enzymes
				Mutational resistance	*mexR, nfxB, mexZ, nalB, ampD*, *oprD, gyrA/gyrB, parC/parE*
Adaptive	-	-	+	Membrane changes	Modification of lipid A (*arnBCADTEF-udg*; *prmE*)
				Overexpression of efflux pumps	MexXY, MexCD
				Overexpression of enzymes	AmpC

+ = yes, - = no, MBL= metallo-β-lactamase, ESBL= extended-spectrum β-lactamase.
Adapted from Breidenstein *et al.*[35] and Gellatly & Hancock[5].

1.3.1 Intrinsic resistance

P. aeruginosa shows an exceptionally high level of intrinsic resistance towards a wide variety of antibiotics, including aminoglycosides, fluoroquinolones and β-lactams. The main underlying mechanism is its decreased outer membrane permeability, caused by the limited permeability of major porins OprF, and the smaller pore size of the other channels, OprD and OprB.[35] This low permeability causes a decreased influx of antibiotics, which increases the efficiency of secondary intrinsic resistance mechanisms such as antibiotic efflux or degradation.[36] *P. aeruginosa* possesses several important efflux pumps that are capable of extruding antibiotics across the membrane. Those contributing to the observed intrinsic resistance are part of the 'resistance-nodulation-division' (RND) family.[37] MexAB-OprM is involved in the efflux of quinolones, tetracyclines, chloramphenicol, sulfamethoxazole, trimethoprim and some β-lactams, while MexXY-OprM plays a primary role in intrinsic resistance towards aminoglycosides and erythromycin.[36]

Further contributing to the observed intrinsic resistance is the presence of the chromosomally-encoded β-lactamases AmpC and PoxB, capable of inactivating β-lactam antibiotics through hydrolysis.[35,38] Other genes involved in intrinsic resistance have been reported, belonging to different functional classes such as cell division, alginate production and DNA replication, recombination and repair, suggesting that *P. aeruginosa* simultaneously uses different systems to achieve high levels of resistance.[35]

1.3.2 Acquired resistance

Besides having a wide arsenal of intrinsic resistance mechanisms, *P. aeruginosa* is capable of obtaining additional resistance mechanisms from other strains and species. Horizontal gene transfer primarily influences aminoglycoside and β-lactam resistance. As such, antibiotic resistance genes can be acquired through conjugation, transformation or transduction of mobile genetic elements such as plasmids and transposons.[16] The flexibility of the *P. aeruginosa* genome together with its presence in varying environments facilitates the uptake of these genetic elements.[39] Examples include the acquisition of plasmids containing aminoglycoside modifying enzymes[40], 16S rRNA methylases[41], new β-lactamases conferring resistance to penicillins and cephalosporins, extended-spectrum β-lactamases (ESBLs) and metallo-β-lactamases (MBLs).[38]

An additional mechanism further increasing intrinsic resistance is mutational resistance. Consequential antibiotic target modifications can occur in *gyrA*, *gyrB* or *parC*, conferring resistance to quinolones. Alternatively, mutations can occur in non-target genes such as efflux pump regulatory genes *nalB, nfxB* and *nfxC*, causing multidrug resistance, *oprD,* resulting in reduced membrane permeability and increased carbapenem resistance, and *ampD* and *ampR*, further increasing AmpC production and thus boosting β-lactam resistance.[16,35,36] Despite the fact that acquired resistance is, by definition, independent of environmental conditions, certain factors such as growth in biofilms or the presence of DNA damaging compounds can further increase the bacterial mutation frequency.[35]

1.3.3 Adaptive resistance

A third mechanism entails so-called adaptive resistance, allowing the bacterium to adapt to the presence of antibiotics and environmental stresses. Unlike intrinsic and acquired resistance, adaptive resistance is characterized by its transient nature and changes in gene expression will be partially reversed when the inducing conditions disappear. Exposure to low doses of β-lactam antibiotics, for example, can trigger induction of chromosomally-encoded β-lactamases while aminoglycosides can increase expression of the MexXY efflux pump.[35,42] Adaptive resistance to polymyxins occurs through upregulation of *arnBCADTEF* and *prmE*, resulting in modification of lipid A and subsequent decreased binding of polymyxins.[38] Other cues that can trigger adaptive resistance include changes in pH, cation or oxygen levels and social conditions such as biofilm formation.[43]

1.4 Biofilm formation and persistence

In addition to the above mentioned mechanisms of resistance to survive antibiotic exposure, *P. aeruginosa* has developed other strategies to increase survival chances upon treatment. In the below paragraphs, relevant characteristics of biofilm and persister formation, both contributing to the difficult treatment of *P. aeruginosa* infections, will be discussed.

1.4.1 Biofilms

A factor greatly contributing to the pathogenicity of *P. aeruginosa* is its ability to form biofilms, aggregations of microbial cells enclosed in a self-produced matrix and attached to

each other and/or a surface.[44] Besides providing mechanical stability and enabling communication amongst the biofilm cells, the presence of degradative enzymes within the extracellular polymeric substance (EPS) matrix creates an external digestive system. DNA from lysed cells not only serves as a carbon, nitrogen and phosphate source but also as a reservoir for horizontal gene transfer.[45]

A well-known feature of bacterial biofilms is their antibiotic tolerance (Figure 1.1), showing 100- to 1000-fold increases in minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC), as compared to the same organism grown planktonically.[46] A first contributing factor is the architecture and composition of the biofilm EPS matrix, creating a physical protection from compounds of the eukaryotic immune system and capable of hindering the penetration of charged antimicrobials. Additionally, biofilms are composed of different bacterial subpopulations due to various local gradients of nutrients, waste, pH, oxygen and metabolic products throughout the EPS.[45] While the non- or slow-growing cells become tolerant to β-lactam antibiotics[47], low oxygen concentrations have been shown to reduce tobramycin and ciprofloxacin bactericidal effects[48] and the combination of lowered pH and oxygen levels impairs the activity of aminoglycosides.[49,50]

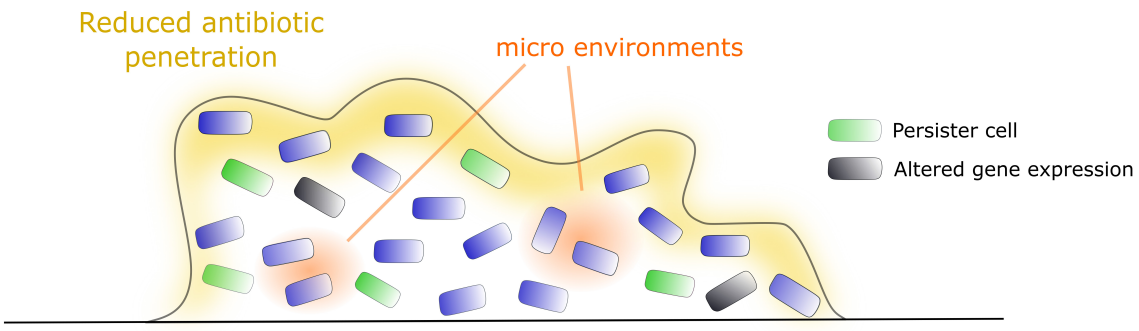


Figure 1.1: Overview of the different mechanisms contributing to the antibiotic tolerance of biofilms. The architecture and composition of the biofilm EPS matrix reduce the efficient penetration of antibiotics into the biofilm. Microenvironments within the biofilm, resulting from gradients in nutrients, waste, pH, oxygen and metabolic products result in bacterial subpopulations showing varying levels of antibiotic sensitivity. Altered levels of gene expression within the biofilm can decrease antibiotic susceptibility while the presence of antibiotic tolerant phenotypical variants, called persister cells, further increases the observed antibiotic tolerance.

Additionally, changes in gene expression between planktonic and biofilm cells, including the increased expression of several two-component regulatory systems and efflux pumps, contribute to the observed antibiotic tolerance.[44,49,51] While this multifactorial biofilm defense mechanism contributes to the observed antibiotic tolerance, it cannot fully explain biofilm recalcitrance. Indeed, research has shown that the presence of an isogenic

subpopulation of antibiotic-tolerant persister cells significantly contributes to the observed antibiotic recalcitrance of biofilms.[52]

Clearly the association of bacterial cells in biofilms greatly enhances their survival, allowing biofilms to successfully colonize higher animals, induce persistent infections in plants and animals and cause severe contamination of medical devices, implants and industrial surfaces.[50,53]

1.4.2 Bacterial persistence

Besides the already covered mechanisms of resistance and biofilm formation, bacterial populations possess an additional strategy to ensure survival in hostile environments or under antibiotic exposure. This phenomenon, where a small subfraction of the isogenic population is transiently tolerant to treatment with high doses of bactericidal antibiotics, is called persistence. Since its discovery in the 1940s[54], persister cells have been identified in many relevant pathogens including *Escherichia coli*, *Staphylococcus aureus*, *P. aeruginosa*[55], *Mycobacterium tuberculosis*[56], *Salmonella* Typhimurium[57] and even the eukaryote *Candida albicans*[58].

General characteristics and clinical importance

Treatment of a bacterial population containing persister cells results in a typical biphasic killing pattern (Figure 1.2). In a first phase, the majority of cells are killed relatively easily while, in the second phase of killing, the so-called persister plateau, cell numbers no longer significantly decrease despite increasing concentrations or treatment times. Consequently, when antibiotic pressure drops, persister cells will switch back to a normal phenotype, resume growth and give rise to a population equally susceptible as the original one.[59]

Accounting for approximately 0.001% to 0.1% of the bacterial population, the number of persister cells is dependent on the bacterial growth phase and species, reaching a maximum level in stationary phase cultures.[55,59,60] Despite earlier beliefs that the persister population is homogenous, recent research revealed different types of persister cells.[61] This was further supported by Allison and co-workers who proposed the presence of different subpopulations of persister cells, each having its own antibiotic tolerance.[62,63]

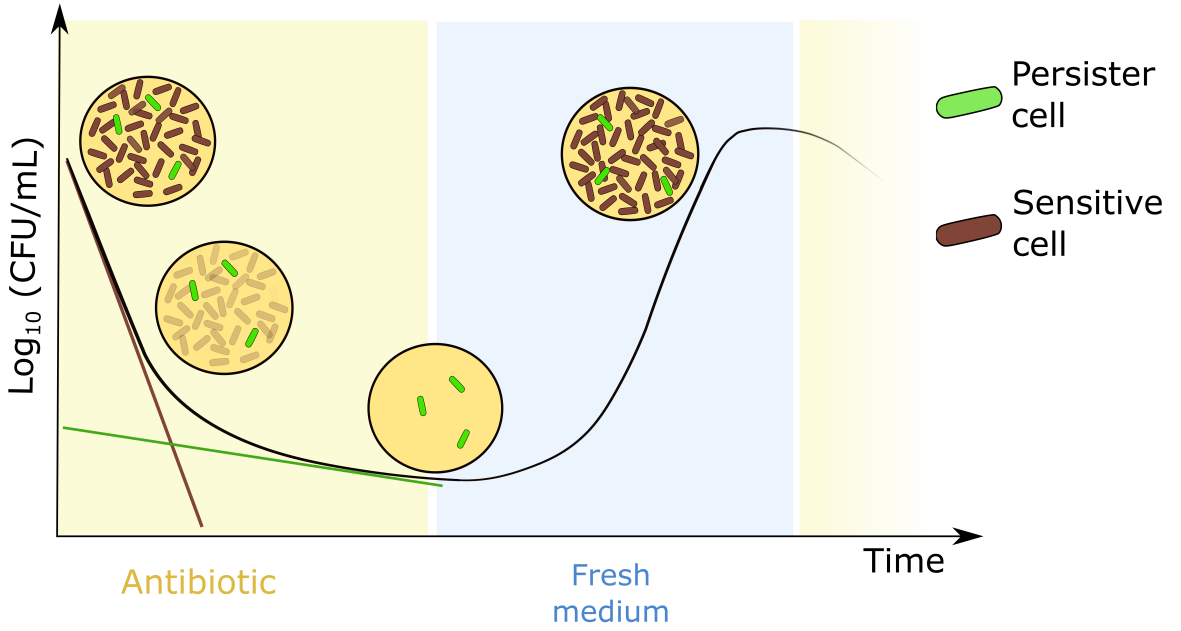


Figure 1.2: Biphasic killing of bacterial populations containing persister cells. When treating a bacterial population consisting of sensitive cells (brown) and persister cells (green), a typical biphasic killing pattern can be observed. The normal, antibiotic-sensitive cells will be killed relatively quickly (brown line) while antibiotic tolerant persister cells will reach a plateau were increasing antibiotic concentrations or treatment times will no longer or more slowly decrease the number of surviving cells, indicated by a drop in killing rate. When antibiotic treatment stops, persister cells will resume growth, giving rise to a population containing both normal and persister cells and equally tolerant to antibiotics as the original one.

While persistence, or antibiotic tolerance, and resistance both aim to increase survival under antibiotic exposure, there are several important differences. First of all, persister cells show a transient phenotypical variation allowing them to survive, while resistant cells undergo permanent genetic alterations that are subsequently passed on to the next generation. While resistant cells actively change their antibiotic targets or increase antibiotic efflux to evade antibiotic killing, persister cells are considered to be insensitive partly due to their low metabolic activity and thus inactivity of antibiotic target.[64] Similarly, resistant cells will be able to grow during antibiotic exposure while persister cells will only switch back to a normal phenotype when the antibiotic pressure is removed.[65] Finally, whereas resistance can clearly be observed by increased MIC values, this is not the case for persistence.[59]

It is generally assumed that, upon successful treatment with a bactericidal antibiotic, the host immune system is capable of eliminating this small fraction of surviving cells. However, in situations where the immune system is compromised or bacteria are able to evade the immune system, persisters become a threat to human health and are considered as a major culprit of chronic infections.[29] Besides their occurrence in biofilms, where they are protected by the physical barrier of the EPS matrix, persister cells can also be present inside

eukaryotic cells and the central nervous system.[66–68] Research has shown that persister cells greatly contribute to the observed antibiotic tolerance in biofilms and are responsible for the recalcitrant nature of chronic infections.[69–71] Recently, evidence is gathering that persister cells can facilitate the emergence of genetic resistance[65,72–74] (also see section 2.2). Effective treatment of persister populations thus has the potential to dramatically improve patient outcomes.

Persistence – active or passive?

The first and still widely accepted theory on persistence implies that persister cells consist of a small subfraction of dormant, metabolically inactive variants in a normal bacterial population.[54] Since most antibiotics interfere with active targets such as cell wall, DNA, RNA and protein synthesis, this could explain the observed antibiotic tolerance of persister cells. Indeed, several researchers reported reduced metabolic activity, mRNA degradation and decreased cell division, protein and ATP synthesis in persister cells.[61,75,76] However, not all dormant cells are persister cells and is has been shown that not all persister cells are dormant. Indeed, several studies observed actively dividing persister cells[77,78] while Orman *et al.* discovered that the majority of a dormant population (> 99%) were not persisters and that 20% of persister cells originated from actively growing cells.[79] Furthermore, it was shown that persister formation can occur as an active response to stress or antibiotic treatment.[74,80,81]

Persistence as a bet-hedging strategy

Balaban and colleagues demonstrated for the first time that a small subpopulation of non-growing persister cells is already present before antibiotic exposure.[61] This allows the bacterial population to anticipate episodes of antibiotic treatment and serves as an example of a bet-hedging strategy; an evolutionary strategy to ensure maximal fitness of an isogenic bacterial population under dynamic conditions.[82–84] Combined with the low level of persisters, this led to the hypothesis that persister formation is a stochastic process. However, increased persister numbers in the late exponential phase[55] and in response to certain stresses[62,63,85] and signal molecules[86] suggests the involvement of a deterministic component.[83] A proposed model combines these two processes and suggests that persister formation is a consequence of stochastic variations in gene expression around a deterministically defined threshold level.[59,60] This hypothesis was experimentally confirmed

for *E. coli*, where expression levels of toxin HipA above the threshold level determine the switch to persistence.[87]

Persister formation in *P. aeruginosa*

Despite their clear clinical importance, interest in persister cells has only revived since the 2000s and the mechanisms behind persister formation are still being unraveled. While persister formation in *E. coli* has been extensively linked to toxin-antitoxin (TA) modules, the limited number of TA modules discovered in *P. aeruginosa* have, up to now, not been shown to influence persister numbers.[16] Mechanisms that have been shown to be involved in *P. aeruginosa* persistence will be discussed shortly in the following paragraphs.

Stringent response

The stringent response is a widespread regulatory mechanism that allows bacteria to react to nutrient limitations including amino acids, iron, carbon and fatty acids. Subsequent induction of *relA* and/or *spoT* results in the production of the alarmone (p)ppGpp, capable of influencing gene expression and metabolism to ensure cell survival.[80] Increased levels of (p)ppGpp have been shown to inhibit protein and cell wall synthesis and replication, resulting in increased survival upon antibiotic exposure.[80,88] Additionally, activation of the stringent response results in induction of antioxidant defenses and reduced pro-oxidant production, thus suppressing oxidative stress.[80]

Oxidative stress

Since endogenous, host-produced or antibiotic-induced reactive oxygen/nitrogen species (ROS/RNS) such as H_2O_2, O_2^- and NO can cause severe cellular damage, bacteria developed the oxidative stress response, eliminating ROS and RNS and restoring the damage suffered. Active suppression of oxidative stress, through increased production of anti-oxidant enzymes such as catalase and superoxide dismutase and decreased production of ROS, increases antibiotic tolerance in *P. aeruginosa*[80,89] and other bacteria[89–91].

SOS response

A third bacterial stress response related to persister formation in *P. aeruginosa* is the SOS response, activated upon DNA damage and using RecA as a sensor for single-stranded DNA. Induction of the SOS response results in the downregulation of genes involved in cell division,

motility, QS, cell permeability and genes coding for proteins involved in general metabolism and DNA replication/repair. Expression of the SOS regulon thus not only repairs damaged DNA but also allows the cells to induce several genetic and phenotypic changes, possibly including persistence formation, to potentiate survival.[85,92,93] This hypothesis was experimentally confirmed when a transposon mutant of *dinG*, part of the *P. aeruginosa* SOS regulon, showed a 16-fold decrease in persister fraction upon antibiotic treatment.[94]

Quorum sensing and signaling molecules

Earlier reports that persister levels depend on the bacterial growth phase already indicated a possible role for QS in persister formation. Indeed, the persister fraction of *P. aeruginosa* was shown to increase upon addition of pyocyanin and 3-oxo-C12 HSL, the QS signal of the pseudomonal Las system.[95] Experiments conducted on *las* mutants further confirmed the involvement of QS in persistence and revealed a role for the global stress response regulator RpoS, inducible by (p)ppGpp, temperature and/or oxidative stress.[96,97] Que and co-workers demonstrated the involvement of an additional QS system in persistence, PQS, through MvfR/PqsR regulated production of 2'-amino acetophenon (2-AA). 2-AA was shown to increase the number of antibiotic-tolerant cells by decreasing transcription of genes involved in translation and thus causing a dormant state.[98]

Redundancy

Extensive research on mechanisms of persister formation in different bacterial species have made it clear that there is not a single mechanism controlling the formation of a heterogeneous persister population. Instead, different mechanisms work together to ensure bacterial survival under several stress conditions. QS and small signal molecules, for example, use intercellular communication to connect different mechanisms of persister formation.[95,99] Furthermore, deletion of one persister-related gene[59,94] or, in the case of *E. coli* oneTA module[100], does not substantially influence the persister fraction, showing that different mechanisms work together to determine whether persistence will be induced. This redundancy not only further complicates the unravelling of the exact mechanisms behind persistence but also impairs the development of targeted anti-persister therapies.

1.5 Combatting *P. aeruginosa* infections

The previous parts of this chapter have made it very clear that *P. aeruginosa* is indeed a life-threatening pathogen and that there is an urgent need for novel therapies to successfully control pseudomonal infections. The lack of effective antibiotics to completely eradicate Gram-negative infections has recently forced physicians to revert to polymyxins, such as polymyxin B and colistin, as last-resort antibiotics. Unfortunately, in addition to some toxic effects associated with these products, the emergence of colistin-resistant *P. aeruginosa* strains has been reported.[101,102] The high risk of *P. aeruginosa* has encouraged researchers to explore novel treatment strategies,[103] several of which will be discussed in the following paragraphs, with a focus on therapies that have already reached the stages of clinical trials.

A first approach is the development **of new antibacterial agents derived from existing antibiotics**. The novel cephalosporin S-649266[104] (NCT02714595) and the aminoglycoside plazomicin[105] (NCT01970371) are currently involved in phase III clinical trials and the new quinolone zabofloxacin (NCT01658020) has successfully finished such trials. While these antibiotics do show slight improvements in susceptibility, chances of resistance development are real since these compounds target the same cellular processes as the currently available antibiotics.

A related strategy uses the **combination therapy** of an existing antibiotic with an antibiotic or non-antibiotic adjuvant, as extensively reviewed in Gill *et al.*[106] Examples of adjuvants include inhibitors of β-lactamases[107–109], efflux pumps, outer membrane permeabilizers and virulence-targeting inhibitors of quorum-sensing, bacterial toxins and secretion systems.[106] Although further research is necessary to study potential negative effects of antibacterial combination therapies, this strategy has the potential to reduce the required concentrations of both compounds, decrease treatment times and, when different mode of actions are combined, lower the risk of resistance development and facilitate the treatment of resistant strains.[110–112]

An alternative approach does not directly target the pathogen itself but rather stimulates the host immune system to deal with the infection. Two types of **immunotherapy** have been developed: passive and active immunization. Active immunization stimulates the human immune system by exposing it to antigens such as the O-glycosylated LPS, cell-surface alginate, flagella, components of the Type III secretion system, outer membrane proteins OprF and OprI, and whole killed cells.[113,114] A promising vaccine is IC43; an *E. coli*-produced

hybrid molecule of outer membrane proteins OprF and OprI.[115] After successful completion of phase I clinical trials a phase II/III study was recently completed assessing the efficacy, immunogenicity and safety of IC43 in ICU patients with a need for mechanical ventilation (NCT01563263). When the response to active immunization might be delayed or impaired, as is the case in critically ill patients such as burn victims and immunocompromised patients, passive immunotherapy could potentially be more effective. Instead of counting on the human immune system, antibodies against *P. aeruginosa* virulence-associated antigens such as flagella, pili, PcrV or exotoxin A are administered.[116] IgY antibodies, showing high affinity for flagellin and interfering with the interaction between *P. aeruginosa* and the epithelial lining, are currently in phase III clinical trials (NCT01455675) and are licensed in Sweden for prophylaxis and treatment of CF patients.[117,118] KBPA101, a human monoclonal IgM antibody, is directed against the LPS O-polysaccharide of the O11 serotype, commonly associated with epidemic strains.[119,120] Despite the *in vitro* successes of several active and passive immunotherapies, few vaccines are reaching clinical trials and none are commercially available. The wealth of potential pseudomonal targets is however promising and, with the possibility of preventing infection, immunotherapy provides an attractive alternative to antibiotic therapy.

A novel class of antibacterial agents gaining attention are the **antimicrobial peptides** (AMP), oligopeptides with varying secondary structures that naturally occur as part of the innate immunity or can be synthetically produced.[121] Targeting the LPS layer of bacterial cell membranes, AMPs show a broad-spectrum activity and are characterized by a rapid killing effect. Despite several AMPs showing good activity against *P. aeruginosa in vitro*, only few have progressed to clinical trials. Besides the well-known polymyxins B and E, LL-37, a human cathelicidin AMP, showed promising anti-biofilm activity *in vitro*[122] and improved wound healing of venous leg ulcers in a phase I/II clinical trial.[123] Additionally, the broad-spectrum Pexiganan completed phase III clinical trials as a topical antimicrobial agent for the treatment of diabetic foot infections (NCT01594762 and NCT01590758).

The first application of lytic **bacteriophages** as an antimicrobial strategy was already reported in 1921 for the treatment of staphylococcal skin infections.[124] However, despite extensive research and promising *in vitro* and *in vivo* results, there are currently no commercial phage therapies available in the field of medicine. Several phase I/II clinical trials demonstrated the efficacy of phages against *P. aeruginosa* infection of burn wounds[125], otitis externa[126] and chronic wounds[127]. Additionally, the European Phagoburn project is currently

recruiting for a phase I/II clinical trial to assess tolerance and efficacy for two anti-infection bacteriophage therapies in the treatment of burn wounds (NCT02116010). A related therapy is the use of isolated, phage-produced endolysins, capable of disrupting the bacterial cell wall.[128] Coupling of these endolysins to LPS destabilizing peptides, termed Artilysin®s, further increases their effect against Gram-negative pathogens[129,130] and was demonstrated to effectively clear *P. aeruginosa* related otitis in dogs.[131] Despite their absence in the field of human medicine, Intralytix has marketed phage therapies for the treatment of respectively *Salmonella* (PLSV-1™) and *Clostridium perfringens* (INT-401™) in poultry and has successfully completed phase I clinical trials to assess the safety of the WPP-201 phage cocktail (NCT00663091).[a]

1.6 Concluding remarks

Pseudomonas aeruginosa is a Gram-negative opportunistic pathogen, well known for causing chronic infections in immunocompromised patients and extensive colonization of the lungs of cystic fibrosis patients, where *P. aeruginosa* is one of the dominant organisms.[5,30,132] The high intrinsic resistance of *P. aeruginosa*, partly caused by the restricted permeability of the outer membrane, together with the presence of antibiotic-tolerant persister cells and its ability to form biofilms, make it a notoriously difficult bacterium to control.[35,59,133] Infections caused by *P. aeruginosa* are commonly treated with antibiotics, which either inhibit bacterial growth or cause bacterial cell death. However, the widespread and excessive use of antibiotics, together with the ability of *P. aeruginosa* to quickly develop antibiotic resistance, have led to an increasing resistance to conventional antibiotics and the emergence of extensively drug-resistant and pandrug-resistant strains.[35,39,134]

Cleary, there is an urgent need for novel therapies capable of effectively treating pseudomonal infections. With the antibiotic crisis in mind, new therapies are currently being investigated and several of these are already demonstrating promising activities in early-phase clinical trials. Other strategies to identify novel antibacterials include the screening of large compound libraries, reverting to the use of natural products, employing rational drug design and further exploring the potential of anti-persister therapies.[103,135] As persisters make up a small but crucial fraction of the bacterial population, contributing to the recalcitrant

[a] http://www.intralytix.com

nature of chronic infections, targeting these antibiotic-tolerant cells could provide an interesting new strategy. Eradication of the persister fraction could facilitate treatment or even prevent the manifestation of chronic infections, decrease the chances of resistance development and greatly increase patient outcomes. The increasing number of literature reports on anti-persister strategies and compounds, as reviewed in Chapter 2, stems us hopeful as they lay the foundation for the development of future anti-persister therapies in the fight against chronic infections.

Chapter 2

Fighting bacterial persistence: current and emerging anti-persister strategies and therapeutics

This chapter will be submitted as a review

Abstract

Besides the well-known principles of antibiotic resistance and biofilm formation, bacterial populations possess an additional survival strategy to cope with hostile environments or antibiotic exposure. A small fraction of transiently antibiotic-tolerant phenotypical variants, called persister cells, is capable of surviving treatment with high doses of antibiotics. When antibiotic pressure drops, persister cells can switch back to a normal phenotype and ensure survival of the bacterial population. Research showed that persister cells, identified in every major pathogen, greatly contribute to the antibiotic tolerance observed in biofilms, are responsible for the recalcitrant nature of chronic infections and recently, evidence is accumulating that persister cells can contribute to the emergence of antibiotic resistance. Effective treatment of persister cells thus has the potential to greatly improve patient outcomes. Despite their small numbers and the redundancy in mechanisms of persistence formation, hampering the target-based development of anti-persister therapies the armory of anti-persister molecules is increasing. This review presents an overview of the anti-persister molecules and strategies described in literature to date and offers perspectives on potential targets and methods for the development of future anti-persister therapies.

2.1 Introduction

Decades of mis- and overuse of antibiotics have sped up the evolution of antibiotic resistance in all major pathogens, resulting in an increase of multi- and even extensive-drug resistant strains. This, combined with the lack of novel antibiotics in the last decades, has resulted in a true antibiotic crisis where once easily curable infections are becoming a serious threat to human health.[136,137] Greatly contributing to the difficult treatment of bacterial infections is the presence of a small fraction of persister cells. Characterized by a transient antibiotic tolerance, these phenotypical variants are capable of withstanding extensive antibiotic treatment and, when antibiotic pressure drops, resume growth.[54,59] Persister cells have been identified in all major pathogens and greatly contribute to the difficult treatment and recalcitrant nature of chronic infections.[29] After being largely forgotten during the golden era of antibiotic discovery, the recent recognition of their clinical importance has stimulated research into persister mechanisms and strategies to eliminate them.[138]

The present review aims to provide an overview of the currently described anti-persister molecules and strategies to combat persistence in different bacterial pathogens. Additionally, we will discuss major targets and approaches that could serve as starting points for the discovery of new, highly needed therapies and the current state and challenges associated with their clinical development. Eradication of persister cells provides an alternative strategy to tackle chronic infections and, with the current antibiotic crisis in mind, can significantly improve patient outcomes.

2.2 Clinical relevance of persister cells

Soon after the discovery of the persistence phenomenon in the 1940s, Joseph Bigger pronounced that penicillin failed to cure staphylococcal infections due to the presence of persister cells.[54] Despite increasing interest in persisters cells after the identification of high persistence (*hip*) mutants and the first persister gene *hipA*[139], the clinical importance of this subfraction was largely ignored until the early 2000s. Kim Lewis was the first to propose a possible role for persister cells in biofilm tolerance,[140] a hypothesis supported by Levin and Rozen who provided mathematical evidence that the presence of persister cells could indeed hamper the effective treatment of bacterial infections.[72] Several independent studies were able to show a direct link between the presence of persister cells and the chronic nature of

different microbial infections. Examples include *Candida albicans* isolates from long-term oral carriage in cancer patients, where remarkably higher persister levels were found in comparison to acute infections.[69] *P. aeruginosa* isolates from CF patients showing chronic lung infections demonstrated increasing persister levels during the course of long-term antibiotic treatment.[70] This was further corroborated in natural and clinical *P. aeruginosa* isolates where the highest persister fractions were found in isolates originating from chronic lung or hospital infections.[82] For *E. coli*, increased numbers of *hipA7* mutants, producing elevated persister levels, were found in patients with urinary tract infections.[141] Similarly, UPEC isolates originating from recurrent infections demonstrated a greater chance of persister formation following sub-inhibitory antibiotic treatment compared to isolates from acute infection, once again confirming that extended antibiotic therapy can select for variants with an increased number of persister cells.[63]

It is generally assumed that, upon successful antibiotic treatment, the human immune system is capable of dealing with the remaining antibiotic-tolerant persister fraction. However, as demonstrated in the above mentioned studies, life threatening infections can occur when the human immune system is compromised, as seen in AIDS/HIV and cancer patients, burn victims and ICU patients[29], or when persister cells can shield themselves from the immune system using the blood-brain barrier, in biofilms or within eukaryotic cells.[66–68] Independent *in vivo* experiments showed the presence of slow growing *S.* Typhimurium in murine cecum lymph nodes[142] and macrophages[143,144], providing evidence for the intracellular presence of persister cells and their ability to reinitiate infection when antibiotic treatment stops. Additional research has shown the occurrence of persister cells in most pathogens associated with chronic or difficult to treat infections, including *Escherichia coli*, *Acinetobacter baumannii, Mycobacterium tuberculosis*, *Staphylococcus aureus*, *P. aeruginosa* and *C. albicans.*[145] To conclude, there is ample evidence that persister cells are responsible for the recalcitrant nature of chronic infections, thereby hampering an effective treatment.

More recently, evidence is gathering that persister cells, partly due to their prolonged stay inside the human body, can facilitate the emergence of genetic resistance.[72,135] A possible explanation for the crosstalk between persistence and resistance are the shared mechanisms these survival strategies employ to overcome antibiotic killing. Indeed, efflux pumps, well known for their prominent role in antibacterial resistance[146] have recently been demonstrated to play a role in persistence of mycobacteria[77] and *E. coli.*[91,147] Other examples are the bacterial stringent and SOS responses, both implicated in persistence. While inhibition

of the stringent response reduced resistance development in *E. coli*[80], activation of both stress responses was shown to accelerate genome-wide mutagenesis and horizontal gene transfer in several pathogens, thereby increasing the probability of resistance development.[74] Genetic mutations accumulated during periodic aminoglycoside treatment and conferring high persistence phenotypes in *E. coli*[148] were also reported to occur in aminoglycoside-resistant strains[149,150], again suggesting an overlap between both survival strategies. Importantly, Balaban and co-workers provided experimental evidence that at high antibiotic concentrations (10 x MIC), the presence of antibiotic tolerance facilitates the establishment of antibiotic resistance mutations.[151] Another factor contributing to resistance development is the biofilm environment; microbial aggregates surrounded by a self-produced matrix showing an increased tolerance to antibiotic treatment. Greatly contributing to this is the increased number of persister cells which are physically protected from the human immune system by the biofilm matrix, thereby ensuring survival and repopulation when antibiotic treatment ceases.[52] Recently, biofilms have been suggested to contribute to the occurrence of adaptive mutations, possibly resulting in antibiotic resistance. Higher mutations rates were found in *P. aeruginosa* and *S. aureus* biofilm cells when compared to planktonic cultures, presumably linked to higher levels of oxidative stress in the biofilm environment.[74] Furthermore, processes involved in horizontal gene transfer were increased, with the biofilm lifestyle additionally increasing plasmid stability and the range of mobile genetic elements.[49]

Together, these studies further corroborate the importance of anti-persister therapies as they have the potential to eradicate the persister reservoir, thereby reducing the evolution of antibiotic resistance during treatment, diminishing the recalcitrant nature of chronic infections, and preventing tissue re-colonization when antibiotic treatment stops.[74,152]

2.3 Anti-persister strategies

Since the acknowledgment of their clinical relevance, the rekindled research interest in persister cells and their eradication resulted in several promising strategies to eliminate this antibiotic-tolerant fraction. Overall, anti-persister therapies can be divided in three groups depending on their anti-persister strategy (Figure 2.1). A first group of anti-persister approaches seeks to directly kill metabolically dormant persister cells, circumventing the need for active cell processes. An alternative approach sensitizes the persister cells for a conventional antibiotic through resuscitation, and thereby activation of the antibiotic targets,

or the stimulation of antibiotic influx. A third and last group of anti-persister therapies comprises molecules capable of interfering with or reducing the formation of persister cells. It must be noted however, that a strict distinction is not always clear and some therapies might belong to multiple categories.

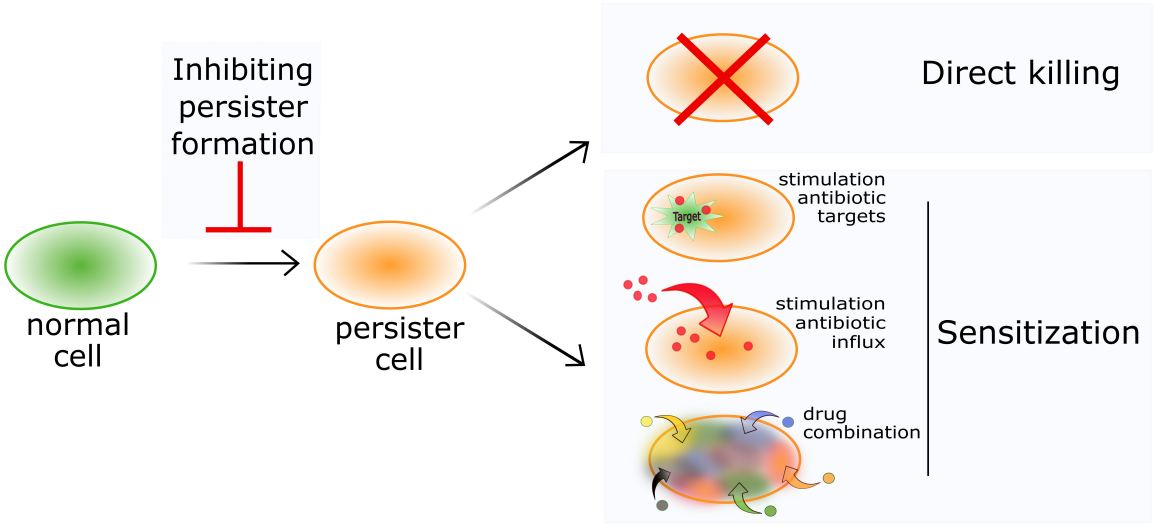


Figure 2.1: Schematic overview of the main strategies employed to kill bacterial persister cells. A first distinction can be made between strategies that inhibit the formation of persister cells and those that kill existing persister cells. The latter can be achieved through direct killing or sensitization of the persister cells. Stimulation of antibiotic targets or influx increase the sensitivity of persister cells to conventional antibiotics. The use of a combination of mechanistically different antibiotics also has the potential to eradicate the persister population.

The following paragraphs will discuss several anti-persister therapies for each approach. Despite the increasing number of anti-persister molecules described to date (Table 2.1 and Figure 2.2), increased research and development efforts will be needed to get these compounds approved for clinical use.

Surprisingly, the first anti-persister strategy mentioned in literature dates back to the 1940s, along with the first description of the persistence phenomenon. Joseph Bigger suggested that fractional or intermittent sterilization would allow the surviving persister cells to resuscitate during the non-treatment period, after which they could be eliminated shortly afterwards during a subsequent treatment period.[54] Mathematical modelling supported this approach in both planktonic[153] and biofilm[154] cultures and experimental evidence demonstrated the existence of a window of timing where antibiotic treatment was effective against persister cells of *S. aureus*.[155] The current antibiotic crisis and lack of commercially available anti-persister therapies has rekindled the interest in this technique, for example to eliminate *Borrelia burgdorferi*[156] and *P. aeruginosa*[52]. However, the practical use of this

strategy is rather limited due to patient-specific parameters[29] and substantial evidence suggests that suboptimal dosing regimens not only select for resistance development[157] but can also lead to increased levels of persister cells, both *in vitro*[148] and *in vivo*[70].

2.3.1 Direct killing of persister cells

Antibiotics kill bacteria by corrupting the normal function of a specific, essential macromolecular target. Likewise, temporary inactivation of these targets is thought to contribute to the persister cells ability to withstand the lethal effect of antibiotics.[64] Consequently, anti-persister compounds aimed at the direct killing of persister cells must target alternative processes to kill cells, *e.g*. the depolarization or destruction of the bacterial membrane, DNA cross-linking, inhibition of enzymes essential for survival of non-replicating bacteria, or generation of reactive oxygen species (ROS).

Hurdle *et al.* suggested the bacterial envelope or proteins integral to membrane function as ideal targets for anti-persister therapies[158], a proposal supported by the abundance of membrane-acting anti-persister compounds that have been described since (Table 2.1). After all, despite their reduced metabolism and dormant phenotype, persister cells still require an intact cytoplasmic membrane to maintain viability. The screening of chemical libraries to identify molecules active against non-multiplying cells represents a novel strategy in antibiotic discovery and yielded several membrane-active compounds. The fluoroquinolone-derived HT61 was specifically selected for activity against non-multiplying *S. aureus* cells. Destruction of the cell wall and depolarization of the cell membrane enables killing of both persister and non-persister cells and enhances the activity of existing antibiotics.[159,160] Similarly, SPI009 (1-((2, 4-dichlorophenethyl) amino)-3-phenoxypropan-2-ol) proved capable of directly killing non-multiplying cells of both Gram-negative and Gram-positive bacteria through extensive membrane damage. Additionally, SPI009 enhanced the activity of mechanistically distinct classes of antibiotics *in vitro* and *in vivo*.[161]

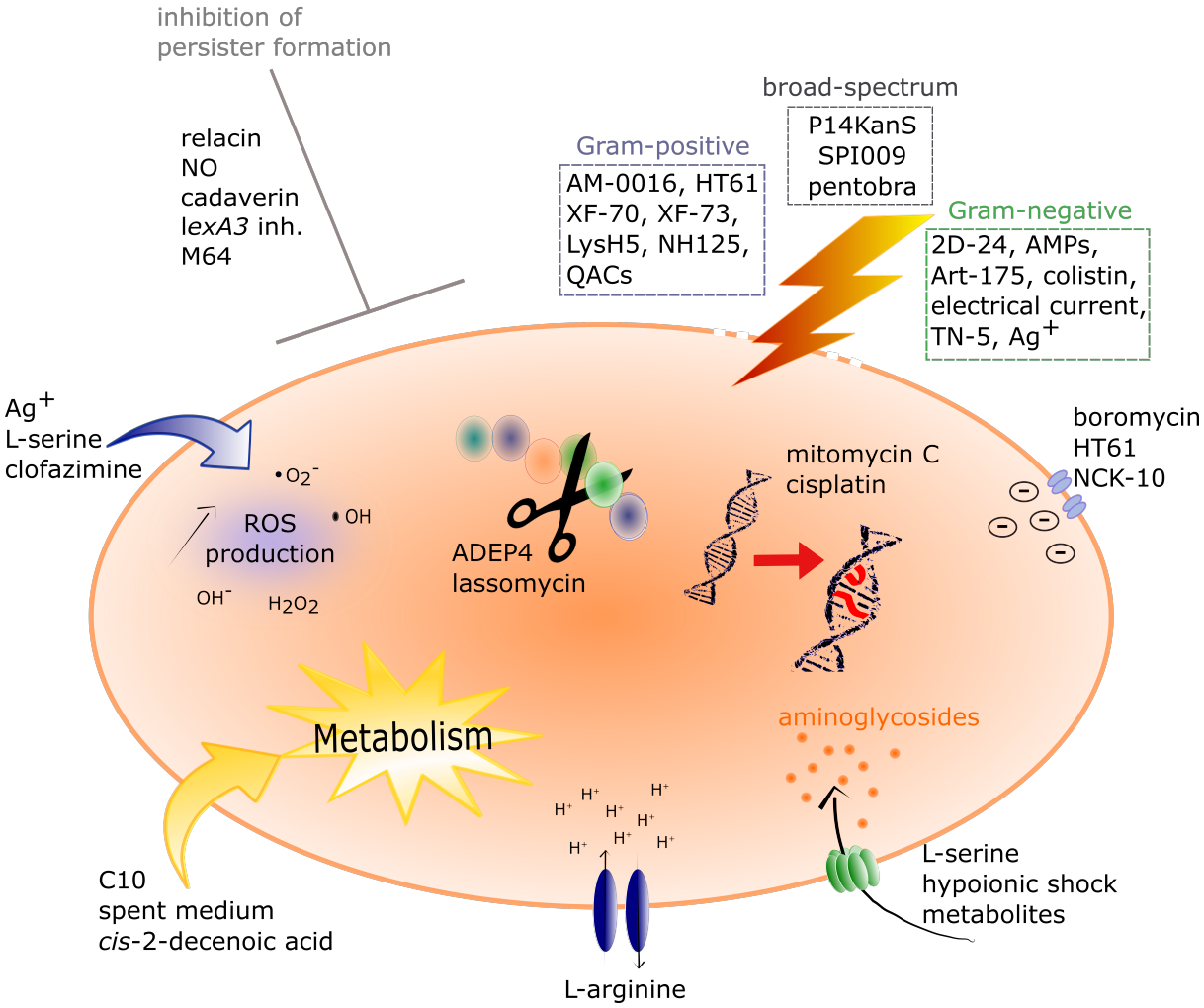


Figure 2.2: Schematic overview of anti-persister molecules described in literature and their cellular targets. Depending on their mechanism of action, anti-persister compounds use different strategies to kill persister cells. Inhibition of persister formation can be obtained through interference with quorum sensing (M64), the SOS-response (*lexA3* inh.), persister specific genes (cadaverin), stationary phase respiration (NO) and the stringent response (relacin). Other strategies include sensitizing persister cells to antibiotics through stimulation of ROS production (Ag^+, L-serine, clofazimine) or stimulation of the bacterial metabolism and thereby antibiotic targets (C10, spent medium, *cis*-2-decenoic acid). Alternatively, antibiotic uptake can be increased through modification of the pH gradient (L-arginine) and direct stimulation of aminoglycoside uptake (L-serine, hypo ionic shock, metabolites). Direct killing of persister cells can be obtained via stimulation of nonspecific protease activity (ADEP4, lassomycin), DNA crosslinking (mitomycin C, cisplatin) or membrane depolarization (boromycin, HT61, NCK-10). The most common strategy to directly kill persister cells is through extensive damage of the bacterial membrane. The different structures of Gram-negative and Gram-positive bacterial membranes typically results in the specific targeting of one of the two groups, as indicated in the figure. However, P14KanS, SPI009 and pentobra were shown to efficiently kill persister cells of both Gram-negative and Gram-positive species.

Well-known for causing cell lysis through extensive peptidoglycan degradation, phage-derived or synthetically produced lysins are also capable of killing persister cells. Bacteriophage-produced LysH5[162] and CF-301[163] efficiently killed growing and non-growing cells of *S. aureus* while demonstrating potent anti-biofilm effects. Engineering of known lysins, as was the case for the peptide-lysin conjugate Art-175[129,130] and the lipidated lysin **9**, containing an octane and decane alkyl chain[164], further expanded their membrane-damaging

activity to persister cells of the Gram-negative *P. aeruginosa*, *A. baumannii* and *E. coli*, respectively. Antimicrobial peptides (AMP), naturally produced antibiotics known for their membrane-damaging activity, have also been reported as scaffolds for the design of antibacterial agents. The dendrimeric peptide 2D-24[165] and Trp/Arg-containing AMPs[166] proved successful in the eradication of *E. coli* persisters, while NCK-10[167] and quaternary ammonium cations, simple AMP mimics,[168] effectively killed *S. aureus* persister cells. Lassomycin[169] and ADEP4[170] are bacterially produced lasso- and acyldepsipeptides, respectively. They exert their anti-persister activity through activation of specific Clp proteases, resulting in nonspecific protease activity and subsequent protein self-digestion in *M. tuberculosis* and *S. aureus,* respectively.

Remarkably, several known anti-cancer drugs were recently shown to possess potent anti-persister activity. Mitomycin C, a DNA cross-linking agent, effectively kills persister cells of several important pathogens including *E. coli*, *P. aeruginosa*, *S. aureus*[171]*, A. baumannii*[172] and *B. burgdorferi*[156]. Possessing several advantageous pro-drug characteristics, mitomycin C is passively transported into the cell after which it is spontaneously reduced to allow cross-linking of adjacent guanine residues.[171] Similarly, the anti-cancer drugs cisplatin, a DNA cross-linker, and anthracyclines were capable of killing persister cells of *E. coli*, *S. aureus*, *P. aeruginosa*[173] and *B. burgdorferi*[174], respectively. While the repurposing of existing drugs provides several advantages, the considerable side effects of these anti-cancer drugs and their relatively high toxicity could impair clinical applications as anti-persister therapies.

2.3.2 Sensitization and resuscitation of persister cells

Stimulation of antibiotic targets

As most antibiotics depend on active cell processes such as macromolecular synthesis to corrupt their targets, stimulation of persister cells could potentiate the activity of conventional antibiotics. As such, C10[175] and the fatty acid signaling molecule *cis*-2-decanoic acid[176] proved capable of reverting the persister phenotype of both *P. aeruginosa* and *E. coli* by stimulating their metabolism, thereby rendering these tolerant cells sensitive to different classes of antibiotics. Likewise, addition of spent medium and the protein and peptide factors therein resuscitated *S. aureus* persister cells, increasing their sensitivity to antibiotic induced killing.[177]

Stimulation of antibiotic influx

Several antibiotics require active transport systems to enter the bacterial cell[178], rendering them ineffective in dormant persister cells. Stimulation of antibiotic influx was demonstrated to accommodate persister eradication through potentiation of different antibiotics. Metabolites such as L-arginine[179], L-serine[180], glucose, fructose, pyruvate and mannitol[181,182] showed capable of sensitizing persister cells of *E. coli*, *P. aeruginosa* and *S. aureus* to gentamicin. Most metabolites presumably increase aminoglycoside uptake by stimulation of the proton-motive force (PMF) through an increase in membrane electric potential. Non-buffered L-arginine, a basic amino acid, was shown to increase the pH , thereby effecting the ΔpH-dependent compound of the PMF and increasing.[179] L-serine presumably inhibits amino acid synthesis, thereby increasing energy levels and subsequent uptake of antibiotics, thus contributing to persister killing.[183] Lysogenic bacteriophages overexpressing the outer membrane protein OmpF, involved in fluoroquinolone uptake[184], were capable of increasing killing by fluoroquinolones in *E. coli* persister cells, as compared to mono treatment.[185]

Another way of stimulating antibiotic influx is by permeabilizing or damaging the bacterial membrane, thereby enabling easy passage of antibiotics into the cell. Silver ions have been reported to increase *E. coli* membrane permeability of both active and dormant cells, enabling mechanistically different classes of antibiotics to kill persister cells.[186] Engineered adaptation of existing antibiotics to enhance influx also proved successful to combat persister cells. Addition of a 12-amino acid sequence, derived from the cell-penetrating peptide penetratin, to tobramycin allowed the disruption of the bacterial membrane, thus bypassing active membrane transport mechanisms, and effectively killing *E. coli* and *S. aureus* persister cells.[187] Similarly, coupling of the polyproline-based P14LRR antibacterial peptide sequence to kanamycin reduced persister fractions of *S. epidermidis, S. aureus*, *A. baumannii* and *P. aeruginosa.*[188]

Combinations of conventional antibiotics to fight persistence

Research showed that the antibiotic-tolerant persister fraction actually consists of several subpopulations of persister cells showing tolerance to one, or a small group, of specific antibiotics.[62,189] The combination of existing antibiotics from mechanistically different classes could thus represent a multi-front attack resulting in the effective eradication of different tolerant subpopulations. Indeed, several drug combinations identified by Feng and co-workers showed potent activity against antibiotic-tolerant *B. burgdorferi* persister cells. By

combining doxycycline with daptomycin and cefoperazone, active against replicating and non-replicating cells, respectively, they confirmed the clinical potential of drug combinations in the treatment of Lyme disease.[190,191] Colistin induced membrane damage enhanced the uptake and thus activity of gentamicin and ofloxacin in uropathogenic *E. coli*[192] while the combination with erythromycin successfully eradicated persister cells of *P. aeruginosa* biofilms both *in vitro* and *in vivo*.[193] The use of antibacterial combination therapies capable of killing persister cells may shorten treatment duration, reduce active concentrations of the different drugs and, by selecting drugs with different modes of action, reduce chances of resistance development.[106,194] Additionally, this approach allows the repurposing of known drugs, thereby reducing costs and development times associated with the marketing of novel therapies.

2.3.3 Reducing persister formation

Increasing knowledge on the mechanisms behind persistence has led to the identification of several approaches that interfere with processes implicated in persister formation, thereby decreasing persister numbers. Examples include inhibition of the quorum sensing regulator MfvR in *P. aeruginosa*[152,195], inhibitors that prevent accumulation of the stringent response alarmone (p)ppGpp in several Gram-positive species[196] and inhibition of the SOS- and oxidative-stress responses in *E. coli* by phage-encoded expression of, respectively, LexA3 and SoxR[185]. Inhibition of stationary phase respiration, obtained through treatment with NO, was also shown to reduce the formation of *E. coli* type I persisters upon addition of fresh medium.[197,198] While elimination of existing persister cells can greatly contribute to an effective treatment, inhibiting or decreasing their formation prior to antibiotic treatment has the potential to prevent chronic infections.

2.4 Potential targets for novel anti-persister therapies

As knowledge on the mechanisms controlling persistence in different pathogens is rapidly increasing, potential targets are being discovered that allow specific engineering of novel anti-persister therapies. Ideal anti-persister molecules should be able to enter the cell without the need for active transport and kill persister cells without requiring other active cell processes. While compounds decreasing or inhibiting persister formation are still rather limited (Table

2.1), the progressive identification of novel persister genes and processes[29,94] could provide a wealth of novel targets for rational drug design.

