OVERCOMING BIOFILM TOLERANCE BY A NOVEL APPROACH TARGETING BACTERIAL PERSISTERS

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Abstract

Multi-drug tolerance is a phenomenon, in which microorganisms normally susceptible to an antimicrobial agent are able to withstand a treatment via phenotypic alteration. The tolerance is conveyed by a microbial subpopulation that is in a non-replicative and metabolically inactive state also known as persistence. Through this kind of dormancy, the subpopulation may survive an otherwise appropriate course of antimicrobials, since the majority of the drugs target cellular division or metabolism. Upon the reduction of the surrounding antimicrobial concentration the multi-drug tolerant cells – persisters – become resuscitated thus allowing repopulation. As opposed to the more widely acknowledged challenge of antimicrobial resistance, the offspring of the specialist survivor cells are genetically identical to the susceptible majority.

Persisters are especially abundant in biofilms, a microbial lifestyle characterized by aggregated microcolonies that are covered in a self-produced slimy matrix known as extracellular polymeric substance (EPS). Partly owning to this protective matrix, biofilms are inherently somewhat tolerant to antimicrobial chemotherapy. Moreover, microbes confined in a biofilm are additionally protected against the components of the host immune system. Conversely, it is assumed that persisters in planktonic, i.e. freely floating state, are easily cleared out by white blood cells. Combined, the immune evasive properties of biofilms and the remarkable multi-drug tolerance of persisters give rise to recalcitrant infections that are immensely difficult to eradicate.

The described phenomenon constitutes crucially to the major healthcare challenge of chronic, treatment-resistant infections. Tuberculosis, cystic fibrosis lung disorder, bacterial endocarditis and infections related to indwelling medical devices are only a few examples of such problems. Despite the need for antimicrobials with anti-persister efficacy, no such therapeutics is available and very few are being investigated – one important factor being the lack of relevant drug discovery platforms. Therefore, the aim of this study was to develop an anti-persister assay and to carry out a pilot screening of natural product derived bioactive compounds.

Based on the notion that persisters are enriched in bacterial cultures that have reached the stationary phase of growth, a persister model was designed using *Staphylococcus aureus* ATCC 25923 as the test strain. The bacteria were grown in liquid cultures until they reached the stationary phase and subsequent experimentation was carried out to confirm the tolerant state. After the stationary phase persister model was validated, a small pilot screening of natural products was undertaken in the hope of finding novel anti-persister activity. Mitomycin C, a cytotoxic drug with an existing anti-cancer indication was assigned as the positive control compound because of its previously established anti-persister activity. Since it is common for all of the persister-related diseases that the target microorganisms reside within a protective biofilm, an additional assay based on biofilm regrowth was designed to characterize the hit compounds on a more clinically relevant platform.

The persister model culture was shown to be tolerant to conventional antibiotics. The re-induction of metabolic activity by diluting into fresh medium recovered the antimicrobial susceptibility expectedly. A total of 4 compounds were identified as anti-persister hits in the pilot screening campaign. Chromomycin A3, dehydroabietic acid, mithramycin A and oleanolic acid were all able to reduce the viable bacterial count in the stationary phase persister model more than 2 logarithmic units at 100 µM. Mithramycin A was the most potent, reducing the viability over 6 log units. The model compound mitomycin C reduced the viable counts 5.49 (± 0.96) logarithmic units. Out of the 4 hits, dehydroabietic acid was selected for the biofilm relapse assay because of its favourable biocompatibility properties. It reduced regrowth for the treated biofilms by 4 logarithmic units, thus demonstrating significant activity.

Keywords

persister, biofilm, antimicrobial, multi-drug tolerance

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Tiivistelmä

Monilääketoleranssi (multi-drug tolerance) on ilmiö, jossa antimikrobilääkkeelle herkät mikro-organismit kykenevät sietämään lääkehoitoa fenotyyppiään muokkaamalla. Toleranssin aikaansaa jakaantumattomassa ja metabolisesti epäaktiivisessa tilassa elävä osapopulaatio. Tämänkaltaisesta horrostilan tuomasta sietokyvystä käytetään nimitystä persistenssi. Sietokykyinen osapopulaatio voi selviytyä asianmukaisesti valitusta antimikrobilääkehoidosta, sillä lääkkeiden vaikutus kohdistuu tyypillisesti juuri solunjakaantumistoimintoihin tai aineenvaihduntaan. Kun ympäröivä lääkeainepitoisuus kuurin päätyttyä laskee, nämä monilääketolerantit solut – persisterit – elpyvät uudelleen kansoittaakseen tuhoutuneen populaation. Toisin kuin paremmin tunnetussa antimikrobilääkeresistenssin ilmiössä, näiden erikoistuneiden selviytyjäsolujen jälkeläiset ovat geneettisesti identtisiä lääkkeelle herkän osapopulaation kanssa.

Persisterisoluja on erityisen runsaasti biofilmeissä. Biofilmi on yhteen takertuneiden bakteerien muodostama yhdyskunta, joka tuottaa ympärilleen tyypillisen, solunulkoisten polymeerien muodostaman limakerroksen (extracellular polymeric substance, EPS). Osittain tämän suojaavan liman ansiosta biofilmit ovat itsessään melko sietokykyisiä antimikrobilääkkeille. Lisäksi biofilmissä elävät mikrobit ovat erityisen hyvin suojattuja isäntäorganismin immuunijärjestelmää vastaan. Sen sijaan planktoniset, yksittäisinä soluina irrallaan kelluvat mikrobisolut ovat helposti valkosolujen hävitettävissä. Biofilmin immunoevasiiviset ominaisuudet yhdistettynä persisterien monilääketoleranssiin mahdollistavatkin erittäin vaikeahoitoisten, kroonisten infektioiden kehittymisen.