A potential target for novel anti-persister therapies receiving much attention are bacterial toxin-antitoxin (TA) modules, initially identified as regulaters of plasmid maintenance through post-segregational killing of plasmid-free strains. Imbalance between the unstable antitoxin and the stable toxin, causing inhibition of DNA replication, mRNA cleaving or inhibition of translation can result in cell death.[199,200] Several chromosomal TA modules of *E. coli* have been demonstrated to be involved in persistence, stimulating research into the possibility of exploiting this system for the development of novel anti-persister therapies.[29,201,202] While deletion or inactivation of the toxin has the potential to decrease persister levels by preventing their formation[100], the first evidence that TA modules could successfully be used as a target for novel anti-persister therapies was only recently provided. Li *et al.* identified a novel inhibitor of the *E. coli* HipA toxin which could indeed interfere with persister formation in an antibiotic-independent manner.[203] The practical use of the approach of targeting a single TA system is however limited since the contribution of TA modules to persistence in other bacterial pathogens is still unclear and research suggests that, due to their redundancy, the inactivation of a single TA module will have only a limited effect on persister levels.[75,76] However, for species containing many TA modules, such as *M. tuberculosis*[204], a single inhibitor could still target several TA modules belonging to the same family. Additional research will also be necessary to investigate the possibility of resistance development or compensatory activation of parallel TA systems when specific toxins are eliminated or antitoxins stabilized as part of an anti-persister approach.

The active suppression of reactive oxygen species (ROS) has previously been implicated in persistence[80,205]. Stimulation of ROS production could therefore stimulate the activity of different bactericidal antibiotics. ROS, such as superoxide and hydrogen peroxide, are produced in the bacterial cell via the Fenton reaction in response to various stresses. Grant *et al*. discovered that clofazimine, which increases ROS production in the cell[206], is capable of successfully decreasing the persister population in *M. smegmatis*.[90] Additionally, silver ions[186], L-serine[183], electrochemically generated H_2O_2[207] and N-acetylcysteine[208] increased sensitivity of persister cells to antibiotics partially through stimulation of ROS production in *E. coli*, *P. aeruginosa* and *M. tuberculosis*. As such, the identification of novel therapies stimulating endogenous ROS or inhibiting ROS-protective genes in the cell could potentiate the effect of conventional antibiotics in both persister and non-persister cells.[205]

Dahl and co-workers recently suggested the microbial polyphosphate synthesis enzyme polyP kinase (PPK) as a possible target for novel antimicrobial therapies. The PPK inhibitor mesalamine resulted in the reduced formation of ampicillin-tolerant persisters of UPEC bacteria and increased their sensitivity towards HOCl while decreasing *P. aeruginosa* colonization of the *Caenorhabditis elegans* gut and reducing biofilm formation in both UPEC and *P. aeruginosa* strains.[209] Gerdes and co-workers discovered an important role for polyphosphates in the (p)ppGpp controlled persistence cascade, inhibiting antitoxins and thus resulting in toxin-mediated induction of persistence.[210] Furthermore, bacteria lacking PPK proved defective in virulence, biofilm formation, oxidative stress response and persistence. Although further research is needed to unravel the mode of action of mesalamine, the overall absence of PPKs in eukaryotic cells and their high level of conservation in many major pathogens[211] could make them attractive targets for novel anti-persister and antimicrobial therapies. Similarly, inhibitors of the stringent response, central to persister induction and virulence, could have therapeutic potential. The search for such potential inhibitors has already begun and synthetic ppGpp analogs have been developed. Inhibition of the (p)ppGpp synthetase activity of Rel proteins was shown to interfere with the long-term survival of Gram-positive bacteria while the 2'-deoxyadenosine-based ppGpp analogue relacin disturbed the bacterial switch into stationary phase, resulting in cell death.[196,212]

Another interesting target associated with persister formation includes the QS system, one of the cell-to-cell communication pathways conserved among different pathogens and previously being linked to virulence and persistence.[95,98,99] The QS-inhibitor BF8 proved capable of sensitizing persister cells of *P. aeruginosa* but presumably via a non-QS target.[213,214] Further research is thus needed to assess the possibility of QS inhibitors as active anti-persister molecules.

Besides using mechanisms involved in persister formation or conservation as novel targets, another possible strategy involves the activation of antibiotic targets, rendering persister cells sensitive towards conventional antibiotics. For example, the upregulation of peptidoglycan synthesis or autolysin activity in persister cells could increase their sensitivity towards β-lactams while therapies that promote DNA replication or inhibit the bacterial SOS-response have the potential to stimulate quinolone effectiveness.[62]

The previous success of inhibiting bacterial efflux pumps, and thereby increasing antibiotic accumulation, as an anti-persister strategy in *E. coli*[147] and *M. marinum*[77] could provide a novel target for anti-persister therapies in other pathogens.

2.5 Anti-persister screenings as an emerging identification tool

Despite the increasing knowledge about persister mechanisms, the target-based development of anti-persister molecules remains challenging. A possible and increasingly used strategy to circumvent this is the use of well-designed screenings for antibacterial activity in persister-enriched or stationary phase cultures.[215] Selecting for activity against non-multiplying cells has led to the discovery of several anti-persister molecules. Besides the use of new chemical libraries, as was the case for C10[175] and the broad-spectrum anti-persister compound SPI009[161], clinical drug or biomolecule libraries also produced several promising candidates including NH125[216], tosufloxacin[217,218], several anthracyclines[191] and different drug combinations capable of eradicating *B. burgdorferi* persisters[190]. Alternatively, structural variant screening of existing antibacterials, such as quinolones, antimicrobial peptides[166] and even the recently reported NH125, resulted in the identification of HT61[159], NCK-10[167] and the N-arylated and 2-hydroxyethyl analogues of NH125[219,220], all showing increased anti-persister activity. Although caution should be taken with known adverse or toxic effects, using drug repurposing libraries or the adaptation of known antibiotic structures could facilitate the development of novel anti-persister compounds.

Additionally, screenings also proved successful in the discovery of inhibitors for several persistence implicated mechanisms such as the QS MvfR regulon[152,195], RelA[212] and the *M. tuberculosis* DlaT enzyme[221]. Increased identification of persistence–associated genes, such as the global regulators *dksA*, *spoT* and *phoU*, provide an alternative use for screenings to identify specific inhibitors in repurposing or novel compound libraries. Representing a versatile and whole cell-based technique, anti-persister screenings thus provide a promising tool for both target and non-target-based identification of future anti-persister molecules.

2.6 Clinical development of novel anti-persister molecules

In theory, anti-persister therapies offer several important advantages over currently available antibiotic therapies. The use of compounds inhibiting persister formation has the potential to prevent the development of a chronic infection. Additionally, anti-persister molecules alone or in combination with conventional antibiotics, can eradicate the entire bacterial population thereby diminishing the recalcitrant nature of infections[152], allow the eradication of biofilm infections[222], and decrease chances of resistance development[74], overall resulting in reduced antibiotic treatment duration and patients' physical burden.

However, the further clinical development and pharmaceutical commercialization of most of the described anti-persister therapies still require extensive testing in *in vivo* models to assess safety, efficacy and pharmacodynamic and pharmacokinetic behavior. While specific animal models for testing efficacy of anti-persister therapies are still lacking, the presence of persister cells was demonstrated in several chronic infection models[223], thus providing an opportunity to evaluate novel anti-persister compounds. The anti-persister activity of ADEP4 for example, was confirmed via the eradication of a *S. aureus* deep-seated thigh infection in neutropenic mice. Mimicking a chronic infection in immunocompromised patients, the lack of bacterial killing between 24h and 48h treatment of infected mice with vancomycin suggested the presence of persister cells.[170] Additional *in vivo* models studying persistence, such as those described for *S.* Typhimurium[142,143,224] could further increase the knowledge on *in vivo* behavior of persister cells. Indeed, additional *in vivo* persistence models and assessment of persister eradication *in vivo* will be crucial to translate the increasingly described anti-persister therapies into clinical use. Additionally, evolution experiments to assess the speed of resistance development and the extensive cytotoxicity testing , especially for membrane targeting compounds, during early phase discovery has the potential to facilitate the transition of future anti-persister candidates to the clinical test phase.

When sufficient evidence for clinical potential is obtained through testing in *in vivo* models, novel compounds can progress to the stages of clinical trials. As demonstrated in Table 2.1, anti-persister molecules have the potential to facilitate treatment of bacterial infections in different medical conditions and, for broad-spectrum agents, caused by different organisms. The strict regulations concerning the set-up of clinical trials in both the US and Europe however, require the description of a very specific disease condition in which the

novel compound will be tested.[2] This will require selection of the most relevant organism, infection site, infection type and, in case of a combination therapy, antibiotic for the evaluation of the novel compound, thereby impeding participant selection and limiting the products' intended use and, consequently, marketing opportunities. Furthermore, development costs may increase dramatically since every novel application or combination of the anti-persister compound for treatment of different organisms or diseases could require additional clinical trials.

Despite these current drawbacks in clinical development, several anti-persister therapies other than the repurposing of known antibiotics are currently enrolled in clinical trials to assess their safety and antibacterial activity in human subjects.[3] The bacteriophage-encoded lysin CF-301[163,225] has successfully completed phase I clinical trials and is currently recruiting for a phase II study to assess safety, efficacy and pharmacokinetics in the treatment of *S. aureus* bloodstream infections (NCT03163446). The nitroimidazole prodrug PA-824[226,227] has recently completed phase II trials to assess antibacterial activity in the oral treatment of early-phase tuberculosis (NCT00567840) and mycobactericidal activity of a PA-824/moxifloxacin/pyrazinamide combination was assessed in patients with drug-sensitive and multidrug-resistant pulmonary tuberculosis (NCT01498419). Additionally, XF-73, a membrane-active porphyrin compound[228], has successfully completed phase I/I-II tests to analyze activity as the active ingredient in nasal gels to reduce *S. aureus* colonization in healthy subjects (NCT01592214 and NCT02282605). Besides increasing attention from the academic world, the clinical relevance of persister cells is making its way into industry and several small-scale pharmaceutical companies, such as Arietis, founded by Kim Lewis, are acknowledging the benefits of anti-persister therapies. Similarly, Helperby Therapeutics has successfully used drug screens targeting non-multiplying *S. aureus* to identify their lead compound HT61.[159] This novel quinolone-derived compound proved effective in phase II clinical trials where it boosted the effect of conventional antibiotics in nasal decolonization (2010-021193-11) and is currently enrolled in phase III trials to compare the effect of a HT61/chlorhexidine combination to the current standard mupirocin (2009-017398-39). Despite these promising developments in the anti-persister field, additional research and development efforts will be needed to achieve commercialization of the anti-persister therapies described above and to combat the increasing threat of bacterial infections.

[2] http://www.fda.gov and http://www.ema.europe.eu
[3] http://www.clinicaltrials.gov and https://www.clinicaltrialsregister.eu/

2.7 Conclusion and future perspectives

In this review, we summarized the different anti-persister molecules and therapies described in literature to date. The general acknowledgement of persister cells and their major clinical importance has boosted persistence research, resulting in a continuously increasing knowledge about the mechanisms controlling persister formation and revealing possible strategies to eradicate them.

Increasing anti-persister research and the identification of compounds capable of targeting non-dividing cells will further facilitate and help overcome the previous hurdles in anti-persister research. The multitude of anti-persister molecules and therapies described in literature is promising, although extensive *in vivo* testing will be required in order to progress to the stages of clinical trials. Indeed, little is known about the effect of anti-persister treatments *in vivo*. *In vivo* infection and persistence models, as reviewed in Van den Bergh *et al.*[223], will be crucial to translate the increasingly described anti-persister therapies to clinical use and stimulate progression to clinical trials.

Future development will not only benefit the treatment of bacterial infections but might also provide insights in the intriguing parallel of drug-tolerant persister cells in cancer.[135] Overall, the development of highly needed anti-persister therapies will greatly improve bacterial infection treatment and subsequently patient outcomes and, with the current antibiotic crisis in mind, might provide a successful alternative in the fight against chronic infections.

Table 2.1: Overview of anti-persister molecules and strategies described in literature.

Compound	Target species	Additional information	Reference
Direct killing of persister cells			
2D-24	*P. aeruginosa*	Membrane-active dendrimeric peptide, kills persister cells by disturbing the bacterial membrane and enhancing the activity of ciprofloxacin, tobramycin and carbenicillin.	[165]
5-Iodoindole	*E. coli,* *S. aureus*	Testing of several halogenated indole derivatives identified 5-iodoindole as a novel anti-persister molecule additionally capable of inhibiting biofilm and persister formation.	[229]
ADC111-ADC113	*E. coli*	Analogs of, respectively, nitrofurantoin and tilbroquinol , together with the unclassified β-diketone were identified in an Alamar Blue based screen of 55 000 prodrugs. All three demonstrate a prodrug-based, potent anti-persister activity in both planktonic and biofilm cultures.	[230]
ADEP4	*S. aureus*	Acyldepsipeptide, activates ClpP protease, causing a nonspecific protease activity and eradicating persister cells in both planktonic and biofilm cultures.	[170]
AM-0016	*M.tuberculosis,* *M. bovis*	Structure-based screening of α-mangostin derivatives identified 3,6-Di[4-(diethylamino)-butoxy]-1-hydroxy-7-methoxy-2,8-bis(3-methylbut-2-enyl)-9H-xanthen-9-one (AM-0016). AM-0016 causes inner membrane damage and rapidly collapsing membrane potential. Selectively kills Gram-positive bacteria without inducing detectable drug resistance.	[231,232]
Anthracyclines	*B. burgdorferi*	Several promising anthracycline/antraquinone compounds were identified in a SYBR Green I/PI based screening of the National Cancer Institute compound collection against stationary phase *B. burgdorferi* cultures, showing excellent activity against both growing and non-growing cells.	[174]
Antimicrobial peptides	*E. coli*	Trp/Arg-containing AMPs show anti-persister activity in planktonic and biofilm-cultures through penetration of the bacterial membrane.	[166]
Art-175	*A. baumannii,* *P. aeruginosa*	Art-175 consists of the peptidoglycan-degrading phage ϕKZ endolysin KZ144 covalently coupled to the sheep myeloid 29-amino acid antibacterial peptide SMAP-29. Other Artilysins have been described but Art-175 is the first to show anti-persister activity.	[129,130]
Boromycin	*M. tuberculosis*, *M. bovis* *S. aureus,* *S. epidermidis,* *E. faecalis*	Acting as a potassium ionophore, the polyether-macrolide boromycin causes membrane depolarization, subsequent decrease of ATP levels and leakage of cytoplasmic proteins. Boromycin kills growing and non-growing *Mycobacterium* cells and shows potent growth inhibition and bactericidal activity against several other gram-positive pathogens.	[233]
Cisplatin	*E.coli,* *S. aureus,* *P. aeruginosa*	Anti-cancer drug (*cis*-diamminodichloroplatinum(II)) that causes growth-independent crosslinking of DNA, thereby effectively killing persister and non-persister cells.	[173]
Clofazimine	*M.tuberculosis,* *M. smegmatis*	Stimulates ROS production through an NADPH-dependent redox-cycling pathway, successfully eradicating persister cells.	[90]
D157070/D155931 (rhodanines)	*M. tuberculosis*	Screening-based identification of dihydrolipoamide acyltransferase (DlaT) inhibitors, selectively killing non-replicating cells.	[221]
DG70	*M. tuberculosis*	The biphenyl amide GSK1733953A (DG70) kills non-growing cells, through inhibition of MenG, and shows synergy with several known anti-TB therapies. Identified through whole-cell based screening for inhibitors of the respiratory pathway.	[234]
Electrical current	*P. aeruginosa*	Low levels of electrical current (70 μA/cm^2) caused substantial changes in membrane structure and integrity, allowing the reduction of persister levels in planktonic and biofilm cultures and synergistic effects with tobramycin.	[235–237]
Electrochemically generated H_2O_2	*P. aeruginosa*	Intracellular increase of ROS production, following electrochemical treatment, eradicated persister cells and increased membrane permeability, thereby potentiating tobramycin.	[207]

Compound	Target species	Additional information	Reference
Halogenated phenazines	MRSA, *M. tuberculosis*	Group of phenazine-inspired small compounds, operating through a unique metal(II)-dependent mechanism, showing a potent anti-persister activity in MRSA and *M. tuberculosis.* Potent biofilm eradication activity was demonstrated in MRSA, MRSE, VRE and *M. tuberculosis.*	[238,239]
HT61	*S. aureus*	The quinolone-derived HT61 kills persister and non-persister cells through depolarization of the cell membrane and destruction of the cell wall. Additionally, HT61 significantly enhanced the activity of gentamicin, mupirocin, neomycin and chlorhexidine *in vitro* and *in vivo.*	[159,160,240]
Lassomycin	*M. tuberculosis*	A microbially produced cyclic lasso peptide showing a bactericidal effect against exponential and stationary phase and persister cells of *M. tuberculosis.* Binding of lassomycin to ClpC1 ATPase results in increased ATPase activity and inhibition of ClpP1/P2 proteolytic activity.	[169]
Lipidated lysine **9**	*E. coli,* *S. aureus*	Synthetic design of lysine-based membrane-active agents containing one amino acid and two lipid tails. The most active compound, **9** (C_8-K-C_{10}) was capable of lysing persister cells of *E. coli* and *S. aureus.*	[164]
LysH5; CF-301	*S. epidermidis,* *S. aureus*	The vB_SauS-phiIPLA88 phage-derived endolysin causes cell lysis of growing and non-growing cells, allowing the eradication of persister cells. The bacteriophage lysin CF-301 efficiently killed persister cells of *S. aureus* and exhibited potent biofilm inhibition and eradication activity.	[162,163,225]
Mitomycin C	*E. coli,* *S. aureus,* *P. aeruginosa,* *B. burgdorferi,* *A. baumannii*	Anti-cancer drug that causes growth-independent DNA crosslinking, thereby effectively killing persister and non-persister cells of several pathogenic bacteria.	[156,171,172]
NCK-10	*S. aureus*	Aryl-alkyl-lysine antibacterial peptoid mimics causing membrane depolarization and permeabilization in persister cells, stationary phase, planktonic and biofilm cultures.	[167]
NH125	*S. aureus*	(1-Hexadecyl-2-methyl-3-(phenylmethyl)-1H-imidazolium iodide) kills persister cells through membrane permeabilization and shows activity in biofilms. Identified in *C. elegans* screening.	[216,241]
NH125 analogues	*S. aureus*	Design and antibacterial analysis of *N*-arylated, alkylated, benzylated and benzimidazole analogues of the previously identified NH125. Analogues **1, 3, 4** and **11** showed an increased anti-persister activity in *S. aureus* (MRSA) with increased biofilm eradication in *S. aureus* (MRSA), VRE, *C. albicans* and *Cryptococcus neoformans* for **1** and **11**.	[219,220]
Nitroxoline	*S. aureus*	Iron and zinc chelator (5-nitroquinolin-8-ol) capable of killing persister and stationary phase cells at high concentrations. Previously characterized as biofilm disperser in *E. coli* and *P. aeruginosa.*	[242]
PA-824	*M. tuberculosis*	Non-replicating *M. tuberculosis* cells proved susceptible to treatment with the 3-substituted nitroimidazopyran prodrug PA-824, acting as an intracellular NO donor.	[226,227]
Pyrazinamide, KKL-35	*M. tuberculosis*	Nicotinamide analog that, after passive diffusion into the cell, is converted to 2-pyrazinecarboxylic acid (POA), capable of corrupting the *trans*-translation process that normally recovers stalled ribosomes. Similarly, the *trans*-translation inhibitor KKL-35 (4-chloro-*N*-[5-(4-fluorophenyl)-1,3,4-oxadiazol-2-yl]-benzamide) was active in growing and non-growing cells.	[243,244]
QACs (QAC-10)	*S. aureus* (MRSA), *E. faecalis*	Quaternary ammonium cations mimicking antimicrobial peptides and capable of disrupting the bacterial membrane. Besides killing persister cells, QAC-10 showed potent biofilm eradication activity.	[168]
SPI009	*P. aeruginosa,* *E. coli, ESKAPE* pathogens, *B. cenocepacia,* *S.* Typhimurium	Propanol-amine derivative (1-((2,4-dichlorophenethyl)amino)-3-phenoxypropan-2-ol) identified in a *P. aeruginosa* anti-persister screening, directly kills persister cells of several Gram-positive and Gram-negative pathogens through extensive membrane permeabilization.	[161]; Defraine *et al.,* 2017 (unpublished)

Compound	Target species	Additional information	Reference
Stevia whole leaf extract	*B. burgdorferi*	Whole leaf extract from *Stevia rebaudiana* was found to outcompete several antibiotics and antibiotic combinations in the effective elimination of *Borrelia* spirochetes and persisters. Anti-persister activity was also seen in long-term subculturing and biofilm experiments. Possibly, Stevia acts as a sugar derivative, priming the uptake of phytochemicals and disrupting the biofilm structure.	[245]
TCA1	*M. tuberculosis*	Identified in a whole-cell screening and shown to downregulate cell wall and molybdenum cofactor biosynthesis-related genes. Activity alone and in combination with rifampicin and isoniazid against both replicating and non-replicating cells.	[246]
TN-5	*E. coli*, *P. aeruginosa*	Synthetically produced 1,3,5-triazine (AMP) derivative showing moderate anti-persister activity, possibly due to interaction with the negatively charged bacterial membrane.	[247]
Colistin	*E. coli*	Screening of a small clinical drug library identified the membrane-damaging polymyxin colistin as possessing potent anti-persister activity in uropathogenic *E. coli*. Colistin was also capable of eradicating TisB-induced persister cells.	[81,217]
Tosufloxacin	*E. coli, S. aureus*	Screening of a small clinical drug library identified the quinolone tosufloxacin as possessing the highest anti-persister activity in uropathogenic *E. coli.* Additionally, tosufloxacin was capable of efficiently killing persister cells of *S. aureus.*	[217,218]
XF-70 and XF-73	*S. aureus*	Dicationic porphyrin compounds showing rapid membrane-perturbing activity in growing and slow-growing bacteria with demonstrated anti-biofilm activity.	[228]
Metronidazole	anaerobic *M. tuberculosis* and *B. burgdorferi*	A synthetic prodrug (2-(2-methyl-5-nitro-1H-imidazol-1-yl)ethanol) that, upon activation by a microbial-specific nitrate reductase, covalently binds to multiple targets including DNA, proteins and the bacterial membrane. Since activation does not require energy, metronidazole could target persister cells.	[248,249]
Sensitization			
BF8	*P. aeruginosa*	QS inhibitor (Z)-4-bromo-5-(bromomethylene)-3-methylfuran-2(5H)-one reverts antibiotic tolerance of persister cells and increases susceptibility to ciprofloxacin and tobramycin, presumably via non-QS targets. Cidal at higher concentrations.	[213,250]
C10	*E. coli, P. aeruginosa*	3-[4-(4-methoxyphenyl) piperazin-1-yl] piperidin-4-yl biphenyl-4-carboxylate causes reversion of dormant persisters to a normal, replicating phenotype. Identified in a screening for norfloxacine potentiators.	[175]
cis-2-decenoic acid	*P. aeruginosa, E. coli*	Fatty acid signaling molecule capable of reverting persister cells to a metabolically active state, rendering them susceptible to different classes of antibiotics.	[176]
Efflux pump inhibitors	*E. coli, M. marinum*	Combining efflux pump inhibitors PaβN and NMP with mechanistically distinct antibiotics significantly reduced persister fractions of *E. coli* through increased antibiotic accumulation. Similarly, the addition of verapamil reduced intracellular growth and macrophage-induced tolerance of *M. marinum,* increasing the efficacy of antibiotic treatment.	[77,147]
Engineered bacteriophages	*E. coli*	Bacteriophages expressing the outer membrane protein OmpF increased killing of persister cells by fluoroquinolones, as compared to mono treatment.	[185]
Glucose	*S. aureus*	Glucose, and the bacterial metabolism thereof, enhances the effect of daptomycin, resulting in increased persister killing.	[251]
GM-CSF	*P. aeruginosa*	The human granulocyte macrophage-colony stimulating factor (GM-CSF) is capable of increasing persister sensitivity to ciprofloxacin and tobramycin through a persister- and species-specific induction of stress- and pyocin-related genes.	[252]
Hypo ionic shock treatment	*E. coli*	Hypo-ionic shock treatment facilitates aminoglycoside killing, possibly through increased uptake via mechanosensitive ion channels, and enabled killing of persister cells.	[253]

Compound	Target species	Additional information	Reference
L-arginine	*P. aeruginosa*, *S. aureus,* *E. coli*	L-arginine induced modification of membrane pH gradient potentiates gentamicin activity in planktonic cultures and *in vitro* and *in vivo* biofilms.	[179]
L-serine	*E. coli*	Addition of L-serine sensitizes persister cells to gentamicin, possibly through the inhibition of amino acid synthesis and increased antibiotic uptake. Furthermore, the combination of L-serine with ofloxacin or moxifloxacin increases the NAD^+/NADH ratio, disrupts the Fe-S clusters and increases production of ROS, thereby potentiating the antibiotic activity against persister cells.	[180,183]
Metabolites	*E. coli*, *S. aureus*	Glucose, fructose, pyruvate and mannitol induced proton-motive force sensitizes persister cells by an increase of gentamicin uptake. Increased persister killing was observed in biofilms and a mouse urinary tract infection model.	[181]
Mannitol	*P. aeruginosa*	Mannitol proved capable of reverting the persister phenotype in *P. aeruginosa*, most likely by inducing metabolism and generating a proton-motive force, thereby enhancing the efficacy of tobramycin in clinical isolates and biofilms.	[182]
Moxifloxacin and gatifloxacin	*M. tuberculosis* *E. coli*	Killing of rifampin-tolerant persister cells increased for both fluoroquinolones in different models of non-replicating *M. tuberculosis,* suggesting bactericidal activity against slow-growing bacilli and rifampin-tolerant persister cells.	[254]
P14LR-kanamycin (P14KanS)	*S. aureus,* *S. epidermidis,* *P. aeruginosa,* *A. baumannii*	Conjugate of the aminoglycoside kanamycin with the proline rich P14LRR antimicrobial peptide. P14KanS displays potent activity against stationary phase and persister cells by selective disruption of the bacterial cell membrane and showed potent anti-biofilm activity.	[188]
Pentobra	*E. coli, S. aureus*	Engineering of the aminoglycoside tobramycin through addition of a 12-amino acid peptide resulted in increased cell penetration and subsequent tobramycin-induced killing of persister cells.	[187]
Silver ions	*E. coli*	Ag^+ particles increase ROS production and permeability of the Gram-negative membrane in both active and dormant cells, allowing the effective killing of persister cells. Increased membrane permeability also potentiates antibiotic activity (ampicillin, gentamicin, ofloxacin) against persister cells in planktonic and biofilm cultures.	[186]
Spent medium	*S. aureus*	Addition of spent medium, and protein/peptide factors therein, resuscitates dormant *S. aureus* cells, making them more prone to antibiotic-induced killing.	[177]
Drug combinations			
Colistin	*E. coli,* *P. aeruginosa*	Colistin-induced membrane damage enhances the activity of gentamicin and, to a lesser extent, ofloxacin in uropathogenic *E. coli.* Combination with erythromycin resulted in complete eradication and repression of antibiotic-tolerant subpopulations of *P. aeruginosa* in biofilms.	[192,193]
Daptomycin, cefoperazone and doxycycline	*B. burgdorferi*	Combination of conventional antibiotics active against replicating (doxycycline) and non-replicating cells (daptomycin, cefoperazone) that successfully eradicated stationary phase cultures, including persister cells.	[190]
Ciprofloxacin and vancomycin	*S. epidermidis*	Combination of high concentrations of ciprofloxacin and vancomycin allowed the complete eradication of persister and biofilms cells in *S. epidermidis.*	[255]
Artemisinin/ cefoperazone/ doxycycline and sulfachlorpyrid-azine/ daptomycin/ doxycycline	*B. burgdorferi*	An FDA-approved drug library was screened for drug combinations that could decrease amoxicillin generated persister cells, using a recently developed SYBR Green I/propidium iodide (PI) assay. Two triple drug combinations showed superior activity against amoxicillin-induced and stationary phase *B. burgdorferi* persisters.	[191]
Polymyxin B and meropenem	*A. baumannii*	Combination of polymyxin B and meropenem proved successful in the eradication of clinical *A. baumannii* strains after 48 hours.	[256]

Compound	Target species	Additional information	Reference
Controlling formation			
Benzamide, benzimidazole compounds	*P. aeruginosa*	Screening-based identification of inhibitors of the QS MvfR regulon, including M56, M34, M59 and the most active M64 compound, which reduced persister formation *in vivo* and could efficiently be combined with ciprofloxacin to obtain full bacterial clearance. Further research on the MvfR antagonist M64 revealed a downregulation of the QS *pqs* regulon, thereby reducing the levels of 2-AA and decreasing persistence formation.	[152,195]
Cadaverine	*P. aeruginosa*	Identification of persister gene PA4115, involved in cadaverine production. Exogenous cadaverine supplementation increased carbenicillin and ticarcillin-induced lysis, possibly by overcoming persister formation.	[257]
Engineered bacteriophages	*E.coli*	Reduction of persister levels was achieved for phages expressing the SOS-response repressor *lexA3*. Combining ofloxacin with engineered SoxR delivering bacteriophages also increased persister killing by interfering with the bacterial response to oxidative stress.	[185]
Mesalamine	*E. coli,* *P. aeruginosa*	Anti-inflammatory drug (5-aminosalicylic acid) capable of increasing bacterial sensitivity to oxidative stress (HOCl), reducing persister formation and decreasing bacterial colonization of the *C. elegans* gut.	[209]
MOPS, osmolytes	*E. coli*	Addition of MOPS (3-(*N*-morpholino)propanesulfonic acid) or low concentrations of osmolytes (trehalose, betaine, glycerol and glucose) decreased the frequency of persister formation by inhibiting the accumulation of protein aggregates.	[258]
N-acetylcysteine	*M. tuberculosis*	Metabolic conversion of cysteine to cystine presumably prevents the cell from entering the persister state through activated respiration and thus metabolism. Additionally, increased ROS generation might explain the potentiation of isoniazid, resulting in sterilization.	[208]
NO	*E. coli*	Nitric oxide treatment upon nutrient deprivation reduced the formation of type I persister cells upon addition of fresh medium through inhibition of stationary phase respiration.	[197,198]
RelA inhibitors, relacin	*E. coli,* *B. subtilis,* *B. anthrachis,* Group A *streptococci*	Preventing the accumulation of the alarmone (p)ppGpp, by inhibiting RelA, interferes with the bacterial stringent response and decreases persister levels. Relacin proved active only in Gram-positive bacteria, and was capable of strongly inhibiting sporulation and biofilm formation.	[196,212]

MRSA = methicillin resistant *S. aureus*, MRSE = methicillin resistant *S. epidermidis* VRE = vancomycin resistant *Enterococcus*

Chapter 3

Identification of SPI009, a novel antibacterial compound active against persisters of *Pseudomonas aeruginosa*

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Contributions of individual authors to the manuscript:

The screening was designed by Veerle Liebens, Maarten Fauvart and Jan Michiels and was executed by Veerle Liebens, Wouter Knapen and Toon Swings. Other experiments described in this paper were designed by **Valerie Defraine**, Maarten Fauvart and Jan Michiels and performed by **Valerie Defraine**. The data was analyzed and the manuscript written by **Valerie Defraine**. The manuscript was revised by Maarten Fauvart, Jan Michiels and Arnaud Marchand.

Abstract

Antibiotics typically fail to completely eradicate a bacterial population, leaving a small fraction of transiently antibiotic-tolerant persister cells intact. Persisters are therefore seen to be a major cause of treatment failure and greatly contribute to the recalcitrant nature of chronic infections. The current study focused on *Pseudomonas aeruginosa*, a Gram-negative pathogen belonging to the notorious ESKAPE group of pathogens (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa*, and *Enterobacter* species) and, due to increasing resistance against most conventional antibiotics, posing a serious threat to human health. Greatly contributing to the difficult treatment of *P. aeruginosa* infections is the presence of persister cells, and elimination of these cells would therefore significantly improve patient outcomes. In this study, a small-molecule library was screened for compounds that, in combination with the fluoroquinolone antibiotic ofloxacin, reduced the number of *P. aeruginosa* persisters compared to the number achieved with treatment with the antibiotic alone. Based on the early structure-activity relationship, 1-((2,4-dichlorophenethyl)amino)-3-phenoxypropan-2-ol (SPI009) was selected for further characterization. Combination of SPI009 with mechanistically distinct classes of antibiotics reduced the number of persisters up to 106-fold in both lab strains and clinical isolates of *P. aeruginosa*. Further characterization of the compound revealed a direct and efficient killing of persister cells. SPI009 caused no erythrocyte damage and demonstrated minor cytotoxicity. In conclusion, we identified a novel anti-persister compound active against *P. aeruginosa* with promising applications for the design of novel, case-specific combination therapies in the fight against chronic infections.

3.1 Introduction

Pseudomonas aeruginosa is a Gram-negative opportunistic pathogen best known as the dominant cause of life-threatening chronic airway infections in cystic fibrosis (CF) patients.[259] Infections are also commonly found in highly vulnerable patients such as burn wound victims, immunocompromised individuals and patients residing in ICUs.[260,261] In addition to an intrinsic resistance towards a wide variety of antibiotics, *P. aeruginosa* has the remarkable ability to acquire additional resistance mechanisms. The rapidly increasing resistance to multiple antibiotic classes, including the so-called last-resort polymyxins, classifies the pathogen as multidrug or even pan-resistant and has caused *P. aeruginosa* infections to become increasingly difficult to treat.[260,262,263] *P. aeruginosa* is fourth most common cause of healthcare-associated infections in Europe (8.9%), with 14% of all isolates reported as being resistant to at least three antimicrobial classes.[21,22] The World Health Organization (WHO) has recently ranked *P. aeruginosa* as critical in the 'Global priority list of antibiotic-resistant bacteria to guide research, discovery and development of new antibiotics', emphasizing its clear clinical importance and encouraging the development of novel antibacterial therapies.[264,265]

Some infections however, prove difficult to eradicate, despite the absence of clinically detectable resistance against the antibiotic used.[70] This can be explained by the presence of persister cells, a small fraction of phenotypic variants in a genetically homogeneous population that is tolerant to treatment with high doses of antibiotics.[60,266] It is generally assumed that, upon successful treatment with a bactericidal antibiotic, the host immune system is able to cope with this small fraction of surviving cells. However, in situations where the immune system is compromised or bacteria are able to evade the immune system, persisters become a threat to human health and are considered a major culprit in chronic infections.[29] Persister cells greatly contribute to the observed antibiotic tolerance in biofilms, are responsible for the recalcitrant nature of chronic infections.[69–71] and recently, evidence is gathering that persister cells can facilitate the emergence of genetic resistance.[65,72–74] Targeting persisters is therefore likely to greatly improve outcomes for patient with chronic infectious diseases. Despite the clear clinical relevance of persisters, few anti-persister compounds have been described in the literature.[223] Contributing to this is the still limited knowledge on the mechanisms behind persister formation in *P. aeruginosa*, the fact that multiple, redundant pathways seem to be involved and the observation that the processes

implicated in persistence seem to be species-specific.[83,135] Therefore, the rational design of target-based anti-persister therapies remains difficult.

Here, we describe the screening of a diverse set of small-molecule compounds that resulted in the identification of the potent anti-persister compound SPI009. This molecule strongly reduces or even completely eradicates persisters of *P. aeruginosa* when administered in combination with mechanistically distinct antibiotics. Importantly, SPI009 retains its activity against several clinical isolates, directly kills persister cells in a highly efficient manner, and causes no hemolytic effects.

3.2 Materials and methods

Bacterial strains, human cell lines and culture conditions. Bacterial strains (see Tal 3.1) were cultured in 1:20 diluted Trypticase Soy Broth (1/20 TSB) at 37 °C with shaking at 200 rpm. For solid medium, TSB was supplemented with 1.5% agar. Human HEK293T cells were cultivated in DMEM medium containing 5% heat-inactivated fetal calf serum and kept at 37°C with 5% CO_2. The following antibiotics were used: ofloxacin, ceftazidime (Sigma Aldrich) and amikacin (Acros). Concentrations are indicated throughout the text. The small-molecule library was provided by the Centre for Drug Design and Discovery (CD3) of KU Leuven.[267,268] Bacterial strains used in this study are listed in Table 3.1.

Table 3.1: Overview of strains used in this study

Strain	Description/ Resistance profile	Source or reference
P. aeruginosa PA14	UCBPP-PA14, wild type	Pierre Cornelis
P. aeruginosa AA249	Clinical isolate from burn wound; CIP^R, CAZ^R, ATM^R, IPM^R, MEM^R	[269]
P. aeruginosa BR642	Clinical isolate from bed pan; ATM^R	[269]
P. aeruginosa BU004	Clinical isolate from throat; ATM^R	[269]
P. aeruginosa BE136	Clinical isolate from bronchus; IPM^R, ATM^R	[269]
P. aeruginosa PA1256	Clinical isolate from CF patient	Françoise van Bambeke (UCL)
P. aeruginosa PA1255	Clinical isolate from CF patient; TIC^R, PIP^R, TZP^R, CAZ^R	Françoise van Bambeke (UCL)
P. aeruginosa PA272	Broncho pulmonary isolate PIP^R, TIC^R	Françoise van Bambeke (UCL)
P. aeruginosa DAF87-203	Sputum isolate from CF patient PIP^R, OFX^R, CST^R	Françoise van Bambeke (UCL)

KAN= kanamycin, CIP= ciprofloxacin, CAZ= ceftazidim, ATM= aztreonam, IPM= imipenem, MEM= meropenem, PIP= piperacillin, TIC= ticarcillin, OFX= ofloxacin, CST= colistin, TZP= piperacillin-tazobactam.

Anti-persister screening assay. The small-molecule library was screened for anti-persister activity as previously described.[270] Briefly, stationary phase cells of *P. aeruginosa* PA14 cultured in 1/20 TSB were treated for 5 hours with a combination of ofloxacin (10 μg/mL; 8 x MIC) and test compound (10 μg/mL, corresponding to 20-50 μM of compound). Treated cells were diluted 100-fold into fresh TSB growth medium and incubated in an automated optical density (OD) plate reader (Bioscreen C, Oy Growth Curves Ab Ltd.) at 37°C with aeration. Previous research revealed a linear relationship between the number of cells incubated in the automated plate reader and the time it took to reach an OD_{600} of 0.6, which was $t(OD_{600} = 0.6)= -2.1574x + 28.792$; the R^2 value was 0.987.[94] On the basis of this relationship, 105 compounds that produced delayed growth and thus decreased the number of surviving persister cells were selected. Of the 15 most interesting compounds producing a significant reduction in persister fraction, five compounds belonging to three structurally distinct families were further confirmed via plate counting. Family 1 proved to be the most active, and 19 chemical analogues of compound SPI001 were subjected to a structure-activity relationship study (Table 3.2).

MIC assay. Minimal Inhibitory Concentrations (MICs) were determined using the EUCAST standards for broth microdilution [271]. The starting inoculum was adapted to approximately 5 x 10^5 cells/mL and incubated in the presence of a 2-fold dilution series of SPI009 for 24 hours with shaking at 37°C. MIC was determined to be the lowest concentration completely inhibiting bacterial growth.

Treatment of isolated persister cells. Persister cells were isolated as described previously, with minor modifications.[213] Stationary phase cells were treated with ofloxacin (10 μg/mL) for 5 h. Higher ofloxacin concentrations or increased incubation times did not lead to a further reduction of the number of surviving cells (data not shown). Persisters were washed twice with 0.85% NaCl (centrifugation at 5200 x *g*, 15 min, 4 °C) and subsequently used for killing assays, as described below.

Killing assays. Killing assays were performed as previously described.[272] Briefly, stationary phase cultures were treated with a combination of antibiotic and compound (to determine the anti-persister effect) or compound alone (to determine the bactericidal effect). Antibiotic concentrations were chosen to allow only persister cells to survive, indicated by a drop in killing rate. Volumes of 200 μL of the treated culture were dispensed in a 96-well plate, and the plate was incubated for 5 hours at 37 °C with shaking at 200 rpm. To explore the effects

of different treatment regimens, SPI009 was added 0, 5 or 24 h after the onset of ofloxacin treatment, and the cells were treated for a total of 72 hours. For time-kill curves, different treatment durations between 15 minutes and 24 hours were chosen, and the treatment comprised DMSO (0.5%; solvent control), ofloxacin (10 µg/mL), SPI009 alone (17 or 34 µg/mL) and the combination of ofloxacin with SPI009. To assess the effect of different classes of antibiotics, stationary-phase or exponential-phase cultures were treated with 75 µg/mL amikacin or 30 µg/mL ceftazidime for 5 h. After treatment, the cells were washed twice in 10 mM $MgSO_4$ (centrifugation at 3300 x g, 10 min, 4 °C) after which appropriate dilutions were plated on solid growth medium to determine the number of viable cells. Plates were monitored for 72 h to ensure detection of slow-growing colonies.

Cytotoxicity assay. Cytotoxicity was evaluated through colorimetric determination of the lactate dehydrogenase (LDH) activity released from the cytosol of damaged cells (Cytotoxicity Detection Kit PLUS). HEK293T cells were seeded at concentrations of 1.25 x 10^4 cells/well in 50 µL of appropriate medium, and adhesion was allowed overnight. The eukaryotic cells were exposed to increasing concentrations of SPI009 for 24 h, after which LDH activity was measured according to the manufacturer's guidelines. The relative cytotoxicity was determined as stated below, with low and high controls consisting of treatment of the eukaryotic cells with medium or lysis buffer, respectively:

$$Cytotoxicity\ (\%) = \frac{(Sample\ value - low\ control)}{(High\ control - low\ control)}\ x\ 100$$

Hemolytic assay. Hemolytic activity was assessed as described previously[273], with minor modifications. Defibrinated whole blood from horses (Oxoid) was washed three times with 10 mM Tris-HCl 0.9% NaCl pH 7.4 (centrifugation at 1000 x *g*, 10 min, 4°C) with sequential removing of the buffy coat. Washed erythrocytes were diluted to a final concentration of 2% in 10 mM Tris-HCl 0.9% HCl pH 7.4 and pre-incubated in 1 mL volumes for 10 min (37 °C). Erythrocytes (190 µL) were mixed with 10 µL of the different testing agents, and incubated at 37°C for 1 h. 10 mM Tris-HCl 0.9% HCl pH 7.4 and 0.1% Triton X-100 were used as negative and positive controls, respectively. The test concentrations of SPI009 ranged from 8.5 to 34 µg/mL. After incubation, the erythrocyte solutions were centrifuged for 5 min (3000 x *g*) and the absorbance of the supernatant was measured at 540 nm (Biotek multimode reader) to assess hemolytic damage. The values for the background controls were subtracted from the OD_{540} values, and % hemolysis relative to that for the positive control (0.1% Triton X-100) was

determined. Statistical analysis was performed on control-corrected OD_{540} values using unpaired, one-way analysis of variance (ANOVA) testing with appropriate correction for multiple comparison (Dunnett's test) (significance level, $\alpha = 0.05$).

Statistical analysis. Unless mentioned otherwise, all statistical calculations were performed on $\log_{10}$-transformed data using GraphPad Prism software (version 6.01). The effect of the different treatments on the number of CFU was analyzed using unpaired, one-way ANOVA testing with appropriate correction for multiple comparison (Dunnett's test) (significance level, $\alpha = 0.05$). Averages are the result of at least three independent repeats.

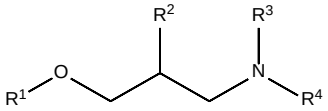
3.3 Results

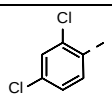
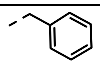
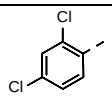
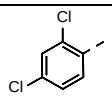
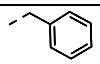
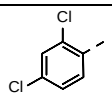
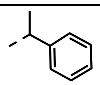
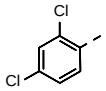
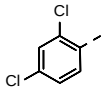
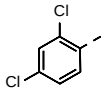
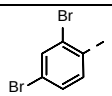
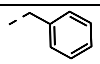
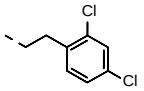
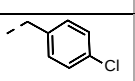
3.3.1 Identification and structure-activity relationship of anti-persister compound SPI009

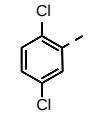
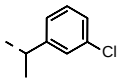
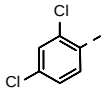
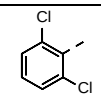
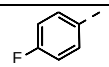
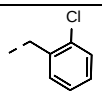
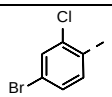
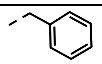
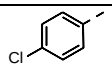
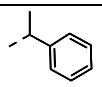
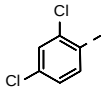
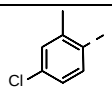
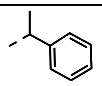
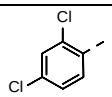
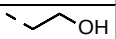
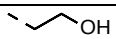
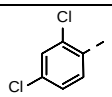
A small-molecule library comprising 23 909 diverse molecules[267] was screened to identify compounds that reduce the persister fraction of *P. aeruginosa* when they are used in combination with the fluoroquinolone antibiotic ofloxacin (10 µg/mL). To confirm the anti-persister activity of the identified compounds, a range of concentrations (0 to 200 µM) was tested and the efficacy was assessed through viable cell counting. From this analysis, SPI001 was selected for further characterization. To explore the effect of chemical modifications of the molecule on the observed activity, commercially available chemical analogues were purchased and evaluated for their anti-persister effect (Table 3.2). On the basis of preliminary experiments (data not shown), the evaluation of the analogues was carried out using a single concentration of 200 µM, corresponding to 68 µg/mL, in combination with 10 µg/mL of ofloxacin. Among the analogues that significantly reduced the persister fraction, only SPI009, SPI015 and SPI016 showed an increase in anti-persister activity compared to the original hit SPI001. SPI009, 1-((2,4-dichlorophenethyl)amino)-3-phenoxypropan-2-ol, had the most pronounced effect, reducing the persister fraction ~7 200-fold ($P < 0.0001$) compared to that achieved with treatment with ofloxacin alone. From a structure-activity relationship (SAR) point of view, it is worth noticing that the 7 most active compounds (SPI001, SPI004, SPI009, SPI010, SPI015, SPI016, SPI018) are all secondary amines (R^3 = hydrogen) whereas most of the other compounds are tertiary amines (except SPI011, SPI014 and SPI020). Moreover, the hydroxyl residue (R^2) does not seem to be essential for good biological activity (see the results

for SPI015), although this should be confirmed by the evaluation of additional analogues. Finally, it is striking that the most active analogue (SPI009) was the only compound bearing a phenethyl residue on R^4 instead of a benzyl residue, indicating that there is flexibility in the chain length. On the basis of all these results, SPI009 was selected for further characterization. The antibacterial activity of SPI009 was also evaluated by determination of the MIC value. The MIC value, defined as the minimal concentration to completely inhibit bacterial growth, as calculated for *P. aeruginosa* in 1:20 TSB was 150 µM (corresponding to approximately 51 µg/mL).

Table 3.2: SAR for SPI001 and chemical analogs



Code	R^1	R^2	R^3	R^4	Log_{10} (CFU /mL)[a]	Fold decrease compared to ofloxacin	P-value[b]
SPI001		OH	H		1.90	2527.1	<0.05
SPI002		OH			5.53	0.6	NS
SPI003		OH			5.57	0.5	NS
SPI004		OH	H		3.05	176.4	NS
SPI005		OH			4.95	2.2	NS
SPI006		OH			5.53	0.6	NS
SPI007		OH			5.14	1.4	NS
SPI008		OH			4.89	2.6	NS
SPI009		OH	H		1.44	7198.2	<0.0005
SPI010		OH	H		3.71	39.2	<0.005

Code	R^1	R^2	R^3	R^4	Log_{10} (CFU /mL)[a]	Fold decrease compared to ofloxacin	P-value[b]
SPI011		OH	H		4.87	2.7	NS
SPI012		OH			4.76	3.5	NS
SPI013		OH			4.85	2.8	NS
SPI014		OH	H		5.08	1.6	NS
SPI015		H	H		1.74	3647.2	<0.0005
SPI016		OH	H		1.71	3913.6	<0.05
SPI017		OH	CH_3		5.68	0.4	NS
SPI018		OH	H		3.15	142.1	<0.0005
SPI019		OH			5.54	0.6	NS
SPI020		OH	H		5.25	1.1	NS
DMSO 1%					5.30	1.0	

[a] Log (CFU/mL) represents the number of bacteria surviving after the 5-h treatment of a stationary-phase culture with the combination of 10 µg/mL ofloxacin and 200 µM compound; [b]P-value compared to the results for the DMSO control treatment. NS, no statistically significant difference (P > 0.05).

3.3.2 SPI009 directly kills isolated persister cells

To investigate whether SPI009 needs to be administered simultaneously with ofloxacin for maximal activity, the compound was added at different time-points during ofloxacin treatment. For this, *P. aeruginosa* stationary-phase cells were first treated with ofloxacin (10 µg/mL) and SPI009 was subsequently added after 0, 5 and 24 hours (Figure 3.1a to c). For each experiment, complete eradication of the bacterial population was achieved within 24 h after adding SPI009. These results indicate that SPI009 can be administrated at any point during treatment without affecting its activity, thus broadening treatment options.

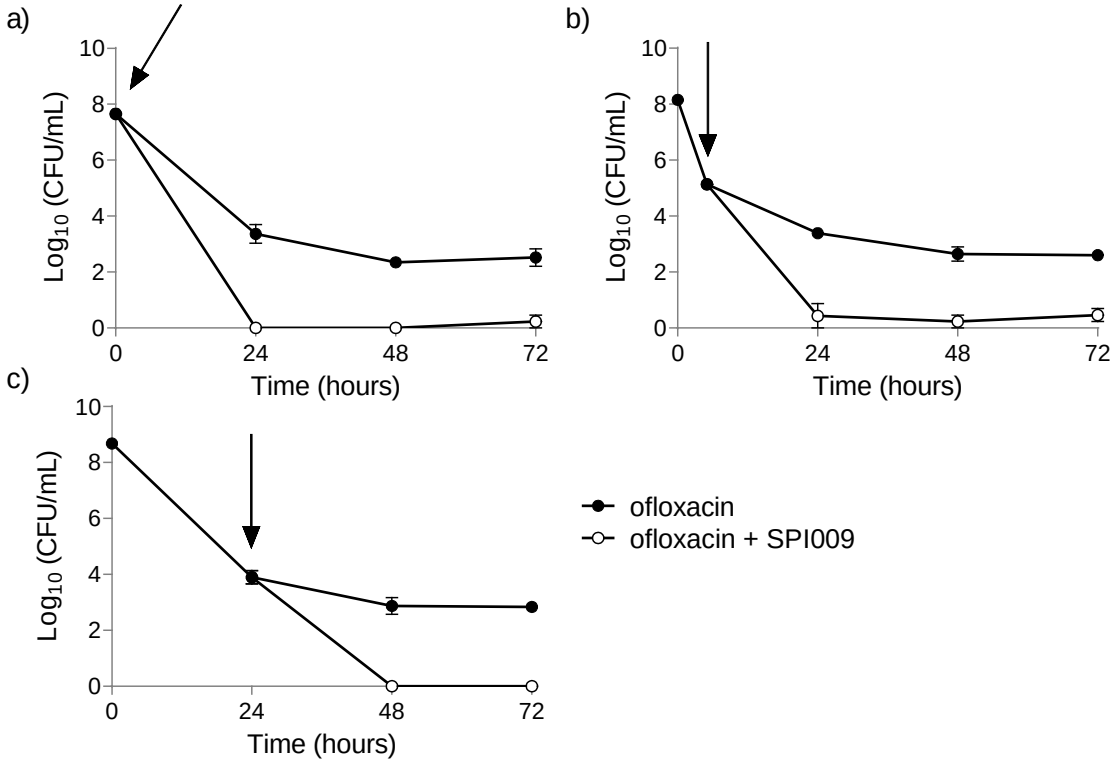


Figure 3.1: Timing of SPI009 addition to ofloxacin treatment does not influence activity. Stationary phase cells of a PA14 WT culture were treated for 72 hours with ofloxacin (OFX; filled circles) or the combination of ofloxacin with 68 µg/mL SPI009 (open circles). SPI009 was added to the cultures at a) the beginning of treatment or b) 5 or c) 24 hours after the onset of treatment. During treatment the number of viable cells was determined at 24, 48 and 72 hours by means of CFU counting. Addition of the solvent DMSO (1%, v:v) did not result in any significant killing of the bacterial cells (data not shown). Data points represent the averages of three independent repeats; error bars indicate Standard Error of the Mean (SEM).

Based on these results, we conclude that SPI009 may act in two different ways. Either SPI009 wakes up persister cells, thereby rendering them sensitive to the bactericidal action of ofloxacin, or SPI009 kills the persister cells directly. Examples of both strategies have been reported in the literature.[129,170,175,250] To discriminate between these two possibilities, persister cells were isolated and treated with either 10 µg/mL ofloxacin, 17 to 68 µg/mL SPI009 or a combination of ofloxacin and SPI009 (Figure 3.2). As expected, treatment of the

isolated persisters with ofloxacin alone only caused a minor decrease in the number of surviving cells, indicating the effective isolation of persister cells. In contrast, treatment of isolated persister cells with SPI009 caused a significant decrease in the number of surviving cells ranging between 0.71 ± 0.24 log units and complete eradication compared to the number of surviving cells with ofloxacin treatment. Treatment with the combination of SPI009 at 34 µg/mL with ofloxacin was able to completely eliminate all bacterial cells (Figure 3.2). These results show that SPI009 is capable of directly killing persister cells, even in the absence of antibiotics.

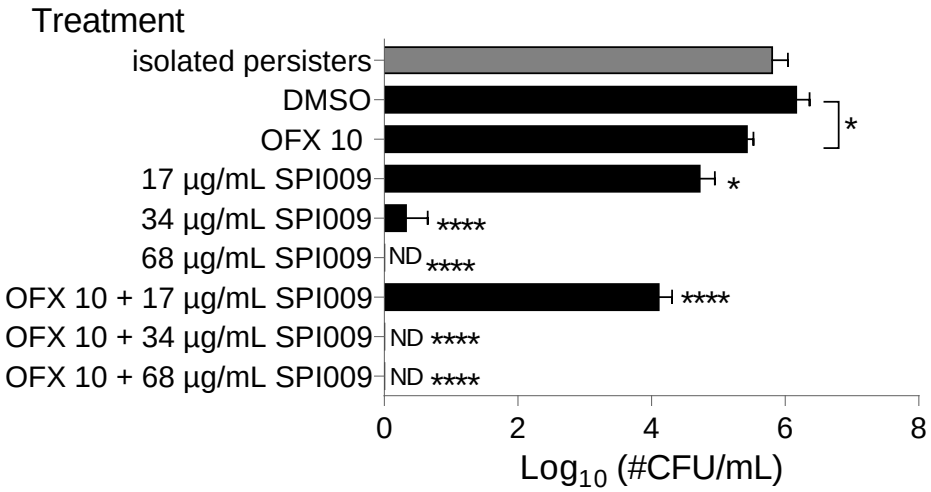


Figure 3.2: SPI009 directly kills isolated persister cells. Persister cells were isolated by means of ofloxacin treatment, after which they were treated for 5 hours with either 1% DMSO, 10 µg/mL ofloxacin (OFX), 17 to 68 µg/mL SPI009 or the combination of ofloxacin with SPI009. After treatment, cells were washed, diluted and plated out to determine the number of surviving persister cells. Data points correspond to the mean of three independent repeats, error bars represent SEM-values. Significant differences are determined on log10-transformed data in comparison to ofloxacin treatment. * $P \leq 0.05$, **** $P \leq 0.0001$. ND, not detected.

3.3.3 SPI009 is capable of killing both normal and persister cells of *P. aeruginosa*

Previous experiments have clearly shown that SPI009 efficiently targets persister cells. To gain more information about the bactericidal effect on normal, non-persister cells, stationary-phase cultures were treated with 10 µg/mL ofloxacin or 17 to 68 µg/mL of SPI009. As expected, treatment with 10 µg/mL of ofloxacin caused a significant decrease in the number of surviving cells and allowed only persister cells to survive. Treatment of the culture with 34 or 68 µg/mL of SPI009 alone also significantly decreased the number of surviving cells as compared to the control, with reductions of 3.22 ± 0.38 and 6.36 ± 0.39 log units, respectively

(Figure 3.3). Since these decreases are larger than the expected persister fraction, the results obtained clearly show that SPI009 is capable of killing both persister and normal cells.

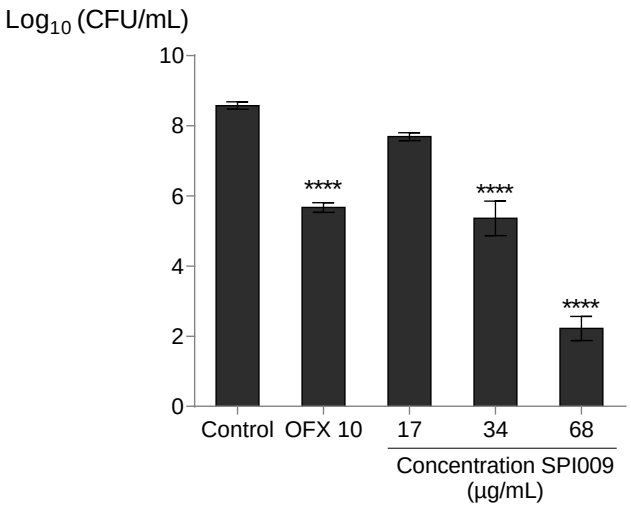


Figure 3.3: SPI009 targets both normal and persister cells. Stationary phase cells of *P. aeruginosa* PA14 were treated for 5 hours with 1% DMSO (control), 10 µg/mL ofloxacin (OFX) or 17 to 68 µg/mL of SPI009. After treatment, cells where washed, diluted and plated onto solid medium to determine the number of surviving cells. Data points represent the mean of three independent repeats. Error bars represent SEM. Statistical analysis was performed on log-transformed CFU data comparing different treatment conditions with the effect of ofloxacin treatment, where **** $P \leq 0.0001$. ND, not detected.

3.3.4 Killing kinetics of SPI009 as mono- and co-therapy

To assess the killing kinetics of SPI009, stationary-phase cultures were exposed to different treatments from 15 minutes to 24 h. In order to evaluate both the antibacterial and the anti-persister effect of the compound, cells were treated with SPI009 alone (17 to 34 µg/mL) or with the combination of SPI009 and ofloxacin (10 µg/mL), respectively. The time-kill curves obtained show a slight biphasic pattern, where most killing was obtained in the first 3 to 4 h (Figure 3.4). When the treatments were compared, treatment with the combination of ofloxacin and SPI009 always outcompeted the monotherapies. The combination of ofloxacin with 17 or 34 µg/mL completely eradicated the culture after 24 h or 5 h of treatment, respectively. In comparison, 5 h treatment with 10 µg/mL ofloxacin or 34 µg/mL SPI009 alone caused a 2.87 ± 0.35 log unit and 4.14 ± 0.35 log unit decrease in the number of surviving cells, respectively. As suspected, use of SPI009 alone also had an effect on the bacterial culture, causing maximal 2.36 ± 0.38 and 4.38 ± 0.42 log unit decrease after 3 h of treatment with 17 or 34 µg/mL, respectively. The effect of SPI009 was comparable to that of ofloxacin, with a minor increase in bacterial CFUs after 3 h for SPI009.

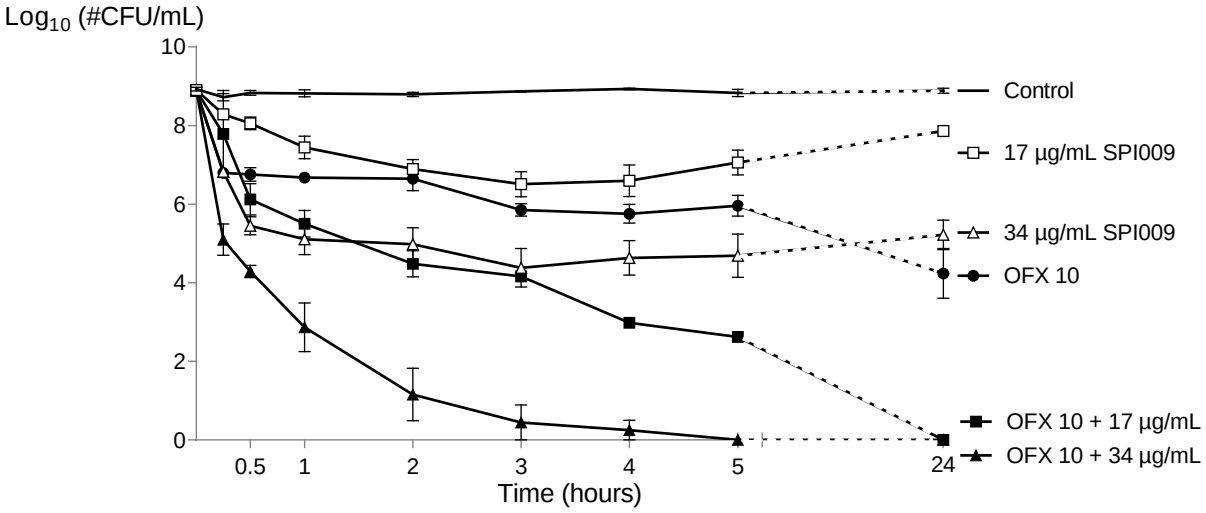


Figure 3.4: Killing kinetics of SPI009 as a mono- or combination therapy. Stationary phase cells were treated with 1% DSMO (control, solid line); 10 µg/mL ofloxacin (OFX; filled circles); 17 or 34 µg/mL SPI009 (open squares and triangles, respectively) or the combination treatment of ofloxacin with 17 or 34 µg/mL SPI009 (filled squares and triangles, respectively). Samples were taken 15', 30', 1 h, 2 h, 3 h, 4 h, 5 h and 24 hours after the onset of treatment after which they were washed and appropriate dilutions were plated out to assess the number of surviving cells. Data points represent the averages of at least three independent repeats with error bars indicating SEM values.

3.3.5 Activity of SPI009 is antibiotic independent

In addition to ofloxacin, other antibiotics used to treat *P. aeruginosa* infections in the clinic include the aminoglycoside amikacin and the cephalosporin ceftazidime.[274] As shown in Figure 3.5a, 5 h of treatment with SPI009 in combination with 75 µg/ml amikacin completely eradicated the stationary-phase culture, while treatment with treatment with the combination of SPI009 at 17 or 34 µg/mL and ofloxacin resulted in significant 3.36 ± 0.45 and 5.58 ± 0.45 log unit decreases, respectively. The treatment with the combination of ceftazidime and 34 µg/mL SPI009 also significantly reduced the persister fraction (> 3 500 fold) in an exponentially growing culture (Figure 3.5b). These results clearly show that SPI009 can be combined with different classes of antibiotics and thus possesses antibiotic-independent activity.

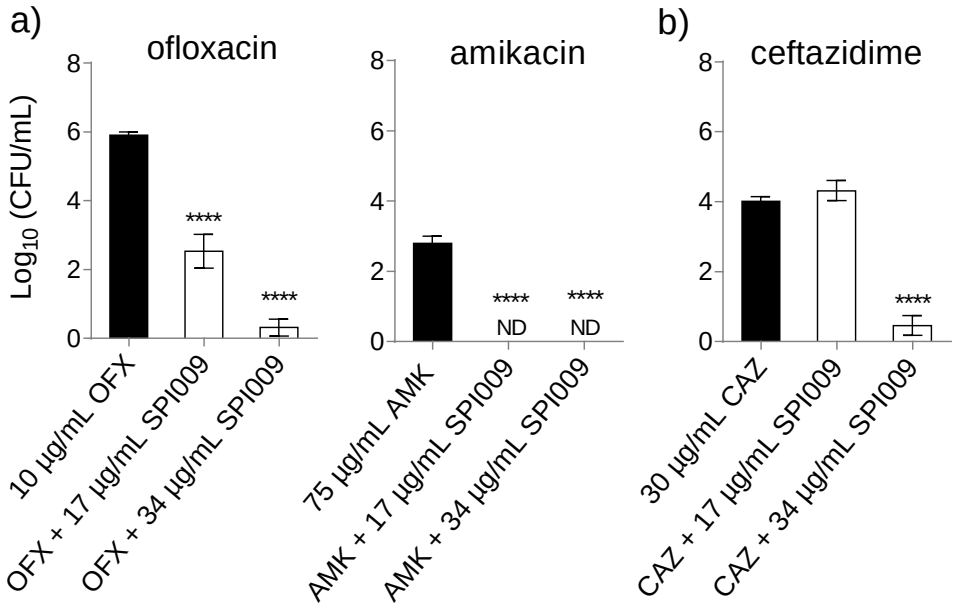


Figure 3.5: The combination of SPI009 with antibiotic of distinct classes reveals an antibiotic-independent effect. a) Stationary phase cells and b) exponential phase cells of *P. aeruginosa* PA14 were treated for 5 hours with a) ofloxacin (OFX, 10 µg/mL), amikacin (AMK, 75 µg/ml) or b) ceftazidime (CAZ, 30 µg/mL), respectively, in combination with 1% DMSO (black bars) or 17-34 µg/mL SPI009 (white bars). Data points correspond to the mean of three independent repeats. Error bars represent SEM. ND, not detected. Statistical analysis compared the effect of antibiotic treatment alone with that of the different combination treatments with significance corresponding to **** $P \leq 0.0001$. ND, not detected.

3.3.6 SPI009 shows potent activity against several clinical isolates

To test whether SPI009 is also active against other clinically relevant strains, we selected five *P. aeruginosa* strains to obtain a panel of isolates that originated from different human sources, such as burn wounds, urine, throat and bronchus, and with different resistance patterns. Being one of the most dominant bacterial species in the lungs of CF patients, several *P. aeruginosa* isolates from the sputum or bronchus of CF patients were included in the panel. Ofloxacin concentrations were optimized for each strain (data not shown). Stationary-phase cells of the different cultures were treated with 10 or 100 µg/mL ofloxacin and the combination of ofloxacin with 17 or 34 µg/mL of SPI009. Six out of the eight isolates proved extremely susceptible to killing by the combination treatment, resulting in a (nearly) complete eradication of the bacterial population (Figure 3.6). For the least sensitive strains AA249, which was resistant to aztreonam, ciprofloxacin, ceftazidime, imipenem and meropenem, the combination of ofloxacin with 17 or 34 µg/mL SPI009 still resulted in a significant 2.06 ± 0.55 and 4.69 ± 0.55 log unit reduction in the number of surviving cells, respectively. Evaluation of isolates derived from CF patients showed modest sensitivity to ofloxacin and demonstrated the potent activity of SPI009, resulting in 4.1 ± 0.3, 4.8 ± 0.3 and 3.7 ± 0.5 log unit decreases

in surviving cells when treated with the combination of ofloxacin and 34 µg/mL SPI009 for DAF87-203, PA1256 and PA1255, respectively. In summary, SPI009 showed potent anti-persister activity against all clinical isolates tested, independently of the source of isolation, and was even capable of strongly reducing the survival of multidrug-resistant strains and isolates from CF patients.

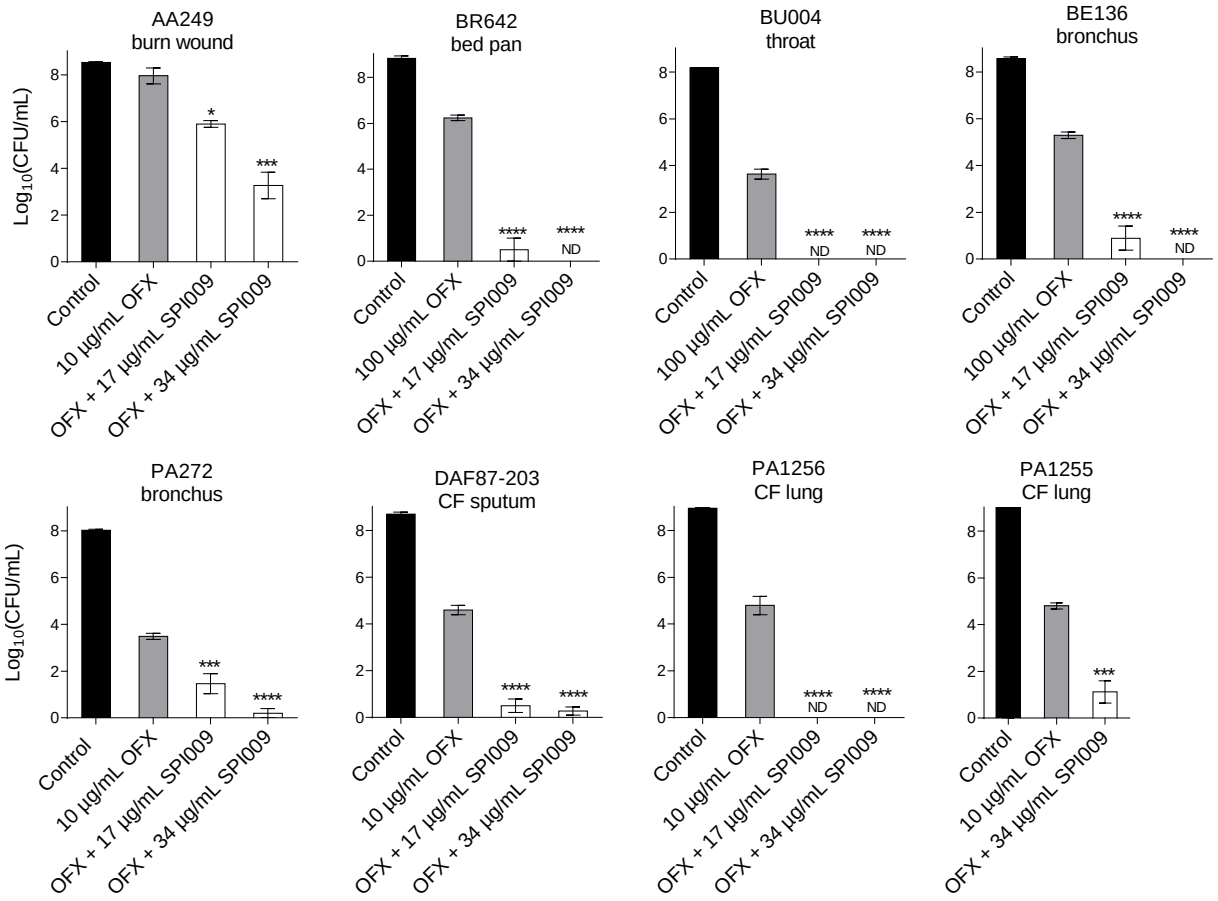


Figure 3.6: The effect of SPI009 against several clinical *P. aeruginosa* isolates. Stationary phase cells of several clinical isolates were treated for 5 h with DMSO (0.5%, black bars), ofloxacin (OFX) alone (grey bars) or combined with SPI009 (white bars). After treatment, cells were washed, diluted and plated onto solid medium to determine the number of surviving cells. Data points represent the means of at least three independent repeats, error bars represent SEM. Statistical analysis was done on log10-transformed CFU data with * $P \leq 0.05$, *** $P \leq 0.001$, **** $P \leq 0.0001$ representing significant differences to the ofloxacin treated control.. ND, not detected.

3.3.7 SPI009 causes no significant cytotoxicity or hemolysis

The *in vitro* activity of SPI009 was clearly demonstrated in different experimental setups. An important factor determining its *in vivo* potential is the cytotoxicity of SPI009 in eukaryotic cell lines. For this, embryonic kidney HEK293T cells were treated with increasing concentrations of SPI009, ranging from 4.25 to 136 µg/mL for 24 h. The cytotoxicity was determined by spectroscopic detection of lactate dehydrogenase (LDH) with three independent repeats resulting in a 50% inhibitory concentration (IC_{50}) value of 32.3 ± 0.81

µg/mL. Another important factor to be considered for the clinical use of SPI009 is possible hemolytic activity. This was evaluated by exposing horse erythrocytes to increasing concentrations of SPI009 (8.5 to 34 µg/mL) for 1 h. The absorbance at 540 nm was measured, and the percent hemolysis relative to that for the positive control (treated with 0.1 % Triton X-100) was determined. The results indicated no significant difference between the results obtained with the negative control or DMSO (carrier control; 0.5%) and concentrations of up to 34 µg/mL SPI009 or between the results obtained with the different testing agents and 0% hemolysis (see Figure 3.7 in the supplemental material). While the lack of any hemolytic activity is promising, additional adaptation of the chemical structure of SPI009 is desirable to further decrease the moderate cytotoxic effects without affecting its antibacterial properties when applications other than topical treatment of infections are envisioned.

3.4 Discussion

The resistance of *P. aeruginosa* to the most commonly used antibiotics is rapidly increasing worldwide and even resistance against colistin and polymyxin B, the antibiotics currently used as a last resort in the treatment of *P. aeruginosa* infections, has been reported.[101,262,275] Besides this rapidly increasing multidrug resistance, treatment of *P. aeruginosa* infections is further compromised by the ability to form biofilms and the presence of an antibiotic-tolerant persister fraction. Persister cells are able to withstand antibiotic treatment, thereby greatly hampering efficient eradication of the bacterial infection, contributing to the recalcitrant nature of chronic infections and increasing the chance of resistance development.[29,59,72] Multiple international organizations and research groups acknowledge the increasing threat of bacterial infections, predicted to cause over 10 million deaths annually by 2050[265], and research into new antibacterial and anti-persister therapies is increasing. In this study, a screen to search for novel anti-persister compounds capable of significantly decreasing the persister fraction in combination with the fluoroquinolone antibiotic ofloxacin led to the identification of 1-((2,4-dichlorophenethyl)amino)-3-phenoxypropan-2-ol (SPI009).

Small persister numbers, the different parallel mechanisms behind their formation and conservation and the still limited knowledge greatly impede the rational design of target-based anti-persister therapies. Nevertheless, several anti-persister molecules have been described in the literature. Theoretically, there are three approaches to combat bacterial persister cells:, (i)directly killing persister cells, (ii) waking up persisters and thereby rendering

them sensitive to an antibiotic, and (iii) preventing the formation of persister cells. Experiments on both isolated persister cells (Figure 3.2) and the population level (Figure 3.3) revealed the ability of SPI009 to significantly decrease the number of surviving cells when it was added alone. These results clearly demonstrate that SPI009 does not depend on ofloxacin to exert its activity and is capable of directly killing persister cells, categorizing SPI009 in the first class of anti-persister molecules. Other examples of this class include membrane-acting molecules such as peptides shown to target *E. coli* cells, including persisters[166], and NH125, a compound identified through large-scale screening capable of eradicating methicillin-resistant *Staphylococcus aureus* persisters through membrane permeabilization.[216] ADEP4, an acyldepsipeptide, promotes self-digestion in *S. aureus* through constitutive activation of the ClpP protease, and combination with rifampicin completely eradicated biofilm infections *in vitro* and *in vivo.*[170] More recently, the anti-cancer drugs mitomycin C was described to actively kill persister cells of *E. coli*, *S. aureus* and *P. aeruginosa.*[171] A more rational approach was used in the development of the Artilysin® Art-175, consisting of a bacteriophage-encoded endolysin coupled to a peptide for guidance through the bacterial outer membrane. Art-175 is capable of puncturing the peptidoglycan, resulting in cell lysis, and was shown to be active against persister cells of both *P. aeruginosa*[129] and *Acinetobacter baumannii*[130]*.* Another target-based approach led to the identification of a group of quorum-sensing (QS) inhibitors that specifically target the *P. aeruginosa* MvfR system. Besides disrupting cell-to-cell communication and decreasing infection, these compounds were the first molecules identified to limit the formation of persister cells in *P. aeruginosa*.[152]

When a normal bacterial population consisting of both persister and non-persister cells was treated with SPI0009, it was observed that the activity of the compound was not restricted to non-dividing persister cells but also encompassed normal, non-persister cells (Figure 3.3). MIC assays however, revealed a relatively high concentration of 150 µM for SPI009, corresponding to 51 µg/mL, well above the MIC values for most conventional antipseudomonal antibiotics.[276] Taken together, these results suggest a primary activity against non-dividing or persister cells, with SPI009 having an advantageous secondary effect against normal, actively dividing cells. Coates and coworkers even suggested the use of alternative, more relevant parameters such as the minimal stationary cidal concentrations (MSC) or minimal dormicidal concentration (MDC) for evaluation of the activities of compounds active against non-dividing cells.[159,215,277] Experiments were further focused on stationary phase cultures since the absence of active growth, a higher persister fraction and

nutritional starvation result in a more pronounced antibiotic tolerance and, partly due to the similarities in biofilms, increased clinical relevance.[29,64] Other anti-persister molecules primarily aimed at targeting non-multiplying cells have been described for *Mycobacterium tuberculosis*[221] and *S. aureus*[194].

While the initial screening was done in combination with ofloxacin, further experiments were undertaken to explore the combination spectrum of SPI009. The limitation of described anti-persister therapies often lies in the fact that they increase the susceptibility of persister cells to one or a limited number of antibiotics. It has, however, been suggested that the multidrug-tolerant persister population is actually composed of several subpopulations of persister cells, each of which is characterized by its own tolerance profile. This hypothesis is supported by the observed differences in persister fraction upon treatment of a population with distinct classes of antibiotics.[55,62,63,278] Previous results already indicated an antibiotic-independent effect and additional experiments showed that SPI009 is capable of reducing the persister fraction in combination with at least two additional mechanistically different classes of antibiotics (Figure 3.5). Combination of SPI009 with the commonly used aminoglycoside amikacin, which acts by inhibiting translation, or ceftazidime, a cephalosporin antibiotic acting on cell wall biogenesis, resulted in complete eradication or significant decreases in the number of surviving cells, proving that SPI009 is capable of targeting multiple subpopulations of persisters. For all antibiotics tested, the addition of SPI009 clearly enhanced bacterial killing, making it a good candidate for the case-specific design of antibacterial co-therapies. The increasing rates of resistance to most antibiotics, together with the current lack of novel antipseudomonal compounds has recently resulted in the increased use of antibiotic combination therapies. Although the possible negative side effects need to be studied in more detail, addition of a different antibiotic or non-antibiotic adjuvant to conventional therapy has the potential to lower the concentrations of both agents, reduce treatment times, combine different mode of actions, and facilitate the treatment of resistant strains.[110,111,279]

The currently described anti-persister molecules can be classified into three broad categories based on their mechanism of action; (i) compounds that directly kill persister cells, (ii) compounds that sensitize persister cells to antibiotic killing, and (iii) compounds that prevent or decrease persister formation. The ability of SPI009 to directly kill persister cells, combined with its excellent activity in combination with distinct classes of antibiotics suggests that SPI009 belongs to this first class. Several compounds capable of directly killing persister

cells have been described and use different strategies to kill persister cells, such as depolarization and destruction of the cell membrane, DNA cross-linking, inhibition of essential enzymes, and generation of reactive oxygen species.[223,280,281] Additional research will be necessary to further unravel the mode of action, but preliminary data suggest that SPI009 acts primarily by causing membrane damage (see Chapter 5).

In conclusion, we identified 1-((2,4-dichlorophenethyl)amino)-3-phenoxypropan-2-ol to be a promising compound for use in the development of future anti-persister therapies. SPI009 is capable of significantly reducing or even eliminating the persister fraction of the Gram-negative pathogen *P. aeruginosa* by direct killing of antibiotic-tolerant persister cells. Due to its activity against both non-dividing and dividing cells and the possibility to combine SPI009 with mechanistically different classes of antibiotics, this molecule has the potential to serve as an adjuvant in antibacterial combination therapies to treat infections containing a mix of bacteria in different growth phases. The absence of any hemolytic effects and the limited cytotoxicity, together with the possibility to further adapt the chemical structure to increase the selectivity index, encourage further research into SPI009 and the development of novel antibacterial strategies in the fight against chronic infections.

3.5 Supplemental material

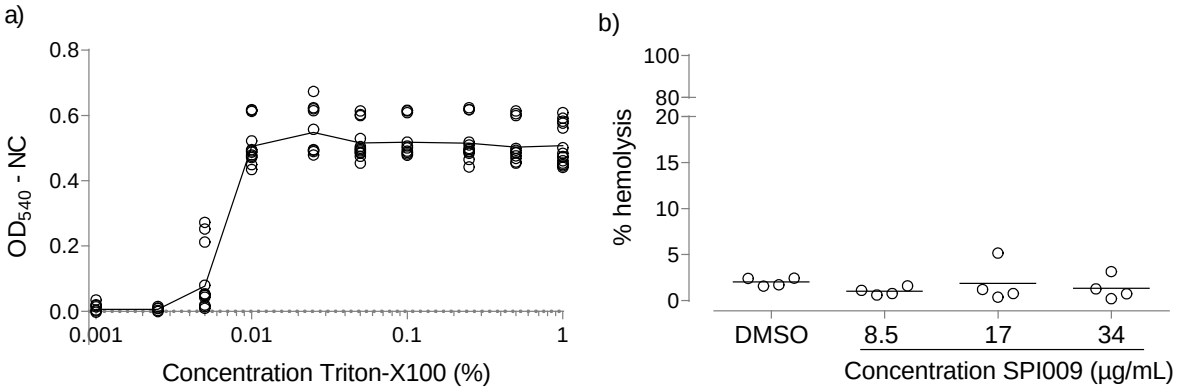


Figure 3.7: SPI009 causes no hemolytic damage. a) Treatment of defibrillated horse erythrocytes with different concentrations of Triton X-100 to evaluate maximal hemolysis. No further increase in hemolysis was detected at concentration exceeding 0.01%. b) Hemolytic effects of DMSO (0.5%) and 8.5 to 34 µg/mL of SPI009. Individual data points represent % hemolysis relative to the positive control, 0.1% Triton X-100, at independent repeats. Statistical analysis revealed no significant differences between the different treatments and the carrier, DMSO. None of the testing agents resulted in an observed hemolysis significantly different from 0 %.

3.6 Additional data to the above-mentioned manuscript

To further confirm the obtained CFU data, the LIVE/DEAD Baclight™ kit (Live Technologies Europe) was used to assess bacterial viability after treatment with SPI009. The bacterial LIVE/DEAD kit consists of two complementary dyes, SYTO 9 and propidium iodide (PI). SYTO 9 is a nucleic acid stain showing a bright green fluorescence and capable of entering both living and dead cells. PI, a red fluorescent intercalating stain, will only enter cells that are dead or have a compromised membrane.[282]

Overnight cultures of *P. aeruginosa* were treated for 15, 30 or 60 minutes with increasing concentrations of SPI009, after which cells were washed and stained with a LIVE/DEAD mixture containing 10 µM SYTO 9 and 60 µM of PI. Stained cells were plated onto 2% agarose pads to avoid bacterial movement and visualized using a Zeiss Axio Imager.Z1 fluorescence microscope equipped with an EC Plan-NEOFLUAR 100 x objective using a green (λ_{ex}= 493 nm, λ_{em}= 513 nm) and red (λ_{ex}= 592 nm, λ_{em}= 614 nm) fluorescent channel. For each time point and treatment, green and red fluorescent cells were counted using Zen 2.0 software and cell viability was determined as the percentage of green cells relative to the total number of cells (Figure 3.8). Statistical analysis was done by means of a two-way ANOVA, where the effect of the different treatments was compared with the control, for every time point.

As shown in Figure 3.8, treatment of *P. aeruginosa* with increasing concentrations of SPI009 causes a clear decrease in the number of viable cells. While one hour treatment with 17 µg/mL of SPI009 reduces bacterial viability by 59%, treatment with 34 µg/mL or 51 µg/mL (1 x MIC) of SPI009 result in 3.6% and 2.1% survival, respectively. Statistical analysis of the obtained results shows a significant decrease in cell viability upon treatment with different concentrations of SPI009, and this for all time points tested. Overall, these experiments corroborate the time-kill curves obtained by CFU counts and microscopically confirm the quick decrease in membrane integrity and bacterial viability caused by SPI009 treatment.

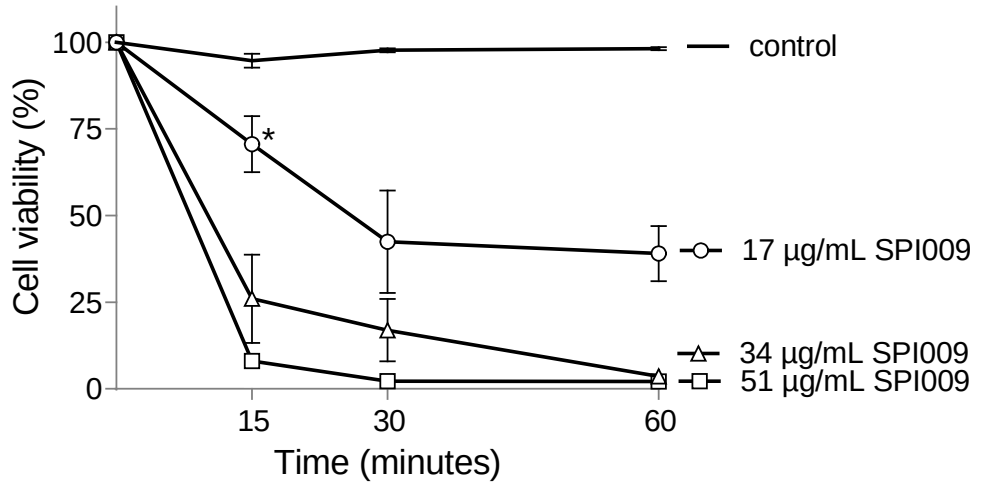


Figure 3.8: Microscopic assessment of cell viability. Overnight cultures of *P. aeruginosa* were treated for 15, 30 or 60 minutes with DMSO (1.5%; carrier control), 17, 34 or 51 µg/mL SPI009. After treatment, cells were stained using the BacLight Live/Dead kit and visualized using a Zeiss Axio Imager Z1 fluorescence microscope equipped with an EC Plan-NEOFLUAR 100 x objective. Data points represent the averages of three independent repeats ± SEM. Statistical analysis was done by means of two-way ANOVA ($\alpha = 0.05$) with Dunnett's correction for multiple comparison. All treatment times and concentrations, unless indicated otherwise (* $P < 0.05$), resulted in $P < 0.0001$.

Chapter 4

Antibacterial activity of SPI009 against antibiotic-resistant strains of diverse bacterial pathogens, biofilms and in pre-clinical infection models

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Contributions of individual authors to the manuscript:

Experiments were designed by **Valerie Defraine**, Maarten Fauvart and Jan Michiels and performed by **Valerie Defraine**. The *in vivo C. elegans* experiments were performed by Laure Verstraete. The intracellular THP-1 model and chronic wound biofilm model were designed by Ahalieyah Anantharajah and Françoise Van Bambeke, and Eleanor M. Townsend and Gordon Ramage, respectively. The data was analyzed and the manuscript written by **Valerie Defraine**. The manuscript was revised by Maarten Fauvart, Jan Michiels, Françoise Van Bambeke and Gordon Ramage.

Abstract

We recently described the novel anti-persister compound 1-((2,4-dichlorophenethyl)amino)-3-phenoxypropan-2-ol (SPI009), capable of directly killing persister cells of the Gram-negative pathogen *Pseudomonas aeruginosa*. This compound also shows antibacterial effects against non-persister cells, suggesting that SPI009 could be used as an adjuvant for antibacterial combination therapy. Here, we demonstrate the broad-spectrum activity of SPI009, combined with different classes of antibiotics, against the clinically relevant ESKAPE pathogens (*Enterobacter aerogenes, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, P. aeruginosa, Enterococcus faecium*), *Burkholderia cenocepacia* and *Escherichia coli.* Importantly, SPI009 re-enabled killing of antibiotic-resistant strains and effectively lowered the required antibiotic concentrations. The clinical potential was further confirmed in biofilm models of *P. aeruginosa* and *S. aureus* where SPI009 exhibited effective biofilm inhibition and eradication. *Caenorhabditis elegans* infected with *P. aeruginosa* also showed a significantly increased survival when SPI009 was added to conventional antibiotic treatment. Overall, we demonstrate that SPI009, initially discovered as an anti-persister molecule in *P. aeruginosa*, possesses broad-spectrum activity and is highly suitable for the development of antibacterial combination therapies in the fight against chronic infections.

4.1 Introduction

Antibiotic resistance is rapidly increasing in the majority of nosocomial pathogens, complicating the effective treatment of bacterial infections and transforming once easily cured diseases into serious human health threats.[21,265] Although selection for resistance in micro-organisms is inevitable, the widespread and excessive use of antibiotics allowed pathogens to efficiently adapt to these stressful conditions, resulting in the occurrence of extensively drug-resistant and pan-drug-resistant strains.[283,284] In an attempt to guide research and development towards the most critical pathogens, the World Health Organization (WHO) recently published their 'global priority list', containing 12 bacterial pathogens that raise particular concern.[19] Among these are the so-called ESKAPE pathogens, *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, P. aeruginosa* and *Enterobacter* spp., which efficiently evade antibiotic treatment and represent new paradigms in pathogenesis, transmission, and resistance.[285] Together, this select group of bacteria is responsible for most of the hospital-acquired infections and, despite increasing research efforts, therapeutic options remain scarce.[261,286] Greatly contributing to the difficult treatment of these bacterial infections is the presence of non-growing persister cells. These phenotypic variants show a reduced metabolic activity, are able to withstand intensive antibiotic treatment, and, when antibiotic pressure drops, are capable of restoring the bacterial population, causing recurrence of infection.[29,223] Persistence is widely acknowledged as a major culprit of treatment failure in chronic and biofilm infections and recent research has identified the persister fraction as a possible reservoir for the development of resistance.[64,74] Effective elimination of persister cells could significantly improve patient outcomes, but their small numbers and the apparent redundancy in persister mechanisms greatly hampers the development of targeted anti-persister therapies.

We recently reported the identification of a novel anti-persister molecule capable of directly killing persister cells of *P. aeruginosa*.[161] In the present study, we explore the activity of SPI009 in several additional pathogens and demonstrate its broad-spectrum activity and the ability to sensitize resistant strains. Furthermore, SPI009 was shown to retain activity in different biofilm models and is capable of significantly improving antibiotic efficacy both in *in vitro* and *in vivo* infection models. Overall, these results further increase the clinical potential of SPI009 and offer compelling perspectives for the use of SPI009 as an adjuvant in effective antimicrobial therapies.

4.2 Materials and methods

Bacterial strains, human cell lines, *C. elegans* and culture conditions. Bacterial strains used in this study are listed in Table 4.1. All strains were cultured in 1:20 diluted Trypticase Soy Broth (1/20 TSB) at 37 °C shaking at 200 rpm. For solid medium, TSB was supplemented with 1.5% agar. Human THP-1 cell lines were cultivated in RPMI-1640 medium containing 10% fetal calf serum at 37°C with 5% CO_2. The *C. elegans* AU37 strain (g*lp-4(bn2); sek-1(km4))* was obtained from the *Caenorhabditis* Genetics Center (CGC) and maintained according to standards.[287] The following antibacterials were used: ofloxacin, ciprofloxacin, rifampicin, polymyxin B, vancomycin (Sigma Aldrich) and 1-((2,4-dichlorophenethyl)amino)-3-phenoxypropan-2-ol (SPI009; CD3) with concentrations indicated throughout the text.

Table 4.1: Strains used in this study

Strain	Description	Source or reference
P. aeruginosa PA14	UBCPP-PA14, KAN^R, wild type	Pierre Cornelis[8]
P. aeruginosa PAO1	Wild type	Dieter Haas (ETH)
P. aeruginosa PA62	Broncho-pulmonary clinical isolate OFX^R, CIP^R, GEN^R, AMK^R, ATM^R, TIC^R, PIP^R, TZP^R, CAZ^R, FEP^R	Françoise van Bambeke (UCL)
P. aeruginosa 9BR	Clinical isolate, PMB^R, MEM^R, CIP^R and FEP^R,CAZ^R or TZP^R	Robert Hancock, [14]
E. aerogenes	ATCC 13048 /LMG 2094	BCCM/LMG, [288]
S. aureus Rosenbach 1844	Wild type, methicillin resistant, ATCC 33591/ LMG 16217	BCCM/LMG, [170]
K. pneumoniae	ATCC 13883	[289]
A. baumannii	RUH134	Jean-Paul Pirnay, [290]
E. faecium	LMG 8148	[291]
B. cenocepacia K56-2	LMG 18863	[292]
E. coli BW25113	F^-,Δ(*araD-araB*)567, ΔlacZ4787(::*rrnB*-3), λ^-, *rph-1*, Δ(*rhaD-rhaB*)568, *hsd*R514	[293]

Resistance profiles determined according to EUCAST MIC breakpoints.[276] KAN= kanamycin, OFX= ofloxacin, CIP=ciprofloxacin, GEN= gentamicin, AMK= amikacin, ATM= aztreonam, TIC= ticarcillin, PIP= piperacillin, TZP= piperacillin-tazobactam, CAZ= ceftazidime, FEP= cefepime, PMB= polymyxin B.

Antibacterial assays. Antibacterial assays were performed on different clinically relevant pathogens as previously described.[161] Briefly, stationary phase cultures were treated for 5 hours with 17 or 34 μg/mL of SPI009 alone or in combination with an appropriate antibiotic to assess anti-bacterial and anti-persister effects, respectively. To evaluate activity against resistant strains, stationary phase cultures were treated for 5 hours with 1 x, 4 x and 8 x MIC

concentrations of the respective antibiotic; 17 or 34 µg/mL SPI009, or the combination of both. After treatment, cells were washed and viability was assessed via plating.

Quantification of biofilm formation and eradication after treatment with SPI009. Overnight cultures of *P. aeruginosa* PA14 or *S. aureus* ATCC 33591 were diluted 1:100 in 1/20 TSB medium supplemented with 2% DMSO (carrier control) or increasing concentrations of SPI009 (4.25 µg/mL to 68 µg/mL). Biofilms were grown for 24 hours at 37°C on the bottom of a polystyrene 96-well plate, non-shaking. Medium and free-living cells were removed and the biofilms were washed, scraped off and passed 5 times through a syringe (0.5 x 1.6 mm) to disrupt any cell clumps and obtain singe cells.[294] Appropriate dilutions made in 1 x PBS were plated on solid TSB agar plates to assess biofilm growth under different conditions.

To explore the biofilm eradicating effects of SPI009, overnight cultures of *P. aeruginosa* PA14 or *S. aureus* ATCC 33591 were diluted 1:100 in 1/20 TSB medium and incubated for 24 hours at 37°C (non-shaking). Mature biofilms were treated for five hours with 2% DMSO and increasing concentrations of SPI009 (8.5 µg/mL to 136 µg/mL) at 37°C, non-shaking, after which the remaining biofilms were processed as described above.

Chronic wound model. A three-dimensional wound biofilm model was used, as previously described.[295] *P. aeruginosa* PA14 coated cellulose matrices, obtained after 2h of adhesion (1 x 10^6 cells/mL), were placed onto the hydrogels after which 3D biofilm development was allowed for 24 hours at 37°C. Mature biofilms were treated for 24 hours with DMSO (1 %), 10 µg/mL ofloxacin, 34 and 69 µg/mL of SPI009 or the combination of ofloxacin and SPI009. Any non-adherent cells were removed by rinsing with 1x PBS, after which biomass was removed by sonication at 35 kilohertz for 10 min and DNA was extracted. After isolation of genomic DNA, samples were split in two, one half being treated with the DNA intercalating dye propidium monoazide. This will prevent the detection of DNA by PCR in cells with compromised membranes. The resulting viability based qPCR, using *Pseudomonas rpoD* specific primers F- GGGCGAAGAAGGAAATGGTC and R- CAGGTGGCGTAGGTGGAGAA, was used to determine live and total fractions of biofilm cells under different treatment conditions.[295,296] Standard curves were used to convert the obtained qPCR values to Colony Forming Estimates (CFE), after which $\log_{10}$-transformed values were used for statistical analysis, as described below. All experiments were carried out in triplicate, each containing three technical repeats.

Intracellular infection model. Infection of human THP-1 cells was performed as described previously, with minor modifications.[66] Since a newly synthesized batch of SPI009 was used for this experiment, cytotoxicity assessment via an LDH enzyme assay was repeated for the THP-1 cell line, as previously described.[161] After THP-1 infection with *P. aeruginosa* PAO1 and subsequent removal of any non-phagocytosed or adherent bacteria, ciprofloxacin and SPI009 were added in final concentrations of, respectively, 0 - 20 µg/mL and 6.8 or 10.2 µg/mL. After 5 h of treatment, eukaryotic cells were collected in three consecutive centrifugation steps and complete cell lysis was obtained by sonication (10 s). Lysates were used for bacterial CFU counting and determination of protein content by Lowry's assay (Bio-Rad DC protein assay kit; Bio-Rad laboratories, Hercules, CA). For analysis of surviving bacterial cells, CFU data were divided by corresponding protein content.

***C. elegans* toxicity testing and survival assay.** AU37 nematodes were synchronized as previously described[297] to obtain L4 worms suitable for toxicity and infection assays.[298] Larvae obtained after bleaching were plated onto solid NGM-OP50 agar plates and incubated at 25°C during 2 days to allow development of the worms to the L4 stage. Worms were transferred to fresh NGM agar plates containing OP50 (toxicity testing and uninfected control), or PA14 (infection) for an additional 24 hours at 25°C.

To evaluate toxicity of SPI009 L4 nematodes grown on OP50 were transferred to 12-well plates (20 - 30 worms/well) containing different concentrations of SPI009 (8.5 – 136 µg/mL) in 1.5 mL NGM:M9 (1:4). Controls consisted of untreated worms and DMSO (2% and 20%). For the infection assay, adult worms were allowed to feed on NGM-PA14 plates for 24 hours, after which residual bacteria were removed and nematodes were divided over a 12-well plate (20 - 30 worms/well). Different treatments were prepared in 1.5mL NGM:M9 (1:4) and consisted of an untreated control, 1.56 µg/mL ciprofloxacin (5 x MIC), 8.5 µg/mL of SPI009 and the combination of ciprofloxacin and SPI009. As an additional control, uninfected worms were included. For both assays, worms were incubated at 25°C and survival was scored visually for 6 days.

Statistical analysis. Unless mentioned otherwise, all statistical analyses were performed on $\log_{10}$-transformed data using GraphPad Prism software (version 6.01). Bacterial survival after different treatments was compared to the untreated or antibiotic control using a one-way ANOVA ($\alpha = 0.05$), with Dunnett's correction for multiple comparisons. Statistical comparison of mono- and combination treatment in resistant strains was done using a two-way ANOVA

(α = 0.05) with Tukey correction for multiple comparisons. Statistical analysis of the *in vivo C. elegans* data was done by means of a log-rank test using GraphPad Prism.

4.3 Results

4.3.1 SPI009 shows broad-spectrum activity against different clinically relevant bacterial species

The anti-persister activity of SPI009 was previously assessed in *P. aeruginosa* PA14 and several clinical isolates, where combination with ofloxacin significantly decreased the persister fraction in all strains tested.[161] In the present study, we challenged a panel of clinically relevant species, including the ESKAPE pathogens (Figure 4.1a), *B. cenocepacia* and *E. coli* (Figure 4.1b). For each species, appropriate concentrations of a conventional antibiotic used in the clinic were selected to allow only persister cells to survive (data not shown). Combination of the antibiotic with 17 µg/mL SPI009 significantly decreased the number of surviving bacteria for five of the eight species with reductions in CFU ranging between 1.5 ± 0.1 and 6.0 ± 0.2 log units and complete eradication of *K. pneumoniae*. Addition of 34 µg/mL completely eradicated the bacterial cultures of five of the eight species tested and resulted in significant 6.6 ± 0.5 log, 6.2 ± 1.3 log and 5.4 ± 0.5 log reductions in bacterial survival for *S. aureus*, *E. faecium* and *B. cenocepacia*, respectively. No significant reduction was survival is observed after treatment with 17 µg/mL for either of the Gram-positive species, *E. faecium* and *S. aureus.* These results suggest that the latter two species, and the Gram-negative *B. cenocepacia*, are slightly less sensitive towards the combination therapy. *K. pneumoniae* proved the most susceptible species towards SPI009. Overall, the obtained results further support the antibacterial effect of SPI009 and reveal a broad-spectrum activity.

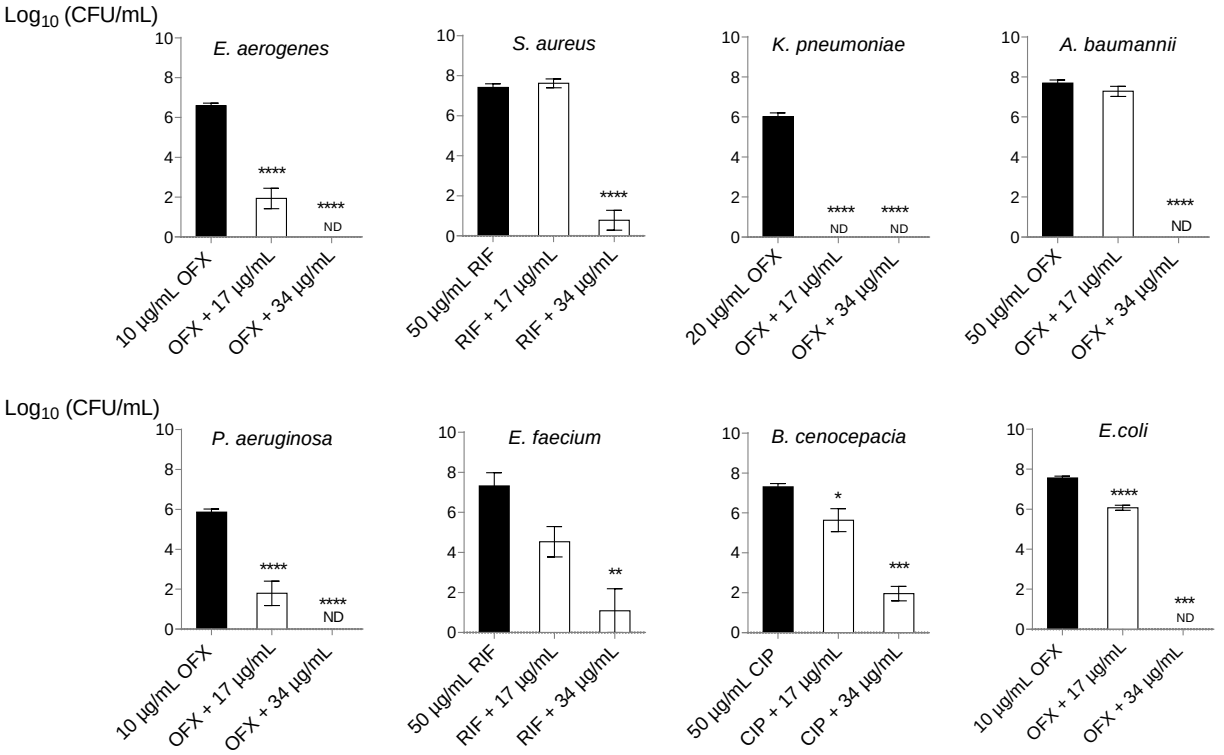


Figure 4.1: SPI009 possesses broad-spectrum activity against different clinically important pathogens. 200 µL volumes of stationary phase cultures of a) ESKAPE pathogens *E. aerogenes, S. aureus, K. pneumoniae, A. baumannii, P. aeruginosa* PA14 and *E. faecium* and b) *B. cenocepacia* and *E. coli* were treated for 5 hours with the combination of a conventional antibiotic; ofloxacin (OFX), ciprofloxacin (CIP) or rifampicin (RIF) and 17 or 34 µg/mL SPI009. Results are the mean of at least three independent experiments with error bars depicting SEM values. One-way ANOVA with Dunnett's correction for multiple comparisons was used to detect significant differences to the antibiotic control with * P ≤ 0.05, ** P ≤ 0.01, ***P ≤ 0.001, **** P ≤ 0.0001. Significant differences between the untreated control and conventional antibiotic treatment are indicated by a horizontal line; ND not detected.

4.3.2 SPI009 sensitizes antibiotic-resistant strains

To investigate the possible use of SPI009 as an adjuvant in antibacterial combination therapies, several (multi)drug-resistant strains were treated with 1 x, 4 x and 8 x MIC concentrations of the antibiotic, alone and in combination with SPI009. While SPI009 alone did not cause a significant decrease in survival of the ofloxacin resistant *P. aeruginosa* PA62 (Figure 4.2a), addition of 17 or 34 µg/mL of SPI009 significantly reduced the number of surviving cells by 5.3 ± 0.9 and 7.8 ± 0.9 log units at 4 x MIC of ofloxacin while combination with 8 x MIC completely eradicated the bacterial culture. In comparison, treatment with ofloxacin alone caused 0.8 ± 0.9 log and 2.8 ± 0.9 log decreases in surviving cells at concentrations of 4 x MIC and 8 x MIC, respectively. A similar trend was observed in the polymyxin B resistant *P. aeruginosa* 9BR (Figure 4.2b). Here, addition of the antibiotic alone had a slightly greater effect but combination with SPI009 still significantly improved the

treatment and 17 µg/mL of SPI009 successfully eradicated the entire bacterial culture in combination with 4 x MIC of polymyxin B. For K56-2, addition of 17 µg/mL and 34 µg/mL of SPI009 alone resulted in significant 1.6 ± 0.5 and 4.5 ± 0.5 log unit decreases in survival, pointing towards a clear antibacterial effect of the compound. Combination of SPI009 with polymyxin B further decreased the number of surviving cells, but to a lesser amount than observed for the PA62 and 9BR strains (Figure 4.2c).

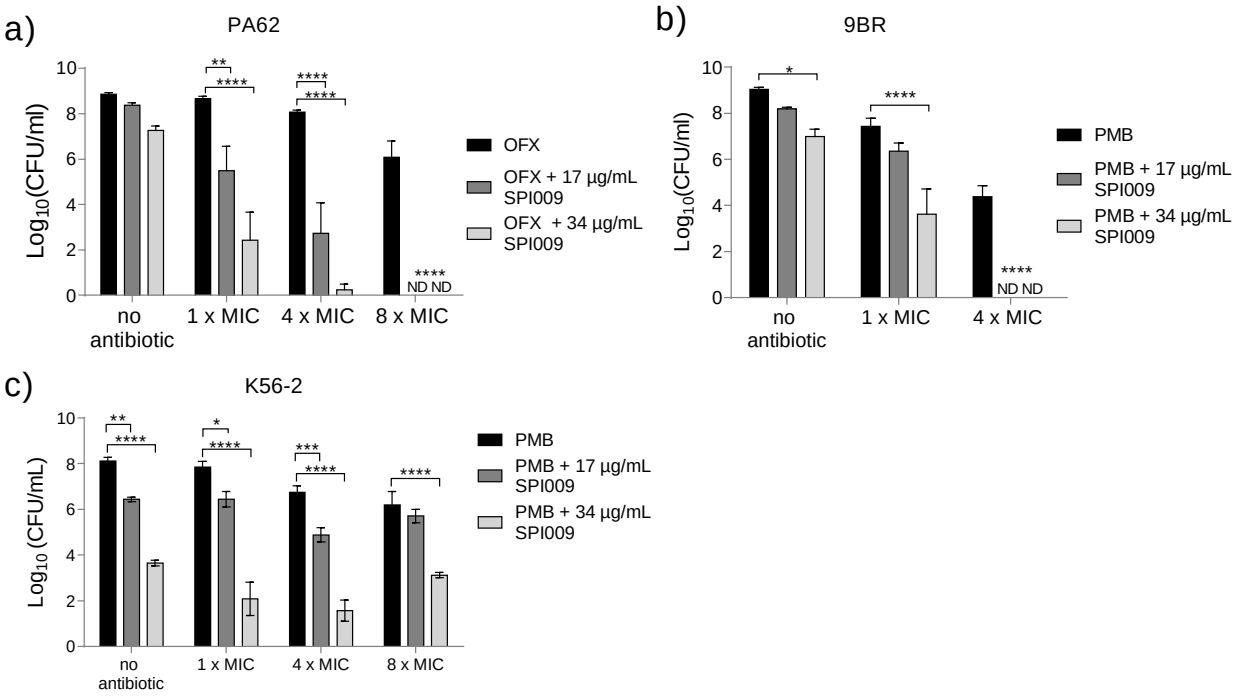


Figure 4.2: SPI009 re-enables the treatment of (multi)drug-resistant strains. Stationary phase cultures of a) *P. aeruginosa* PA62 (OFX^R), b) *P. aeruginosa* 9BR (PMB^R) and c) *B. cenocepacia* K56-2 (PMB^R) were treated for 5 hours with 1 x MIC, 4 x MIC and 8 x MIC concentrations of the respective antibiotic alone and in combination with 17 or 34 µg/mL of SPI009. Data points represent the average of at least three biological repeats. SEM values are shown as error bars. Statistical analysis was done by means of two-way ANOVA (α= 0.05) with Tukey correction for multiple comparisons and * P ≤ 0.05, ** P ≤ 0.01, ***P ≤ 0.001, **** P ≤ 0.0001; ND not detected.

The obtained results clearly demonstrate the effective use of SPI009 as an adjuvant for antibacterial therapy thereby facilitating the treatment of different antibiotic-resistant strains. Furthermore, SPI009 retains activity in multidrug-resistant strains, revealing the lack of cross-resistance. Importantly, resensitization of resistant strains could restore the effectiveness of established antibiotics.

4.3.3 Biofilm inhibition and eradication effects of SPI009

To assess biofilm inhibiting properties of SPI009 in *P. aeruginosa* PA14 and *S. aureus* ATCC 33591, biofilm growth was allowed in the presence of increasing concentrations of SPI009 (Figure 4.3a). Analysis of the obtained results clearly show an effective inhibition of biofilm growth in both *P. aeruginosa* PA14 and *S. aureus.* For *P. aeruginosa,* a steep increase in inhibitory activity was observed at concentrations above 8.5 µg/mL, resulting in 1.8 ± 0.5 log and 2.4 ± 0.4 log decreases at 17 or 34 µg/mL SPI009, respectively, and complete inhibition of biofilm growth at 68 µg/mL. *S. aureus* showed a more gradual decrease in biofilm formation with 34 µg/mL and 68 µg/mL resulting in significant 6.2 ± 0.6 log and 6.4 ± 0.6 log decreases in biofilm formation, respectively. These results clearly demonstrate the potent biofilm inhibiting activity of SPI009 mono treatment for both Gram-negative and Gram-positive model pathogens.

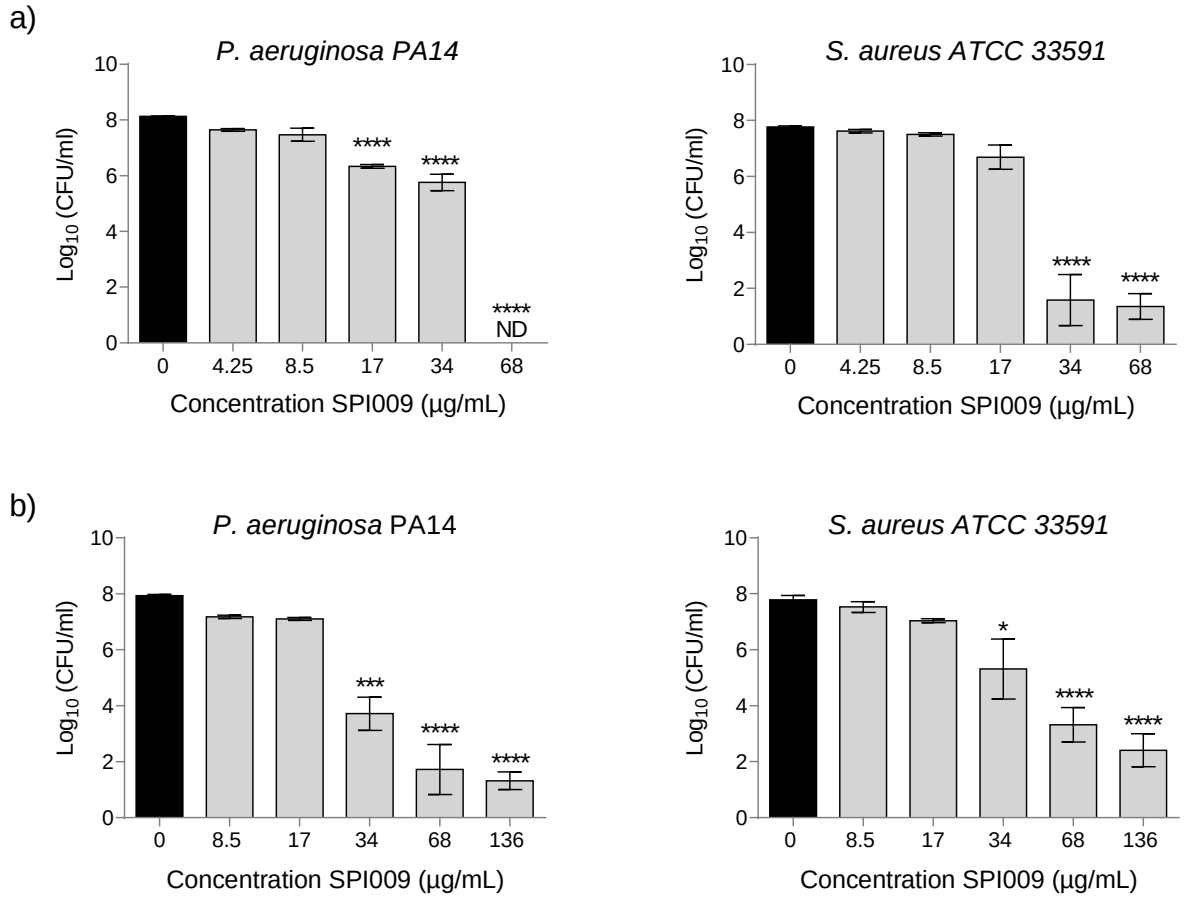


Figure 4.3: Anti-biofilm effects of SPI009 in *P. aeruginosa* PA14 and *S. aureus* ATCC 33591. Increasing concentrations of SPI009 (4.25-136 µg/mL; grey bars) and a DMSO control (black bar) were added for a) 24 hours to 1:100 diluted cultures or b) 5 hours to 24 hour old biofilms in 96-well microtiter plates to assess biofilm inhibition and eradication, respectively. After treatment, biofilms were washed, disturbed and plated out to calculate the number of surviving cells. Data points represent the average of at least three biological repeats, each containing three technical repeats. Error bars depict SEM values. Statistical significance was calculated on log_{10} transformed CFU counts using a one-way ANOVA with Dunnett's correction for multiple comparisons. * P ≤ 0.05, ***P ≤ 0.001, **** P ≤ 0.0001. ND, not detectable.

To explore biofilm eradication, SPI009 was added to mature biofilms and survival was assessed after five hours of treatment. For *P. aeruginosa,* the lower concentrations (8.5 and 17 µg/mL) caused a decrease in biofilm survival of about 0.8 log units (Figure 4.3b). Doses of 34 µg/mL or higher significantly decreased the number of surviving biofilms cells, resulting in 4.2 ± 0.6, 6.2 ± 0.6 and 6.6 ± 0.6 log reductions. In comparison, 10 µg/mL of the conventional antibiotic ofloxacin caused a significant 4.5 ± 1 log decrease in the number of surviving biofilm cells (see Figure 4.7a in the supplemental material). For *S. aureus*, the treatment of mature biofilms with lower concentrations of SPI009 proved slightly less effective than for *P. aeruginosa*. Treatment with higher concentrations did cause extensive damage, resulting in significant decreases in biofilm survival ranging from 2.5 ± 0.7 to 5.4 ± 0.6 log. For the 96-well biofilm models used in this study, the combination of SPI009 with a conventional antibiotic did not further decrease the number of surviving cells as compared to mono-treatment with SPI009 (supplemental Figure 4.7).

Overall, SPI009 shows potent antibacterial activity in biofilms of both Gram-negative and Gram-positive species and is capable of significantly inhibiting biofilm formation and decreasing survival of mature biofilms.

4.3.4 SPI009 reduces bacterial load in a chronic wound model

After confirming the biofilm eradication capacity of SPI009 in a standard biofilm set-up, a clinically more relevant model was used to assess the clinical potential of SPI009 as a topical antibacterial treatment. Using a porous cellulose matrix placed upon a moist hydrogel allowed the growth of a complex, three-dimensional hydrated structure, effectively mimicking biofilms in a chronic wound environment.[295,299] Assessment of viability was performed by means of live/dead quantitative PCR (Figure 4.4). For the viable cells, treatment with increasing concentrations of SPI009 alone resulted in significant 1.6 ± 0.5 log (34 µg/mL) and 2.0 ± 0.5 log (68 µg/mL) decreases in the number of surviving cells. As was shown for the previous biofilm experiments, the combination of SPI009 with an antibiotic was not capable of further decreasing the level of surviving cells when compared to SPI009 mono treatment. The obtained results confirm the biofilm eradication capacity of SPI009 as an antimicrobial and this in a more complex, realistic biofilm environment.

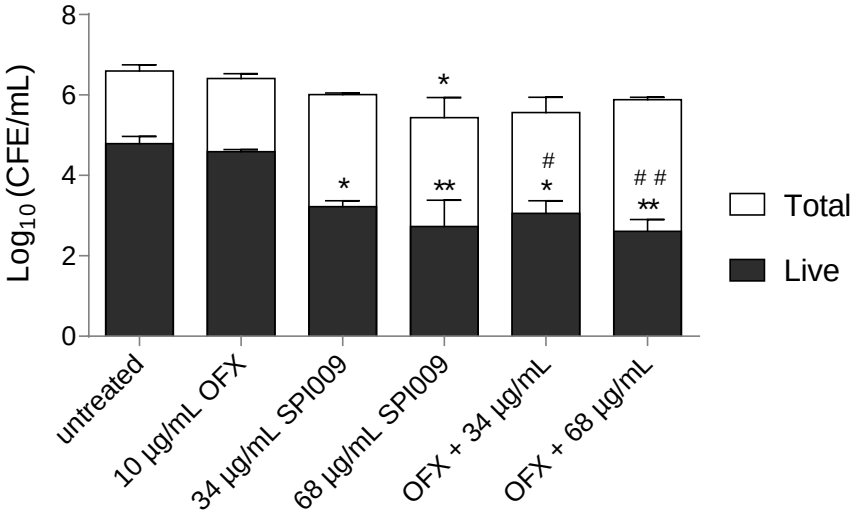


Figure 4.4: Effect of SPI009 in a chronic wound model. Mature *P. aeruginosa* PA14 biofilms, grown on hydrogels supporting a cellulose matrix, were treated for 24 hours with 10 µg/mL ofloxacin, 34 or 68 µg/mL of SPI009 alone or in combination with ofloxacin. Washed biofilms were removed by sonication and viability was assessed by means of Live/Dead PCR. Values are shown as the $\log_{10}$ transformation of Colony Forming Estimates (CFE), as determined for three biological repeats each containing three technical repeats. Error bars represent SEM values. Significant differences relative to untreated control are depicted by *, # represent significant differences relative to ofloxacin treatment with */# $P \leq 0.05$, **/## $P \leq 0.01$, as determined by means of one-way ANOVA with Dunnett's correction.

4.3.5 SPI009 potentiates antibiotic activity in an intracellular infection model.

Next, the anti-persister and antibacterial activities of SPI009 were verified in a recently developed *P. aeruginosa* intracellular infection model.[66] Due to the higher virulence of *P. aeruginosa* PA14, human THP-1 cells were infected with PAO1 cells (MOI 10) and treated for 5 hours with different concentrations of ciprofloxacin, alone or in combination with 6.8 or 10.2 µg/mL of SPI009. Concentrations of SPI009 were chosen to be well below the determined IC_{50} value of 24.5 ± 1.4 µg/mL. After treatment, both the number of surviving PAO1 cells and the amount of eukaryotic proteins present was assessed as this can provide information about the possible toxic effect of the different treatments and the infecting bacteria. While treatment with SPI009 alone did not significantly decrease the number of surviving bacteria, addition of SPI009 to ciprofloxacin greatly improved the antibacterial effect for all concentrations tested and this in a dose-dependent manner (Figure 4.5). Maximal antibacterial activity for the combination therapy with 10.2 µg/mL of SPI009 occurs at ciprofloxacin concentrations of 10 µg/mL, resulting in near complete eradication of the bacterial culture. Moreover, all combinations tested significantly reduced the bacterial load

as compared to ciprofloxacin alone. Combination treatment with 6.8 µg/mL SPI009 showed a maximal 0.78 ± 0.6 log decrease as compared to antibiotic alone at a ciprofloxacin concentration of 20 µg/mL. These results clearly show that SPI009 can effectively penetrate the eukaryotic cell membrane, without causing extensive damage, to eradicate the intracellular *P. aeruginosa* infection.

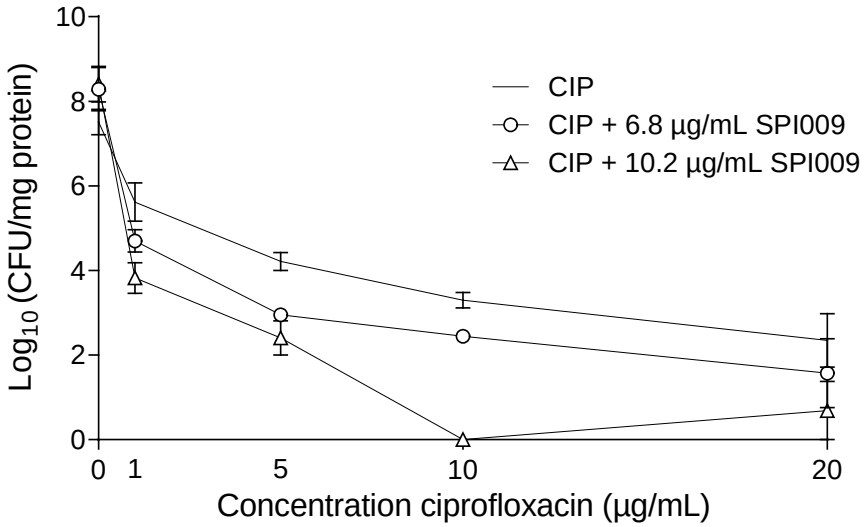


Figure 4.5: Addition of SPI009 enhances antibiotic treatment of intracellular infections. Human THP-1 cells were infected for 5 hours with *P. aeruginosa* PAO1 (MOI 1:10) after which extracellular bacteria were removed and cells were treated with increasing concentrations of ciprofloxacin, alone or in combination with 6.8 µg/mL or 10 µg/mL of SPI009. Data points represent the average of at least two biological repeats, each containing three technical repeats. Error bars depict SEM values. Statistical comparison between ciprofloxacin treatment and the different combination treatments was done by means of one-way ANOVA testing with correction for multiple comparisons (Dunnett) ($\alpha = 0.05$).

4.3.6 SPI009 combination therapy significantly improves *in vivo* survival

Since the anti-persister and antibacterial effects of SPI009 were demonstrated extensively *in vitro*, a next step was to assess the effect of this new compound in an *in vivo C. elegans* gut infection model. Toxicity testing of SPI009 in *C. elegans* revealed minor levels of toxicity at 68 µg/mL and > 80% killing at 136 µg/mL (supplemental Figure 4.8), excluding these concentrations from further experiments. Analysis of the different DMSO concentrations suggests that the observed toxicity is mainly caused by increasing concentrations of the solvent.

Infection of nematodes with PA14 resulted in 91.5% killing within 6 days after the start of infection, confirming the highly virulent nature of the PA14 strain in this model (Figure 4.6). Addition of 8.5 µg/mL of SPI009 alone slightly improved survival but not as good as 5 x MIC of ciprofloxacin, resulting in survival rates of 19.0% ($P = 0.045$) and 46.6% ($P < 0.0001$),

respectively. However, addition of 8.5 µg/mL of SPI009 to ciprofloxacin greatly increased survival, resulting in 73.8% nematode survival after 6 days. These results show a significant improvement in antibacterial effect of the combination therapy compared to the untreated ($P < 0.0001$) and ciprofloxacin-treated ($P = 0.0001$) controls (Supplemental Table 4.2). Since a low dose of SPI009 can greatly enhance the effect of conventional antibiotic treatment, resulting in more than 73% survival, these results indicate the highly efficient antibacterial and potentiating effect of SPI009 as part of a combination therapy.

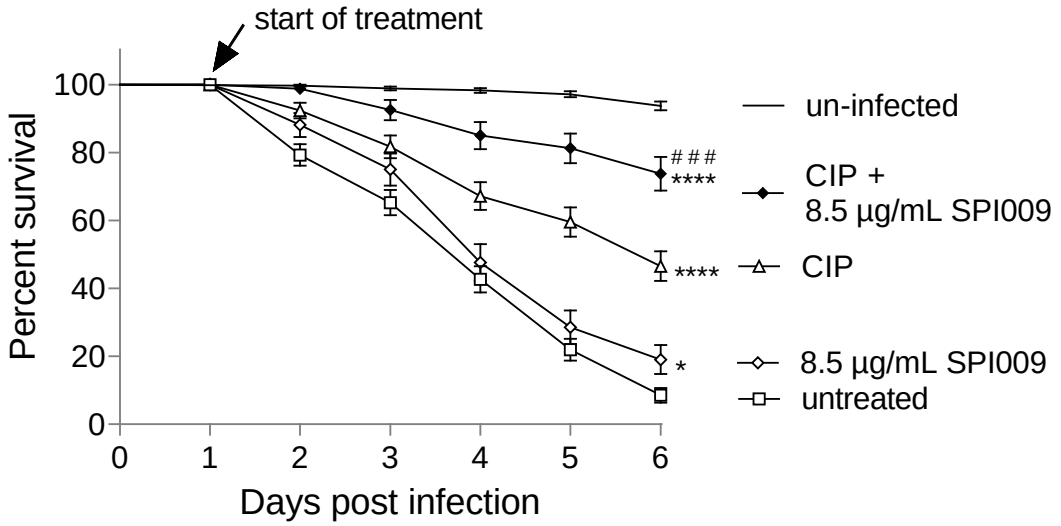


Figure 4.6: SPI009 combination therapy enhances *C. elegans* survival in a *P. aeruginosa* PA14 infection assay. Δ*glp-4(bn2)*/Δ*sek-1(km4) C. elegans* worms were infected with *P. aeruginosa* PA14 by feeding them on NGM-PA14 plates for 24 hours. Worms were treated with 8.5 µg/mL SPI009 (open diamonds), 1.56 µg/mL ciprofloxacin (5 x MIC; open triangles) or the combination of SPI009 with ciprofloxacin (filled circles). Untreated worms (open squares) and uninfected worms (solid line) served as controls. Worms were counted daily for 6 days with nematode survival expressed as a percentage relative to the viability on day 1. Data points represent the mean of at least three independent repeats ± SEM. Statistical analysis was performed on Kaplan-Meier plots by means of the log-rank test (α=0.05). Significant differences in survival as compared to the untreated or ciprofloxacin treated controls are depicted with * or #, respectively. * $P < 0.05$, # # # $P < 0.001$ and **** $P < 0.0001$.

4.4 Discussion

Decades of excessive drug prescription, misuse of antimicrobials and extensive agricultural applications have caused a massive increase in drug resistance. Conventional antibiotic therapies are losing the battle against emerging extensively drug-resistant strains, resulting in 25 000 annual deaths in the European Union.[300] A group of pathogens raising particular concern are the so-called ESKAPE pathogens, *E. faecium, S. aureus, K. pneumoniae, A. baumannii, P. aeruginosa* and *Enterobacter* spp.. Responsible for the majority of nosocomial infections, these pathogens show significant rises in resistance rates and are becoming increasingly difficult to treat with currently available antibiotics.[261,301] Since it is becoming

alarmingly difficult to identify novel antibiotic targets, combination therapies could provide an alternative strategy for the effective treatment of bacterial infections. When different mode of actions are combined, they can lower the risk of resistance development and extend the life span of currently available antibiotics. [106,111] An additional advantage of combination therapies is their potential use in the treatment of persister cells, a small reservoir of phenotypical variants that tolerate antibiotic treatment and reinitiate bacterial infection when the antibiotic pressure drops. The antibiotic-tolerant phenotype of persister cells contributes to the recalcitrant nature of chronic infections, greatly complicates treatment and increases the chances of resistance development.[29,64,135]

We recently described the discovery of the propanol-amine derivative SPI009, a novel anti-persister molecule capable of directly killing persister cells of *P. aeruginosa*.[161] Most anti-persister molecules described in literature are only active against one or a very limited number of bacterial species, which can be explained by a very specific mode of action or the sensitizing of persister cells to a specific class of antibiotics.[223,280] In this study, we showed that SPI009 possesses broad-spectrum activity and is capable of significantly decreasing or even eradicating the bacterial culture for all pathogens tested, including the notorious ESKAPE pathogens. In addition, combination therapy of conventional antibiotics with SPI009 allowed the efficient treatment of polymyxin B and ofloxacin resistant *P. aeruginosa* strains and could lower the required concentration of antibiotics, thereby enabling their use in resistant strains.

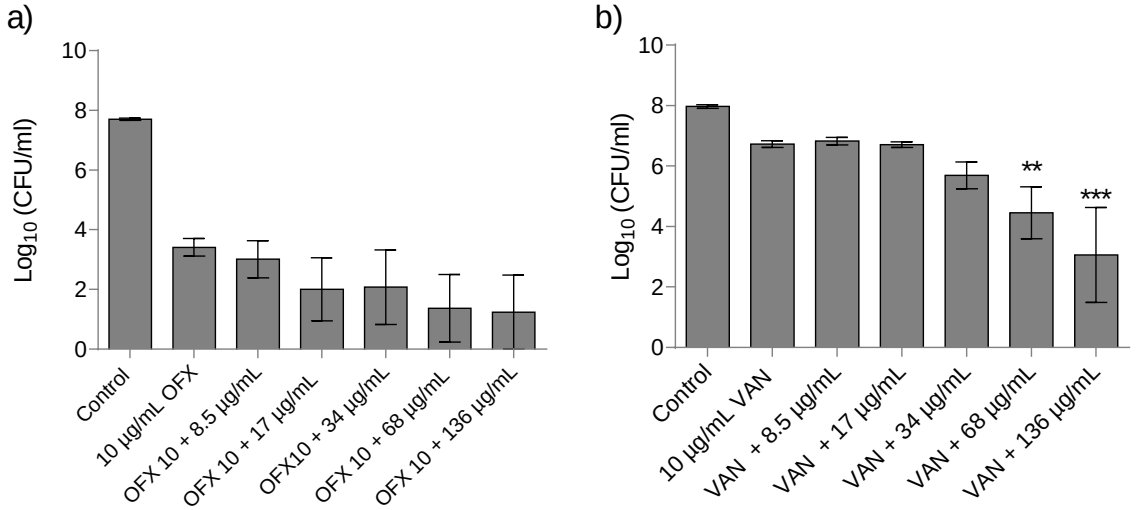
The close relationship between persisters and chronic infections[58,70] is partly caused by their presence in biofilms. The presence of the biofilm matrix is capable of physically protecting the persister cells against the human immune system, thereby enabling the persister cells to resume growth when antibiotic pressure drops and cause recurrence of infection. When compared to other anti-biofilm compounds or conventional antibiotics, SPI009 monotherapy shows a promising anti-biofilm effect, both decreasing biofilm formation and causing a strong reduction in the number of surviving biofilm cells, for both Gram-negative and Gram-positive species. A more clinically relevant biofilm model was obtained by *P. aeruginosa* growth on cellulose matrices and hydrogels, providing a three-dimensional structure and moist environment closely mimicking the environment of a chronically infected wound. In this 3D model, clinical treatments have been shown to have less impact on the viability of biofilms in comparison to traditional 2D models, which are more susceptible to eradication.[295,299] Therefore this further supports the ability of SPI009 mono-treatment to eradicate cells in a more complex biofilm model and suggests the possible use

of SPI009 in the topical treatment of chronically infected wounds. For all biofilm experiments executed, the addition of SPI009 to a conventional antibiotic did not further decrease the biofilm population as compared to SPI009 alone. In comparison to planktonic cultures, where combination therapy with antibiotics strongly enhances the antibacterial effect, the specific architecture and environment of the bacterial biofilm, including a possibly reduced penetration of antibacterials, could impair the cooperation between both antibacterials.

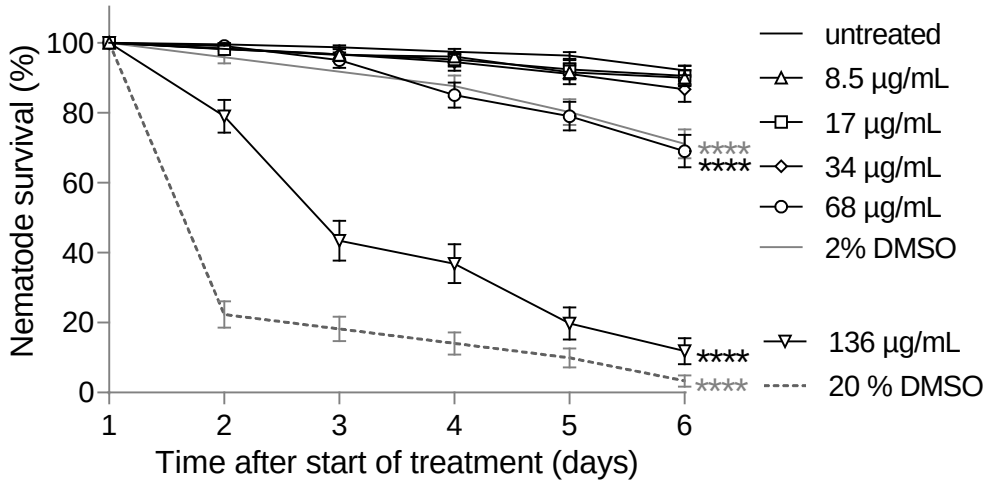
Besides the biofilm matrix, persister cells have also been shown to use eukaryotic cells to shield themselves from the human immune system. The presence of intracellular persister reservoirs has been confirmed *in vivo* and can be associated with the chronic nature of infections.[66,144] The ability of SPI009 to effectively target the intracellular bacteria further confirms the potential of SPI009 as an adjuvant in combination therapies. Capable of increasing nematode survival to more than 70% when combined with ciprofloxacin, the *in vivo C. elegans* model further contributes to the clinical potential of SPI009. The *C. elegans* model has been extensively used in the identification and clinical assessment of novel antibacterials and antifungals with ample studies confirming the consistent correlation between protective effects in *C. elegans* and mammalian models.[302]

In conclusion, we demonstrated that the anti-persister molecule SPI009 possesses a broad-spectrum antibacterial activity and, taken into account that it can be combined with different classes of antibiotics, shows great potential for the development of case-specific antibacterial combination therapies. The clinical potential of SPI009 was further confirmed by the observation of an excellent anti-biofilm activity, successful eradication of an intracellular infection in human eukaryotes and the significant increase in *C. elegans* survival after treatment with the combination of SPI009 and ciprofloxacin. Additional *in vivo* experiments will be required to assess the future applicability of SPI009 but its excellent activity in antibacterial combination therapies holds great promise.

4.5 Supplemental material



Supplemental Figure 4.7: Effect of conventional antibiotics in the treatment of bacterial biofilms. 24 hour, mature biofilms of a) P. *aeruginosa* PA14 and b) *S. aureus* ATCC 33591 were treated for 5 hours with a conventional antibiotic, ofloxacin (OFX) and vancomycin (VAN) respectively, or the combination of this antibiotic with increasing concentrations of SPI009. Assessment of biofilm survival was done by CFU counting and statistical differences to the control treatment were detected by means of a one-way ANOVA (α= 0.05) with Dunnett's correction for multiple comparisons. Data points represent the average of three independent repeats, each containing three technical repeats, ± SEM with ** P < 0.01 and *** P < 0.001. No significant decreases in biofilm survival were observed for the combination therapy, as compared to treatment with equal concentrations of SPI009 alone.



Supplemental Figure 4.8: Toxicity testing of SPI009 in *C. elegans.* OP50 fed nematodes were monitored for 5 days in the presence of increasing concentrations of SPI009 (8.5 - 134 µg/mL). Controls consisted of untreated worms (negative control, black line), 2% DMSO (carrier control, red solid line) and 20% DMSO (positive control, red dotted line). Data point represent the average of at least two independent experiments, as determined by means of Kaplan-Meier survival plots. Statistical analysis was done using log-rank test, showing significant differences between the untreated control and 68 µg/mL; 136 µg/mL; 2% DMSO and 20% DMSO. No toxicity was observed for SPI009 concentrations of 8.5 µg/mL (P=0.359), 17 µg/mL (P=0,556) and 34 µg/mL (P= 0.094).

Supplemental Table 4.2: Statistical analysis of *C. elegans* survival data. The Kaplan-Meier method was used to analyze the obtained survival data. N represents the total number of subjects, median lifespan is considered >6 if, at the end of the experiment > 50% of the population is still alive. Kaplan-Meier curves (Figure 4.6) were statistically analyzed using a log-rank test to compare between treatments and control or different treatments, as indicated in the table. $P_{log\text{-}rank}$ indicates the P-value as a result of the log-rank analysis ($\alpha = 0.05$); the last column indicates significance levels after the Bonferroni correction for multiple comparisons ($\alpha = 0.01$).

Group	N	Median lifespan (days)	$P_{log\text{-}rank}$	Bonferroni correction
un-infected	351	>6		
untreated	165	4	< 0.0001 vs un-infected	****
8.5 µg/mL SPI009	85	4	0.0452 vs untreated	ns
CIP (5 x MIC)	132	6	< 0.0001 vs untreated	****
CIP + 8.5 µg/mL SPI009	81	>6	< 0.0001 vs untreated	****
			0.0001 vs CIP	****

4.7 Additional data to the above-mentioned manuscript

4.7.1 SPI009 MIC determination in clinically relevant pathogens

Despite the clinical importance of non-dividing cells, such as the dormant phenotypical variants called persister cells, assessment of antibacterial activity is still commonly done using MIC assays. However, since MIC assays are performed in exponentially growing cultures, these results are more indicative of the growth inhibitory effect of the compound and give no information regarding activity against non-growing or dormant cells. Coates and co-workers have previously suggested the use of more relevant tests and terminology for this sub fraction of cells such as the 'minimum stationary-cidal concentration' (MSC) and the 'minimum dormicidal concentration' (MDC).[159,215] Despite their ability to correctly assess the ability of old and new antibacterials to target stationary phase and dormant bacteria, respectively, the actual use of this terminology is still rather limited. To allow comparison with other, known antibiotics MIC (and MBC) values of SPI009 in different clinically relevant pathogens are provided in Table 4.3.

MIC values were determined in 1/20 TSB using the Broth Microdilution method, as described previously.[276] Corresponding MBC values were obtained by plating out 10 µL of bacteria, originating from the wells of the MIC assay that did not show bacterial growth, onto solid TSB

agar plates.[303,304] Colonies were allowed to grow for 2 days and MBC was determined as the lowest concentration for which no colony forming units (CFU) were detected.

Table 4.3: Overview of SPI009 MIC values for different pathogens

Strain	MIC (µg/mL)	MBC (µg/mL)
Acinetobacter baumannii	34	51
Burkholderia cenocepacia	51	102
Enterobacter aerogenes	34	34
Escherichia coli	34	34
Klebsiella pneumoniae	34	34
Pseudomonas aeruginosa PA14	51	102
Enterococcus faecium	51	51
Staphylococcus aureus	51	102

Evaluation of the obtained results shows comparable MIC values for the different pathogens with slightly higher values for the Gram-positive *Enterococcus* and *Staphylococcus* species and the Gram-negative *B. cenocepacia* and *P. aeruginosa*.

As mentioned before, MIC values are not the best characteristic to evaluate activity against non-dividing cells, which is corroborated by the fact that sub-MIC levels of SPI009 are capable of greatly decreasing persister fractions for all pathogens tested (see section 4.3.1). For example, combination treatment of ofloxacin with 0.5 x MIC concentrations of SPI009 (17 µg/mL) completely eradicates stationary phase *K. pneumoniae*. MBC values allow the evaluation of the 'minimum bactericidal activity', thus providing information about the concentration that actually kills bacterial cells. Since antibacterials are usually regarded bactericidal if the MBC is no more than four times the MIC[305], these results further confirm the direct killing of persister and non-persister cells by SPI009.

4.7.2 Activity of SPI009 in the food pathogen *Salmonella* Typhimurium

In addition to the pathogens mentioned in the above manuscript, SPI009 anti-persister activity was also assessed in the Gram-negative *Salmonella enterica* serovar Typhimurium. While healthy adults must ingest a large number of bacteria to get sick, infants, elderly, organ-transplant and immunocompromised patients are at risk for acquiring potentially severe salmonellosis (food poisoning). *Salmonella* infections predominantly present as gastrointestinal diseases but, if the bacterium is able to escape the stomach, serious

bloodstream infections can occur.[306,307] *Salmonella* is a facultative intracellular pathogen and it was recently shown that internalization by macrophages stimulated the formation of non-replicating persister cells, providing reservoirs of bacterial cells capable of re-initiating infection.[144] In addition, *Salmonella* is capable of forming biofilms which, due to increased antibiotic tolerance and the presence of persister cells, are often very hard to eradicate.[307,308] Since SPI009 already proved active against several other Gram-positive and Gram-negative species, *S.* Typhimurium was added to the test panel.

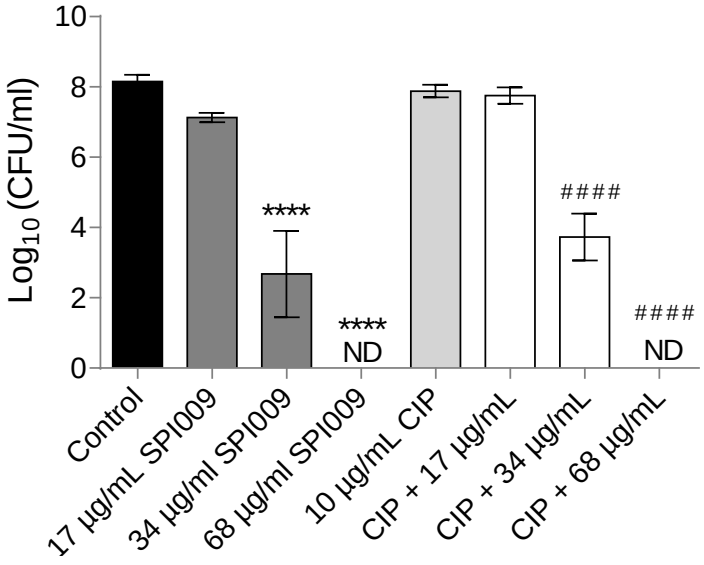


Figure 4.9: SPI009 activity in the food pathogen *S.* Typhimurium. Stationary phase cells of *S.* Typhimurium SL1344 were treated for 5 hours with DMSO (carrier control; black bar), 17 – 68 µg/mL SPI009 (dark grey bars), 10 µg/mL ciprofloxacin (CIP; light grey bar) or the combination of ciprofloxacin with SPI009 (white bars). Data points represent the averages of three independent repeats ± SEM; ND, not detected. Statistical comparison between ciprofloxacin and combination treatments was done by means of an ordinary one-way ANOVA using Dunnett's correction for multiple comparisons. Significant differences as compared to the DMSO control or ciprofloxacin treated control are depicted with, respectively * and # with ****/ #### indicating $P < 0.0001$. Tests on *S.* Typhimurium were conducted with a different batch of SPI009, possibly explaining the slight decrease in activity in the combination therapy.

In contrast to *P. aeruginosa*, where the anti-persister effect of SPI009 strongly increases killing by conventional antibiotics, SPI009 mainly exerts antibacterial effect in *S.* Typhimurium. Indeed, treatment with SPI009 alone resulted in 1.0 ± 0.8, 5.5 ± 0.7 log units decrease in bacterial cells compared to the DMSO treated control and complete eradication of the bacterial culture for 17 µg/mL, 34 µg/mL and 68 µg/mL, respectively. Despite the fact that fluoroquinolones are used in the treatment of *S.* Typhimurium[d], ciprofloxacin did not

[d] Antibioticagids UZLeuven – Editie 2015 (https://www.uzleuven.be/antibioticagids/)

seem to have any substantial effects. Combination of the fluoroquinolone SPI009 clearly enhances bacterial killing as compared to ciprofloxacin alone, but not to the level of SPI009 mono treatment.

Overall, we can conclude that SPI009 mono treatment is capable of efficiently killing *S.* Typhimurium persister and non-persister cells and could provide future treatment options for *Salmonella* associated gastroenteritis.

4.7.3 Effect of SPI009 on catheter-associated *S. aureus* biofilms

As described in the above manuscript, SPI009 showed potent biofilm inhibition and biofilm eradication activity in a 96-well microtiter plate biofilm model of *S. aureus.* (Methicillin resistant) *S. aureus* is still one of the most frequently isolated nosocomial pathogens and is known for causing catheter-associated bloodstream infections [22,309]

To assess the activity of SPI009 in a foreign-body biofilm model, *S. aureus* ATCC 33591 biofilms were grown on human catheters (Certofix® Trio S730; B. Braun) as previously described.[310] Briefly, 1 cm pieces of catheter were submerged in FCS (Life Technologies, Europe) for 24 hours to facilitate bacterial adhesion. An overnight culture of *S. aureus* ATCC 33591 was washed in 1 x PBS and adjusted to 1 x 10^6 cells/mL after which rinsed catheters were placed in the bacterial solution for 90 minutes. Each catheter was washed twice with 1 x PBS after which fresh 1/20 TSB was added and biofilms were allowed to grow for 24 hours (37°C, non-shaking). Again, catheters were washed and 1 mL volumes of rifampicin or rifampicin with SPI009, prepared in 1/20 TSB, were added in a 12-well plate. After 24 hours of treatment, catheters were washed and remaining bacteria were removed by means of sonication (10', 40 000 Hz) and vortexing. Bacterial solutions were plated on solid TSB agar plates and CFU was counted to estimate the number of surviving *S. aureus*.

To assess the anti-persister effect of SPI009 as an adjuvant in combination therapy, attached *S. aureus* was treated with 50 µg/mL rifampicin alone, or in combination with 17 µg/mL SPI009 (Figure 4.10). Concentrations were chosen based on previously obtained *in vitro* data (data not shown). The increase in CFUs between 'adhesion' and 'untreated control' clearly shows the successful adherence and demonstrates the ability of *S. aureus* to form biofilms on these catheters. Treatment with 50 µg/ml rifampicin caused a 2.6 ± 0.3 log decrease in surviving bacteria ($P < 0.0001$). Addition of 8.5 µg/mL or 17 µg/mL SPI009 further decreased survival with significant 0.6 ± 0.3 and 0.9 ± 0.3 log units reduction, as compared to

rifampicin alone. To correctly assess the antipersister or antibacterial effect of SPI009 in this set-up, the effect of SPI009 mono treatment should be evaluated.

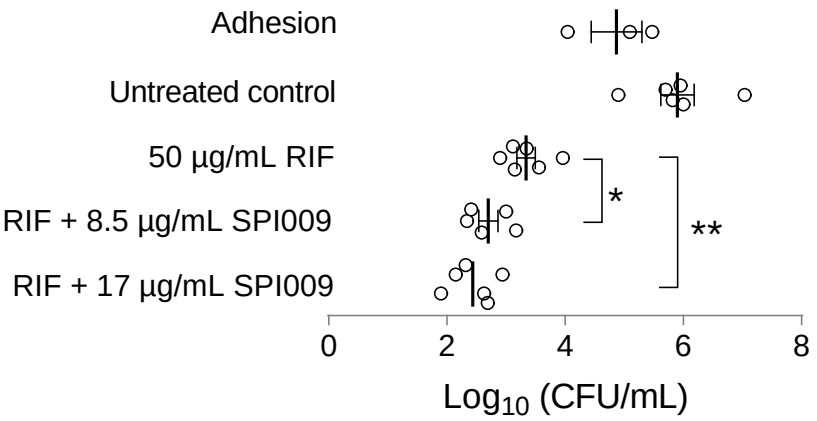


Figure 4.10: SPI009 significantly reduces catheter associated *S. aureus* in combination with rifampicin. Catheter associated, 24 h old *S. aureus* biofilms were treated for 24 hours with MilliQ (untreated control), 50 µg/mL rifampicin (RIF) or the combination of rifampicin with 8.5 or 17 µg/mL of SPI009. Data points represent the mean of three independent repeats ± SEM. Statistical differences between the combination therapies and rifampicin alone were determined using a one-way ANOVA (α=0.05) with Dunnett's correction for multiple comparison. * $P < 0.05$, ** $P < 0.01$.

Overall, we can conclude that, although not as effective as previous experiments in stationary phase cultures, addition of SPI009 further decreases bacterial survival in *S. aureus* biofilms as compared to antibiotic treatment alone.

4.7.4 Conclusion

Overall, the presented results further corroborate the findings in the above-mentioned manuscript, showing that SPI009 can successfully be combined with mechanistically distinct classes of antibiotics, thereby significantly decreasing or even completely eradicating the bacterial population of diverse human pathogens.

Chapter 5

SPI009 kills bacterial cells through extensive membrane damage

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This chapter was submitted as:

Defraine, V. & Liebens, V., Loos, E., Swings, T., Sharkey, L., O'Neill AJ., Weytjens, B., Fierro, C., Marchal, K., Corbau, R., Marchand, A., Chaltin, P., Fauvart, M. and Michiels, J. (2017). 1-((2,4-dichlorophenethyl)amino)-3-phenoxypropan-2-ol kills bacterial cells through extensive membrane damage

Contributions of individual authors to the manuscript:

The experiments described in this paper were designed by **Valerie Defraine**, Veerle Liebens, Maarten Fauvart and Jan Michiels. RNA sequencing and SUV experiments were performed by Veerle Liebens, SPI009 resistant mutants were generated by Evelien Loos and remaining experiments were performed by **Valerie Defraine**. Processing of RNA sequencing data was performed by Bram Weytjens and Caroline Fierro. Processing of whole genome sequencing data was performed by Toon Swings and **Valerie Defraine**. The data was analyzed and the manuscript written by **Valerie Defraine**. The manuscript was revised by Maarten Fauvart, Jan Michiels, Veerle Liebens, Alex O'Neill, Arnaud Marchand, Liam Sharkey and Carolina Fierro.

Abstract

The ever-increasing multidrug-resistance of clinically important pathogens and the lack of novel antibiotics have resulted in a true antibiotic crisis where many antibiotics are no longer effective. Further complicating the treatment of bacterial infections are antibiotic-tolerant persister cells. Besides being responsible for the recalcitrant nature of chronic infections, persister cells greatly contribute to the observed antibiotic tolerance in biofilms and even facilitate the emergence of antibiotic resistance. Evidently, eradication of these persister cells could greatly improve patient outcomes and provide an alternative approach in combatting chronic infections. We recently characterized 1-((2,4-dichlorophenethyl)amino)-3-phenoxypropan-2-ol (SPI009), a novel anti-persister molecule capable of directly killing persister cells from both Gram-negative and Gram-positive pathogens. SPI009 potentiates antibiotic activity in several *in vitro* and *in vivo* infection models and possesses potent anti-biofilm activity. Strikingly, SPI009 restores antibiotic sensitivity even in resistant strains (section 4.3.2). In this study, we investigated the mode of action of this novel compound using several parallel approaches. Genetic analyses and a macromolecular synthesis assay suggest that SPI009 may exerts its action by causing membrane damage. This hypothesis was confirmed by liposome leakage assay and membrane permeability studies, demonstrating that SPI009 rapidly impairs the integrity of the bacterial outer and inner membranes. Evaluation of SPI009-resistant mutants, which only could be generated under severe selection pressure, suggested a possible role for the MexCD-OprJ efflux pump. Overall, our results demonstrate the extensive membrane-damaging activity of SPI009 and confirm its clinical potential in the development of novel anti-persister therapies.

5.1 Introduction

Modifying existing antibiotic scaffolds upon emergence of resistance has proven a successful strategy to extend a drug class's utility in the past. However, recent data suggest that multidrug resistance increases at an alarming rate while few novel antibacterials reach the market.[311,312] A particular issue are multidrug-resistant Gram-negative pathogens, posing additional challenges to antibiotic discovery due to their highly impermeable outer membrane, and the so-called ESKAPE pathogens, efficiently evading antibiotic treatment and responsible for the majority of bacterial infections.[38,184,285] Since no new antibiotic scaffolds active against Gram-negative pathogens have been identified in the last decades, physicians are reverting to the use of polymyxins, once avoided due to cytotoxic effects, as a last-resort treatment for these strains. Therefore, new antibacterial scaffolds are desperately needed.[313,314]

Contributing to the difficult treatment of bacterial infections is the presence of persister cells, which constitute a small but important fraction of phenotypic variants tolerant to treatment with high doses of antibiotics. [59] Their occurrence in many bacterial pathogens combined with a demonstrated link between persistence and the recalcitrant nature of chronic bacterial infections renders persisters a serious threat to immunocompromised patients and effective anti-persister treatments are much needed.[29,315,316] Previous research revealed the potent activity of the novel anti-persister molecule SPI009 as an adjuvant in antibacterial combination therapies against different bacterial pathogens. In addition, the compound proved highly successful in the treatment of intracellular and *in vivo P. aeruginosa* infections when combined with the fluoroquinolone ciprofloxacin. SPI009 sensitizes bacteria to antibiotic activity and, strikingly, restores antibiotic sensitivity even in resistant strains. In addition, SPI009 monotherapy exhibited potent inhibition and eradication activity in biofilms of *P. aeruginosa* and *S. aureus.* The current need for novel antibacterials active against Gram-negative species, together with the unique characteristic of SPI009 to kill both normal and persister cells, prompted us to further investigate the mode of action of this compound.

Current approaches to identify the next generation of antibacterials involve high-throughput screenings of natural and chemical products, genome hunting, whole-cell based assays and the targeting of non-multiplying bacteria.[215] As these options offer interesting alternatives that can bypass the identification of novel antibiotic targets, mechanism of action

studies become increasingly important to characterize and select interesting candidates after initial discovery.[317]

In this study, we set out to determine the mode of action of a recently discovered antibacterial compound, SPI009, showing a broad spectrum antibacterial effect and capable of killing both dividing cells and non-dividing or dormant persister cells.[161] While generating useful information for the further development of this compound as an antibacterial therapy, determination of the mode of action could also greatly assist in the development of future anti-persister therapies. A combination of genetic and cellular approaches was employed, revealing the membrane damaging activity of SPI009 and suggesting the ability of SPI009 to attack the bacterial membrane(s) from both the cytoplasm and extracellular environment.

5.2 Materials and methods

Bacterial strains, media and growth conditions. Bacterial strains were cultured in 1:20 diluted Trypticase Soy Broth (1/20 TSB) at 37 °C, shaking at 200 rpm. For solid medium, TSB was supplemented with 1.5% agar. The following antibacterials were used: ofloxacin, ciprofloxacin, erythromycin, polymyxin B, rifampicin (Sigma Aldrich), fosfomycin, meropenem (TCI Europe), triclosan (Merck Chemicals), melittin (Bachem) and SPI009. Concentrations are indicated throughout the text. Bacterial strains used in this study are listed in Table 5.1.

Table 5.1: strains used in this study.

Strain	Description[a]	Source/ reference
Pseudomonas aeruginosa		
PA14	Wild type, UCBPP –PA14	Pierre Cornelis; [8]
YM WT	PAO1 WT, K767	[318]
YM64	*mexAB-oprM::FRT, mexXY::FRT, mexCD-oprJ::FRT, mexEF-oprN::FRT*	[319]
K1521	K767 Δ*mexCD-oprJ*	[318]
K1525	K767 Δ*mexXY*	[318]
Liberati WT	Wild type UCBPP-PA14 (Lib WT)	[320]
ΔGalU	PA14_38350::Mar2xT7 (52640)	[320]
ΔnfxB	PA14_60860::Mar2xT7 (55219)	[320]
*Δ*PA14_0812	PA14_08120::Mar2xT7 (47659)	[320]
Acinetobacter baumannii		
Ab-84	MDR clinical isolate	[321]
Ab-84R	Ab-84::40nt insertion at nt 321 of *lpxC*	[321]

Screening of a *P. aeruginosa* mutant library. A genetic screen of the *P. aeruginosa* PA14 transposon mutant library[320] was performed to identify single gene knockouts sensitive or resistant for SPI009. Stationary phase mutant cultures were split in two and treated for 5 hours with either 10 µg/mL ofloxacin or the combination of ofloxacin and 51 µg/mL of SPI009. Treated cultures were diluted 1:100 in fresh TSB medium and incubated at 37°C, shaking at 200 rpm. Growth was monitored over a total period of 40 hours by means of periodic OD_{595} measurements. Average OD_{595} was calculated for each 96-well plate and used to correct mutant OD_{595} values. Mutants were identified as sensitive if the OD_{595} after 24 hours of growth was ≤ 0.3 x average OD_{595} after 24 hours. Alternatively, mutants having an OD_{595} > 3 x average were defined as resistant. The screening was performed twice to prevent false-positive hits and, to allow identification of SPI009 specific effects, selected mutants showing a clear sensitivity or resistance for ofloxacin were excluded. Resistant hits were additionally confirmed via detailed monitoring of growth in the presence of 51 µg/mL SPI009, using an automated OD reader (Bioscreen C). Functional enrichment analysis was performed based on PseudoCAP classifications and using Fisher's exact test.

RNA sequencing and data analysis. Overnight cultures of *P. aeruginosa* were diluted 1:100 in fresh 1/20 TSB medium and allowed to grow until late-exponential phase (OD_{595} = 0.2). Cells were treated for 15 minutes with 50 µM SPI009, 50 µM of the inactive analogue SPI014 or 1% DMSO. Total RNA isolation was performed in triplicate for each sample, as previously described.[272] The Ribo-Zero™ rRNA Removal Kit for Gram-negative bacteria (Epicentre) was used to deplete ribosomal RNA and RNA samples were sent to the Genomics Core facility of EMBL (Heidelberg, Germany). The quality of the raw sequencing reads was verified using FastQC after which genomic alignments of the reads were performed with Bowtie2, using the *P. aeruginosa* UCBPP-PA14 genome as a reference (NC_008463.1). Differential expression analysis between treated and control samples was done using the DESeq2 package with a False Discovery Rate threshold of 5%[322]. Genes with a $\log_2$ fold-change above one and no differential expression under the inactive compound treatment were selected, allowing the detection of SPI009 specific effects on gene expression. Functional annotation of the obtained results was performed using PseudoCAP functional classes obtained from www.pseudomonas.com[323] and functional enrichment was assessed using Fisher's exact test. A UCBPP-PA14 interaction network was created using the STRING database[324] where only reactions with a minimum reliability score of 0.8 were retained. PheNetic was run using both this network and the obtained omics data to generate a downstream interaction network,

using the standard parameters and a cost of 0.25[325]. Obtained networks containing more than two genes were visualized using Cytoscape.[326]

Macromolecular synthesis assay. An overnight culture of *P. aeruginosa* PA14 was diluted 1:10 in 1/20 TSB and grown to an OD_{600} of 0.3 at 37°C (late exponential phase). Radiolabeled precursors for DNA (1 µCi/mL [Methyl-^{3}H]-thymidine), RNA (2.5 µCi/mL [5,6-^{3}H]-uridine), protein (2.5 µCi/mL L-[4,5-^{3}H]-leucine), peptidoglycan (2.5 µCi/mL D-[6-^{3}H(N)]-glucosamine hydrochloride) and fatty acids (1 µCi/mL [2-^{3}H]-glycerol) were added after which cultures were treated with 17 µg/mL SPI009 or 8 x MIC concentrations of relevant control antibiotics. 30 minutes after onset of treatment, 100 µL samples were added to 3.5 mL of ice-cold 10% TCA and precipitates were collected under vacuum on 25 mm glass microfiber filters (Whatman® Grade GF/C). Filters were washed twice with 4 mL ice-cold distilled water and added to 3.5 mL scintillation liquid (Ultima-Flo M, Perkin Elmer). Incorporation of the different radiolabels was assessed using a Hidex 300SL scintillation counter and resulting Counts Per Minute (CPM) at different treatment conditions were used to evaluate the incorporation of radiolabeled precursors relative to the untreated control.

Fluorescein leakage assay. Small unilamellar vesicles (SUV) mimicking the Gram-negative membrane and loaded with carboxyfluorescein (CaF) were produced as described previously.[327,328] The total phospholipid concentration was kept at 25 µM, containing a mixture of 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine(DOPE)/1,2-dioleoyl-sn-glycero-3-phospho-(1'-rac-glycerol)(DOPG) (4:1) (Avanti-Polar Lipids, Inc.). Liposomes were treated with increasing concentrations of SPI009 and an inactive chemical analog, SPI005, keeping the final DMSO concentration at 1 % (v: v). Release of CF (λ_{ex} = 485 nm, λ_{em} = 520 nm) was measured in function of time. The percentage of CF leakage was determined relative to the treatment with 0.5% Triton X-100.

Assessment of inner and outer membrane permeabilization. Inner membrane permeabilization was examined using a SYTOX Green uptake assay, as previously described.[327] A PA14 culture was grown until late exponential phase and corrected to a final OD_{595} of 0.5 in 1 x phosphate buffered saline (PBS) supplemented with 1 µM SYTOX Green. Cultures were treated with MilliQ (untreated control), DMSO (1%; carrier control), 10 µg/mL melittin (1 x MIC) or increasing concentrations of SPI009 and transferred to the wells of a black microtiter plate (clear bottom). Fluorescence (λ_{ex} = 504 nm, λ_{em} = 523) and absorbance (OD_{595}) were measured every minute, using a Synergy MX multimode reader (Biotek) at 37°C.

P. aeruginosa outer membrane permeabilization by SPI009 was measured using a 1-*N*-phenylnaphtylamine (NPN, Sigma, US) uptake assay.[327] Briefly, a *P. aeruginosa* PA14 culture was grown until late exponential phase, after which the OD_{595} was corrected to 0.5 in 5 mM HEPES (pH=7.2). 150 µL volumes of culture were treated with MilliQ (untreated control), DMSO (1%; carrier control), 0.625 µg/mL polymyxin B (1 x MIC) and different concentrations of SPI009 (4.25 – 34 µg/mL) and transferred to the wells of a black microtiter plate (clear bottom). 50 µL of a 40 µM NPN solution in 5 mM HEPES (pH=7.2) was added and fluorescence was measured immediately using a Synergy MX multimode reader (Biotek) at 37°C.

Independent assays for outer and inner membrane permeabilization assessment were performed three times. Measured fluorescence signals were divided by well-specific OD_{595} to correct for cell density after which the values of the respective untreated controls were subtracted. Results are expressed in relative fluorescence units.

Microscopic visualization of membrane damage. Overnight cultures of *P. aeruginosa* and *S. aureus* were treated for 20 minutes with 0.5% DMSO (carrier control) or 34 µg/mL SPI009, centrifuged and stained with 10 µg/mL *N*-(3-triethylammoniumpropyl)-4-(6-(4-(diethylamino) phenyl) hexatrienyl) pyridinium dibromide (FM®4-64, Molecular Probes). Samples were spotted on 2% agarose pads for imaging with Zeiss Axio imager Z1 fluorescence microscope, using an EC Plan-NEOFLUAR 100 x objective (λ_{ex} = 540-580 nm; λ_{em} = 593 -668 nm).

Interaction of SPI009 with LPS. To assess possible interaction between SPI009 and the lipid A compound of Gram-negative LPS layers, a whole cell BODIPY™ TR Cadaverine displacement assay was performed. Briefly, a late-exponential PA14 culture was adjusted to an OD_{595} of 0.3 in 50 mM Tris-HCl and added to the BODIPY™ TR Cadaverine conjugate (BC, 5 µM; Life Technologies) in the wells of a black microtiter plate (clear bottom). Cultures were incubated for 2 hours to allow BC binding after which equimolar amounts of Tris-HCl (negative control), meropenem, polymyxin B and SPI009 were added and fluorescence (λ_{ex}=580 nm, λ_{em}=620 nm) was measured continuously for one hour. Fluorescence values from the negative control (Tris-HCl) were used to correct for background fluorescence.

Antibacterial assay. The effect of SPI009 on different bacterial cultures was assessed as described previously.[161] Briefly, stationary phase cultures were treated for 5 hours with DMSO (carrier control) and different concentrations of SPI009. After treatment, cultures were washed twice in 10 mM $MgSO_4$ and appropriate dilutions were plated onto solid agar plates to assess the number of colony forming units.

Generation and whole genome sequencing of SPI009-resistant mutants. Resistant mutants were generated as previously described, with minor modifications.[129,329] An initial MIC test was performed in three independent *P. aeruginosa* PA14 cultures with ofloxacin and SPI009, according to EUCAST standards.[271] After 24 hours of growth at 37°C, shaking, the MIC value was determined as the minimal concentration that completely inhibited bacterial growth. A new MIC assay was prepared using 1:100 diluted cells at MIC/4 as a starting condition. The assay was repeated for 10 passages with daily assessment and, if necessary, adjustment of antibiotic and compound concentrations. Intermediate and endpoint cultures were stored at -80°C in glycerol (25 % v/v) for further analysis. Genomic DNA of the *P. aeruginosa* PA14 strain and the three evolved resistant mutants was isolated from overnight cultures grown in 1/20 TSB using the DNeasy Blood & Tissue Kit (Qiagen) following the manufacturer's instructions. DNA quantity and purity were verified using a NanoDrop ND-1000, after which samples were sent to the Genomics Core Facility of EMBL (Heidelberg, Germany) for whole genome sequencing on the Illumina HiSeq 2500 platform. Assembly of the 125 bp paired-end reads and further analysis was performed using CLC Genomics Workbench v8.0. Genome sequences of the resistant mutants were aligned with the genome of the PA14 WT strain in order to detect genetic differences, taking into account a coverage above 10x and cutoff frequency of 75%. Identified non-synonymous mutations were confirmed via PCR amplification and Sanger sequencing (GATC Biotech).

5.3 Results

5.3.1 Genetic analysis of the SPI009 mode of action

To gain more information about the mode of action of SPI009 and allow the detection of possible persister-specific effects, individual single-gene knockouts from a *P. aeruginosa* PA14 mutant library were treated with ofloxacin alone or in combination with SPI009, after which mutants showing an increased or decreased sensitivity only to SPI009, were selected. Analysis of the obtained screening results revealed a total of 118 and 37 different mutants that showed an increased or decreased sensitivity for SPI009, respectively. Functional enrichment analysis of the sensitive mutants based on their PseudoCAP functions,[323] revealed an over-representation for genes involved in 'Adaptation and protection' and 'Cell wall/LPS/Capsule' (Figure 5.1a and table A1 in Appendix A). The relatively low levels of resistance for the selected mutants, as seen in more detailed growth curves, combined with the functional enrichment analysis (Figure 5.1a) suggest that single-gene knockouts are not sufficient to obtain significant resistance towards the anti-persister effects of SPI009 and point to a more general effect of the compound.

Next, we compared genome-wide gene expression levels of *P. aeruginosa* PA14 following treatment with either SPI009 or an inactive analogue. RNA sequencing analysis generated a list of 297 genes that were specifically differentially expressed (log_2-fold change >1) upon treatment with SPI009 (Table A2 in Appendix A). Functional enrichment analysis of up-and downregulated genes (Figure 5.1b), combined with network analysis (Figure 5.9) revealed a first group of SPI009 upregulated genes to be involved in antibacterial efflux and multidrug resistance, suggesting the increased efforts of the cell to protect itself against SPI009. Another group of mostly upregulated genes is involved in fatty acid metabolism and degradation, suggesting altered amounts of available fatty acids upon treatment of the cell with SPI009. Furthermore, there is a downregulation of multiple genes involved in virulence; including phenazine biosynthesis, pilus assembly and protein secretion and the bacterial Type VI secretion system and biofilm formation.[330]

Taking both genetic analyses into account, there does not appear to be a single process or pathway that emerges as the primary target for SPI009. Instead, membrane related functions are perturbed, supplemented with more general effects in different regulatory and metabolic pathways. This could point to SPI009 causing membrane damage.

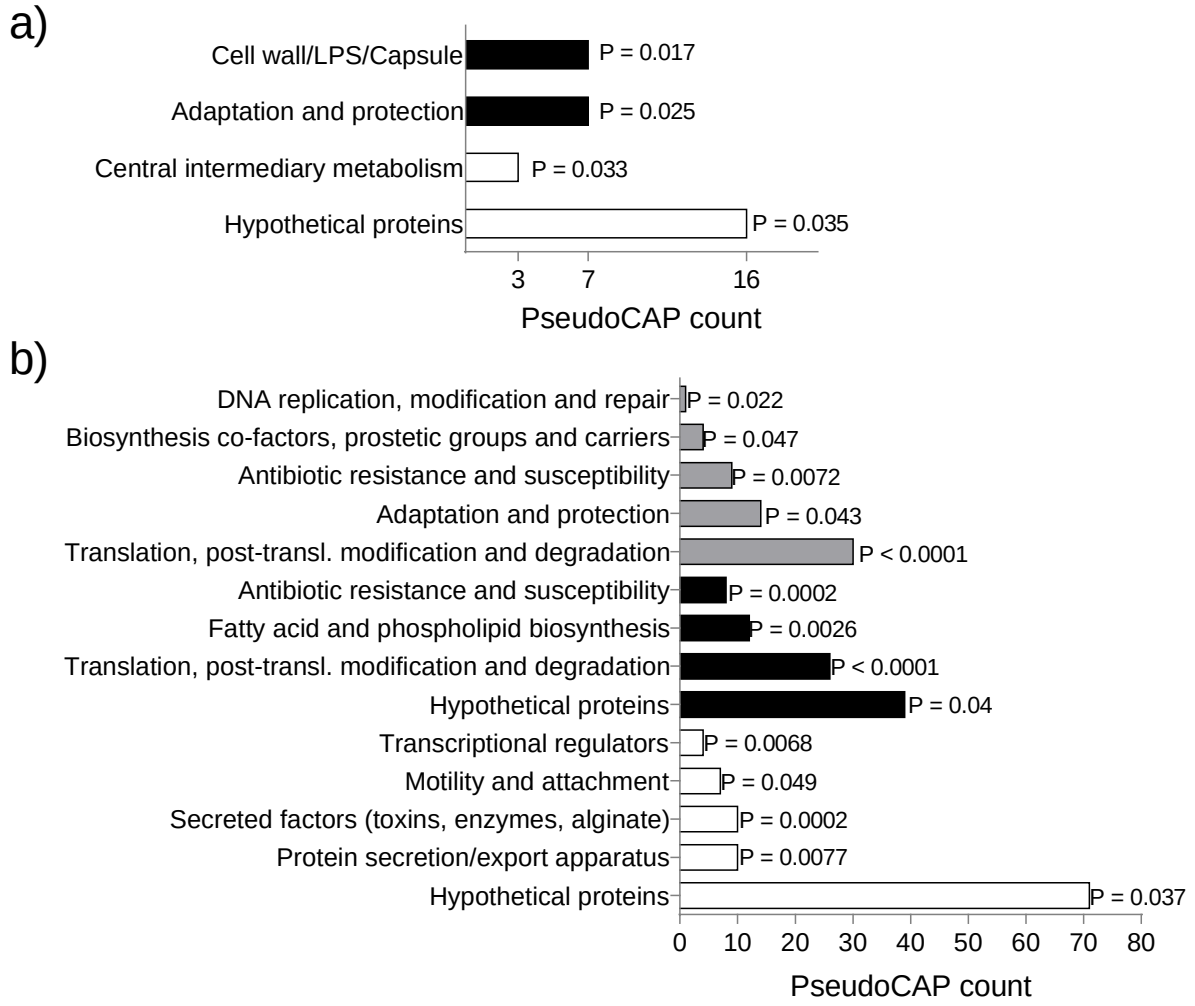


Figure 5.1: Genetic analysis of SPI009 mode of action. a) Functional enrichment of the obtained set of sensitive (black bars) and resistant (white bars) mutants using Pseudocap classifications. Mutants were selected after comparison of the effect of ofloxacin treatment and the combination treatment of ofloxacin and SPI009, to allow the detection of SPI009 specific effects. P-values were determined using a two-tailed Fisher's exact test (α=0.05). Pseudocap counts represent the number of mutants in the respective classes. b) Functional enrichment analysis of the up- and downregulated differentially expressed genes (log2 fold change >1) as determined after RNA sequencing upon treatment with SPI009 alone. A two-tailed Fisher's exact test was used to determine P-values for the classes of downregulated genes (white bars), upregulated genes (black bars) and the total set (grey bars).

5.3.2 SPI009 inhibits macromolecular biosynthesis in a non-specific manner

To further explore the hypothesis of SPI009-induced membrane damage and rule out other bacterial mechanisms targeted by the compound, a macromolecular synthesis assay was performed.[331] Addition of 17 µg/mL of SPI009 strongly reduced incorporation of radio-labeled precursors for DNA, RNA, proteins, fatty acids and peptidoglycan, resulting in a more than 50% decrease in synthesis for all macromolecules tested (Figure 5.2). When compared to different antibiotics, known to inhibit incorporation of precursors, 1/3 x MIC concentrations of SPI009 show a generally stronger inhibitory effect, a pattern previously reported for membrane-damaging compounds.[327,332–334]

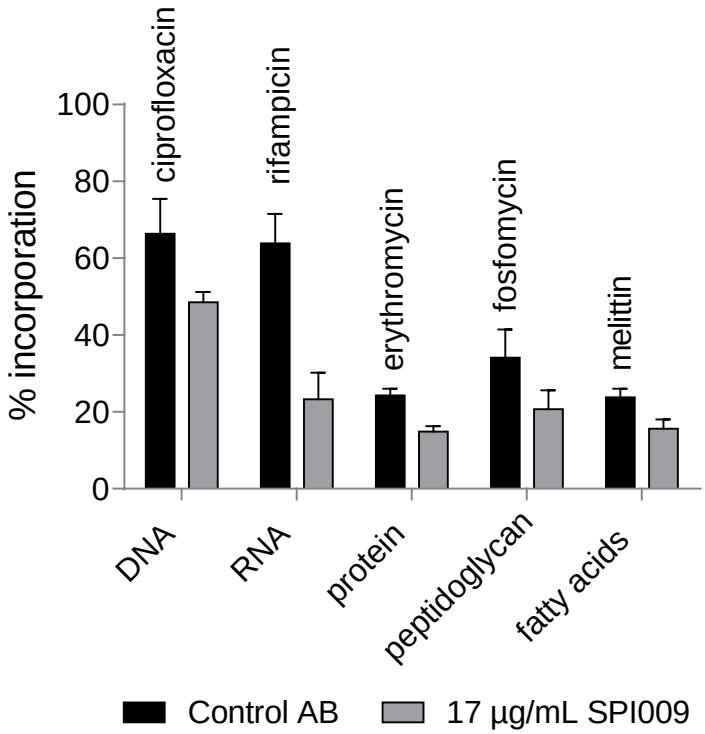


Figure 5.2: SPI009 inhibits macromolecular synthesis. Incorporation of [methyl-3H]-thymidine (DNA), [5,6-3H]-uridine (RNA), L-(4,5-3H)-leucine (protein), D-[6-3H(N)]-glucosamine (peptidoglycan), 2-3H-glycerol (fatty acids) by *P. aeruginosa* PA14 after treatment with relevant control antibiotics (AB, black bars) at 8 x MIC concentrations or 17 µg/mL of SPI009 (grey bars). Incorporation was measured after 30 minutes and expressed relative to an untreated control. Bars represent the average of at least three independent repeats ± SEM

5.3.3 SPI009 is capable of disrupting artificial bacterial bilayers

To further confirm the suggested membrane damaging effect of SPI009, its capacity to disrupt lipid bilayers that mimic the Gram-negative inner membrane was tested. Increasing concentrations of SPI009 clearly induced CaF leakage in a concentration-dependent manner, while the inactive analogue SPI023, displaying no antibacterial or anti-persister effect, and the conventional antibiotic ofloxacin, did not cause any significant leakage (Figure 5.3). When comparing SPI009 with the membrane damaging antibiotic polymyxin B, 50% CaF leakage was obtained at concentrations of 11.92 ± 0.07 µg/mL and 1.16 ± 0.04 µg/mL, representing 0.185 x MIC and 1.85 x MIC concentrations of SPI009 and polymyxin B (Figure 5.10), respectively. These results indicate that SPI009 is indeed capable of efficiently disturbing an artificial lipid bilayer and further support the membrane-damaging hypothesis.

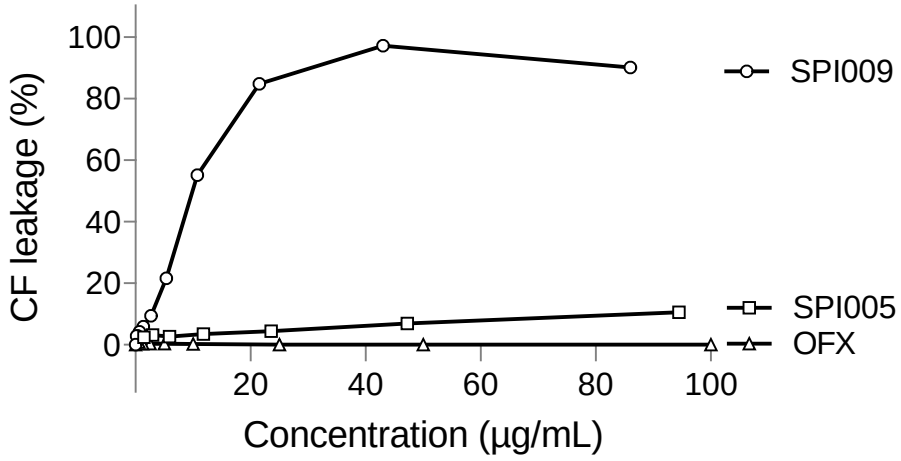


Figure 5.3: SPI009 causes extensive CaF leakage. SUVs were treated for 15 minutes with increasing concentrations (x-axis) of SPI009 (open circles), the inactive analogue SPI005 (open squares) or the conventional antibiotic ofloxacin (OFX; open triangles). % CaF leakage was determined by fluorescence measurements, corrected for background fluorescence and expressed relative to the positive control (0.5% Triton X-100). Data points represent the mean of three independent repeats ± SEM.

5.3.4 Membrane permeabilization studies

To evaluate membrane disruption activity of SPI009 on whole *P. aeruginosa* cells, NPN and SYTOX Green assays were carried out, allowing the investigation of respectively outer and inner membrane permeabilization. SYTOX green shows a strong increase in fluorescence upon binding to DNA. This is however only possible when the structure of the inner membrane of the cell is compromised, thus correlating the observed fluorescence with the amount of inner membrane damage.[335] 30-minute treatment of *P. aeruginosa* with increasing concentrations of SPI009 caused a strong increase in fluorescence as compared to the untreated control (Figure 5.4a). At concentrations of 17 µg/mL (= 0.33 x MIC), the observed membrane damage was comparable to the effect of treatment with 1 x MIC concentrations of melittin, the active compound in bee venom known to induce inner membrane damage.[336]

Next, permeabilization of the outer membrane was assessed by means of the hydrophobic fluorescent probe NPN. Bacterial cells normally exclude NPN. Consequently, increasing fluorescence caused by the insertion of the probe in the phospholipid bilayer is indicative of damage to the bacterial outer membrane. [337] Fluorescence measurements revealed a rapid outer membrane permeabilization by SPI009 in a clear concentration-dependent manner (Figure 5.4b). In comparison, treatment with 1 x MIC concentration of polymyxin B resulted in a comparable fluorescence level at 1/6 x MIC concentrations of

SPI009. These results strongly support the hypothesis that SPI009 is capable of disrupting the bacterial membrane and this for both the inner and outer membrane of *P. aeruginosa*.

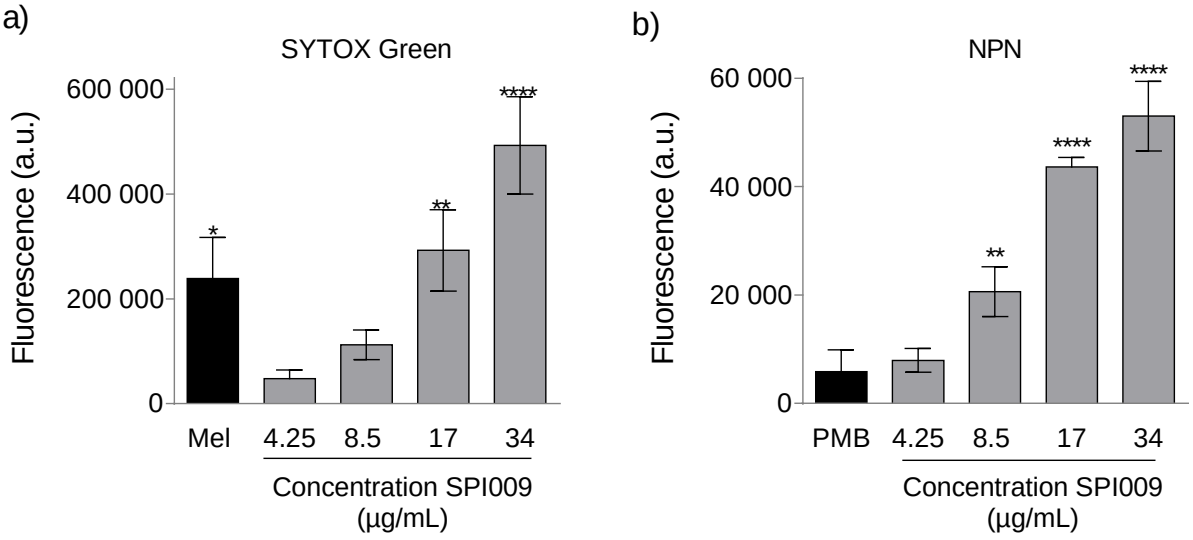


Figure 5.4: SPI009 extensively permeabilizes both inner and outer membrane of *P. aeruginosa* PA14. a) Effect of increasing concentrations of SPI009 on the inner membrane permeability, as measured by the SYTOX Green uptake assay. Cells were treated for 30 minutes using melittin (Mel; 1 x MIC) as a positive control. b) Outer membrane permeability after treatment with increasing concentrations of SPI009, using polymyxin B (PMB, 1 x MIC) as a positive control. Data for both assays represent the mean of at least three independent repeats ± SEM. Statistical comparisons with the untreated control were performed using a one-way ANOVA (α=0.05) with Dunnett's correction for multiple comparison (* $P < 0.05$, ** $P < 0.01$, **** $P < 0.0001$).

5.3.5 Microscopic confirmation of membrane damage

Since the bacterial membrane is such a critical part of the cell's architecture, membrane stains are commonly used for microscopic visualization of cell integrity. Treatment of *P. aeruginosa* and *S. aureus* with DMSO (1%, carrier control) resulted in uniformly stained membranes while addition of 34 µg/mL SPI009 induced brightly fluorescent membrane accumulations (Figure 5.5). In the Gram-negative *P. aeruginosa* another phenotype was visible: stained membrane blebs, possibly originating from severe outer membrane deformations.[338] The observed membrane accumulations after treatment with SPI009 confirm a direct effect of the compound on the bacterial membranes for both Gram-negative and Gram-positive species.

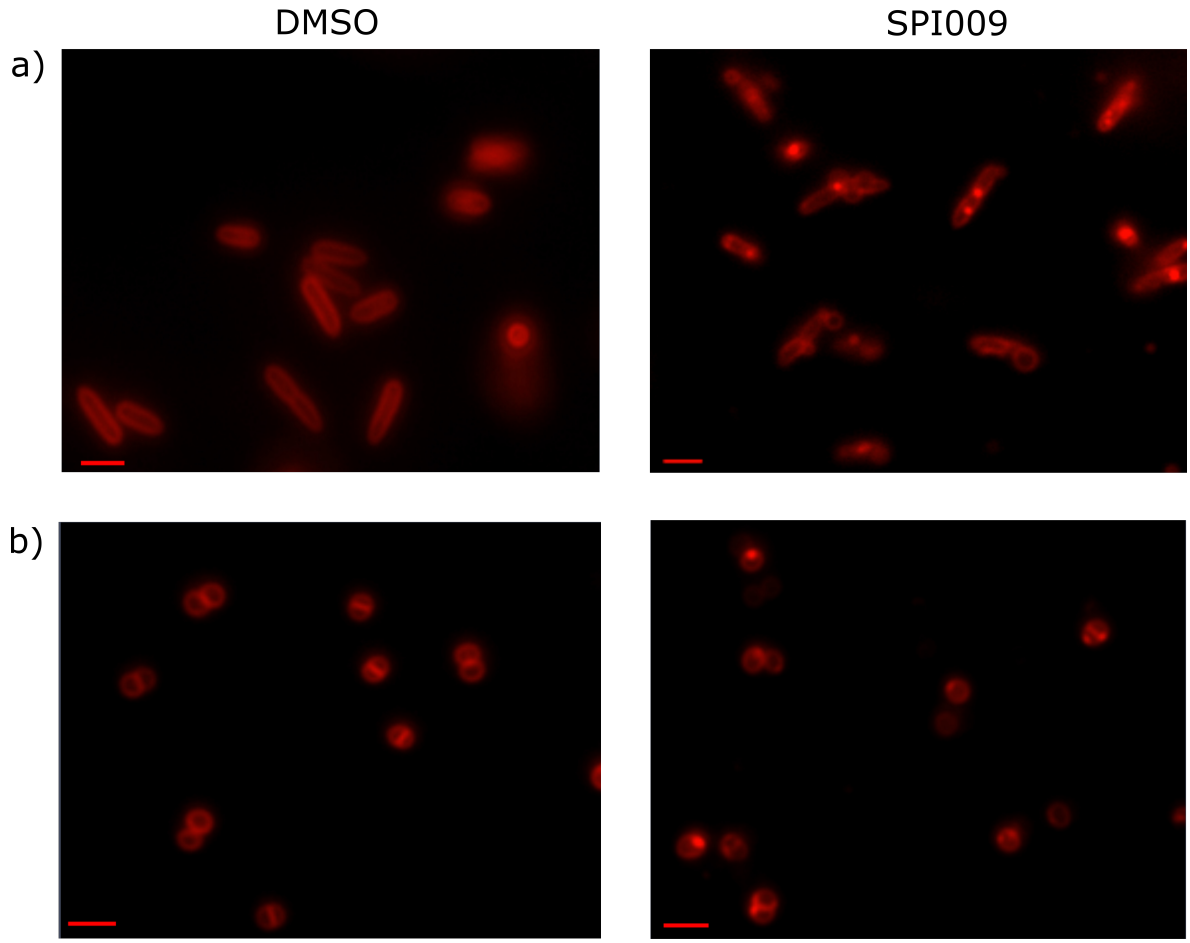


Figure 5.5: Microscopic confirmation of SPI009 induced membrane damage. Overnight cultures of a) *P. aeruginosa* PA14 and b) *S. aureus* cultures were treated for 20 minutes with 1% DMSO (carrier control) or 34 µg/mL of SPI009. Treated cells were stained with 10 µg/mL of FM® 4-64 and visualized using a Zeiss Axio imager Z1 fluorescence microscope equipped with a EC Plan-NEOFLUAR 100x objective (λex= 540-580 nm; λem= 593-668 nm). Scale bars correspond to 2 µM. Pictures are representatives of repeated experiments.

5.3.6 SPI009 interacts with the lipid A compound of the bacterial LPS layer

The BODIPY™-TR-cadaverine probe (BC) was used to reveal possible interactions of SPI009 with the bacterial LPS layer. If compounds are added that have the ability to bind lipid A, BODIPY™-TR cadaverine will be displaced, resulting in a strong increase in fluorescence[327,339] Positive and negative controls consisted of, respectively, polymyxin B, known to use the interaction with lipid A for self-promoted uptake and resulting in cell lysis[340], and meropenem, not capable of interacting with LPS. Comparison of the effects of equimolar amounts of SPI009 and these controls demonstrated a clear time and concentration-dependent interaction between SPI009 and lipid A (Figure 5.6). However, since it was previously shown that SPI009 maintains its antibacterial and anti-persister activity in Gram-positive bacteria, the interaction with lipid A in the bacterial LPS layer cannot be the sole mechanism of SPI009-induced membrane damage.

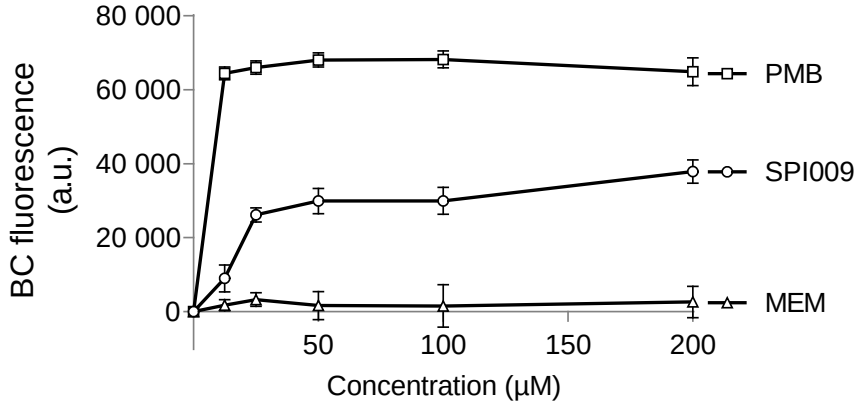


Figure 5.6: SPI009 interacts with the LPS layer. BC bound *P. aeruginosa* PA14 cultures were treated with equimolar amounts of SPI009, polymyxin B (positive control; PMB) and meropenem (negative control; MEM). Fluorescence was measured continuously, using λex=580 nm and λem=620 nm, and corrected for background fluorescence. Results represent the average of four independent repeats ± SEM with BC fluorescence values obtained after one hour.

5.3.7 The role of efflux pumps in SPI009 activity

The SPI009-induced inner membrane damage, as indicated by the SYTOX Green uptake assay, could be a secondary effect resulting from extensive outer membrane damage caused by SPI009. Alternatively, the compound may enter the bacterial cell and cause membrane damage from within. To explore these possibilities, different efflux mutants were evaluated for their sensitivity towards SPI009 (Figure 5.7).

Upon treatment with SPI009, the PAO1-derived YM64 mutant, lacking the four major *mex* operons of *P. aeruginosa*; *mexAB-oprM*, *mexCD-oprJ*, *mexEF-oprN* and *mexXY-oprM*[319], showed a significantly decreased survival for all concentrations tested. Treatment with 8.5, 17 and 34 µg/mL SPI009 caused significant 2.0 ± 0.3, 4.5 ± 0.9 and 3.7 ± 0.4 log unit decreases in survival as compared to the YM WT, respectively, while 68 µg/mL of SPI009 was capable of completely eradicating the efflux mutant. To further explore the role of the different efflux pumps missing in YM64, separate Δ*mexCD-oprJ* and Δ*mexXY* mutants were also analyzed. For these, only the Δ*mexCD-oprJ* strain showed decreased survival compared to the YM WT after treatment with 34 µg/mL SPI009. The obtained results clearly show that some *P. aeruginosa* efflux pumps, including MexCD-OprJ, are capable of actively removing SPI009 from the bacterial cell and suggest the involvement of other Mex pumps, most likely not MexXY-OprM. These experiments confirm the inner membrane as an important target of SPI009, in addition to the observed outer membrane permeabilization.

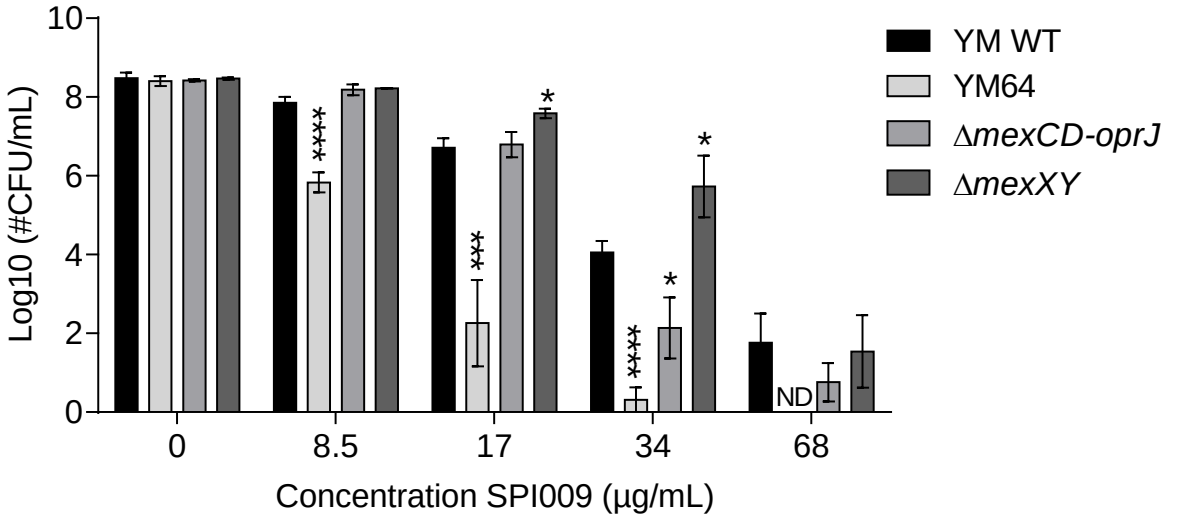


Figure 5.7: Activity of SPI009 in efflux mutants. *P. aeruginosa* efflux mutants YM64, Δ*mexCD-oprJ* and Δ*mexXY*, together with the YM WT strain, were treated for 5 hours with increasing concentrations of SPI009. Significant differences in sensitivity towards SPI009 between the wild type and different mutants were detected using multiple t tests (α = 0.05) with Holm-Sidak correction for multiple comparisons and represented by * $P < 0.05$; *** $P \leq 0.001$ and **** $P < 0.0001$. Values represent the average of at least three independent repeats with error bars depicting SEM values. ND, not detected.

5.3.8 Role of natural membrane permeability in SPI009 sensitivity

Since the natural membrane permeability of different bacterial species contributes to their intrinsic antibiotic resistance[35], we investigated whether this also affected the activity of SPI009. *P. aeruginosa* Δ*galU,* is no longer capable of synthesizing UDP-glucose, a precursor required for the formation of the glycosyl residues found in the bacterial LPS layer.[341] Natural membrane permeability was assessed by measuring NPN fluorescence of MilliQ (MQ) treated samples and revealed a significantly higher permeability for Δ*galU* (Figure 5.8a). Treatment with 17 or 34 µg/mL SPI009 revealed respective 1.4 ± 0.3 log and 2.9 ± 0.7 log unit decreases in survival as compared to the WT strain.

To rule out any *P. aeruginosa*-specific effects, we also tested *Acinetobacter baumannii* Ab-84R, containing a 40 bp insertion in *lpxC* which results in the complete loss of the LPS layer.[321] As compared to the wild-type strain Ab-84, the absence of the LPS layer greatly increased the observed natural membrane permeability (Figure 5.8b). The differential sensitivity of these strains for SPI009 shows a more pronounced character than for *P. aeruginosa* since treatment with 17 µg/mL of SPI009 already completely eradicated the LPS-deficient Ab-84R strain. Together, these results indicate that the structure of the bacterial LPS layer, which partly determines membrane integrity and strength, strongly influences the susceptibility of the cell towards SPI009.

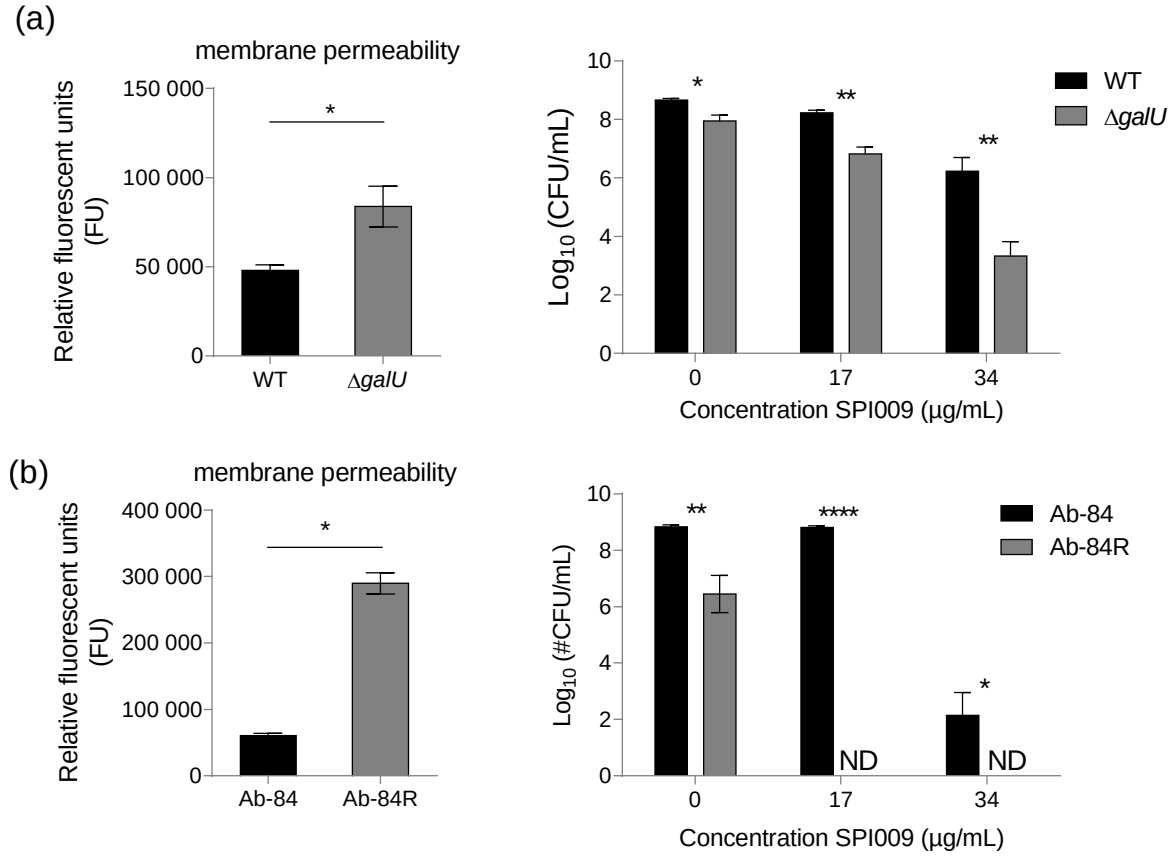


Figure 5.8: Cells displaying increased membrane permeability show higher sensitivity towards SPI009. Mutants with LPS biosynthesis malfunction or complete loss of the LPS layer were selected for a) *P. aeruginosa* PA14 and b) *A. baumannii*, respectively. Membrane permeability was assessed by means of NPN assays of untreated cultures (left panel). Statistical analysis was done by means of a single unpaired, two-tailed t test (α=0.05). Next, stationary phase cultures of wild type and mutant strains were treated for 5 hours with increasing concentrations of SPI009 (right panel). Statistical analysis was done by means of multiple t tests (α=0.05) to compare the antibacterial effect of SPI009 between the WT and mutant strain, with Holm-Sidak correction for multiple testing. Displayed data points show the average of at least three independent repeats ± SEM. *P< 0.05; ** P< 0.01; ***P< 0.001 and **** P≤ 0.0001; ND, not detected.

5.3.9 Generation and analysis of SPI009-resistant mutants

Being so far unable to generate spontaneous SPI009-resistant mutants on solid growth medium (our own unpublished data), an evolution experiment using the MIC broth dilution method was used to generate mutants showing a 10-fold increase in the SPI009 MIC. MIC values slowly increased for the strains grown in the presence of SPI009 while resistance to the conventional antibiotic ofloxacin showed a more abrupt transition with a first plateau of 16-fold increase in MIC being reached after just two days (Figure 5.11a). The results obtained for ofloxacin are in agreement with previous fluoroquinolone resistance evolution experiments.[129,329,342] Decreased sensitivity for SPI009 of the evolved strains was confirmed via MIC and plate assays, where treatment with 68 µg/mL of SPI009 caused a maximal 1.46 log decrease in the number of surviving cells (Figure 5.11b in the supplemental material). While the evolution of resistance development would suggest the consecutive acquisition of

several mutations, whole genome sequencing and analysis of the evolved strains revealed two identical non-synonymous SNPs in each of the parallel lines. A first mutation involved a 47A>C (Asp16Ala) change in PA14_08120 (PA0625), a phage tail length determination protein located in the outer membrane or outer membrane vesicles.[323,343] PA0625 is part of a 16-ORF gene cluster coding for R-type phage tail-like pyocins in *P. aeruginosa*, bacteria-produced bacteriocins that are capable of depolarizing the cytoplasmic membrane in sensitive cells and inhibiting active transport.[252,344,345] A second SNP, 88G>A, was identified in *nfxB*, the negative regulator of the MexCD-OprJ efflux system. Interestingly, this SNP is located in a predicted helix-turn-helix region responsible for DNA-binding. Other similar mutations in this region have been shown to disturb the binding to the MexCD-OprJ promotor and thus cause overexpression of this efflux pump and active expulsion of SPI009 from the cell.[346,347]

A transposon mutant of *nfxB* showed a significant decrease in sensitivity towards SPI009 resulting in 1.7 ± 0.4 and 3.1 ± 0.9 log unit increases in survival as compared to the wild-type after treatment with 17 µg/mL and 34 µg/mL of SPI009, respectively (Figure 5.11b). In contrast to the whole genome sequencing results, transposon gene knockout of PA14_08120 did not cause a significant increase in survival. Taken together, these results confirm the hypothesis that the MexCD-OprJ efflux is capable of protecting the cell against SPI009-mediated membrane damage. Further research will however be necessary to unravel the exact role of PA14_08120 in this mechanism.

5.4 Discussion

Increased understanding of persister formation mechanisms and the general acknowledgment of their clinical importance has resulted in a growing number of reports on anti-persister molecules, contributing to potential future treatment options in the fight against bacterial infections.[223,280] Targeting persisters is likely to greatly improve patient outcomes but unfortunately, the rational target-based design of anti-persister therapies remains a great challenge. Contributing to this are the limited numbers of persister cells, the incomplete knowledge and redundancy in mechanisms controlling persister formation and the observation that these processes are often species-specific.[83,135,223] A possible way of bypassing these issues is the use of well-designed whole cell screenings that can identify novel compounds based on anti-persister activity rather than target.[215] We recently reported the use of such a screening in the identification of SPI009 (1-((2,4-dichlorophenethyl)amino)-3-phenoxypropan-2-ol), a small molecule capable of directly killing persister cells of clinically relevant Gram-negative and Gram-positive pathogens in different *in vitro* and *in vivo* set-ups. Other anti-persister compounds reported to directly kill bacterial persister cells use varying strategies such as depolarization and destruction of the cell membrane, DNA cross-linking, inhibition of essential enzymes, and generation of reactive oxygen species.[223,280,281] Several characteristics of SPI009, such as its broad-spectrum activity, ability to tackle both dividing and non-dividing cells and the potentiation of mechanistically different antibiotics presented a first indication of a non-specific target. Identifying the mechanism of action for this novel anti-persister and antibacterial compound is not only important for the further development of possible therapies but also increases our knowledge about persister cells and contributes to the identification of possible targets for future anti-persister therapies.[317]

In this study, we present the detailed exploration of the mode of action of SPI009, combining complementary genetic and cellular approaches. Several lines of evidence support that SPI009 kills persister and non-persister cells by causing extensive membrane damage. The overall inhibition of macromolecular synthesis, together with the obtained genetic data, provided us with the indication that SPI009 induced membrane damage.[327,332–334] Several membrane and whole-cell based assays confirmed this hypothesis and revealed the possibility of SPI009 to efficiently and extensively damage both the outer and inner bacterial membrane. Furthermore, microscopic analysis revealed membrane damage in both Gram-positive and Gram-negative bacteria upon treatment with SPI009, while Gram-negative cells additionally showed severe outer membrane deformations and blebs. Since the integrity of the bacterial

envelope remains crucial for the viability of persister cells, this have previously been suggested as potential targets for anti-persister strategies.[158] Several anti-persister compounds described in literature, such as the peptidoglycan-degrading Artilysin® Art-175[129,130], membrane-acting peptides[166], HT61[159,240] and AM-0016[231] use this strategy to efficiently tackle antibiotic-tolerant persister cells of both Gram-negative and Gram-positive species.

Although SPI009 proved capable of interacting with the lipid A moiety of the bacterial LPS layer, the demonstrated activity in Gram-positive species and LPS-deficient strains excludes LPS as the primary binding target of SPI009. However, the architecture of the LPS layer and resulting membrane permeability do have a strong influence on SPI009 activity and could propose LPS as an initial binding target in Gram-negative species. Possible explanations include the physical barrier formed by the LPS sugars, its influence on overall membrane strength or the changes in membrane charge due to the absence or presence of sugars and phosphate groups.[348,349] Additionally, the increased SPI009 sensitivity of the *P. aeruginosa* YM64 mutant, lacking the four major Mex efflux pumps[319], provided evidence that SPI009 can cause cytoplasmic membrane damage and suggests the possible use of efflux pump inhibitors to further increase SPI009 activity.

Additional experiments revealed a possible role for MexCD-OprJ in the efflux of SPI009, but the substantial difference in sensitivity between YM64 and Δ*mexCD-oprJ* suggests that additional efflux mechanisms are involved. Interestingly, both the genetic screen and RNA sequencing revealed genes belonging to the *mexCD-oprJ* system and its regulator *nfxB*, previously reported to be inducible by membrane-damaging agents and best known for its role in fluoroquinolone resistance.[347,350,351] Besides increasing the efflux of antibacterial compounds through MexCD-OprJ, Δ*nfxB* also influences cell membrane permeability[346], thus suggesting an alternative role for *nfxB* in SPI009 resistance. The involvement of this bacterial efflux pump was corroborated through genetic analysis of the evolved SPI009-resistant strains, where all three independent mutants showed SNPs in *nfxB* and PA14_08120. While inactivation of *nfxB* strongly decreased sensitivity towards SPI009, this was not the case for PA14_08120. The R2 pyocin region of *P. aeruginosa* has previously been linked with antibiotic resistance. Fluoroquinolone induced production and release of pyocins was shown to cause cell lysis, while deletion of several R2 genes induced significant resistance to ciprofloxacin.[352,353] However, the lack of upregulation of any of the R2 genes in response to SPI009 (Tabel A.2 in Appendix A), suggests a different role for PA14_08120 in SPI009

susceptibility. Differences in survival between the *nfxB* knockout mutant and the SPI009-resistant strains could suggest the enhancement of the observed SPI009 resistance after accumulation of both mutations. The high selection pressure needed to generate SPI009-resistant mutants, combined with the lack of any clear resistance phenotypes after single-gene knockout, predicted fitness defects of Δ*nfxB*[354] and possibility of using efflux pump inhibitors[37] all decrease the chances of SPI009 resistance emerging in *in vivo* situations.

Overall, the use of different combined approaches resulted in compelling evidence that the novel antibacterial compound SPI009 is capable of directly killing both Gram-negative and Gram-positive bacterial cells as a consequence of severe membrane damage. Further experiments revealed the ability of the compound to impair both the outer and inner membrane of *P. aeruginosa*, the latter being a direct consequence of SPI009 entry into the cell. The crucial importance of membrane integrity for survival of both active and metabolically inactive bacterial cells combined with the suggested limited resistance potential of membrane-damaging compounds[158] and the previously reported limited cytotoxicity[161], further support the clinical potential of SPI009 and its role as a scaffold in the development of future anti-persister therapies.

5.5 Supplemental material

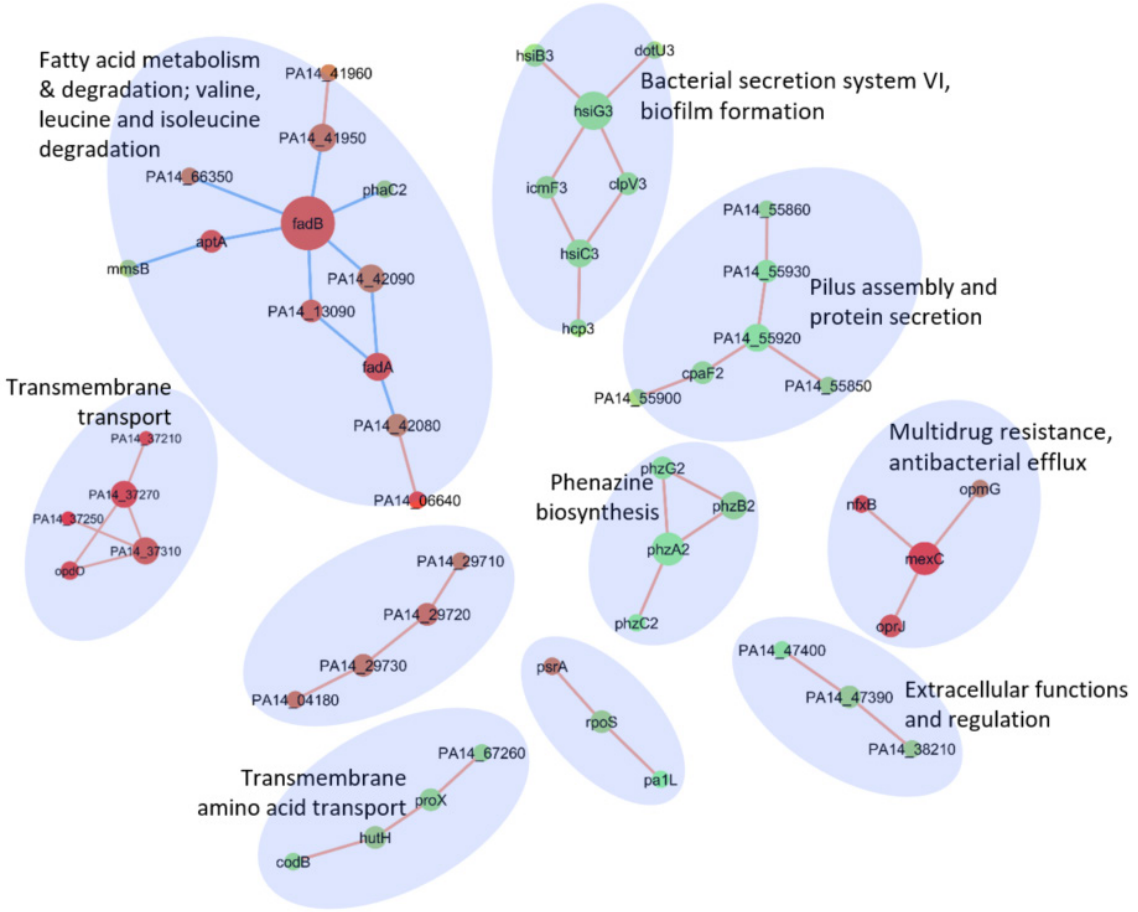


Figure 5.9: PheNetic network analysis of differential expression data upon treatment with SPI009. PheNetic network analysis was performed on obtained RNAseq data using PheNetic (downstream, cost = 0.25) and the custom-made UCBBP-PA14 interaction network. Sub-networks of only two genes were excluded from analysis. Nodes and connecting lines represent genes and interactions differentially active upon treatment with 50 µM SPI009. Red and green circles represent up- and downregulated genes with color intensity correlated to the absolute log-fold change. Orange and blue lines represent protein-protein and metabolic interactions, respectively.

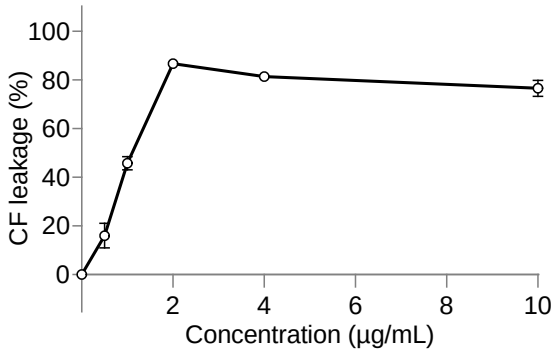


Figure 5.10: Disruption of artificial bacterial membranes by polymyxin B. SUVs were treated for 15 minutes with increasing concentrations of polymyxin B. % CF leakage, as determined relative to the positive control (0.5% Triton X-100) is represented on the y-axis. Data points represent the mean of three independent experiments ± SEM.

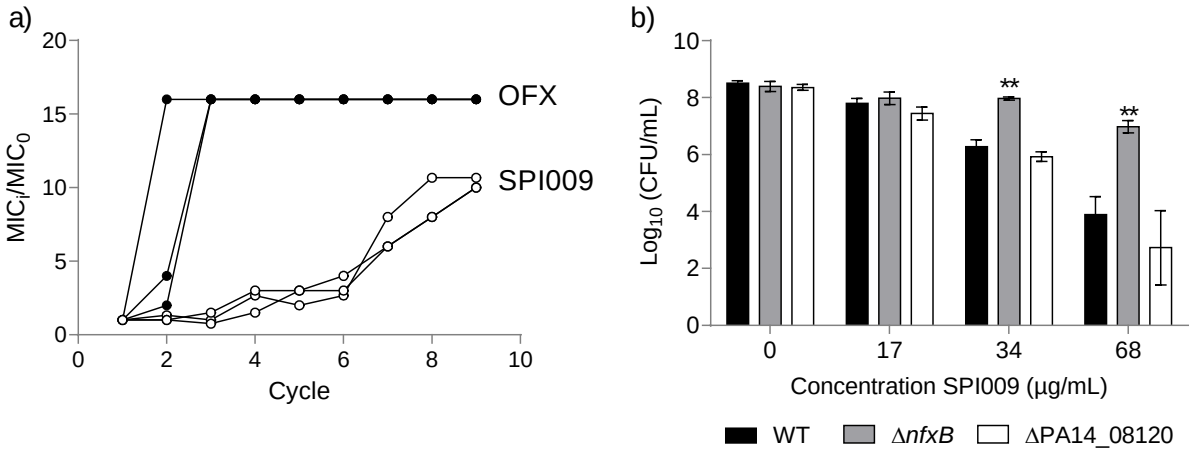


Figure 5.11: High selection pressure resistance development in SPI009. a) Six independent PA14 strains were treated daily with, if necessary, increasing concentrations of ofloxacin (filled circles) or SPI009 (open circles) in a MIC-based evolution experiment. The y-axis represents the fold increase in MIC-value relative to the MIC at the start of the experiment. Three separate strains are shown for each antimicrobial. b) Stationary phase cultures of PA14 (black bars) and transposon mutants of *nfxB* (grey bars) and PA14_08120 (white bars), the two SNP's identified in all SPI009 resistant strains, were treated for 5 hours with increasing concentrations of SPI009. Data points represent the average of at least three independent repeats ± SEM. Statistical differences between the WT and mutants were determined by means of multiple t tests using the Holm-Sidak correction for multiple comparisons. ** $P < 0.01$.

5.6 Additional data to the above mentioned manuscript

5.6.1 LPS characterization of Δ*galU*, Ab-84 and Ab-84R

As described in the above manuscript, natural membrane permeability can influence the activity of SPI009. Different approaches were used to confirm that strains with an increased membrane permeability were more sensitive to the antibacterial effects of SPI009. Selection of the strains used in section 5.3.8, *P. aeruginosa* Δ*galU* and *A. baumannii* strains Ab-84 and Ab-84R, was done based on literature and therein reported defects in membrane structure.

galU encodes an ATP-glucose-1-phosphate uridylyltransferase and is highly conserved among *Pseudomonas* species.[323] Inactivation of *galU* blocks the biosynthesis of UDP-glucose from glucose 1-P, a precursor needed for the production of the outer core glycosyl oligosaccharides, resulting in a truncated core oligosaccharide and lack of O-polysaccharides.[341,355] Multi-drug resistant *A. baumannii* clinical isolates Ab-84 and Ab-84R were chosen based on a previously reported 40-nucleotide insertion at position 321 in *lpxC* of Ab-84. Mutations in any of the LPS biosynthesis genes *lpxA*, *lpxC* or *lpxD* can lead to complete loss of LPS expression. Loss of this protective barrier of the Gram-negative cell confers resistance to colistin, due to loss of the initial interaction target, but susceptibility to other antibiotics has been show to change as well.[321]

To confirm the changes in LPS structures for both mutants, LPS profiles were determined for wild-type and mutant strains, as previously described.[356,357] Briefly, overnight bacterial cultures were diluted to an OD_{600} of 0.5 and washed twice with 1 x PBS. Pellets were dissolved in 250 µL Hitchcock and Brown Lysis Buffer and heated to 95°C for 30 minutes. After cooling down, 1.5 µL volumes of proteinase K were added and mixtures were incubated overnight at 55°C. Samples were heated to 95°C for 5 minutes, after which 5 µL volumes were loaded onto a Bolt™ 12% Bis-Tris Plus gel for SDS-PAGE. Electrophoresis was allowed for 1 hour at 150 V after which LPS was visualized using the SilverQuest Silver Staining Kit according to the manufacturer's instructions. A representative LPS profile gel for the different strains is shown in Figure 5.12.

Analysis of the obtained LPS profiles confirms the expected changes in LPS structure for the different mutants. *ΔgalU* shows a clear reduction in O-antigen chains and a less clear core band, indicative of a faster migrating lipid A core (Figure 5.12). The Ab-84R shows an even more pronounced difference with the corresponding wild type since no clear O-antigen

or core structure could be observed, corroborating the *lpxC* inactivation and subsequent loss of LPS biosynthesis. The obtained LPS profiles confirm the described mutations in these strains and their effects on LPS structures. The increased sensitivity of *ΔgalU* and Ab-84R to SPI009, as described in section 5.3.8, can thus be explained by their altered LPS structure and subsequent influence on membrane integrity.

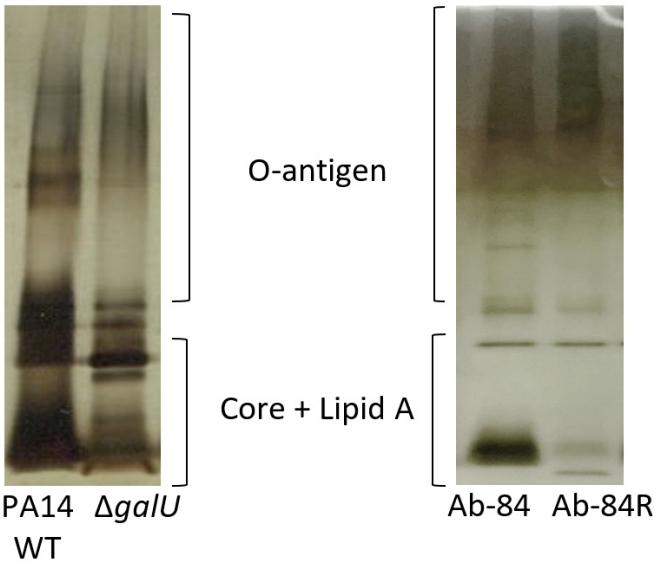


Figure 5.12: LPS profiles of P. aeruginosa WT, Δ*galU* and *A. baumannii* Ab-84 and Ab-84R. Thick bands at the bottom of the gel represent the Lipid A core. The higher located bands show O antigen side chains of varying lengths. The *galU* mutant lacks O-antigen side chains in comparison with the WT and shows a faster migrating Lipid A core. The *A. baumannii* Ab-84R mutant lacks any clear LPS structures in comparison with the Ab-84 wild-type strains. The results shown are representative of three independent repeats.

5.6.2 Influence of Mg^{2+} on the sensitivity to SPI009 antibacterial activity

The combination of parallel genetic and molecular approaches used to unravel the mechanism of action of SPI009 provided plentiful evidence of extensive damage to both the outer and inner bacterial membrane (section 5.3). As described above, alterations to the bacterial LPS layer can influence the sensitivity towards SPI009.

The specific architecture of the LPS layer functions as a selective permeation barrier and causes intrinsic resistance in many Gram-negative bacteria towards hydrophobic antibiotics, detergents and hydrophobic dyes.[358,359] Possible explanations for this include the role of LPS in the overall membrane strength and permeability, the physical barrier it presents and the differences in membrane charge when sugar and phosphate groups are adapted.[348,349] It is generally assumed that addition of positively charged divalent cations, such as Mg^{2+} and Ca^{2+} can strengthen the bacterial outer membrane of Gram-negative species

by interacting with the negatively charged phosphate groups and reinforcing lateral interactions between different LPS molecules.[359,360] Since chelation of these cations, for example by EDTA, can increase the activity of conventional antibiotics by increasing membrane permeability[361], we wanted to see whether increased membrane strength after addition of Mg^{2+} could reduce the activity of membrane-damaging compounds such as SPI009. For this, cultures of *P. aeruginosa* PA14 were grown to stationary phase in increasing concentrations of $MgSO_4$ (0 µM, 2 µM, 20 µM and 2 mM), after which excess Mg^{2+} was washed away and cells were treated for 5 hours with increasing concentrations of SPI009 (Figure 5.13).

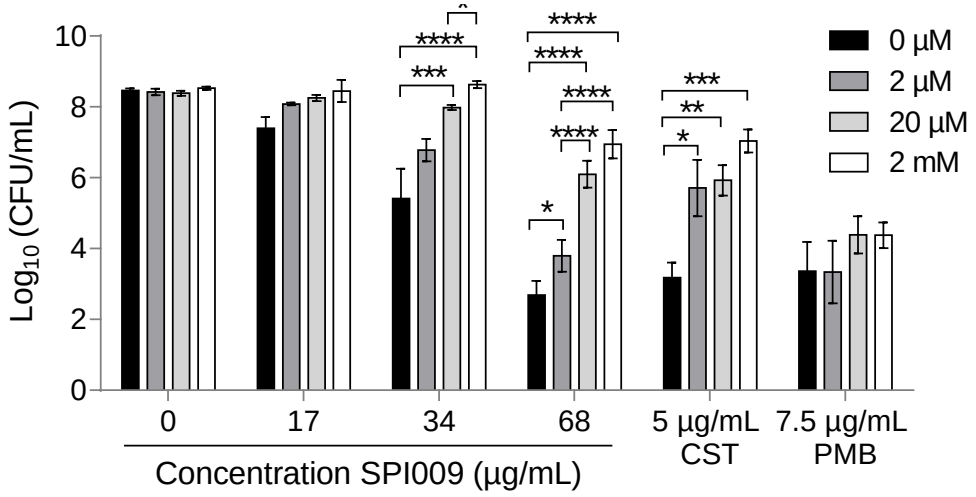


Figure 5.13: Effect of Mg^{2+} on SPI009 activity. *P. aeruginosa* PA14 was grown to stationary phase in the presence of increasing concentrations of $MgSO_4$ (different shades of grey). After washing away excess Mg^{2+} cations, cultures were treated for 5 hours with increasing concentrations of SPI009 (0 - 68 µg/mL; x-axis), 5 µg/mL colistin (CST) or 7.5 µg/mL of polymyxin B (PMB). Survival was assessed by means of CFU counting (y-axis). Data points represent the averages of at least four independent repeats ± SEM. Statistical significant differences in survival for different $MgSO_4$ concentrations were determined using a Two-way ANOVA with Tukey correction for multiple comparisons and * $P < 0.05$; ** $P < 0.01$, *** $P < 0.001$; **** $P \leq 0.0001$.

The obtained results show a clear influence of Mg^{2+} supplementation on the antibacterial effect of SPI009. While varying concentrations of $MgSO_4$ already show some variation in bacterial survival after treatment with 17 µg/mL SPI009, 2 mM $MgSO_4$ causes significant 3.2 ± 0.6 and 4.3 ± 0.4 log unit decreases in bacterial survival upon treatment with 34 and 68 µg/mL SPI009, respectively, as compared to 0 µM of $MgSO_4$. Additional analysis shows a strong decrease in SPI009-mediated killing at increasing concentrations of $MgSO_4$, suggesting that increased amounts of positively charged Mg^{2+} cations indeed interrupt SPI009 activity. A similar trend, although less pronounced, could be observed for 5 µg/mL colistin (Figure 5.13). Since colistin kills bacterial cells by self-promoted uptake via electrostatic interaction with the LPS layer and subsequent disturbance of the cell membrane[314], it was

expected that membrane alterations such as those suggested for Mg^{2+} addition indeed have the potential to reduce colistin activity. Despite the fact that colistin and polymyxin B belong to the same antibiotic class of polymyxins, and share a very similar mechanism of action, differences in polymyxin B induced bacterial killing could only be observed between low (0 and 2 µM) and higher (20 µM and 2 mM) concentrations of $MgSO_4$.

5.6.3 Effect of $MgSO_4$ on the bacterial membrane

The above results demonstrate the reduced activity of SPI009 in cultures with Mg^{2+} reinforced membranes. On the one hand, Mg^{2+} can interact with the negatively charged phosphate groups in the bacterial membrane, reducing the membrane damaging potential of SPI009. Alternatively, Mg^{2+} could interact with SPI009 itself, resulting in the formation of inactive Mg-SPI009 complexes that are no longer capable of interacting with the bacterial membrane.
The latter hypothesis could however be rejected. First of all, washing the bacterial cultures after growth in $MgSO_4$, to remove any excess Mg^{2+} that could possibly interact with SPI009, showed no remarkable differences in bacterial survival with unwashed cultures. Additionally, NPN experiments were performed as previously described,[161,327] on cultures grown in increasing concentrations of $MgSO_4$ to determine the influence of Mg^{2+} cations on natural membrane permeability (Figure 5.14).

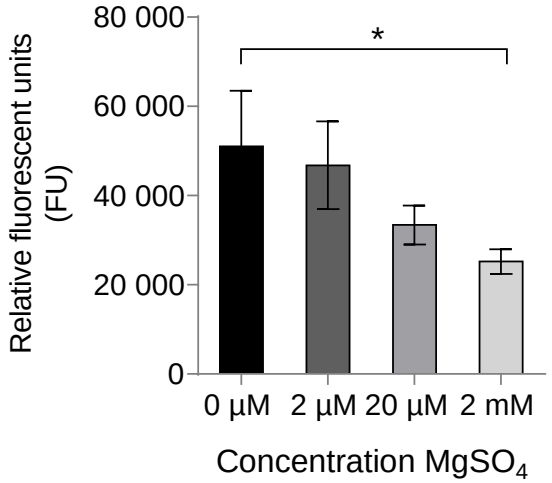


Figure 5.14: Influence of $MgSO_4$ on natural membrane permeability. Cultures grown in increasing concentrations of $MgSO_4$ (x-axis) were assessed for natural membrane permeability via an NPN assay upon treatment with MilliQ. Data points represent the average of four independent repeats ± SEM. Statistical differences in membrane permeability were calculated using a Two-way ANOVA (α=0.05) and Tukey correction for multiple comparisons. * $P < 0.05$.

Despite the fact that significant differences in natural membrane permeability could only be observed between the extreme concentration values, there is a clear trend. Increasing concentrations of $MgSO_4$/Mg^{2+} are indeed capable of reinforcing the bacterial membrane, resulting in decreased NPN fluorescence and thus membrane permeability.

5.6.4 Conclusion

Overall, these results once again confirm the membrane damaging activity of SPI009 and suggest that alterations to the bacterial membrane have the potential to strongly influence SPI009-induced bacterial killing. Furthermore, the specific effect of Mg^{2+} on SPI009 activity could suggest a possible chelating effect of SPI009, actively removing Mg^{2+} from the outer leaflet. EDTA, a well-known chelator, has been shown to disrupt electrostatic cross-links of divalent cations within the LPS inner core, thereby disturbing the membrane's structural integrity and possibly causing ruptures in the outer membrane that facilitate entry and thus the effect of bactericidal antibiotis.[359–362] However, degradation of phospholipids and fatty acids upon SPI009 treatment, as measured in the previously mentioned macromolecular synthesis assays (section 5.3.2) did not show any changes in measured radioactivity and could thus not confirm any SPI009-mediated degradation. Additionally, comparison of the chemical structure of the compound (section 3.3.1) with known chelating agents, such as EDTA, suggests a lack of negative charges to efficiently interact with the metallic Mg^{2+}. Alternatively, the previously shown lipid A interaction, together with the presence of aromatic groups could suggest that SPI009 disrupts the hydrophobic stacking of the membrane phospholipids.

Further experiments should be performed to elucidate the exact membrane damaging mechanism of SPI009. Possible experiments include Atomic Force Microscopy, previously shown capable of visualizing the specific membrane alterations caused by EDTA[362], titration experiments or measurement of LPS release upon SPI009 treatment.

Chapter 6

Genetic analysis and characterization of clonal *P. aeruginosa* isolates collected from cystic fibrosis patients and showing differences in SPI009 sensitivity

6.1 Introduction

In the exploration of the mode of action and activity spectrum of the novel anti-persister compound SPI009, several clinical isolates were tested for their sensitivity towards the compound. These included a set of nine clonal pairs isolated from the sputum of the same CF patient between 0.5 and 14 months apart and proven to be genetically related. *P. aeruginosa* is known for causing life-threatening infections in CF patients and 85-90% of all patients will eventually die due to the consequences of chronic *P. aeruginosa* infection.[27,29] During infection, the bacterium is capable of undergoing different genetic mutations to adapt to the specific CF lung environment. Typical changes occurring over time include increased antibiotic tolerance, alginate production and biofilm formation to support long-term survival of the pathogen in the human lung. Another common evolutionary adaptation is the reduced virulence and the shift towards a host-adapted chronic virulence.[11,259,363–365] Not only do these genetic alterations change the susceptibility of the strain towards different antibiotics, research by the Lewis group also indicated an increased production of persister cells in late CF isolates of *P. aeruginosa*.[70]

To evaluate the ability of SPI009 to kill *P. aeruginosa* strains isolated from CF sputum, a set of nine clonal pairs was used to determine SPI009 activity. The 18 clinical isolates were treated with the combination of ofloxacin and SPI009 and while this treatment could successfully eradicate most of the isolates, two pairs showing an interesting evolution in sensitivity towards the combination therapy were selected for further research. Of these, the SSF65 pair, showing a significant decrease in sensitivity towards SPI009 alone in the late isolate, was selected for whole genome sequencing. Additionally, a strain-specific antibiogram was constructed and the production of several quorum sensing and virulence related molecules was assessed. The combination of the observed genetic and molecular differences between the early and late isolate of SSF65 could provide more information about the mechanism of action of SPI009.

6.2 Materials and methods

Bacterial strains and culture conditions. Bacterial strains were cultured in 1/20 diluted Trypticase Soy Broth (1/20 TSB) at 37 °C shaking at 200 rpm. For solid medium, TSB was supplemented with 1.5% agar. Clinical isolates consisted of confirmed clonal pairs isolated between 0.5 and 14 months apart, as indicated in Table 6.1.

Table 6.1: Overview of strains used in this study. All clinical isolates refer to clonal pairs of *P. aeruginosa* isolated from the sputum of CF patients.

Strain	Description	Time between isolations (months)	Source or reference
P. aeruginosa PAO1	Wild type		Dieter Haas (ETH)
BJM85-267 & 544	CF sputum isolate	3	Françoise van Bambeke (UCL)
BSM70-467 & 612	CF sputum isolate	1	Françoise van Bambeke (UCL)
DAF87-203 & 341	CF sputum isolate	13	Françoise van Bambeke (UCL)
DJM92-552 & 672	CF sputum isolate	1	Françoise van Bambeke (UCL)
MBM00-236 & 419	CF sputum isolate	14	Françoise van Bambeke (UCL)
NFM63-245 & 555	CF sputum isolate	3	Françoise van Bambeke (UCL)
RMF84-122 & 219	CF sputum isolate	1	Françoise van Bambeke (UCL)
SSF65-443 & -563	CF sputum isolate	1	Françoise van Bambeke (UCL)
TCM51-177 & 229	CF sputum isolate	0.5	Françoise van Bambeke (UCL)

Anti-persister and anti-bacterial assay. The effect of SPI009 on persister cells of different strains was determined as described previously.[161] Briefly, stationary phase cultures were treated for 5 hours with 10 µg/mL ofloxacin or the combination of ofloxacin and 34 µg/mL of SPI009. Survival assessment was done by means of CFU counting after plating of the appropriate dilutions on solid agar plates. Strains selected for further research were also treated with increasing concentrations of SPI009 (17 - 34 µg/mL) alone and in combination with 10 µg/mL ofloxacin to assess both antibacterial and anti-persister effect, respectively, in more detail.

Whole genome sequencing and variant detection of selected strains.[5] To detect genetic differences between the early and late isolate, genomic DNA of an overnight culture was purified using the DNeasy Blood & Tissue Kit (Qiagen) and sent out for whole genome

[5] Initial processing of sequencing data and contig assembly as described in this chapter was performed by Toon Swings

sequencing to the Genomics Core Facility of EMBL (Heidelberg, Germany). After paired-end sequencing on a Illumina HiSeq2000, CLC Genomics Workbench version 6.5.1 was used for analysis of the obtained sequences. Trimmed reads were used for *de novo* assembly using the CLC Assembly Cell version 4.0 algorithm, generating a set of contigs for each of the strains. Reads of the early and late isolate were mapped onto the contigs of the late and early isolate, respectively, after which variant detection and InDel analysis were performed to identify genetic differences between the early and late isolate. Additional approaches to determine relevant differences between the early and late isolate consisted of Rapid Annotation of microbial genomes using Subsystems Technology (RAST), an online server allowing the annotation and functional or genetic comparison of draft genomes or contigs.[366] The Mauve Contig Mover allowed the ordering of contigs of the early and late isolate to create draft genomes while Mauve Aligner, using the progressive Mauve alignment algorithms, facilitated visual mapping and identification of large genetic alterations between both isolates.[367]

LPS profiling. LPS was isolated as previously described[356], using the method of Hitchcock and Brown. Purified LPS was loaded on a NuPAGE® Novex® Bis-Tris Mini Gel for SDS-PAGE and subsequent visualization with the SilverQuest Silver Staining Kit (Invitrogen), using the basic staining protocol.

MIC determination. MIC values for different classes of antibiotics were determined according to EUCAST standards using the broth microdilution method.[271] Briefly, a starting inoculum of 5 x 10^5 cells/mL was used to set up a two-fold dilution series of antibiotic or compound. Cultures were grown for 24 hours at 37°C, shaking, after which the OD_{595} was measured. MIC was defined as the lowest concentration to completely inhibit bacterial growth.

Pyocyanin and pyoverdin quantification. Levels of pyocyanin production were estimated for the PAO1 strain and SSF65-443 and SSF65-563 clinical isolates, as described previously.[368] Standardized overnight cultures were grown for 18 hours in LB medium after which pyocyanin was extracted from the culture supernatant using chloroform and 0.2M HCl. The absorbance was measured at 520 nm and the resulting pyocyanin concentrations in µg/mL cell culture were obtained through multiplication by an extinction coefficient of 17.072. Pyoverdin quantification was performed as previously described with minor modifications.[369] Cultures were grown for 24 hours in supplemented casamino acids medium, OD_{600} was measured and

the cultures were centrifuged for 30 minutes. After measuring the OD_{420} of the supernatant, pyoverdin production was calculated as the ratio of OD_{420}/OD_{600}.

Quantification of short- and long-chain AHLs.[6]Production of short-and long-chain AHLs was assessed through an adaptation of the previously described QSIS2 assay.[370] Briefly, overnight cultures of *P. aeruginosa* QSIS2, a Δ*lasR*Δ*rhlI*-pLasB-SacB1 strain encoding an AHL-induced killing system, was diluted in fresh minimal AB medium supplemented with 0.5% glucose, 0.5% casamino acids, 2.5 µg/mL thiamine and 80 µg/mL of gentamicin. The OD_{590} was adapted to 0.1. 50 µL of QSIS2 cultures was dispensed in the wells of a 96-well microtiter plate after which 50 µL of LB supplemented with sucrose (56 mg/mL) was added to each well. Gentamicin was added at a concentration of 80 µg/mL. QSIS2 cells were allowed to grow for 6 hours in the presence of MilliQ water (negative control), supernatant of PAO1, SSF65-443 or SSF65-563, or 200 nm concentrations of 3-oxo-C12-HSL and C4-HSL (Sigma) as a positive control. Absorbance was measured at 450 nm and the difference in growth between the negative and positive control was set at 100%. The assay was repeated five times, with six technical repeats of every condition.

[6] Experiments described in this paragraph were performed by Gilles Brackman, Laboratory of Pharmaceutical Microbiology, Ghent University, in collaboration with prof. Tom Coenye.

6.3 Results

6.3.1 Identification of clonal strains with altered sensitivity to SPI009

The set of clinical isolates consisted of nine clonal pairs, isolated between 0.5 and 14 months apart from the sputum of CF patients. To test their sensitivity towards the anti-persister effect of SPI009, the strains were treated for 5 hours with 10 µg/mL ofloxacin or the combination of ofloxacin and 34 µg/mL of SPI009 (Figure 6.1). Combination treatment with SPI009 was capable of successfully eradicating 13 of the 18 clinical isolates, highlighting the possible clinical use of SPI009 in the treatment of chronic *P. aeruginosa* infections. Further analysis of the obtained results revealed two clonal pairs for which a difference in SPI009-mediated killing could be observed between the early and the late isolate. For the first pair, DAF87-203 and DAF87-341, combination therapy with SPI009 lost its ability to completely eradicate the bacterial culture in the late isolate, resulting in a 2.4 ± 1.4 log difference in survival between the two isolates (Figure 6.1). For the second pair, SSF65-443 and SSF56-563, an opposite evolution was observed where the early isolate was less sensitive towards the combination therapy compared to the late isolate, resulting in a 3.2 ± 1.7 log difference in survival (Figure 6.1).

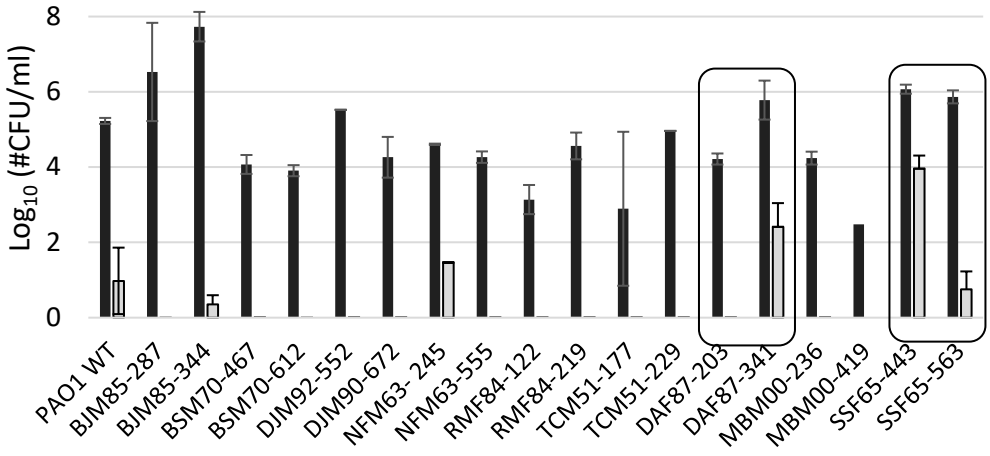


Figure 6.1: Anti-persister effect of SPI009 on nine *P. aeruginosa* clonal pairs of clinical isolates. 18 clinical isolates and the PAO1 strain were treated for 5 hours with 10 µg/mL ofloxacin (black bars) or the combination of ofloxacin with 100 µM of SPI009 (34 µg/mL; grey bars). Data points represent the average of at least two independent repeats. Rectangles highlight the clonal pairs showing an evolution in SPI009 sensitivity.

To obtain a more complete picture about the activity of SPI009 in the selected clonal pairs, additional experiments were carried out to assess both the anti-persister and antibacterial effect of SPI009. For this, stationary phase cultures were treated for 5 hours with

1% DMSO (carrier control), 17 µg/mL and 34 µg/mL of SPI009 and the combination of 10 µg/mL ofloxacin with 17 µg/mL or 34 µg/mL of SPI009 (Figure 6.2). Analysis of the obtained results revealed differences in the response to SPI009 for both clonal pairs. While DAF87-203 showed a significantly greater sensitivity towards the combination therapy, treatment with SPI009 alone did not reveal any significant differences between the early and late isolate. For the SSF65 clonal pair, treatment with 17 and 34 µg/mL of SPI009 alone did result in significant 0.78 ± 0.72 log and 3.42 ± 0.72 log unit decreases in survival for the late isolate SSF65-563, as compared to the early isolate. Since previous mode of action studies focused on the effect of SPI009 alone, the SSF pair was selected for further research.

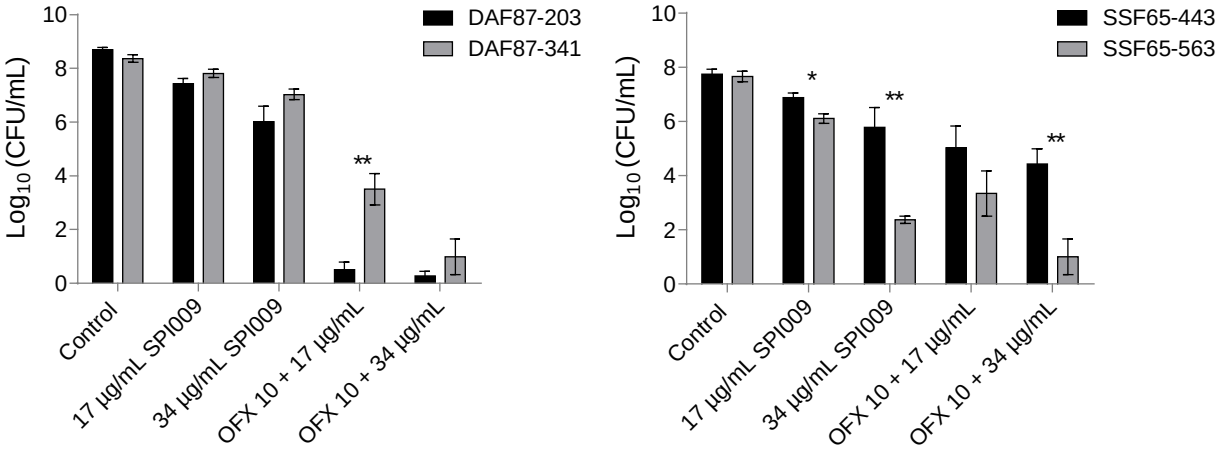


Figure 6.2: Analysis of SPI009 anti-persister and antibacterial effect in clonal pairs DAF87 and SSF65. Stationary phase cultures were treated for 5 hours with increasing concentrations of SPI009 alone, or in combination with 10 µg/mL ofloxacin. Early isolates DAF87-203 and SSF65-443 are shown in black, late isolates in grey. Data points represent the averages of at least three independent repeats ± SEM. Statistical analysis was done by means of multiple t tests, including Holm-Sidak correction for multiple comparisons. * $P < 0.05$; ** $P < 0.01$.

6.3.2 Genetic analysis of early and late clinical isolates

Since all clinical pairs were classified as clonal, this provided the unique opportunity to study the genetic differences occurring in *P. aeruginosa* during infection of the CF lung. Since the goal is to obtain more information about the mode of action of SPI009, analysis is focused on the differences between the early and late isolate of pair SSF65. The applied workflow resulted in the identification of several small and large genetic alterations, as depicted in Table 6.2.

Table 6.2*:* Overview of the genetic differences between early isolate SSF65-443 (6 352 955 bp) and late isolate SSF65-563 (6 267 601 bp).

Mutation	Reference	Allele	Gene	Description [g]
Insertion	-	T	*hslU* (PA5054)	ATP dependent protease ATP-binding subunit of heat-shock protein HslUV
Deletion	C	-	PA2269	Hypothetical membrane protein; MFS superfamily
SNV	T	G	*pauA4* (PA2040)	Glutamylpolyamine synthetase
Deletion	85 kb	-		

SNV = Single-nucleotide variant

Despite the fact that isolation of both strains was done only three months apart, a large-scale genomic deletion of 85 kb was observed, underscoring the adaptability of *P. aeruginosa* under these specific conditions. The rapid changes in genomic composition of CF isolates is often attributed to mutations in the DNA mismatch repair system and more specifically the *mutS* gene, resulting in mutator phenotypes.[365] In general, the genetic changes reported to occur in *P. aeruginosa* CF lung infections result in decreased biofilm formation and dispersion, an increase in the ability to form antibiotic-tolerant persister cells, loss of quorum sensing and reduced production of virulence factors with a subsequent transition to chronic virulence and adaptation of metabolic pathways. All these changes allow *P. aeruginosa* to thrive in the specific lung environment and avoid detection by the human immune system.[365,371–373] In the next paragraphs, the genetic differences between the early and late isolate, and their possible role in SPI009 sensitivity will be examined.

HslU, the ATP-binding subunit of the HslUV ATP-dependent protease, plays an important role in the pseudomonal heat stress response and the protection of the cell against indole and tobramycin.[374,375] While HslU could be linked to QS[376] and the adaptation of *P. aeruginosa* to the CF lung [377], no clear evidence was found for a possible role in SPI009 sensitivity.PA2040 Although no previous record is found of adaptations in the glutamine synthetase PA2040 in CF isolates, its role in the microbial metabolism in diverse environments provides a possible link with the further adaptation of the SSF strain to the specific lung environment[323] but no link was identified to the decrease in SPI009 sensitivity. PA2269 is a predicted membrane protein acting as a permease in an MFS transporter. Mutations in

[g] Descriptions are based on gene sequences and combined information obtained through RAST[366], NCBI Blast and Pseudomonas.com.[323]

transporter proteins could influence SPI009 uptake or efflux, thereby increasing intracellular concentrations of the compound and resulting in an increased bacterial killing.

Despite possible links with stress responses and changing environments for *hslU* and PA2040, no clear evidence could be found for the small-scale mutations to greatly influence SPI009 sensitivity. Changes in general stress responses and efflux could however contribute to the described increase in SPI009-mediated killing observed in the late isolate SSF65-563.

Further efforts were aimed at unraveling the role of the large genetic deletion in SPI009 observed sensitivity. Genetic analysis, combined with annotation of the obtained contigs by RAST[366] revealed the presence of 72 different genes involved in different cellular processes such as virulence, quorum sensing, metabolism,…. (See table B.1 in Appendix B). Comparison with literature revealed several genes reported to be deleted in later stage *P. aeruginosa* lung infections. This includes the 'putative exopolysaccharide-related' cluster spanning PA1378 - PA1393, one of the genomic islands mentioned to vary between different CF isolates[378] and the partially overlapping PA1381 - PA1393 cluster encoding exopolysaccharide biosynthesis functions that are non-essential for biofilm formation.[379,380] Although loss of O antigen from the LPS is a common feature among *P. aeruginosa* CF isolates[371,381,382] , the deleted cluster did not contain any of the predicted O-antigen biosynthesis cluster genes (PA3141 - PA3160). Network analysis of the genes in these clusters did predict possible interactions of PA1385 and PA1386 with several of the LPS biosynthesis related genes including *wbpW*, *wzt* and *wzm.* Deletion of these two genes could thus influence the correct synthesis of the LPS layer and more specifically the common polysaccharide antigen.[383]

Mutation of PA1396, a sensor kinase involved in *cis*-2-dodecenoic acid recognition, has previously been demonstrated to cause increased levels of different proteins involved in bacterial stress tolerance and polymyxin resistance.[384] Deletion of *bdlA* prevents biofilm-dispersal, causes increased adherent properties and contributes to the persistence of *P. aeruginosa* infections in the lung.[385,386] The *lapD-lapG* operon was also deleted in the late isolate SSF65-563. Both genes have only recently been discovered in *P. aeruginosa* and are believed to play a role in starvation-mediated biofilm dispersal.[387] Also several genes previously mentioned to be downregulated in CF isolates were present, including PA1404[378], *rsaL*[378], *lasI*[377,378], *lasR.*[12,363,388]

To gain more information about which of these genes is involved in the protection of the cell against the effect of SPI009, it was attempted to identify the causal mutations. For this, the 85 kb region was divided into 9 sub regions, each comprising 7-10 kb (Figure B.1 in Appendix B). Next, 600bp homologues fragments flanking these nine sub regions were created and coupled to a kanamycin resistance cassette. This enabled the use of the lambda Red system[389], which uses a phage derived recombinase system to swap the targeted genomic region for a kanamycin cassette, allowing selection of successfully mutated strains. Unfortunately, creation of the intended SSF65-443 mutants, each missing a part of the 85 kb deletion region, was not successful.

6.3.3 LPS profiling

Previous results indicated the importance of membrane strength on the antibacterial activity of SPI009 with increased killing being observed in cells with a weaker membrane or higher membrane permeability. An important factor contributing to membrane strength is the bacterial LPS layer, a structure previously reported to be adapted in *P. aeruginosa* CF isolates.[371,381,382]

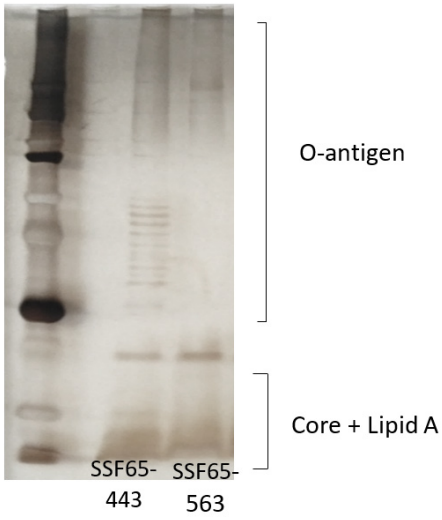


Figure 6.3: LPS profiling of *P. aeruginosa* CF isolates. The thick bands at the bottom represent the Lipid A core, followed by the different O antigen polysaccharides of varying lengths. The SSF65-563 late isolate is lacking part of the O-antigen structures as compared to the early isolate SSF65-443. Figure 3 shows a representative view of three independent repeats.

Despite the absence of any clear genetic changes in the LPS biosynthesis gene cluster, LPS profiles of both isolates did show some differences in the O-antigen structures (Figure 6.3). The obtained profiles corroborate the genetic data which suggest a possible influence of PA1385 and PA1386 on the common polysaccharide or A-band synthesis of the cell.

6.3.4 Antibiogram

To further study the differences between the early and late isolate of the SSF65 clonal pair, MIC assays were used to create an antibiogram (Table 6.3). This not only provides more information about the differences between both strains but can also reveal possible cross-resistance between SPI009 and conventional antibiotics. For most antibiotics, no significant changes were observed in MIC value between the early and late isolate. However, similar trends were seen for SPI009, colistin and both fluoroquinolones where respective 2-, and 4-fold decreases in MIC were detected for the late isolate. Furthermore, no relevant increases in resistance (> 2-fold increase in MIC) were observed for the late isolate SSF65-563.

Table 6.3: Overview of MIC values obtained for SSF65-443 and SSF65-563.

	MIC (µg/mL)	
Antibiotic	**SSF65-443**	**SSF65-563**
Aminoglycosides		
Tobramycin	0.313	0.313
Amikacin	0.313	0.625
Fluoroquinolones		
Ofloxacin	20	5
Ciprofloxacin	5	1.25
β-lactam antibiotics		
Ceftazidime	50	100
Meropenem	0.039	0.019
Piperacillin	200	200
Polymyxins		
Polymyxin B	0.625	1.25
Colistin	0.781	0.19
SPI009	300 µM	150 µM

Although no literature or genetic evidence was found to support the increased fluoroquinolone sensitivity, previous research already showed a link between SPI009 and fluoroquinolone susceptibility in *P. aeruginosa* (Chapter 5), which could imply the involvement of similar systems in the cellular response to SPI009 and this class of antibiotics. Further analysis of the colistin-resistant *Acinetobacter baumannii* Ab84-R however, revealed no cross-correlation between both antibacterials and even showed an increased sensitivity towards SPI009 (Chapter 5).

6.3.5 Quantification of pyocyanin and pyoverdin production

The deletion of several genes involved in QS and virulence in the late isolate, including *lasR* and *lasI,* prompted us to further look into the effect of these systems on SPI009 activity. In order to investigate the possible role of QS molecules in the sensitivity towards SPI009, the production of two virulence factors regulated by the QS system, pyocyanin and pyoverdin, was determined (Figure 6.4).

The obtained results clearly show a significant difference in both pyocyanin and pyoverdin production between the early (SSF65-443) and late (SSF65-563) isolate. When looking at overall pyocyanin production, there is no statistical difference between the PAO1 strain and the early isolate SSF65-443. This indicates the probable lack of any mutations in the SSF65-443 strain influencing the *phzA1* and *phzA2* operons responsible for phenazine production in the early isolate.[368]

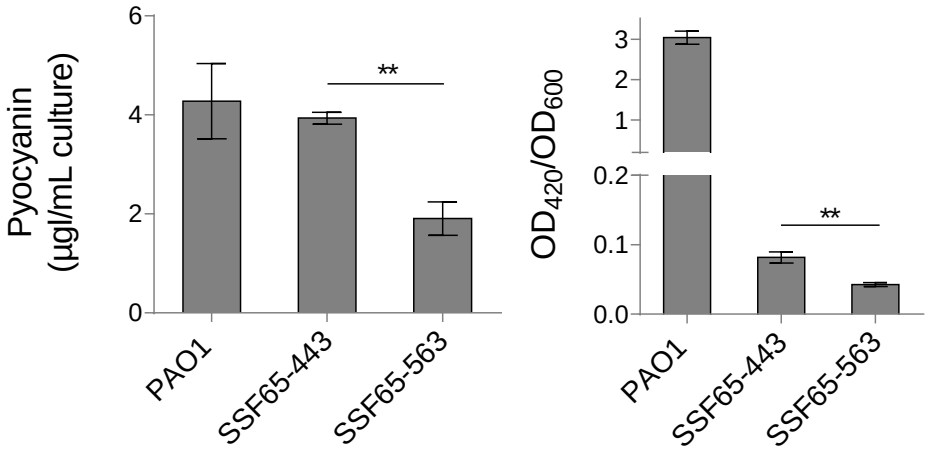


Figure 6.4: Pyocyanin and pyoverdin quantification for clonal isolates SSF65-443 and SSF65-563. Pyocyanin (left panel) and pyoverdin (right panel) were extracted from culture supernatant. Data points represent the average of three independent repeats ± SEM. Statistical analysis was done by an unpaired, one-tailed *t* test with ** $P < 0.01$.

The decrease in pyocyanin production in the late isolate could be attributed to the deletion of the Las system, one of two dominant QS systems in *P. aeruginosa* and with *lasR* frequently reported to be inactivated in CF lung infections.[363,365] The *las* system acts as a first step in the QS cascade and, via regulation of *rhlR*, controls the expression of several virulence factors, including pyocyanin.[390] Decreases in pyocyanin production in sequential CF isolates have been reported previously.[391]

When comparing production of the siderophore pyoverdin, there is a strong decrease in both clinical isolates, as compared to the PAO1 strain, suggesting the rather advanced character of this specific *P. aeruginosa* infection. Genetic comparison of the SSF65-443 draft genome with the PAO1 strain revealed no mutations in the different *rhl* genes but did show some discrepancies in the *pvd* region, one of two loci known to be associated with pyoverdin biosynthesis in *P. aeruginosa*.[392] When comparing both isolates, there is a significant reduction in pyoverdin production in the late isolate. Two-fold decreases in pyoverdin production have been described previously in *P. aeruginosa lasR* and *lasI* mutants where reduced expression of the homoserine lactone effector molecule PAI-1 , synthesized by LasI hampers the synthesis of pyoverdin.[393]

6.3.6 Quantification of AHL production

A last group of QS molecules to be studied was the *N*-acyl homoserine lactones (AHLs), which are produced by the LuxI family of AHL synthases in Gram-negative bacteria. AHLs can be divided in two groups based on the length of their side chains; short-chain and long-chain AHLs, and act as transcriptional activators through binding of LuxR homologues.[376,394] A first attempt to quantify AHL production was done by means of a *Chromobacterium violaceum* CV026 biosensor assay. CV026 induction and inhibition assays allow the detection of short-chain and long-chain AHLs through the visual assessment of violacein production or inhibition, respectively.[395] However, based on this assay neither SSF65-443 nor SSF65-563 seemed to produce any AHLs (data not shown).

Since detection of violacein production or inhibition in the CV026 biosensor assay is based on visual interpretation, an adaptation of the previously described QSIS2 assay[370] allowed the more quantitative detection of AHL production (Figure 6.5). The obtained results clearly indicate the absence of AHL production in the late isolate SSF65-563, as compared to the SSF65-443 strain. Since no significant difference could be identified between the early isolate SSF65-443 and the PAO1 strain, the genetic changes of *P. aeruginosa* in the CF lung are probably responsible for the observed drop in QS activity. As indicated previously, decreased QS activity is probably caused by the deletion of *lasR*, *lasI* and *rsaL* genes.[396]

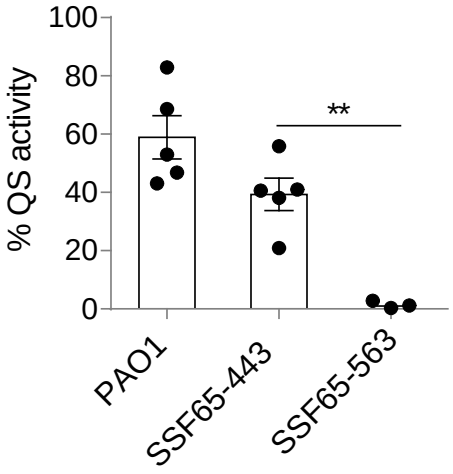


Figure 6.5: Assessment of AHL production using the QSIS2 system. Cell culture supernatant of the PAO1 strain and both clinical isolates was added to the QSIS2 strain and incubated for 6 hours. OD_{450} measurements were used to predict the level of AHL production relative to the difference in QSIS2 growth between the positive and negative controls, as described in the Materials & Methods section. Data points represent five independent repeats, each containing six technical repeats. Statistical analysis was done by means of an unpaired, two-tailed t-test with ** $P<0.01$.

6.4 Discussion

While assessing the effect of the anti-persister molecule SPI009 on several clinical isolates, two clonal pairs of CF sputum isolates showed an intriguing difference in SPI009 sensitivity between the early and late isolate. The evolution seen in the SSF65 pair provides evidence that SPI009 could be used in the treatment of chronic *P. aeruginosa* infections in CF patients since conventional treatment did not result in cross-resistance towards the compound. However, since the late DAF isolate showed a decreased sensitivity towards the combination of ofloxacin and SPI009, influence of previous antibacterial therapy administered to the patient should be taken into account upon selection of an appropriate combination treatment.

Further research on the SSF pair confirmed a 3.4 log difference in survival after treatment with 34 μg/mL SPI009, clearly indicating the increased sensitivity in the late isolate SSF65-563. In order to identify the reason for this observed difference and learn more about the mode of action of our compound, the early and late isolate were characterized both genetically and phenotypically. Genetic analysis revealed several small-scale mutations and a large-scale 85kb deletion containing 73 genes involved in efflux, QS, virulence, …. The effect of several of these deletions could be coupled to phenotypic difference in the production of virulence factors pyocyanin and pyoverdin and the QS molecules AHL. Reduction of virulence is a phenomenon commonly observed in mucoid CF isolates of *P. aeruginosa* and reduces detection and subsequent clearing of the pathogen by the host.[373] Interesting mutations regarding the mode of action of SPI009 include a frameshift in the ABC transporter permease PA2269, possibly limiting SPI009 efflux, and the mutations of the PA1378 - PA1393/PA1381 - PA1393 clusters, which might influence the correct assembly of the pseudomonal LPS layer. LPS profiling of the early and late isolate confirmed an absence of O-polysaccharides for the late isolate SSF65-563. Previous research showed a correlation between membrane permeability and sensitivity for SPI009. Reduced synthesis of the LPS layer and the resulting decreased membrane integrity could thus provide the key in explaining why the late isolate SSF65-563 shows a significantly higher sensitivity towards SPI009.

Overall, we successfully used a combination of whole genome sequencing and several phenotypical approaches to confirm the presence of extensive genetic changes between two sequential *P. aeruginosa* CF isolates, indicating the further adaptation of this strain to the specific CF lung environment. Phenotypical changes resulting from the observed 85 kb

deletion could be confirmed through the decreased production of several virulence and QS related molecules. No direct genetic cause for the observed difference in SPI009 sensitivity could be identified so far but the observed differences in LPS structure suggest that slight adaptations of the LPS layer to facilitate survival in the CF lung could potentially sensitize the bacterium for treatment with SPI009. In addition, successful eradication of the majority of the clinical isolates, together with the observed increase in SPI009 sensitivity for the late isolate SSF65-563 demonstrate the lack of cross-resistance with conventional antibiotics used in the treatment of *P. aeruginosa* CF lung infections and provide interesting perspectives for the clinical application of SPI009 in chronic infection control.

Chapter 7

Efficacy of Artilysin Art-175 against resistant and persistent *Acinetobacter baumannii*

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Contributions of individual authors to the manuscript:

The experiments were designed by **Valerie Defraine**, Yves Briers, Sander K. Govers and Rob Lavigne. The experiments were performed by **Valerie Defraine**, Joris Shuermans, Barbara Grymonprez and Sander K. Govers. The data was analyzed by **Valerie Defraine** and Yves Briers. The manuscript was written by **Valerie Defraine** and Yves Briers. The manuscript was revised by Sander K. Govers, Abram Aertsen, Maarten Fauvart, Jan Michiels and Rob Lavigne.

Abstract

Bacteriophage-encoded endolysins have shown promise as a novel class of antibacterials with a unique mode of action, i.e., peptidoglycan degradation. However, Gram-negative pathogens are generally not susceptible due to their protective outer membrane. Artilysin®s overcome this barrier. Artilysin®s are optimized, engineered fusions of selected endolysins with specific outer membrane-destabilizing peptides. Artilysin® Art-175 comprises a modified variant of endolysin KZ144 with an N-terminal fusion to SMAP-29. Previously, we have shown the high susceptibility of *Pseudomonas aeruginosa* to Art-175. Here, we report that Art- 175 is highly bactericidal against stationary phase cells of multidrug-resistant *Acinetobacter baumannii*, even resulting in a complete elimination of large inocula (>10^8 CFU/ml). Besides actively dividing cells, Art-175 also kills persisters. Instantaneous killing of *A. baumannii* upon contact with Art-175 could be visualized after immobilization of the bacteria in a microfluidic flow cell. Effective killing of a cell takes place through osmotic lysis after peptidoglycan degradation. The killing rate is enhanced by the addition of 0.5 mM EDTA. No development of resistance to Art-175 under selection pressure and no cross-resistance with existing resistance mechanisms could be observed. In conclusion, Art-175 represents a highly active Artilysin® against both *A. baumannii* and *P. aeruginosa*, two of the most life-threatening pathogens of the order *Pseudomonadales*.

7.1 Introduction

Acinetobacter baumannii is a Gram-negative, opportunistic pathogen which has become one of the most important pathogens responsible for hospital-acquired infections including urinary tract infections, secondary meningitis, (burn) wound infections, and ventilator-associated pneumonia. Particularly, patients admitted to the intensive care units are affected. Treatment options are becoming increasingly limited due to the ongoing spread of antibiotic resistance mechanisms. Combined with a high intrinsic resistance, this has resulted in the emergence of multidrug-resistant clinical isolates, leaving colistin as the only therapeutic option. However, an outbreak of pandrug-resistant (including colistin) strains has been recently reported in Spain.[261,262,397] Not only does the presence of a protective outer membrane limits the passage or diffusion of many currently available therapeutic antibiotics, most novel antibiotics in the development pipeline are too large to pass this barrier.[184] *A. baumannii* infections often have a chronic nature, which can be attributed to the formation of persister cells, a small fraction of phenotypic variants highly tolerant to different classes of antibiotics.[59,398] For many antibiotics, persisters remain unaffected because of their dormant, non-dividing and metabolically quiescent state. They are, however, also tolerant to antibiotics targeting non-dividing cells and evidence is accumulating that dormancy is not the only reason for the observed tolerance. The role of active mechanisms, such as those related with the suppression of oxidative stress, is currently gaining more support.[78–80] When antibiotic treatment ceases, persisters may resuscitate and initiate a new infection. Eradication of persisters would thus lead to an improved long-term recovery.[29,59]

We have recently reported the development of a novel class of enzyme-based antibacterials, coined Artilysin®s.[298] Their mode of action relies on the fast enzymatic degradation of peptidoglycan by endolysins, resulting in osmotic lysis. Endolysins are produced by bacterial viruses (or bacteriophages). At the end of the infection cycle, they pass the cytoplasmic membrane through holes formed by small membrane proteins (holins) in a precisely timed manner, causing a sudden lysis of the infected host cell to disperse the newly formed viral particles.[399] Exogenous addition of recombinantly produced endolysin molecules is successfully being used to kill Gram-positive pathogens, but they are too large to pass the outer membrane of Gram-negative bacteria such as *P. aeruginosa* and *A. baumannii*. Artilysin®s consist of optimized fusions of a selected endolysin and a specific lipopolysaccharide (LPS) destabilizing peptide that effectively facilitates the outer membrane translocation of the endolysin moiety, eventually resulting in a physical cell lysis.[131]

In this study, the antibacterial effect of Artilysin® Art-175 against highly prevalent epidemiological clones of multidrug-resistant *A. baumannii* and persisters thereof was analyzed. Art-175 combines a mutated variant of endolysin KZ144, encoded by the giant *P. aeruginosa* bacteriophage ϕKZ, and the sheep myeloid antibacterial peptide 29 (SMAP-29). Art-175 has previously been shown to be highly bactericidal against multidrug-resistant *P. aeruginosa* with no cross-resistance with existing resistance mechanisms of 21 different antibiotics. In addition, resistant strains could not be selected after serial exposure to sub-inhibitory doses of Art-175.[129] In this study, we found that Art-175 represents a highly active antibacterial capable of killing large inocula of multidrug-resistant stationary phase cells of *A. baumannii*, including their persisters, with a high killing rate.

7.2 Materials and methods

Bacterial strains and culture conditions. *A. baumannii* strains used in this study are presented in Table 7.1 in the supplemental material. Eighteen strains were received from the Ruhr-Universität Bochum (Nationales Referenzzentrum für gramnegative Krankenhauserreger), one *A. baumannii* and one *A. Iwoffii* strain were received from the Universitätsklinikum Regensburg and seven strains from the Queen Astrid Military Hospital, Brussels.[290] All strains were grown in 1:20 Tryptic Soy Broth (TSB), unless mentioned otherwise, while shaking at 30°C. For solidified medium, 1.5% agar was added to TSB.

MIC determination. Minimum inhibitory concentrations (MIC) for Art-175 (0.25-20 µg/ml), ciprofloxacin (Sigma-Aldrich) (0.01-17 µg/ml) and tobramycin (0.125 - 64 µg/ml) were determined by the microdilution method in 96-well microtiter plates, according to the CLSI guidelines. The respective overnight bacterial cultures were adjusted to 0.5 McFarland standard, corresponding to 1 x 10^8 CFU/ml (OD_{625} ~ 0.08 - 0.1). Dilutions (100 x) were made in 2 x Mueller-Hinton (MH) medium with or without 0.5 mM EDTA-Na_2 to obtain a start inoculum of 1 x 10^6 CFU/ml. Bacterial cultures were distributed over the required number of wells and 50 µl volumes of cells were incubated for 24 h in the presence of 50 µl of a 2-fold dilution series of Art-175 or antibiotic, prepared in 20 mM HEPES-NaOH 100 mM NaCl (pH 7.4). 2 x MH medium (0.5 mM EDTA-Na_2) with cells and 2 x MH medium (0.5 mM EDTA-Na_2) with 50 µl 20 mM HEPES-NaOH 100 mM NaCl (pH 7.4) were included as positive and negative control, respectively. MICs were determined as the minimum concentration at which no bacterial growth was detected. MIC values for colistin were determined with the Etest® strips

for colistin of bioMerieux SA (Marcy l'Etoile, France) according to the manufacturer's instructions.

Antibacterial assay. *A. baumannii* overnight cultures were diluted 1:100 in 50 ml 1:20 Tryptic Soy Broth (TSB) and grown overnight (18 h) to achieve stationary phase cultures. RUH134 was selected as a reference strain and the MICs of ciprofloxacin, tobramycin and Art-175 in the presence of 0.5 mM EDTA were determined as 0.5 µg/ml, 2 µg/ml and 2 µg/ml, respectively. Stationary phase cultures of RUH875, LUH5875, RUH134 and NCTC 13423 were treated with 5 x to 30 x MIC_{RUH134} concentrations of ciprofloxacin, tobramycin, Art-175 or the mixture of Art-175 with these antibiotics in total volumes of 200 µl, both in the presence and the absence of 0.5 mM EDTA-Na_2. After 20 min, 40 min, 1 h, 2 h or 24 h for RUH134 and 1 h or 24 h for RUH875, LUH5875 and NCTC 13423, respectively, treated cultures were washed in 20 mM HEPES-NaOH (pH 7.4) and appropriate dilutions were plated on TSB agar plates. Colonies were counted after 48 h of growth at 37°C. Each experiment was repeated independently at least three times.

Persister killing. Persister cells were isolated from a stationary phase culture as described previously, with minor modifications.[166] An overnight culture of *A. baumannii* RUH134 was inoculated in 50 ml 1:20 TSB. After 18 h of growth at 37°C, the culture was treated with 60 x MIC concentrations of tobramycin for 5 h at 37°C, while shaking at 200 rpm. Persister cells surviving antibiotic treatment were isolated by centrifugation (5,250 x g, 15 min, and 4°C) and the cell pellet was washed twice with 20 mM HEPES-NaOH (pH 7.4). Cells were resuspended in 1:10 the initial volume of 20 mM HEPES-NaOH (pH 7.4), after which a killing assay was performed as described previously,[272] with minor modifications. A volume of 100 µl of the isolated persister fraction was mixed with ciprofloxacin, Art-175 (final concentrations of 30 x MIC) or an equimolar amount of KZ144, in the absence or presence of 0.5 mM EDTA-Na_2 (final concentration). Control treatments with Art-175 storage buffer (20 mM HEPES-NaOH 0.5 M NaCl [pH 7.4]), 0.5 mM EDTA and tobramycin (30 x MIC) were performed in parallel. The mixtures were shaken (200 rpm) for 1 h at 37°C. Treated persister cells were washed twice with 20 mM HEPES-NaOH (pH 7.4), appropriate dilutions were plated on TSB agar plates and incubated at 37°C. The number of surviving persister cells was determined after 72 h incubation to allow complete resuscitation of all surviving persisters. Each experiment was independently repeated at least three times.

Time-lapse microscopy. *A. baumannii* RUH134 cells were grown to mid-exponential phase (OD_{600} = 0.6) in lysogeny broth (LB) at 37°C, washed three times with 20 mM HEPES-NaOH (pH 7.4) (for cells with high turgor pressure) or 20 mM HEPES-NaOH 0.5 M NaCl (pH 7.4) (for cells without turgor pressure), and finally concentrated five times by resuspension in the corresponding wash buffer, supplemented with 0.5 mM EDTA-Na_2. Cells were kept on ice until use. Bacterial cells (with high or low turgor pressure) were loaded into a ONIX B04A-03 Microfluidic Plate (EMD Millipore Corporation, Hayward, CA, USA), placed under the microscope and subsequently exposed to Art-175 to monitor cell inactivation and lysis. Art-175 (0.4 mg/ml) was dialyzed before use against 20 mM HEPES-NaOH (pH 7.4) (for cells with high turgor pressure) or 20 mM HEPES-NaOH 0.5 M NaCl (pH 7.4) (for cells with a lower turgor pressure) and was supplemented with 50 mM EDTA-Na_2 to a final concentration of 0.5 mM EDTA-Na_2. Visualization of cells with high turgor pressure was done by immobilization of the cells in the inlet channel of Art-175 to ensure high Art-175 concentrations. The cells with lower turgor pressure, however, were visualized in the middle of the flow cell where, due to diffusion effects, the concentration of Art-175 is lower. Time-lapse microscopy experiments were performed in a temperature controlled (Okolab, Ottaviano, Italy) Eclipse Ti-E inverted microscope (Nikon, Champigny-sur-Marne, France), equipped with a Ti-CT-E motorized condenser and a CoolSNAP HQ2 FireWire CCD camera.[400] Images were acquired using NIS-Elements (Nikon), and the resulting pictures were further handled with open-source software, ImageJ (http://rsbweb.nih.gov/ij/). The CellASIC® ONIX Microfluidic System was operated using the CellASIC® ONIX FG Software, provided by the supplier (EMD Millipore Corporation).

Resistance development. The MIC of Art-175 and ciprofloxacin was determined against *A. baumannii* RUH134, RUH875 and LUH5875 as described above. Briefly, cultures were grown in Mueller-Hinton broth and adjusted to 5 x 10^5 CFU/ml. Twofold dilution series of Art-175 and ciprofloxacin were prepared in 20 mM HEPES-NaOH-100 mM NaCl (pH 7.4) in a round bottom 96-well microtiter plate, in the presence of the bacterial cells and a final concentration of 250 µM EDTA. After 24 h, the MICs were recorded as the minimal concentration that completely inhibited growth. A new cycle was initiated for each strain/compound combination using a 1:100 dilution of cell suspensions at the highest concentration with full growth. This was repeated for 20 cycles.

Hemolytic assay. A standardized hemolytic assay was performed by Pharmacelsus® GmbH to assess the level of erythrocyte damage caused by Art-175. Human erythrocytes were exposed to increasing concentrations of Art-175 for 0.5 to 4 h on an orbital shaker. Positive controls consisted of 2% Tween 80 and 2% Triton X-100; 20 mM HEPES-NaOH - 0.5 M NaCl (pH 7.4) was used as negative control. The hemolytic effect of Art-175 was determined as the fraction of hemolysis relative to that of 2% Triton X-100.

7.3 Results

7.3.1 Art-175 is an effective antibacterial against relevant *A. baumannii* strains

Growth inhibition by Art-175 was tested against a panel of thirty-three *A. baumannii* strains and one *A. Iwoffii* strain from different sources, containing both lab strains and clinical (multidrug-resistant) isolates (see Table 7.1 in the supplemental material). MICs of Art-175 range between 4 µg/ml and 20 µg/ml, showing the broad inhibitory activity of Art-175 against different (multidrug-resistant) *A. baumannii* strains. Addition of 200 µM of EDTA reduced the MIC value of Art-175 and improved its antibacterial effect for all strains tested (ranging from ≤4 to 10 µg/ml), providing a potentially interesting feature for topical applications. Colistin resistance (>2 to 4 µg/ml) arises through addition of phosphoethanolamine or 4-amino-4-arabinose to the phosphate groups of lipid A moiety of the lipopolysaccharides[401,402] and may thus directly affect the susceptibility for Art-175. However, no correlation was found between the MIC values for colistin and Art-175, respectively (n = 28; R^2 = 0.0043), indicating the lack of cross-resistance (see Table 7.2 in the supplemental material).

The increased number of outbreaks of *A. baumannii* infections in clinical settings, in which the involved strains often share a genetic background, is believed to be linked to the global spread of a number of highly successful clones.[403,404] The strains tested in this study include the reference strains of EU clones I (RUH875), II (RUH134) and III (LUH5875) and the NCTC 13423 strain, with MICs of 8 µg/mL, 10 µg/mL, 12 µg/mL and 6 µg/mL, respectively, all multidrug-resistant strains showing resistance against aminoglycosides, quinolones and carbapenems.[290] The NCTC 13423 or T strain was first isolated in 2003 from a casualty returning from the Iraq war, deeming it the nickname Iraqibacter. Despite not having greatly contributed to an increase in *A. baumannii* infections, it possesses all characteristics of a

highly successful outbreak strain and has become a major cause of concern in conflict zones.[405,406] In sum, Art-175 shows a good antibacterial activity against all tested *A. baumannii* strains, independent of the presence of multidrug-resistance mechanisms.

7.3.2 Art-175 outcompetes conventional antibiotics in bactericidal activity and killing rate against *A. baumannii*

Time-kill experiments were done for Art-175 and the results were compared to those of ciprofloxacin and tobramycin, two first-line antibiotics against *A. baumannii* infections (Figure 7.1 a and b).

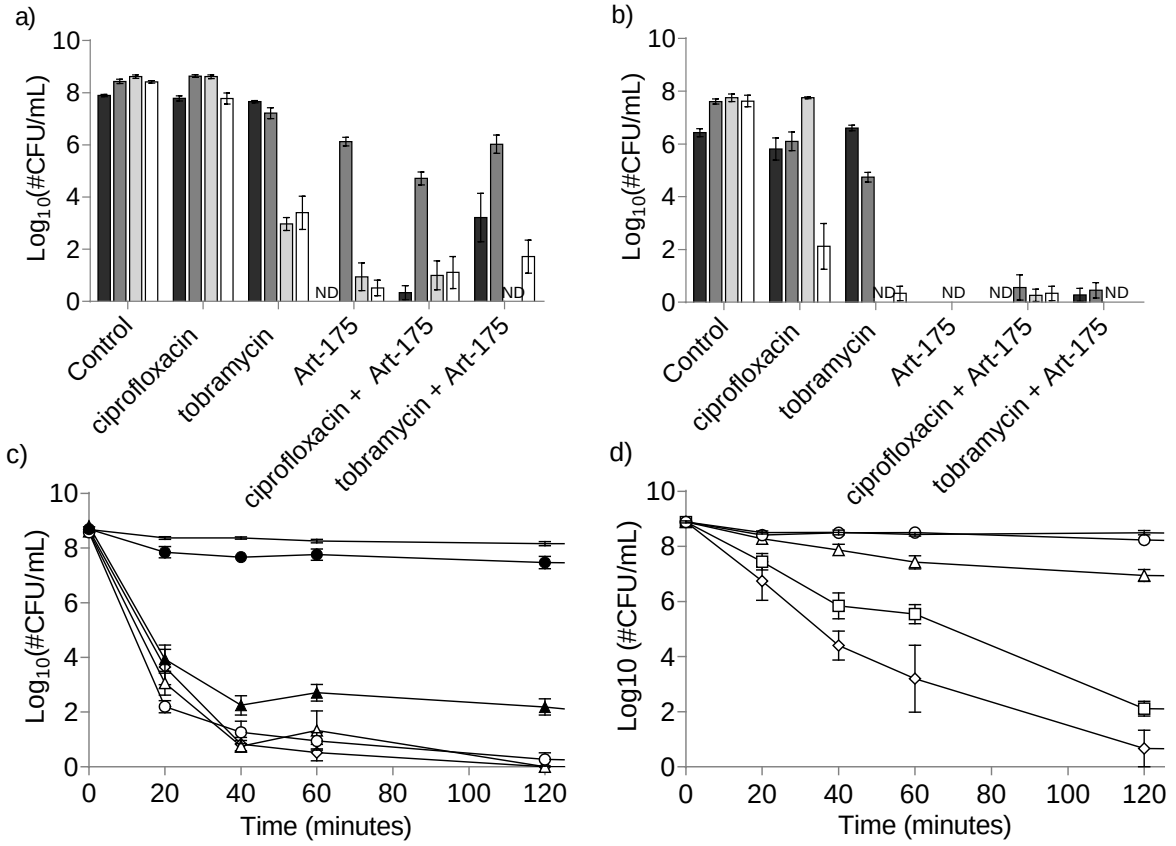


Figure 7.1: Antibacterial effect of Art-175 and combinations of conventional antibiotics with Art-175 on different *A. baumannii* reference strains. a) and b) Stationary phase cultures of respectively RUH875 (black), LUH5875 (dark grey), NCTC 13423 (light grey) and RUH134 (white) were treated for a) 1 hour or b) 24 hours with ciprofloxacin (15 µg/mL), tobramycin (60 µg/mL), Art-175 (60 µg/mL) or the combination therapy of Art-175 with these antibiotics. All concentrations correspond to 30 x MIC_{RUH134}. Bacterial survival after 1 h and 24 h treatment is expressed in log_{10} CFU per milliliter. ND, not detected. c) Time-kill curves were obtained for RUH134 with stationary phase cells that were treated for 20 min, 40 min, 1 h or 2 h with buffer (control; black line), ciprofloxacin (filled circles; 15 µg/mL), ciprofloxacin + Art-175 (open circles; 15 µg/mL and 60 µg/mL respectively), tobramycin (filled triangles; 60 µg/mL), tobramycin + Art-175 (open triangles; 60 µg/mL and 60 µg/mL, respectively) or Art-175 (open diamonds; 60 µg/mL). All concentrations correspond to a 30 x MIC_{RUH134}. Data points represent the mean value (± SEM) from at least three independent repeats. d) Stationary phase cells of RUH134 were treated with increasing concentrations of Art-175 for 20 min, 40 min, 1 h and 2 h. The control consists of buffer (black line). Art-175 was administered at 5 x MIC (open circles; 10 µg/mL), 10 x MIC (open triangles; 20 µg/mL), 20 x MIC (open squares; 40 µg/mL) and 30 x MIC (open diamonds; 60 µg/mL). Data points represent the mean value (± SEM) of three independent repeats.

Stationary phase cultures of RUH134, RUH875, LUH5875 and NCTC 13423 (~10^7 to 10^8 CFU/ml) were treated for 1 and 24 h with 30 x MIC_{RUH134} concentrations of Art-175, ciprofloxacin or tobramycin. A single dose of Art-175 eradicated all strains completely within 24 h (approximately 8- to 9-log reduction), with a strain-dependent killing rate. RUH875 was already completely eliminated after 1 h, while RUH134 and NCTC 13423 still showed minute surviving fractions (approximately 7- to 8- log reductions), which was completely eliminated after 24 h. Art-175 had the slowest antibacterial effect against LUH5875, with a 2.5-log reduction after 1 h. The bactericidal effect of an Art-175 treatment significantly exceeded the effect of either ciprofloxacin or tobramycin, both in extent and in rate. Ciprofloxacin and tobramycin killed between 1.0 and 4.5 log units in 24 hours, with the exception of RUH134 and NCTC 13423. Both strains were completely eliminated by tobramycin after 24 hours, but still at a slower rate than in the case of Art-175 (Figure 7.1 a and b).

Subsequently, the effects of the combination of a conventional antibacterial and an experimental antibacterial were evaluated to assess the usefulness of Art-175 as a supplement to currently used antibiotics and to exclude possible antagonistic effects (Figure 7.1 a and b). The same four strains were exposed to either a mixture of either Art-175 and tobramycin or Art-175 and ciprofloxacin (each 30 x MIC). The antibacterial effect of the mixtures was significantly better ($P < 0.005$) than those of the respective conventional antibiotics alone. No significant differences ($P > 0.05$) were observed compared to the value of Art-175 treatment alone. However, one exception is observed; strain LUH5875, which was affected by Art-175 at a lower rate than the three other strains, was killed faster by a mixture of ciprofloxacin and Art-175 (4-log reduction after 1 h) than by Art-175 alone (2.5-log reduction after 1 h). After 24 h of treatment with both combinations, the surviving fractions of all bacterial strains tested were reduced well below the detection limit. In conclusion, the efficacy of a mixture of Art-175 and ciprofloxacin or tobramycin was comparable to Art-175 alone. No antagonistic effects are observed, and it is likely that most cells are already killed by Art-175 before ciprofloxacin and tobramycin could exert their action.

To evaluate the dose dependence of the antibacterial effect exerted by Art-175 in the absence of 0.5 mM EDTA, RUH134 was exposed to increasing concentrations of Art-175 for 20 min, 40 min, 1 h and 2 h (Figure 7.1 d). A clear dose- and time-dependent effect was observed, with significant killing starting from 10 x MIC of Art-175. After 2 h of treatment reductions of 0.26 ± 0.08, 1.55 ± 0.23, 6.38 ± 0.28 and 7.83 ± 0.67 log units were obtained for 5 x MIC, 10 x MIC, 20 x MIC and 30 x MIC, respectively. Comparison of the effect of 30 x MIC

Art-175 in the presence (Figure 7.1 c) and absence (Figure 7.1 d) of 0.5 mM EDTA shows that EDTA mainly has an accelerating effect and the extents of killing after 2 h were similar. Also, stationary phase cells of RUH875 and NCTC 13423 can be completely eradicated within 24 h with Art-175 (20 x MIC) in the absence of EDTA, whereas LUH5875 was slightly less affected with a decrease in surviving cell number of 6.93 ± 0.49 log units (data not shown).

7.3.3 Real-time time lapse microscopy shows bactericidal effect upon contact

The rapid bactericidal effect of Art-175 previously hindered real-time monitoring of the mode-of-action, unless Art-175 was sufficiently diluted.[129] Therefore, a flow cell was used to immobilize *A. baumannii* RUH134 cells in a microfluidic channel, and the immobilized cells were monitored in real-time when a flow of Art-175 (0.4 mg/ml) made contact (Figure 7.2a; Movie S1A[h]).

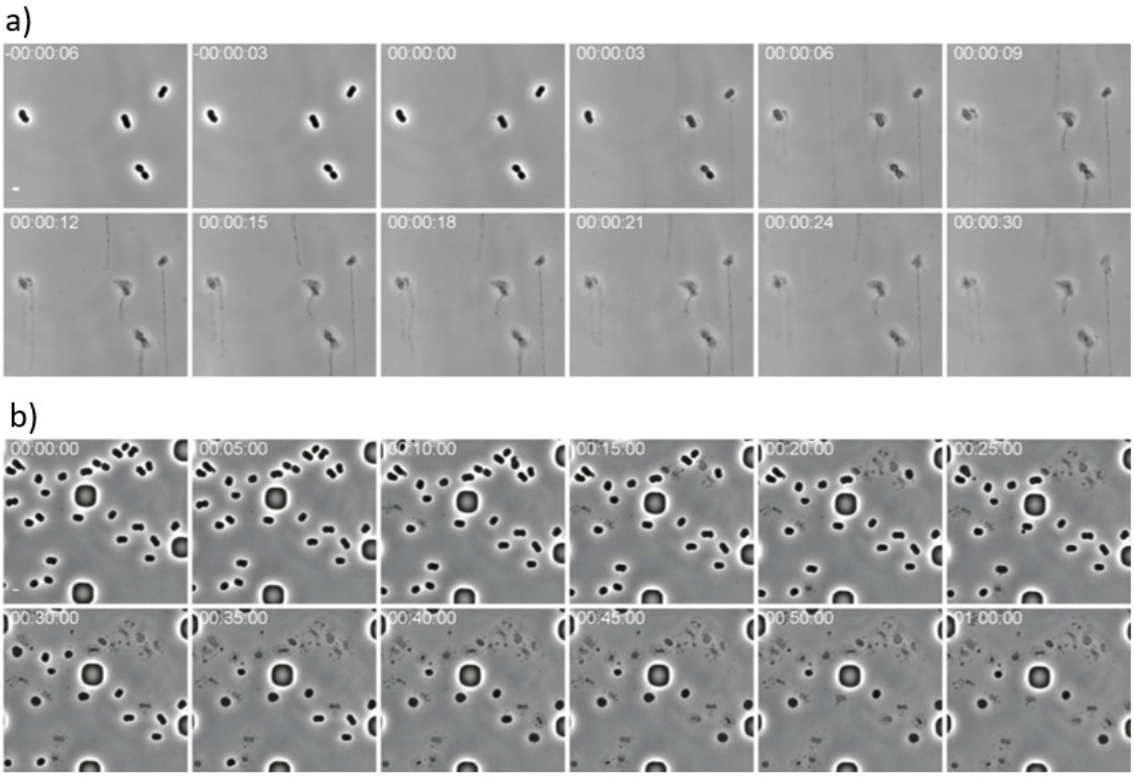


Figure 7.2: Real-time monitoring of osmotic lysis induced by Art-175. Exponential phase cells of *A. baumannii* RUH134 with a) a high internal osmotic pressure (washed with 20 mM HEPES-NaOH (pH 7.4) 0.5 mM EDTA) or b) a low internal osmotic pressure (washed with 20 mM HEPES-NaOH (pH 7.4) 0.5 M NaCl 0.5 mM EDTA) were immobilized in a flow cell and exposed to Art-175 (0.4 mg/ml with 0.5 mM EDTA). A time-lapse series is presented with intervals of a) 3 sec or b) 5 min. To visualize the rapid lysis under high osmotic pressure conditions, three frames before contact with Art-175 are shown (from -00:00:06). The flow of Art-175 is started at 00:00:00). Total duration is a) 36 seconds and b) 1 h. Scale bars correspond to 2 µm. Movie S1A and S1B show the full time-lapse series.

[h] http://aac.asm.org/content/60/6/3480/suppl/DCSupplemental

All cells lysed immediately in the first frame (3 s), and the cell contents were dispersed in the flow of Art-175 solution. This clearly demonstrates that Art-175 can act upon contact. Cells were resuspended in a buffer with low ionic strength (20 mM HEPES-NaOH [pH 7.4]), resulting in a high internal osmotic pressure and thus likely contributing to a drastic lysis phenomenon. In a similar experiment but with cells resuspended in a buffer with high ionic strength (20 mM HEPES-NaOH [pH 7.4] supplemented with 0.5 M NaCl) and a lowered Art-175 concentration, different observations were made (Figure 7.2b; see also Movie S1b in the online supplemental material). Lysis took place again but was slower (<1 h), and the cells first adopted a spherical shape before becoming unstable and lysing. Under these conditions, the contribution of two separate effects caused by Art-175 could be visualized, i.e. peptidoglycan degradation resulting in the adoption of a spherical shape and osmotic lysis due to the lack of a protective cell wall.

7.3.4 Art-175 is a highly efficient anti-persister compound

Persister cells constitute a small subfraction of the bacterial population transiently tolerant to treatment with high doses of antibiotics.[59] Art-175 is capable of completely eradicating the persister fraction of *P. aeruginosa.*[129] This effect can be attributed to the mode of action of Artilysin®s that physically cleave the peptidoglycan, instead of inhibiting an essential step in the metabolism. Consequently, there is no need for an active metabolism, making them very suitable for the elimination of persister cells.[129] To investigate whether the anti-persister effect of Art-175 is broadly conserved, *A. baumannii* RUH134 persister cells were isolated through 5 h treatment with high doses of tobramycin. After isolation, the remaining fraction was treated for 1 h with a concentration corresponding to 30 x MIC of ciprofloxacin or Art-175, respectively, in the absence or presence of 0.5 mM EDTA. In addition, equimolar amounts of the endolysin KZ144 alone were tested. As controls, treatments with 20 mM HEPES-NaOH (pH 7.4) (storage buffer), 0.5 mM EDTA and 30 x MIC tobramycin were included.

The isolated persister cells could not be killed by a new tobramycin exposure, confirming that the actual persister subfraction was isolated. Controls such as the storage buffer, EDTA, and ciprofloxacin cause a small but significant reduction of the persister fraction, which may be best explained by the occasional waking up of persister cells during sequential washing steps. Treatment of the persister cells with 30 x MIC Art-175, in the

absence and presence of 0.5 mM EDTA, significantly reduced the remaining persister fraction by 2.35 ± 0.42 and 2.66 ± 0.45 log units, respectively. Under these conditions, EDTA did not significantly enhance the anti-persister effect. KZ144, on the other hand, caused reductions of only 1.24 ± 0.43 and 1.93 ± 0.49 log units in the absence and presence of 0.5 mM EDTA, respectively (Figure 7.3).

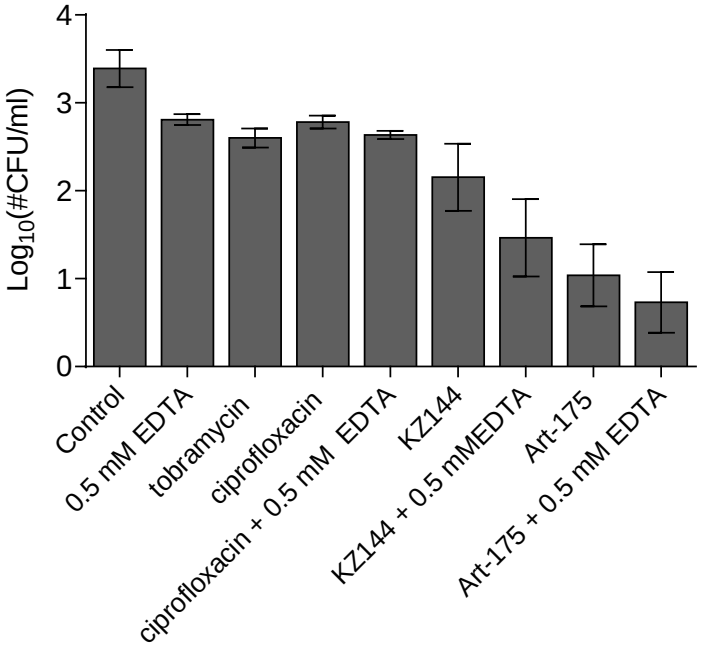


Figure 7.3: Art-175 kills isolated persister cells. Stationary phase cultures of RUH 134 were treated with high doses of tobramycin to isolate the surviving persister fraction. The remaining persister cells were treated with 30 x MICs of ciprofloxacin (15 µg/mL), Art-175 (120 µg/mL) and an equimolar dose of KZ144 (12 µg/mL), all in the absence and presence of 0.5 mM EDTA. As a control, untreated persister cells and treatment with 0.5 mM EDTA and 30 x MIC tobramycin (60 µg/mL) were taken into account. Persister survival after 5 hours is expressed in $\log_{10}$ CFU per milliliter. Mean values are shown (± SEM) for at least three independent repeats.

In conclusion, Art-175 reduces the surviving persister fraction most compared to the controls, demonstrating a potent anti-persister effect. Although the occurrence of persister cells has been described in *A. baumannii*[398,407], Art-175 and KZ144 are, to our knowledge, the first antibacterial compounds described to exert an anti-persister effect against *A. baumannii*.

7.3.5 Art-175 shows no hemolytic activity

The antibacterial effect of SMAP-29 is explained by its membrane pore formation.[408] Membrane pore formation requires oligomerization, and this effect is also correlated with the hemolytic activity of SMAP-29 (7.9% hemolysis at 50 µM or 0.16 ng/mL).[408] Although it is expected that SMAP-29 oligomerization cannot take place in a fusion with the endolysin KZ144 due to steric hindrance, we have evaluated the hemolytic potential of Art-175 by assessing the damage of erythrocytes after exposure to Art-175. No hemolysis was observed

for doses up to 200 µg/ml (100 x MIC with 200 µM EDTA; 10 to 50 x MIC without EDTA) in contrast to the positive controls Tween 80 and Triton X-100 (data not shown).

7.3.6 Resistance of *A. baumannii* against Art-175 cannot be provoked *in vitro*

The rate of resistance development is an important parameter to evaluate the potential of a new antibacterial compound. *A. baumannii* is acknowledged as a multi-drug resistant organism, capable of developing resistance rapidly compared to other clinically relevant pathogens.[409,410] To analyze the resistance development of *A. baumannii* against Art-175, the epidemiological European reference strains (RUH134, RUH875, and LUH5875) were serially exposed to sub inhibitory doses of Art-175. Ciprofloxacin was included as a control antibiotic. A maximal 2-fold increase of the MIC of Art-175 was observed for all strains after 20 cycles (Figure 7.4b).

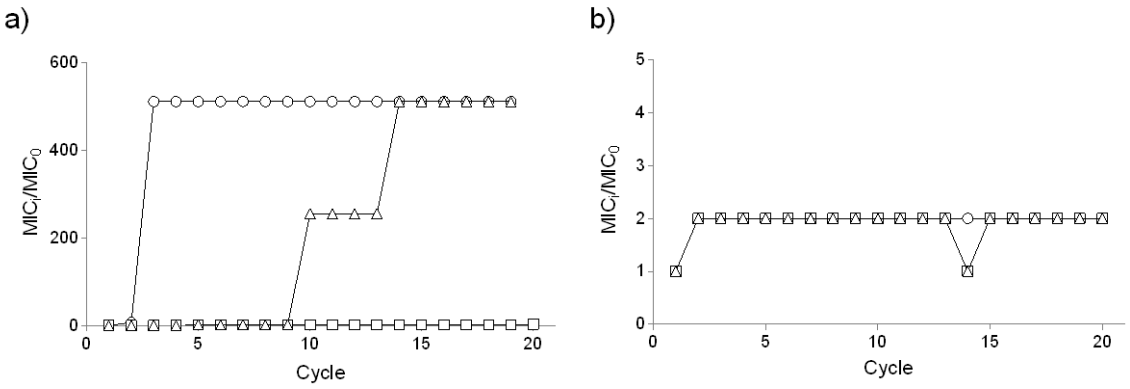


Figure 7.4: Art-175 is highly refractory to resistance development. *A. baumannii* strains RUH134 (triangle), RUH875 (circle), and LUH5875 (square) were serially treated with subinhibitory concentrations of a) ciprofloxacin or b) Art-175 to select for decreased susceptibility. Values are represented as the ratio of the MIC value after cycle i (MIC_i) over the MIC value at the start of the experiment (MIC_0). After 20 cycles, the MIC of Art-175 increased maximally 2-fold, whereas the MIC of ciprofloxacin increased 512-fold (RUH134 and RUH875) or 4-fold (LUH5875).

In contrast, sub inhibitory doses of ciprofloxacin resulted in the recovery of highly resistant strains (according to the breakpoints defined by EUCAST) of both RUH134 and RUH875 after 8 and 3 cycles, showing 256- and 512-fold increases in MIC value, respectively (Figure 7.4a). The MIC of RUH134 further developed to a 512-fold increase in cycle 14. For LUH5875 a 4-fold increase in MIC was observed after 20 cycles. However, this strain already showed a relatively high initial resistance against ciprofloxacin, i.e., 85 µg/mL versus 1.3 µg/mL (RUH134 and RUH875), corresponding to a 64-fold higher MIC. Resistance against Art-175 does not appear to be provoked through genetic mutations that are typically selected upon exposure to sub-inhibitory doses.

7.4 Discussion

A. baumannii causes a wide range of infections and is becoming an important risk in health care settings. Besides its great intrinsic resistance, extensive antibiotic use has fostered the selection for resistance against all known antimicrobials.[410] Despite its increasing clinical relevance, few or no compounds are currently found in late stages of clinical development for the treatment of multidrug-resistant *A. baumannii* infections.[261] Increasing multidrug resistance in most bacterial pathogens and lack of new antibiotics have increased the need for alternative treatment options to combat these infections.

The ability of purified, phage-derived endolysins to kill bacteria was discovered in 1959 and, over the years, research on phage endolysins and their ability as new antibacterials has greatly progressed.[128,411] The unique nature of endolysins makes them highly attractive antibacterials for Gram-positive bacteria and provides several advantages over conventional antibiotics. Indeed, endolysins do not disturb the normal human microflora due to their high specificity, they provide novel antimicrobial mechanisms, have a small chance of developing bacterial resistance and are able to kill colonizing pathogens on mucosal surfaces.[412,413] The potent effect of these phage encoded endolysins against different Gram-positive pathogens has been extensively reviewed.[128,414–416]

The majority of known endolysins are unable to cross the highly impermeable outer membrane of Gram-negative species. The engineering of endolysins towards Artilysin®s allowed us to overcome this barrier. Optimized fusions of phage endolysins with a peptide component capable of guiding the endolysin through the bacterial outer membrane results in a strong bactericidal effect against Gram-negative pathogens.[298] Previous work described the development of Art-085 and its optimized homolog Art-175, composed of a fusion between the SMAP-29 peptide and the N-terminus of the full-length KZ144 endolysin containing three Cys→Ser mutations. Art-175 proved very successful in the eradication of *P. aeruginosa* and its persister cells, and its potent antibacterial activity has since then been demonstrated *in vivo* in dogs.[129,131,298]The success of Art-175 prompted us to further expand and verify this Artilysin® as a novel antimicrobial for the treatment of *A. baumannii*, another clinically relevant pathogen within the order of the *Pseudomonadales*.

Compounds showing a broad activity without displaying cross-resistance with existing drug resistance mechanisms show great promise as novel antibacterials. Therefore, a diverse panel of 33 *A. baumannii* strains, including strains from different origins and with different

antibiotic susceptibilities, was evaluated. Art-175 inhibits the growth of all these strains (MIC between 4 and 20 µg/ml). No correlation between the MIC value of Art-175 and the source, isolation site, resistance spectrum (aminoglycosides, quinolones and carbapenems), or the MIC for colistin was observed, demonstrating the lack of cross-resistance and the broad activity of Art-175. Addition of 0.2 mM EDTA generally reduces the MIC value.

Four epidemiological reference strains that are resistant towards at least two classes of first-line antibiotics (aminoglycosides, quinolones and carbapenems) were selected for further studies to evaluate the *in vitro* efficiency of Art-175. In time-kill experiments, Art-175 shows a significantly increased reduction of cell number compared to those obtained with two conventional antibiotics, ciprofloxacin and tobramycin. All therapeutics show a strain-dependent killing rate but a single dose of Art-175 eradicates stationary phase cells of all bacterial strains within 24 h (between 8- and 9-log reduction).

Different endolysins have been reported to have intrinsic antibacterial activity without the need for engineering, especially against *A. baumannii.*[417,418] The outer membrane of *A. baumannii* therefore appears to be less a barrier for large enzyme molecules than in other Gram-negative species. Endolysin PlyF307 derived from an *A. baumannii* prophage is effective against exponentially growing *A. baumannii* cells (>3 log units), but its activity is reduced when stationary phase cells are exposed to the antibacterial (approximately 1.5 log unit).[419] In fact, endolysins have been reported to be generally less active against stationary phase cells when compared to logarithmically growing cells.[420] The complete elimination of stationary phase cells by Art-175, partially due to its anti-persister activity, is therefore one of the most remarkable features of Art-175.

Combination therapy has often been suggested for the treatment of Gram-negative infections, including *A. baumannii.*[111,421] Combinations of endolysins and antibiotics have been reported to be superior to antibiotics alone.[163] To evaluate the potential of Art-175 as a supplement to existing antibiotic therapies, Art-175 was combined with ciprofloxacin and tobramycin, and its effect was compared to monotherapy. The combined therapy results in an improved antibacterial effect for both mixtures over that obtained with conventional antibiotic monotherapy. However, there is no significant improvement compared to Art-175 alone, suggesting that Art-175 kills the bacterial cells before either ciprofloxacin or tobramycin can exert its action.

The rapid mode of action of Art-175 is confirmed with the complete eradication of RUH134 (approximately 9 log reduction) after 2 h of treatment, outcompeting both

ciprofloxacin and tobramycin. A low concentration of EDTA, which chelates divalent cations that contribute to the stabilization of the outer membrane, further accelerates the killing rate. We introduced the use of microfluidic flow cells to immobilize bacteria to allow real-time observation. Using this method, we showed that an immediate, rapid and intense lysis takes place. Under low osmotic pressure conditions (high ionic strength of the medium) and a lowered concentration of Art-175, the mode of action of Art-175 can be visualized in slow motion with the full enzymatic degradation of the shape-determining peptidoglycan sacculus, followed by a destabilization of the cell and spontaneous lysis. Enzymatic degradation of the peptidoglycan layer is only possible when Art-175 has first passed the outer membrane. Since the endolysin KZ144 is not able to pass the outer membrane efficiently, this can be explained only by the fused SMAP-29 moiety that interferes with the electrostatic interactions between adjacent LPS molecules. This is in contrast to the receptor-mediated uptake mechanism as described for a chimeric endolysin, comprising the FyuA binding domain fused to the N-terminus of T4 lysozyme. This hybrid protein kills *Yersinia* and pathogenic *E. coli* strains, which express the FyuA receptor.[422] Once past the outer membrane, enzymatic degradation takes place, resulting in a rod-to-sphere transformation. Nevertheless, loss of the cell wall does not necessarily kill the cell, as has been extensively described for cell wall-deficient bacteria or L forms, which are able to survive and even multiply if there is an appropriate isotonic environment.[423,424] The peptidoglycan layer can also be removed by peptidoglycan-degrading enzymes (such as the commonly used hen egg white lysozyme) to prepare so-called protoplasts. These “naked” cells remain viable, are competent to take up extracellular DNA and can reconstruct an intact cell wall after transformation. This process requires that the cells are resuspended in an isotonic solution, i.e. a solution with the same osmolarity as inside the cell to eliminate the cellular osmotic pressure. This technique is commonly used in molecular microbiology to modify the genetic content of a bacterial cell. Therefore, the actual cause of death can be defined as a physical process called osmotic lysis. We propose that depending on the osmolarity of the medium, osmotic lysis will take place immediately or only after extensive peptidoglycan degradation until the cells get too destabilized by the internal osmotic pressure. Given this dependence on osmolarity, it would be interesting to evaluate Art-175 under serum conditions in the future.

The presence of so-called persister cells is increasingly being recognized as an important cause of the chronic nature of bacterial infections.[69,70] Despite *A. baumannii* being a clinically relevant pathogen known to cause chronic infections, the observation of the

persister phenotype in this species has only recently been described. 94.6% of the tested clinical isolates showed a detectable persister fraction, varying between 0.0007% and 10.1%, indicating that the persister phenotype is broadly distributed among *A. baumannii* strains.[398] Unlike most traditional antibiotics, Art-175 does not require an active metabolism to exert its antibacterial activity, making it an ideal candidate for novel anti-persister therapies. Treatment of stationary phase cultures of *A. baumannii* with high doses of tobramycin confirmed the presence of a persister fraction in RUH134 of about 0.0004%. Exposure of this isolated persister fraction to Art-175 (plus 0.5 mM EDTA) results in an efficient 2.4-log (2.7-log) reduction in surviving cells. However, in the case of *P. aeruginosa* persisters, a complete elimination of the persister fraction could be achieved with similar doses. Despite the clear clinical relevance of persister cells, anti-persister therapies described in literature are still limited, Art-175 being the first molecule, to our knowledge, exerting an activity against *A. baumannii* persister cells.

No resistance development is observed after serial exposure of three different *A. baumannii* strains to sub-inhibitory concentrations of Art-175. On the contrary, repeated exposure to ciprofloxacin, a first-line antibiotic in healthcare settings, resulted in the quick recovery of highly resistant strains for both RUH875 and RUH134 with 512- and 265-fold increases in MICs, respectively. Similar findings have been reported for endolysins.[425,426] The immutable nature of peptidoglycan and the high selection pressure for endolysin optimization during the long and intense co-evolution between phages and bacteria have been proposed as explanations for similar observations with endolysins and appear to hold true for Artilysin®s.[129] Also, the extracellular location of peptidoglycan excludes many potential resistance mechanisms such as efflux pumps and inactivation of the antibacterial.[415]

In conclusion, Art-175 shows high and rapid *in vitro* bactericidal activity against two of the most dangerous Gram-negative pathogens posing risks to our current health care systems, *A. baumannii* and *P. aeruginosa*, both belonging to the order of *Pseudomonadales*. Art-175 clearly differentiates from other classes of antibiotics in completeness of killing (including persisters), killing rate, and the mode of action.

7.5 Supplemental material

Table 7.1: MIC values of Art-175 against *A. baumannii* and *A.Iwoffii* strains. The source of thirty-three *A. baumannii* and one *A.Iwoffii* strain is given, together with the corresponding MIC-value for Art-175 both in the absence and the presence of 0.2 mM EDTA. When available, present resistance mechanisms against three major classes of antibiotics (A= aminoglycosides, Q= quinolones, C= carbapenems) are indicated.[290] S= susceptible, R= resistant. [a] Universitätsklinikum Regensburg, Germany; [b] Ruhr-Universität Bochum, Germany (Nationales Referenzzentrum für gramnegative Krankenhauserreger); [c] Queen Astrid Military Hospital, Brussels, Belgium.

Species	Strain	Source	Remarks and reference	MIC Art-175 (µg/ml)		Antibiotic Susceptibility [290]		
				no EDTA	0.2 mM EDTA	A	Q	C
A. baumannii	2671	Patient isolate	[a]	7.4				
A. baumannii	AC54	Propagation strain		8				
A. baumannii	DSM 30011	Strain collection DSMZ		10				
A. baumannii	DSM 30007	Strain collection DSMZ		3,7				
A. baumannii	DSM 30008	Strain collection DSMZ		10				
A. baumannii	LUH 5875	Patient isolate (blood)	Reference strain of EU clone III, [b]	12	8	R	R	R
A. baumannii	LUH 5908	Patient isolate (subclavia catheter tip)		20	8			
A. baumannii	LUH 9270	Patient isolate (sputum)		10	8			
A. baumannii	LUH 9278	Patient isolate (sputum)	[b]	10	10	S	S	S
A. baumannii	LUH 9695	Patient isolate (sputum)	Reference strain for 'genomic species gsp3' (since 2011: *A. pittii*),[b]	6		S	S	S
A. baumannii	LUH 9825	Patient isolate (bronchial swab)	[b]	10		S	S	S
A. baumannii	NCTC 13423	Strain collection PHE	'Iraqibacter'[b]	6		R	R	R
A. baumannii	NRZ-00013	Patient isolate	[c]	6				
A. baumannii	NRZ-00025	Patient isolate	[c]	15.4				
A. baumannii	NRZ-00028	Patient isolate	[c]	13.2				
A. baumannii	NRZ-00037	Patient isolate	[c]	19.8				
A. baumannii	NRZ-00042	Patient isolate	[c]	8				
A. baumannii	NRZ-00066	Patient isolate	[d]	3,7				
A. baumannii	NRZ-00091	Patient isolate	[c]	12	≤ 4			
A. baumannii	NRZ-00135	Patient isolate	[c]	4				
A. baumannii	NRZ-00163	Patient isolate	[c]	14	≤ 4			
A. baumannii	NRZ-00247	Patient isolate	[c]	8	≤ 4			
A. baumannii	NRZ-00297	Patient isolate	[c]	8				
A. baumannii	NRZ-00323	Patient isolate	[c]	12				
A. baumannii	NRZ-00346	Patient isolate	[c]	12	≤ 4			
A. baumannii	NRZ-00362	Patient isolate	[c]	10	-			
A. baumannii	NRZ-00419	Patient isolate	[c]	18	≤ 4			
A. baumannii	NRZ-00449	Patient isolate	[c]	11	-			
A. baumannii	NRZ-00518	Patient isolate	[c]	11	-			

Species	Strain	Source	Remarks and reference	MIC Art-175 (µg/ml) no EDTA	MIC Art-175 (µg/ml) 0.2 mM EDTA	Antibiotic Susceptibility [290] A	Q	C
A. baumannii	RUH 134	Patient isolate (urine)	Reference strain of EU clone II, [c]	10	-	R	R	S
A. baumannii	RUH 875	Patients isolate (urine)	Reference strain of EU clone I, [b]	8	-	R	R	S
A. baumannii	S717	Isolate from burn wound		-	6			
A. Iwoffii	3135	Patient isolate	[a]	18.5	-			

Table 7.2: MIC values of colistin and Art-175 against *A. baumannii* strains. The susceptibility of twenty-eight *A. baumannii* strains for colistin and Art-175 was compared. No significant correlation (R^2=0.0043) can be identified.

Strain	MIC colistin (µg/ml)	MIC Art-175 (µg/ml)
NRZ-00066	1	7
NRZ-00330	4	12
NRZ-00518	1	11
NRZ-00449	12	11
NRZ-00013	4	6
NRZ-00025	9	15
NRZ-00042	4	8
NRZ-00091	3	12
NRZ-00135	4	4
NRZ-00163	4	14
NRZ-00247	6	8
NRZ-00297	3	8
NRZ-00323	8	12
NRZ-00346	4	12
NRZ-00362	6	10
NRZ-00419	4	18
2671	1.5	7.4
DSM 30011	4	10
RUH 134	6	10
LUH 5875	1	12
LUH 5908	1.5	20
LUH 9270	4	10
LUH 9278	8	10
LUH 9695	6	6
LUH 9825	4	10
NCTC 13423	1	6
RUH 875	1	8
AC54	1	8

7.6 Additional data to the above mentioned manuscript[i]

7.6.1 Art-175 has a superior bactericidal effect against *P. aeruginosa* persisters

Materials and methods

Planktonic persister cells were isolated from a stationary-phase culture as described previously[166], with minor modifications. An overnight culture of P. aeruginosa PA14 or PA1255 (a cystic fibrosis isolate) was inoculated in 50 ml 1:20 Trypticase soy broth (TSB). After 48 h of growth at 37°C, the culture was treated with ofloxacin at a final concentration of 10 µg/ml (5 x MIC) for 5h at 37°C, while shaking at 200 rpm. The persister cells surviving this antibiotic treatment were isolated by centrifugation (5 250 x *g* for 15 min at 4°C), and the cell pellet was washed twice with 20 mM HEPES (pH 7.4). Prior to treatment, the cells were resuspended in the same volume of 20 mM HEPES (pH 7.4). A persister killing assay was performed as described previously[166], with minor modifications. A volume of 100 µL of the isolated persister fraction was mixed with either Art-175 or ciprofloxacin (final concentrations of 10 x or 30 x MIC against the respective strains), in the absence or presence of 0.5 mM EDTA-Na^2 (final concentration) up to a final volume of 200 µL. Control treatments with 20 mM HEPES (pH 7.4), 0.5mM EDTA, or ofloxacin (10 x and 30 x MIC) were performed in parallel. The mixtures were shaken (200 rpm) for 1h at 37°C. The treated persisters were washed twice with 20 mM HEPES (pH 7.4), and the appropriate dilutions were plated on TSB plates and incubated at 37°C. The number of persisters was determined after 72 h of incubation to allow for complete resuscitation of all surviving persisters. Each experiment was independently repeated ≥ 3 times.

Results

Persisters are a subpopulation of antibiotic-tolerant cells and have been linked to chronic and recurrent infections[59]. The bactericidal activity of Art-175 was tested against this dormant subpopulation (Fig. 7.5). Persister fractions of two different strains (PA14 and PA1255, an isolate of a cystic fibrosis patient) were collected after 5-h exposure to 5 x MIC ofloxacin. The lack of killing after further exposure of the isolated fractions to increased ofloxacin

[i] The additional data mentioned in this section are part of the following publication:
Briers Y., Walmagh M., Grymonprez B., Biebl M., Pirnay J., Defraine V., Michiels J., Cenens W., Aertsen A., Miller S., Lavigne R. (2014). Art-175 is a highly efficient antibacterial against multidrug-resistant strains and persisters of Pseudomonas aeruginosa. *Antimicrobial Agents and Chemotherapy*, 58 (7), 3774-3784.

concentrations (10 x and 30 x MIC) confirmed the persistent (antibiotic-tolerant) nature of both isolated persister fractions. The persisters were incubated with different concentrations of Art-175 or ciprofloxacin (10 x MIC and 30 x MIC) in the absence or presence of 0.5 mM EDTA. Art-175 (10 x and 30 x MIC) completely eradicated the remaining persisters (approximately 5-log reduction) in the presence of 0.5 mM EDTA, whereas similar doses of ciprofloxacin (with and without EDTA) or 0.5 mM EDTA alone did not affect the persisters. In the absence of EDTA, a 30 x-MIC dose of Art-175 reduced the persister fractions of PA14 and PA1255 by 2.16 ± 0.42 log and 4.35 ± 1.15 log units, respectively. In summary, the overall activity of Art-175 against these persister fractions is extremely pronounced.

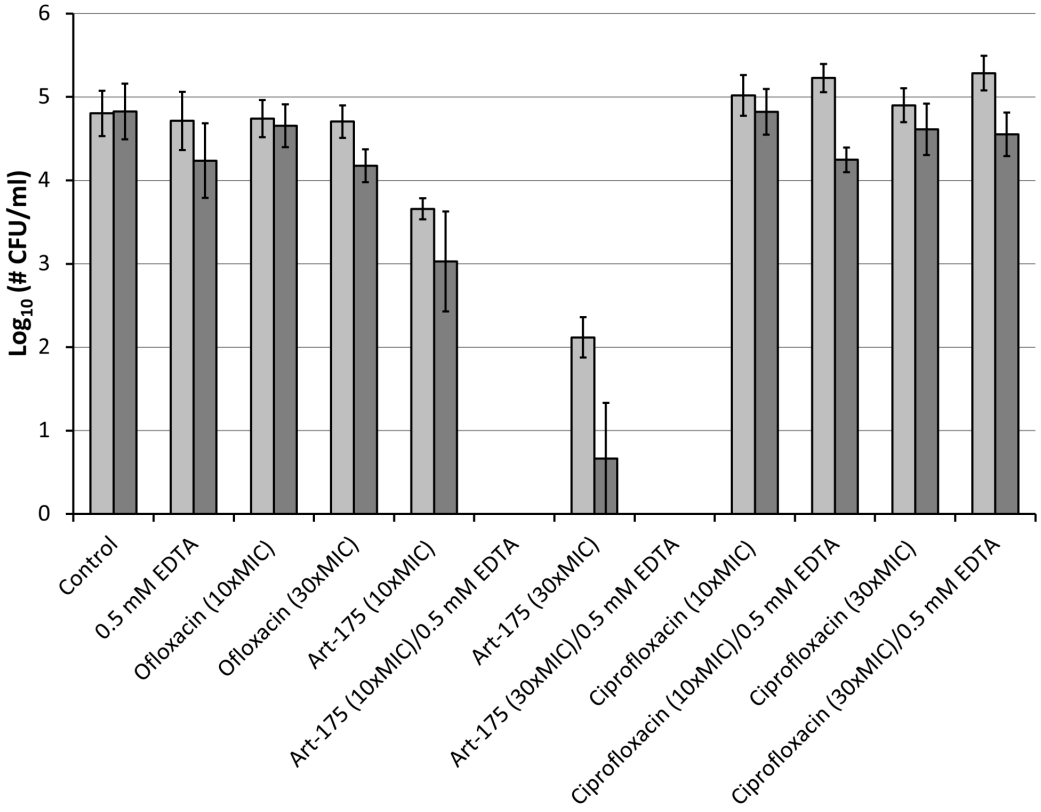


Figure 7.5: Persister killing by Art-175. The bactericidal effects of Art-175 at 10 x and 30 x MIC on isolated persister fractions *of P. aeruginosa PA14* (light gray) and PA1255 (dark gray) are compared to those of ciprofloxacin at 10 x MIC and 30 x MIC, in both the absence and the presence of 0. 5mM EDTA. Controls include 0.5 mM EDTA and ofloxacin (10 x MIC and 30 x MIC). Mean values (±standard error of the mean [SEM]) are shown for at least three independent replicates.

Chapter 8

Conclusions and future perspectives

This PhD thesis focused on the comprehensive characterization and determination of the mode of action of the novel anti-persister molecule 1-((2,4-dichlorophenethyl)amino)-3-phenoxypropan-2-ol (SPI009). Comprehensive characterization revealed a broad-spectrum anti-persister effect in different clinically relevant pathogens and a potent activity in several preclinical model systems. Mode of action studies were employed to discover how SPI009 is capable of directly killing both persister and non-persister cells and revealed extensive membrane-damaging activity. Overall, these findings make SPI009 an attractive scaffold for the development of novel anti-persister therapies.

The need for alternative therapies

While selection for drug resistance in micro-organisms is inevitable from an evolutionary point of view, the rate of resistance development is becoming increasingly worrisome and has long surpassed the current rate of antibiotic development[409,427], causing infections that were once simple to cure, to pose a serious threat to human health.

Causing additional difficulties in the effective treatment of bacterial infections is the presence of so-called persister cells, antibiotic-tolerant variants that are capable of surviving treatment.[52] Persister cell represent a serious threat to human health when the immune system is compromised or bacteria are able to evade the immune system. Nowadays, persistence is widely acknowledged as a major culprit of treatment failure in chronic and biofilm infections and recent research has even identified the persister fraction as a possible reservoir for the development of resistance[64,74] (Chapter 2).

Clearly, there is an urgent need to focus efforts on the design of alternative therapies that not only tackle the problem of resistance but also allow the eradication of persister cells. Complete killing of the persister fraction will prevent reoccurrence of chronic infections, reduce long-term exposure to antibiotics thereby diminishing the development of resistance and greatly improve patient outcomes.

SPI009 as a novel, broad-spectrum anti-persister compound

To address these issues, our research efforts focused on the characterization and determination of the mode of action of the recently discovered anti-persister molecule 1-((2,4-dichlorophenethyl)amino)-3-phenoxypropan-2-ol (SPI009). SPI009 possesses a potent anti-persister and secondary antibacterial effect against several clinically relevant pathogens. Indeed, in addition to (multidrug resistant) *P. aeruginosa,* effective persister killing was observed in *Burkholderia cenocepacia*, found in chronic CF lung infections[428], *Escherichia coli*, associated with urinary tract infections[429], the food-born pathogen *Salmonella* Typhimurium[306] and the ESKAPE pathogens, responsible for the majority of nosocomial infections[285] (Chapter 4). This is in stark contrast with the majority of the anti-persister molecules found in literature with reported activity against one or a very limited number of species.[223] Additionally, SPI009 monotreatment possesses potent anti-biofilm activity and the observed antibiotic-independent effect allows successful combination with antibiotics from mechanistically distinct classes to further increase antibacterial activity of SPI009.

Several parallel approaches were used to unravel the compound's mechanism of action, confirming the hypothesis of membrane damage and demonstrating that SPI009 is capable of efficiently and extensively damaging both the outer and inner membrane of Gram-negative pathogens. While the observed decrease in antibacterial effect of SPI009 upon addition of Mg^{2+} could point towards a possible chelation effect[361], displacement assays revealed an interaction with the lipopolysaccharides of the Gram-negative membrane. Additionally, the chemical structure of the compound suggests disruption of the hydrophobic stacking of the membrane phospholipids. Further research, including the measurement of LPS degradation, titration experiments and atomic force microscopy[362] can be employed to explore the effect of SPI009 on the membrane. Techniques such as surface plasmon resonance[430] or compound-lipid overlay assays[431] could provide more information on the interaction of SPI009 with different components of the bacterial membrane. Furthermore, additional experiments on *P. aeruginosa* efflux pump mutants could unravel the exact role of different bacterial pumps in SPI009 efflux and explore the observed link with fluoroquinolone resistance. Further unraveling of the mechanism of action of SPI009 provides useful information on possible anti-persister targets and may assist in the development of future anti-persister strategies.

Evaluating the clinical potential of SPI009

When evaluating the future clinical potential of SPI009 we can identify several beneficial characteristics including its extensively demonstrated antibacterial and anti-persister activity against both Gram-negative and Gram-positive clinically relevant pathogens and in different model systems, both *in vitro* and *in vivo*. Indeed, the combination therapy of SPI009 with the conventional antibiotic ciprofloxacin was shown to eradicate intracellular *P. aeruginosa* infections and increase *C. elegans* survival to more than 70% in the first SPI009 *in vivo* model. Additional advantages include the absence of any hemolytic activity and the possible optimization of its chemical structure to further improve the selectivity index of SPI009.

When looking at the extensive *in vitro* and even *in vivo* data obtained for SPI009, several clinical applications can be envisioned for this novel anti-persister molecule. When taking into account the currently relatively high active concentrations of SPI009, systemic applications are not yet possible since it will be difficult to achieve these concentrations within infected tissues. However, as demonstrated in Chapter 4, SPI009 monotherapy exhibits potent anti-biofilm activity in both *P. aeruginosa* and *S. aureus* and decreases bacterial load in a hydrated 3D *P. aeruginosa* biofilm model. As such, SPI009 presents a solid candidate for the topical treatment of chronically infected wounds such as those seen in burn patients[27], foot and leg ulcers[432] and surgical site infections[22]. Additionally, the higher compound concentrations that can be reached in topical wound treatment could possibly circumvent the need for further adaptation of the SPI009 structure.

Furthermore, SPI009 has potential in antibiotic lock therapies (ALT), where high concentrations of antibiotics are inserted into catheters for sterilization purposes without the risk of systemic toxicity or the need to monitor drug levels.[433] Biofilms residing on catheters are responsible for 48.3% of all ICU-acquired bloodstream infections with the three most frequently occurring pathogens being *Enterococcus* spp., *S. aureus* and *K. pneumonia*[23], all sensitive to SPI009. Taking into account the biofilm-inhibiting activity of SPI009 against both Gram-positive and Gram-negative bacteria, SPI009 may serve as a good candidate for antibacterial coatings on surgical or dental implants. While physical immobilization would require additional research into the structure and active moiety of SPI009 to ensure optimal antibacterial activity, this strategy has previously been successfully employed in our group. SPI031, a related membrane-damaging small molecule, was shown to eradicate *P. aeruginosa* and *S. aureus* biofilms when covalently bound to titanium surfaces both *in vitro* and *in vivo*.[434]

Furthermore, the development of passive coatings that do not release antibacterials but rather inhibit microbial adhesion could circumvent the current toxicity issues. When envisioning systemic applications for SPI009, a possibility already demonstrated to be highly successful *in vitro* is its use in antibacterial combination therapies. As was shown for *P. aeruginosa*, 0.2 x MIC concentrations of SPI009 (10.2 µg/mL) successfully eradicated intracellular infections well below the limit of detection in combination with the conventional antibiotic ciprofloxacin, illustrating the reduced active concentration upon combination treatment. Another example is the eradication of the polymyxin B resistant *P. aeruginosa* 9BR strain (MIC_{SPI009} = 102 µg/mL) with the combination of polymyxin B and 1/6 x MIC concentrations of SPI009. Although more research is needed to examine possible negative effects, using a combination of a conventional antibiotic together with a (non-)antibiotic adjuvant could extend the life-time of current antibiotics, decrease concentrations of both agents, reduce treatment times and, when combining different modes of action, facilitate the treatment of antibiotic-resistant strains.[110,111,279] While SPI009 was typically combined with conventional antibiotics, the use of non-antibiotic adjuvants such as EDTA or even Art-175 could offer interesting possibilities. The combination of the peptidoglycan-degrading Art-175[129,130] with the outer and inner membrane damaging SPI009 could provide a successful combination in the eradication of both Gram-positive and Gram-negative bacteria.

With a broad activity and combination spectrum, the selection of a specific disease condition and antibiotic combination for clinical testing of SPI009 would severely limit its practical applications after commercialization. A significant disadvantage of this approach thus represents the requirement of separate registration and clinical trials to assess safety and specificity of each combination of antibiotic and SPI009, significantly increasing development time and costs.[10] Alternatively, SPI009 could be marketed as a single product, designed for combination with conventional antibiotics. While allowing one clinical trial to assess the antibacterial effect in combination with several conventional antibiotics, the safety, stability, PK/PD behavior and efficacy of SPI009 monotherapy could be difficult to demonstrate without further adaption of its chemical structure, limiting its chances of registration.

When looking at the different clinical options for SPI009, future work on this compound could include the additional optimization of its chemical structure. Evaluation of

[10] http://www.fda.gov; http://www.ema.europe.eu; https://www.fagg-afmps.be/nl

the 2*S* and 2*R* enantiomers of SPI009 revealed no difference in MIC values for *K. pneumonia*, and even showed a slight increase in MIC (2 x) for both enantiomers separately as compared to the racemic mixture for *P. aeruginosa*. While an initial SAR study already suggested the limited influence of the hydroxyl residue (R2, Table 3.2) and a flexibility in the chain length at position R4, more extensive SAR studies will be necessary to identify derivatives of SPI009 with a higher selectivity index.[435,436] Since the knowledge of organic chemistry required for these analyses is not within our expertise, future structural adaptations will be done in collaboration with specialized labs. Additionally, further unravelling of the membrane-interacting moiety might provide additional insights for possible chemical modifications and the development of future anti-persister molecules.

To further assess the possibilities of SPI009 as an antibacterial therapy, important parameters such as solubility, metabolic stability and serum protein binding will have to be determined and additional animal models will have to be used to assess the safety, PK/PD behavior and efficacy of this novel anti-persister molecule. As discussed in Chapter 2, the use of *in vivo* persistence or chronic infection models will be of great help to translate the current *in vitro* and *in vivo* results of this promising compound to clinical use. Unfortunately, a golden standard for *in vivo* persistence research and the assessment of novel anti-persister therapies is currently lacking. However, chronic infection models in mice have been used to study persister cells of *S. aureus*[170] and *P. aeruginosa*[80] and their treatment in a deep-seated thigh infection and intraperitoneal and subcutaneous biofilm infection model, respectively. However, the highest mortality of *P. aeruginosa* chronic infections is recorded in the lung, for which persister models are still lacking. Our group is currently investing in the development of a chronic lung infection model in mice to allow the screening of new antimicrobials and anti-persister compounds. Other interesting models might include a chronic urinary tract infection model of *S. aureus* or *E. coli* with the possibility to evaluate SPI009-coated catheters to prevent infection.[437,438] As stated above, realistic applications for SPI009 would include the topical treatment of chronically infected wounds. As such, *in vivo* models of chronic skin or burn wound infection would allow the long-term evaluation of SPI009 antibacterial killing and provide information about the feasibility of SPI009 therapy for clinical use.[439–441]

Artilysin®s as alternative antibacterial strategy

In a second part of this PhD thesis I had the opportunity to explore the possible anti-persister activities of another alternative antibacterial treatment strategy, Artilysin®s. The idea of using bacteriophages and phage-encoded endolysins as antibacterial therapy is well known and, with the current antibiotic crisis could provide an attractive alternative.[399,414,442] With the design of Artilysin®s, endolysins coupled to specific LPS-destabilizing peptides, a major hurdle was overcome, providing another weapon in the fight against Gram-negative pathogens.[131,298]

Within the scope of my PhD project, I investigated the anti-persister capabilities of Art-175, a well characterized Artilysin®. Previous research demonstrated a potent antibacterial activity against *P. aeruginosa* and *A. baumannii* via puncturing of the peptidoglycan layer and subsequent cell lysis.[130,298] Probably due to its peptidoglycan-damaging activity, Art-175 efficiently kills both dividing and non-dividing cells of *P. aeruginosa*[129] and *A. baumannii*[130]. This, combined with the predicted absence of resistance development and lack of cross-resistance as evaluated in a panel of 33 *A. baumannii* strains, proposes Art-175 as an ideal candidate for novel anti-persister therapies.[130]

The use of Artilysin®s poses several advantages over traditional antibiotics including their novel mechanisms of action, low resistance potential and fast-acting antibacterial effect. Additionally, they remain active in biofilms, have a limited potential for side effects and, due to their high specificity, do not disturb the normal human microflora.[130] Potent antibacterial activity after topical application was already shown in several *in vivo* case studies; including two dogs suffering from bacterial otitis [131] and a coma patient suffering from non-treatable MRSA-infected decubitus[11]. However, extended clinical trials further assessing both safety and efficacy will be necessary to develop Artilysin®s as novel antibacterials.

[11] http://www.lysando.com

Concluding remarks

With the current antibiotic crisis in mind, academic research into novel antibiotics and alternative therapies is booming and different national and international incentives are stimulating antibacterial research. Several pharmaceutical companies such as Roche, GSK, Johnson & Johnson, AstraZeneca, Novartis and Pfizer are reinvesting in antimicrobial research and alternatives for the current antibiotics in the fight against chronic infections. Overall, the work presented in this thesis has contributed to the discovery and understanding of a novel anti-persister molecule, SPI009, capable of clearing bacterial infections containing both normal and antibiotic-tolerant persister cells. While still in a very early drug development stage and facing some important hurdles in eventual commercialization, we have demonstrated the potential of this alternative therapy in the combat against Gram-negative and Gram-positive bacterial infections and provided a scaffold for the development of future anti-persister therapies.

Appendix A

Supplementary tables to Chapter 5

Table A.1: Functional enrichment analysis of *P. aeruginosa* transposon mutants showing increased sensitivity towards SPI009. PseudoCAP classes showing a functional enrichment, as determined via Fisher's exact test, are highlighted in grey.

Functional class	Liberati mutants in class	Sensitive mutants	Fisher's exact test P-value	Gene list
2-component systems	139	4	0.547598326	*phoP, algR,* PA14_27800, PA14_11630
Amino acid biosynthesis and metabolism	235	9	0.102488053	*argR, carB, thrC, argH, hisH, aguA, hutH,* PA14_05480, PA14_63120
Adaptation and protection	132	7	0.024742372	*lon, dksA, katA, bfrB, ppk, pa1L,* PA14_57840
Antibiotic resistance and protection	60	1	1	*mexD*
Carbon compound catabolism	144	5	0.245592847	*msuD, pa1L, pcaG, pykA, treA*
Cell wall/LPS/capsule	122	7	0.016787118	*comL, lpxC, pa1L, wzz, arnT, wbpW,* PA14_66200, PA14_08540
Central intermediary metabolism	112	4	0.310421356	*tpiA, nasA, hutH,* PA14_04150
DAN replication, modification and repair	97	3	0.471023797	*phr, dksA, sbcB*
Energy metabolism	217	8	0.146264308	*nuoJ, lldD, tpiA, ccpR, pykA,* PA14_69110, PA14_42120, PA14_44360
Export apparatus	102	5	0.071430469	*pscL, xcpT, xcpR,* PA14_40170, PA14_66580
Fatty acid and phospholipid metabolism	157	1	0.264525635	PA14_38640
Membrane proteins	456	14	0.17871873	*shaB, dnpA, nasA,* PA14_38320, PA14_36170, PA14_40170, PA14_63970, PA14_45070, PA14_52350, PA14_65030, PA14_68830
Motility and attachment	112	2	1	*cupC1*, PA14_61530
Nucleotide biosynthesis & metabolism	62	3	0.152827949	*carB, ppk, PA14_05790*

Functional class	Liberati mutants in class	Sensitive mutants	Fisher's exact test P-value	Gene list
Putative enzymes	432	11	0.604476392	*nagZ*, PA14_35880, PA14_69110, PA14_62420, PA14_49070, PA14_04150, PA14_16620, PA14_67440, PA14_07780, PA14_53420, PA14_40030
Related to phage, transposon, plasmid	67	4	0.057868633	PA14_61840, PA14_67810, PA14_48880, PA14_53570
Secreted factors	79	1	1	*rhlB*
Synthesis of co-factors	139	3	1	*mvfR, thrC,* PA14_02420
Transcription, RNA processing and degradation	44	2	0.249555074	*rho, pcnB*
Transcriptional regulators	357	9	0.575666494	*phoP, algR, argR, dksA, mvfR, PA14_06400, PA14_68920, PA14_51340, PA14_56430*
Translation, post-translational modification, degradation	146	6	0.136993652	*selD, pheT, nusA, lon,*PA14_67810, PA14_70480
Transport small molecules	600	19	0.100567331	*mexA, sppB,bfrB, shaB, znuC, nasA, gabP, corA, fepG,* PA14_69070, PA14_47330, PA14_19540, PA14_38320, PA14_69070, PA14_57870, PA14_40170, PA14_45070, PA14_47560, PA14_30590
Hypothetical	1557	36	0.681128806	*dnpA, argH, wzz,pykA,* PA14_62420, PA14_47330, PA14_42160, PA14_36170, PA14_07240, PA14_57840, PA14_16620, PA14_21490, PA14_08490, PA14_14430, PA14_08330, PA14_63970, PA14_63970, PA14_67440, PA14_07780, PA14_07780, PA14_53100, PA14_51570, PA14_59070 PA14_03370, PA14_15140, PA14_42160, PA14_52350, PA14_70480, PA14_07360, PA14_69010, PA14_24570, PA14_66580, PA14_32750, PA14_40030, PA14_21480, PA14_61010, PA14_03250, PA14_08540

Table A.2: Differentially expressed genes in P. aeruginosa upon treatment with SPI009. RNA sequence analysis revealed a set of 297 genes showing a differential up- or down-regulation specifically for SPI009 and with log_2-fold changes >1.

Gene name	Corresponding PAO1 locus	Product name and/or function	Fold change (Log_2)
PA14_26850	PA2880	hypothetical protein	3.64
gpo	PA2826	glutathione peroxidase	3.38
PA14_37250	PA2114	major facilitator transporter	3.25
gcvH2	PA2446	glycine cleavage system protein H	2.99
mexC	PA4599	multidrug efflux RND membrane fusion protein	2.91
PA14_37210	PA2116	hypothetical protein	2.70
PA14_06640	PA0508	acyl-CoA dehydrogenase	2.70
PA14_27530	PA2825	MarR family transcriptional regulator	2.66
PA14_37270	PA2112	LamB/YcsF family protein	2.65
opdO	PA2113	porin	2.63
nfxB (PA14_60810)	PA4600	transcriptional regulator NfxB	2.54

PA14_44230	/	hypothetical protein	2.51
gcvP2	PA2445	glycine dehydrogenase	2.46
PA14_06600	PA0506	acyl-CoA dehydrogenase	2.43
oprJ	PA4597	outer membrane protein OprJ	2.39
fadA	PA3013	3-ketoacyl-CoA thiolase	2.33
aptA	PA0132	beta alanine--pyruvate transaminase	2.27
PA14_32950	PA2448	hypothetical protein	2.25
htpX	PA2830	heat shock protein HtpX	2.24
fadB	PA3014	multifunctional fatty acid oxidation complex subunit alpha	2.14
PA14_47640	PA1282	major facilitator transporter	2.08
PA14_37310	PA2110	hypothetical protein	2.08
PA14_71900	PA5446	hypothetical protein	2.05
PA14_36020	/	paraquat-inducible protein B	2.03
nfxB (PA14_60860)	PA4596	transcriptional regulator NfxB	1.96
PA14_16640	PA3691	lipoprotein	1.93
PA14_64520	PA4880	bacterioferritin	1.90
PA14_32470	PA2487	hypothetical protein	1.87
PA14_13090	PA3925	acyl-CoA thiolase	1.83
PA14_62390	PA4714	hypothetical protein	1.82
PA14_25840	PA2953	electron transfer flavoprotein-ubiquinone oxidoreductase	1.80
PA14_53400	PA0840	oxidoreductase	1.77
PA14_36030	/	paraquat-inducible protein A	1.74
PA14_68840	PA5212	hypothetical protein	1.73
glyA2	PA2444	serine hydroxymethyltransferase	1.73
aspA	PA5429	aspartate ammonia-lyase	1.71
PA14_44311	PA1559	hypothetical protein	1.61
PA14_24360	/	hypothetical protein	1.61
PA14_47120	PA1324	hypothetical protein	1.60
PA14_29720	PA2658	hypothetical protein	1.60
PA14_00620	PA0049	hypothetical protein	1.53
pfpI	PA0355	protease PfpI	1.52
oprH	PA1178	PhoP/Q and low Mg2+ inducible outer membrane prote	1.50
PA14_72930	PA5528	hypothetical protein	1.49
PA14_53410	PA0839	transcriptional regulator	1.49
osmC	PA0059	osmotically inducible protein OsmC	1.48
deaD	PA2840	ATP-dependent RNA helicase	1.45
PA14_40890	PA1828	short chain dehydrogenase	1.45
PA14_04180	PA0320	hypothetical protein	1.40
PA14_02060	PA0165	hypothetical protein	1.39
prmF or *arnC*	PA3553	glycosyl transferase family protein	1.39
PA14_41950	PA1748	enoyl-CoA hydratase	1.37
PA14_40880	PA1829	hypothetical protein	1.37
rplJ	PA4272	50S ribosomal protein L10	1.35
PA14_22730	PA3206	two-component sensor	1.30
pstC	PA5368	membrane protein component of ABC phosphate transporter	1.29
etfB	PA2952	electron transfer flavoprotein subunit beta	1.29
pstA	PA5367	phosphate ABC transporter permease	1.28

arnB	PA3552	UDP-4-amino-4-deoxy-L-arabinose--oxoglutarate aminotransferase	1.28
yccA	PA2604	hypothetical protein	1.27
PA14_29730	PA2657	two-component response regulator	1.27
aceA	PA2634	isocitrate lyase	1.27
PA14_04710	/	hypothetical protein	1.27
PA14_47130	PA1323	hypothetical protein	1.27
ohr	PA2850	organic hydroperoxide resistance protein	1.26
osmE	PA4876	DNA-binding transcriptional activator OsmE	1.25
PA14_33160	PA2433	hypothetical protein	1.24
rplA	PA4273	50S ribosomal protein L1	1.24
yegH	PA1331	TerC family protein	1.24
PA14_53500	PA0833	hypothetical protein	1.23
PA14_31870	PA2528	RND efflux membrane fusion protein	1.23
PA14_57610	/	/	1.21
typA	PA5117	GTP-binding protein TypA	1.19
psrA	PA3006	transcriptional regulator PsrA	1.19
nuoI	PA2644	NADH dehydrogenase subunit I	1.19
tsf	PA3655	elongation factor Ts	1.18
rplL	PA4271	50S ribosomal protein L7/L12	1.17
PA14_05040	PA0386	coproporphyrinogen III oxidase	1.17
PA14_29710	PA2659	hypothetical protein	1.16
PA14_68110	PA5157	transcriptional regulator	1.16
rplX	PA4252	50S ribosomal protein L24	1.16
nuoD	PA2639	bifunctional NADH:ubiquinone oxidoreductase subunit C/D	1.16
PA14_66350	PA5020	acyl-CoA dehydrogenase	1.15
PA14_00570	PA0045	lipoprotein	1.15
emrA	PA5159	multidrug resistance protein	1.15
rplN	PA4253	50S ribosomal protein L14	1.14
rplE	PA4251	50S ribosomal protein L5	1.14
rpsA	PA3162	30S ribosomal protein S1	1.13
PA14_37200	PA2117	hypothetical protein	1.13
ordL	PA2776	hypothetical protein	1.13
rplK	PA4274	50S ribosomal protein L11	1.13
*fmt*or *arnA*	PA3554	bifunctional UDP-glucuronic acid decarboxylase/UDP-4-amino-4-deoxy-L-arabinose formyltransferase	1.13
mocA	PA3795	oxidoreductase	1.13
rplI	PA4932	50S ribosomal protein L9	1.12
PA14_16370	PA3712	hypothetical protein	1.12
rplW	PA4261	50S ribosomal protein L23	1.12
rplR	PA4247	50S ribosomal protein L18	1.12
PA14_64490	PA4877	hypothetical protein	1.12
mdr	PA3136	secretion protein	1.12
ybiT	PA1964	ABC transporter ATP-binding protein	1.11
etfA	PA2951	electron transfer flavoprotein subunit alpha	1.11
rpsQ	PA4254	30S ribosomal protein S17	1.11
rpsB	PA3656	30S ribosomal protein S2	1.10
PA14_04270	PA0327	transcriptional regulator	1.10
PA14_42090	PA1736	acetyl-CoA acetyltransferase	1.09
PA14_41960	PA1747	hypothetical protein	1.08

ycaL	PA0277	Zn-dependent protease with chaperone function	1.08
fadH1	PA3092	2,4-dienoyl-CoA reductase	1.07
PA14_40860	PA1830	hypothetical protein	1.07
rpmC	PA4255	50S ribosomal protein L29	1.07
rplP	PA4256	50S ribosomal protein L16	1.07
potA	PA3607	polyamine transport protein PotA	1.06
PA14_54720	/	hypothetical protein	1.06
galU	PA2023	UTP-glucose-1-phosphate uridylyltransferase	1.06
ycfJ	PA3819	hypothetical protein	1.06
PA14_69270	PA5246	hypothetical protein	1.05
rplD	PA4262	50S ribosomal protein L4	1.05
PA14_41690	PA1768	hypothetical protein	1.05
PA14_23520	PA3137	MFS transporter	1.04
glnA	PA5119	glutamine synthetase	1.04
emrB	PA5160	drug efflux transporter	1.04
opmG	PA5158	outer membrane protein	1.03
gpmA	PA1831	hypothetical protein	1.03
pitA	PA4292	phosphate transporter	1.03
PA14_07550	PA0578	hypothetical protein	1.02
rplB	PA4260	50S ribosomal protein L2	1.02
rplV	PA4258	50S ribosomal protein L22	1.02
PA14_21640	PA3277	short chain dehydrogenase	1.02
PA14_42080	PA1737	3-hydroxyacyl-CoA dehydrogenase	1.02
rpsN	PA4250	30S ribosomal protein S14	1.02
rpsS	PA4259	30S ribosomal protein S19	1.01
rpsE	PA4246	30S ribosomal protein S5	1.00
ugd	PA2022	nucleotide sugar dehydrogenase	1.00
PA14_56700	PA4360	hypothetical protein	1.00
PA14_41710	PA1767	hypothetical protein	1.00
rplF	PA4248	50S ribosomal protein L6	1.00
rpsC	PA4257	30S ribosomal protein S3	1.00
PA14_32490	PA2485	hypothetical protein	1.00
PA14_22130	/	hypothetical protein	-1.00
PA14_68280	PA5168	dicarboxylate transporter	-1.00
PA14_37745	PA2069	carbamoyl transferase	-1.01
orfH	/	UDP-N-acetyl-D-mannosaminuronate dehydrogenase	-1.01
qscR	PA1898	transcriptional regulator	-1.01
PA14_37760	PA2068	MFS transporter	-1.02
PA14_70780	PA5363	hypothetical protein	-1.02
PA14_65090	PA4929	hypothetical protein	-1.02
proW	PA5095	ABC transporter permease	-1.02
mmsB	PA3569	3-hydroxyisobutyrate dehydrogenase	-1.02
PA14_54870	/	hypothetical protein	-1.02
PA14_49330	PA1166	hypothetical protein	-1.03
PA14_38190	PA2036	hypothetical protein	-1.03
coIII	PA0108	cytochrome c oxidase subunit III	-1.03
PA14_06130	PA0468	hypothetical protein	-1.04
PA14_42150	PA1731	hypothetical protein	-1.04
PA14_01780	PA0144	nucleoside 2-deoxyribosyltransferase	-1.04
PA14_58360	PA4497	ABC transporter substrate-binding protein	-1.04

napE	PA1177	periplasmic nitrate reductase NapE	-1.04
lasB	PA3724	elastase LasB	-1.05
arcD	PA5170	arginine/ornithine antiporter	-1.05
fepC	PA4158	ferric enterobactin transport protein FepC	-1.05
clpA	PA0459	ClpA/B protease ATP binding subunit	-1.05
PA14_55200	PA0704	amidase	-1.05
PA14_12680	PA3957	short chain dehydrogenase	-1.05
PA14_35700	/	hypothetical protein	-1.06
PA14_48530	PA1221	AMP-binding protein	-1.06
lpdV	PA2250	dihydrolipoamide dehydrogenase	-1.06
PA14_32630	PA2475	cytochrome P450	-1.07
phaD	PA5059	TetR family transcriptional regulator	-1.07
PA14_46550	/	ribonuclease	-1.07
PA14_46540	/	hypothetical protein	-1.07
phaC2	PA5058	poly(3-hydroxyalkanoic acid) synthase 2	-1.07
PA14_48600	PA1215	AMP-binding protein	-1.08
PA14_21190	PA3311	hypothetical protein	-1.08
exbB1	PA0198	transport protein ExbB	-1.08
ynfM	PA5030	MFS transporter	-1.08
PA14_21510	PA3289	hypothetical protein	-1.09
hslR	PA5195	heat shock protein	-1.09
hutT	PA5097	amino acid permease	-1.09
PA14_08060	PA0621	tail fiber assembly protein	-1.10
PA14_67370	PA5101	hypothetical protein	-1.10
phnC	PA3384	ABC phosphonate transporter ATP-binding protein	-1.10
PA14_36860	/	hypothetical protein	-1.10
tauD	PA3935	taurine dioxygenase	-1.11
PA14_73090	PA5542	hypothetical protein	-1.11
PA14_35710	/	hypothetical protein	-1.12
PA14_07020	PA0540	hypothetical protein	-1.12
PA14_38210	PA2034	hypothetical protein	-1.12
PA14_12910	PA3939	hypothetical protein	-1.13
PA14_04330	PA0332	hypothetical protein	-1.14
PA14_50880	PA1041	hypothetical protein	-1.14
PA14_54010	PA0791	transcriptional regulator	-1.14
PA14_62250	PA4703	hypothetical protein	-1.15
PA14_19480	PA3451	hypothetical protein	-1.15
PA14_32280	PA2504	hypothetical protein	-1.15
PA14_28190	/	tRNA-Val	-1.16
PA14_00080	PA0007	hypothetical protein	-1.16
PA14_36470	PA2174	hypothetical protein	-1.16
PA14_51600	PA0981	hypothetical protein	-1.16
pyoS3I	/	immunity protein S3I structural gene	-1.16
PA14_46780	PA1353	hypothetical protein	-1.16
PA14_11140	PA4078	nonribosomal peptide synthetase	-1.17
PA14_61500	PA4648	hypothetical protein	-1.17
rpoS	PA3622	RNA polymerase sigma factor RpoS	-1.18
PA14_64920	PA4915	methyl-accepting chemotaxis protein	-1.18
PA14_48560	PA1218	hypothetical protein	-1.18
cpg2	PA2787	glutamate carboxypeptidase	-1.19

PA14_10360	PA4141	hypothetical protein	-1.19
PA14_47390	PA1301	transmembrane sensor	-1.19
PA14_13920	/	hypothetical protein	-1.19
PA14_20000	PA3409	transmembrane sensor	-1.20
hupA	PA5348	HU family DNA-binding protein	-1.20
PA14_10770	PA4112	sensor/response regulator hybrid	-1.20
PA14_54240	PA0776	hypothetical protein	-1.21
PA14_55780	PA4293	two-component sensor	-1.21
PA14_23400	/	hypothetical protein	-1.21
nlpD	PA3623	hypothetical protein	-1.21
PA14_55900	PA4303	hypothetical protein	-1.22
fdhD	PA5180	formate dehydrogenase accessory protein FdhD	-1.22
PA14_60960	PA4607	hypothetical protein	-1.22
PA14_65040	PA4925	hypothetical protein	-1.23
PA14_13110	PA3924	long-chain-fatty-acid--CoA ligase	-1.23
PA14_12940	PA3937	taurine ABC transporter ATP-binding protein	-1.23
PA14_55850	PA4299	pilus assembly protein	-1.24
PA14_68440	PA5181	oxidoreductase	-1.25
PA14_58740	/	hypothetical protein	-1.25
orfE	/	polysaccharide biosynthesis protein	-1.26
PA14_64930	PA4916	hypothetical protein	-1.26
fptB	PA4220	hypothetical protein	-1.26
PA14_39270	PA1951	hypothetical protein	-1.28
dotU3	PA2362	hypothetical protein	-1.29
coxG	PA0107	cytochrome C oxidase assembly protein	-1.29
PA14_61940	PA4682	hypothetical protein	-1.30
PA14_49410	PA1159	cold-shock protein	-1.30
ssuB	PA3442	aliphatic sulfonates transport ATP-binding subunit	-1.31
PA14_15280	PA3772	hypothetical protein	-1.32
PA14_38220	PA2033	hypothetical protein	-1.32
PA14_61920	PA4681	hypothetical protein	-1.32
PA14_28790	/	hypothetical protein	-1.33
PA14_37690	PA2072	sensory box protein	-1.33
PA14_34100	PA2363	hypothetical protein	-1.35
bfd	PA3530	hypothetical protein	-1.35
PA14_21480	PA3291	hypothetical protein	-1.36
PA14_29330	/	hypothetical protein	-1.37
PA14_48620	PA1213	clavaminic acid synthetase	-1.37
PA14_34080	PA2364	hypothetical protein	-1.38
pmtA	PA0798	phospholipid methyltransferase	-1.40
PA14_40240	PA1876	ABC transporter ATP-binding protein/permease	-1.41
PA14_40260	PA1874	hypothetical protein	-1.42
PA14_59230	PA0984	colicin immunity protein	-1.42
PA14_39860	PA1907	hypothetical protein	-1.42
PA14_40740	/	hypothetical protein	-1.42
pqqA	PA1985	coenzyme PQQ synthesis protein PqqA	-1.42
PA14_55790	PA4294	hypothetical protein	-1.42
rhlC	PA1130	rhamnosyltransferase 2	-1.43
icmF3	PA2361	hypothetical protein	-1.44
opdB	PA2700	porin	-1.44

phzE1	PA4214	phenazine biosynthesis protein PhzE	-1.44
PA14_46530	/	hypothetical protein	-1.45
proX	PA5096	ABC transporter substrate-binding protein	-1.46
phzB2	PA1900	phenazine biosynthesis protein	-1.46
PA14_27990	PA2794	sialidase	-1.46
PA14_01330	PA0109	hypothetical protein	-1.46
PA14_67260	PA5093	histidine/phenylalanine ammonia-lyase	-1.46
apaH	PA1906	hydrolase	-1.47
PA14_55860	PA4300	hypothetical protein	-1.48
hsiB3	PA2365	hypothetical protein	-1.49
trbF	/	conjugal transfer protein TrbF	-1.50
ssuF	PA3441	molybdopterin-binding protein	-1.51
PA14_12260	PA3986	hypothetical protein	-1.52
PA14_13210	/	hypothetical protein	-1.55
codB	PA5099	cytosine/purines uracil thiamine allantoin permease	-1.55
cpaF2	PA4302	type II secretion system protein	-1.55
PA14_26020	PA2939	aminopeptidase	-1.56
PA14_61010	PA4611	hypothetical protein	-1.57
clpV3	PA2371	ClpA/B-type protease	-1.60
hcp3	PA2367	hypothetical protein	-1.60
PA14_37670	/	hypothetical protein	-1.63
hsiC3	PA2366	hypothetical protein	-1.64
rmf	PA3049	ribosome modulation factor	-1.65
hsiG3	PA2369	hypothetical protein	-1.67
PA14_58040	PA4471	hypothetical protein	-1.67
hvn	PA1914	hypothetical protein	-1.73
PA14_40750	/	hypothetical protein	-1.79
PA14_55880	PA4301	hypothetical protein	-1.81
PA14_28780	/	hypothetical protein	-1.83
PA14_55930	PA4305	pilus assembly protein	-1.85
phzG2	PA1905	pyridoxamine 5'-phosphate oxidase	-1.85
PA14_47400	PA1300	RNA polymerase ECF-subfamily sigma-70 factor	-1.89
PA14_55920	PA4304	type II secretion system protein	-1.93
phzA2	PA1899	phenazine biosynthesis protein	-1.99
PA14_60480	PA4570	hypothetical protein	-1.99
phzC2	PA1901	phenazine biosynthesis protein PhzC	-2.00
PA14_33930	PA2375	hypothetical protein	-2.05
yeiH	PA5383	hypothetical protein	-2.12
PA14_55940	PA4306	hypothetical protein	-2.18
pa1L	PA2570	PA-I galactophilic lectin	-2.19
PA14_61380	/	hypothetical protein	-2.98

Appendix B

Supplementary information to Chapter 6

Table B.1: Overview of the genes identified in the 85 kb deletion region in *P. aeruginosa* SSF65-563. Functions are denoted as identified by RAST. Correct assignment of a function was checked using the corresponding protein sequence and the Pseudomonas database (http://www. pseudomonas.com). PAO1 and corresponding PA14 loci were determined based on protein sequence and verified in Geneious. .

PAO1 locus	PA14 locus	Gene	RAST assigned function
PA1278	/	*cobP*	Adenosylcobinamide-phosphate guanylyltransferase (EC 2.7.7.62)
PA1279	PA14_47680	*cobU*	Nicotinate-nucleotide--dimethylbenzimidazole phosphoribosyltransferase (EC 2.4.2.21)
PA1280	PA14_47660		Alpha-ribazole-5'-phosphate phosphatase (EC3.1.3.73)
PA1281	/	*cobS*	Cobalamin synthase (EC 2.7.8.26)
PA1282	PA14_47640		Permeases of the major facilitator superfamily
PA1283	PA14_47610		Transcriptional regulator, TetR family
PA1284	PA14_47600	*Ptpre*	Acyl-CoA dehydrogenase (EC 1.3.8.7)
PA1285	PA14_47580		Transcriptional regulator, MarR family
PA1282	PA14_47560		Permeases of the major facilitator superfamily
PA1287	PA14_47550		Glutathione peroxidase family protein
PA1288	PA14_47540		Long-chain fatty acid transport protein
PA1289	PA14_47530		hypothetical protein
PA1290	PA14_47520		Transcriptional regulator, TetR family
PA1291	PA14_47510		Hydrolases of the alpha/beta superfamily
PA1292	PA14_47500	*sseA*	Thiosulfate sulfurtransferase, rhodanese (EC2.8.1.1)
PA1382	/		General secretion pathway protein D; probable Type II secretion system protein
PA1383	/		hypothetical protein
PA1384	/	*galE*	UDP-glucose epimerase
PA1385	/		Lipid carrier : UDP-N-acetylgalactosaminyltransferase (EC 2.4.1.-) / Alpha-1,3-N-acetylgalactosamine transferase PglA (EC2.4.1.-)
PA1386	/		SN-glycerol-3-phosphate transport ATP-binding protein UgpC (TC 3.A.1.1.3)
PA1387	/		hypothetical protein
PA1388	/		hypothetical protein
PA1389	/		Twin-arginine translocation protein TatC; probable glycosyl transferase
PA1390	/		probable glycosyl transferase
PA1391	/		hypothetical protein; probable glycosyl transferase
PA1392	/		probable glycosyl transferase
PA1393	/	*cysC*	Adenylylsulfate kinase (EC 2.7.1.25); CysC
PA1394	PA14_46390		FIG00953120: hypothetical protein
PA1395	PA14_46380		FIG00958383: hypothetical protein
PA1396	PA14_46370		Sensor histidine kinase/response regulator
PA1397	PA14_46360		DNA-binding response regulator, LuxR family
PA1398	PA14_46340		FIG00955022: hypothetical protein
PA1399	PA14_46330		Transcriptional regulator, LysR family
PA1400	PA14_46320		Biotin carboxylase (EC 6.3.4.14)
PA1401	PA14_46310		FIG00956228: hypothetical protein
PA1402	PA14_46300		O-methyltransferase (EC 2.1.1.-)
PA1403	PA14_46290		Transcriptional regulator, TetR family
PA1404	PA14_46280		FIG00955989: hypothetical protein
PA1405	PA14_46270		UvrD/REP helicase family protein
PA1406	PA14_46260		Predicted nucleotidyltransferases
PA1407	PA14_46250		Methyltransferase (EC 2.1.1.-)
PA1408	PA14_46240		potassium efflux system KefA

PAO1 locus	PA14 locus	Gene	RAST assigned function
PA1409	PA14_46230	*aphA*	Deacetylases, including yeast histone deacetylase and acetoin utilization protein
PA1410	PA14_46220		Putrescine ABC transporter putrescine-binding protein PotF (TC 3.A.1.11.2)
PA1411	PA14_46200		Permease of the drug/metabolite transporter (DMT) superfamily
PA1412	PA14_46180		Transporter, MFS superfamily
PA1413	PA14_46170		Transcriptional regulators, LysR family
PA1414	PA14_46160		hypothetical protein
PA1415	PA14_46150	*halS*	SoxH protein, homolog - transcription factor
PA1416	PA14_46140		D-2-hydroxyglutarate dehydrogenase
PA1417	PA14_46120		Acetolactate synthase large subunit (EC2.2.1.6)
PA1418	PA14_46110		sodium-solute symporter, putative
PA1419	PA14_46100		Cytosine/purine/uracil/thiamine/allantoin permease family protein
PA1420	PA14_46080		Undecaprenyl pyrophosphate synthase
PA1421	PA14_46070	*gbuA*	Agmatinase (EC 3.5.3.11) - gbuA (guanidinobutyrase)
PA1422	PA14_46060	*gbuR*	Transcriptional regulator GbuR
PA1423	PA14_46030	*bdlA*	Methyl-accepting chemotaxis protein - bdlA (biofilm dispersion locus A)
PA1424	PA14_46020		FIG00957985: hypothetical protein
PA1425	PA14_46010		ABC transporter ATP-binding protein
[1]/	/		ABC-type antimicrobial peptide transport system, ATPase component
PA1428	PA14_45980		Histone acetyltransferase HPA2 and related acetyltransferases
PA1429	PA14_45970		Ca ion transporting P-type ATPase
PA1430	PA14_45960	*lasR*	N-3-oxododecanoyl-L-homoserine lactone quorum-sensing transcriptional activator @ Transcriptional regulator LasR
PA1431	PA14_45950	*rsaL*	Regulatory protein RsaL
PA1432	PA14_45940	*lasI*	N-acyl-L-homoserine lactone synthetase LasI
PA1433	PA14_45930		Membrane bound c-di-GMP receptor (LapD)
PA1434	PA14_45920		T1SS associated transglutaminase-like cysteine proteinase LapP
PA1435	PA14_45910	(*mexM*)[2]	Probable RND efflux membrane fusion protein precursor
PA1436	PA14_45890	(*mexN*/*muxC*)[3]	Probable RND effluxtransporter
PA1437	PA14_45880		DNA-binding heavy metal response regulator
PA1438	PA14_45870		Heavy metal sensor histidine kinase

[1]: The protein sequence as proposed by RAST does not match with PAO1 or PA14, BLAST however identified fully identical protein sequences in *Pseudomonas aeruginosa*. The DNA sequence of the proposed protein partly overlaps with PA1426 and PA1427.

[2]:Gene name as proposed by String-db, based on the corresponding protein sequence.

[3]: While identified as 'Cobalt-zinc-cadmium resistance protein CzcA; Cation efflux system protein CusA' by RAST, the corresponding DNA sequence of both genes did not map in the deletion region and the deletion protein Fsequence was identified as *mexN* or *muxC* by, respectively, String-db and Pseudomonas.com.



Figure B.1: Graphical overview of the deletion region in *P. aeruginosa* SSF65-563. The ± 85 kb region is denoted with a grey bar while individual genes are shown in yellow, together with their through RAST predicted function. To investigate the causal mutations responsible for increased SPI009 sensitivity, the deletion region was split up in 9 sub regions (orange). 600 bp homologues regions flanking each subregion were created via PCR and linked to a kanamycin resistance cassette for use in the lambda-Red recombinase system, as described in Lesic *et al.*(2008).[389]

Appendix C - List of publications

Articles in internationally reviewed academic journals

Defraine V., Verstraete L., Van Bambeke F., Anantharajah A., Townsend E.M., Ramage G., Corbau R., Marchand A., Chaltin P., Fauvart M., Michiels J. (2017). Antibacterial activity of 1-((2,4-dichlorophenethyl)amino)-3-phenoxypropan-2-ol against antibiotic-resistant strains of diverse bacterial pathogens, biofilms and in pre-clinical infection models.
Accepted for publication in Frontiers in Microbiology.

Liebens V.*, **Defraine V.***, Knapen W., Swings T., Beullens S., Corbau R., Marchand A., Chaltin P., Fauvart M., Michiels J. (2017). Identification of 1-((2,4-dichlorophenethyl)amino)-3-phenoxypropan-2-ol, a novel antibacterial compound active against persisters of *P. aeruginosa*. *Antimicrobial Agents and Chemotherapy*, 61 (9).

Gerits E., **Defraine V**., Vandamme K., De Cremer K., De Brucker K., Thevissen K., Cammue B., Beullens S., Fauvart M., Verstraeten N., Michiels J. (2017). Repurposing toremifene for the treatment of oral bacterial infections. Antimicrobial Agents and Chemotherapy, 61 (3).

Defraine V., Schuermans J., Grymonprez B., Govers S., Aertsen A., Fauvart M., Michiels J., Lavigne R., Briers Y. (2016). Efficacy of Artilysin® Art-175 against resistant and persistent *Acinetobacter baumannii*. *Antimicrobial Agents and Chemotherapy*, 60 (6), 3480-3488.

Briers Y., Walmagh M., Grymonprez B., Biebl M., Pirnay J., **Defraine V**., Michiels J., Cenens W., Aertsen A., Miller S., Lavigne R. (2014). Art-175 is a highly efficient antibacterial against multidrug-resistant strains and persisters of *Pseudomonas aeruginosa*. *Antimicrobial Agents and Chemotherapy*, 58 (7), 3774-3784.

* Co-first author

Liebens V., **Defraine V**., Van der Leyden A., De Groote V., Fierro Gutiérrez A., Beullens S., Verstraeten N., Kint C., Jans A., Frangipani E., Visca P., Marchal K., Versées W., Fauvart M., Michiels J. (2014). A putative de-N-acetylase of the PIG-L superfamily affects fluoroquinolone tolerance in *Pseudomonas aeruginosa*. *Pathogens and Disease*, 71 (1), 39-54

Contributions to internationally oriented books

Liebens V., **Defraine V.,** Fauvart M. (2015). A Whole-Cell-Based High-Throughput Screening Method to Identify Molecules Targeting Pseudomonas aeruginosa Persister Cells. In: Michiels J., Fauvart M. (Eds.), bookseries: Methods in Molecular Biology, Bacterial Persistence Methods and Protocols Springer.

Meeting abstracts presented at international conferences

Defraine V., Verstraete, L., Liebens V., Corbau R., Marchand A., Chaltin P., Van Bambeke F., Anantharajah A., Fauvart M., Michiels J. (2017). A small molecule candidate for antibiotic co-therapy in the fight against bacterial persistence. *FEMS 2017* (July 9 – July 13, Valencia, Spain).

Defraine V., Liebens V., Swings T., Corbau R., Marchand A., Chaltin P., Van Bambeke F., Anantharajah A., Fauvart M., Michiels J. (2016). A small molecule candidate for antibiotic co-therapy in the fight against persistence. *Antimicrobial resistance in microbial biofilms and options for treatment*. (October 5 – October 7, Ghent, Belgium).

Defraine V., Liebens V., Swings T., Corbau R., Marchant A., Chaltin P., Fauvart M., Michiels J. (2015). Analysis of the mode of action of a new *Pseudomonas aeruginosa* anti-persister compound. *Keystone Symposium on Gram-Negative Resistance*. (March 29 - April 2, Tahoe City, California, USA).

Meeting abstracts presented at national conferences

Defraine V., Liebens V., Swings T., Corbau R., Marchand A., Chaltin P., Van Bambeke F., Anantharajah A., Fauvart M. (2015). Analysis of the mode of action of a *Pseudomonas aeruginosa* anti-persister compound. *Commun Agric Appl Biol Sci;* 80(1): 137-43.

*__Oral presentation__ at *20th National Symposium of Applied Biological Sciences*. (Louvain-la-neuve, January 30).

Liebens V., Knapen W., Swings T., **Defraine V.**, Corbau R., Marchand A., Chaltin P., Fauvart M., Michiels J. (2014). Identification of small molecules targeting *Pseudomonas aeruginosa* persisters. *Knowledge for Growth*. (Ghent, May 8).

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