Kuvattu sietokykymekanismi kroonistuneiden, hoitoon vastaamattomien infektioiden taustalla muodostaa merkittävän terveysuhan. Tuberkuloosi, kystisen fibroosin keuhko-ongelmat, bakteeriendokardiitti ja lääkinnällisiin laitteisiin liittyvät infektiot ovat joitakin esimerkkejä tällaisista hoidollisista haasteista. Erityisesti persistereihin tepsivien antimikrobilääkkeiden huutavasta tarpeesta huolimatta yhtäkään ei ole käytettävissä ja vain harvoja tutkitaan. Soveltuvien lääkekehitysalustojen puute on yksi olennainen syy tähän uusien keksintöjen puutteeseen. Sen vuoksi tämän työn tarkoituksena onkin kehittää menetelmä persisterilääkkeiden tunnistamiseksi ja tehdä pienen mittakaavan pilottiseulonta luonnontuotteista eristetyille, bioaktiivisille yhdisteille.

Persisterien on havaittu rikastuvan stationäärivaiheen bakteeriviljelmissä. Sen vuoksi persisterimallia ryhdyttiin kehittämään *Staphylococcus aureus* ATCC 25923 -bakteerikannalla, jota kasvatettiin nesteviljelminä staattiseen kasvuvaiheeseen saakka. Malliviljelmän lääketoleranssi tutkittiin, minkä jälkeen se altistettiin valikoiduille luonnontuotteille tarkoituksena löytää persisteribakteereihin tehoavia yhdisteitä. Positiiviseksi kontrolliksi valittiin mitomysiini C – syöpälääkkeenä käytettävä sytotoksinen yhdiste, jonka on aiemmin todettu tuhoavan myös persisteribakteereita. Koska kliinisesti merkittävät persisteri-ilmentymät tavataan juuri biofilmeissä, päätettiin lisäksi kehittää kasvun uudelleenkäynnistymistä biofilmistä mittaava menetelmä seulontalöytöjen tarkempaan kartoittamiseen hoidollisesti relevanteissa olosuhteissa.

Persisterimalli havaittiin sietokykyiseksi perinteisille antibiooteille. Metabolisen aktiivisuuden uudelleenkäynnistäminen tuoreeseen ravinneliuokseen laimentamalla palautti lääkeherkkyyden odotetusti. Seulonta löysi yhteensä 4 persistereihin tehoavaa yhdistettä: kromomysiini A3, dehydroabietiinihappo, mitramysiini A ja oleanolihappo kykenivät kaikki laskemaan elävien bakteerien lukumäärää persisterimallissa yli 2 logaritmiyksikköä 100 µM pitoisuuksina. Mitramysiini A oli yhdisteistä tehokkain vähentäen bakteerilukua yli 6 yksikköä. Malliyhdiste mitomysiini C vähensi elävien bakteerien lukua vastaavasti 5.49 (± 0.96) logaritmiyksikköä. Dehydroabietiinihappo valittiin jatkotutkimukseen biofilmin uudelleenkasvukykykokeeseen sen suosiollisen toksisuusprofiilin perusteella. Se vähensi uudelleenkasvua hoidetusta biofilmistä 4 logaritmiyksikköä osoittaen siten merkittävää aktiivisuutta.

Avainsanat

persisteri, biofilmi, antimikrobilääke, monilääketoleranssi

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Helsinki, August 15th 2016

Ilkka Miettinen

Table of Contents

List of Abbreviations

(p)ppGpp	guanosine tetra- or pentaphosphate
ADEP	acyldepsipeptide
ATP	adenosine triphosphate
BrdU	5-bromo-2'-deoxyuridine
BSA	bovine serum albumin
c-di-AMP	cyclic diadenosine monophosphate
CF	cystic fibrosis
CFU	colony forming unit
DHA	dehydroabietic acid
DNA	deoxyribonucleic acid
ELISA	enzyme-linked immunosorbent assay
EPS	extracellular polymeric substance
FDA	Food and Drug Administration
LD	Lyme disease
MHB	Müeller-Hinton broth
MIC	minimum inhibitory concentration
MMC	mitomycin C
mRNA	messenger RNA (ribonucleic acid)
PBS	phosphate buffered saline
RNA	ribonucleic acid
RSH	RelA-SpoT homologue
TA	toxin-antitoxin
TSA	tryptic soy agar
TSB	tryptic soy broth
VBNC	viable but nonculturable

1 INTRODUCTION

Multidrug tolerance is a phenomenon in which bacteria or other microorganisms can transiently alter their behaviour to survive virtually any regular antimicrobial treatment (Keren et al. 2004a). As opposed to the more widely understood challenge of antimicrobial drug resistance, the basis of multidrug tolerance is in phenotypic rather than genotypic variability. A stochastically determined, transient subpopulation within a microbial community is constantly in a survival-oriented state known as persistence that is characterized by slowed-down growth and metabolic inactivity (Balaban et al. 2004). The respective cells are termed persisters. An antimicrobial treatment may readily eliminate the susceptible majority of cells from such a culture, but the tolerant (persistent) phenotypes prevail to be reverted back to the metabolically active, replicative state after the stressor has passed (Keren et al. 2004b). A population regrown from the surviving persisters is genetically identical to the one previously eradicated.

When in planktonic i.e. freely floating state, infective persisters are not particularly challenging to overcome (Lewis 2007). An accordingly selected antimicrobial treatment will eliminate the susceptible bulk of cells, while the immune system clears out the remaining persisters. The vast clinical significance of multidrug tolerance arises from persister involvement in another singular microbial lifestyle known as biofilms (Lewis 2001). Biofilms are defined as surface-adherent microbial aggregates that are enclosed within a protective layer of self-produced matrix made of extracellular polymeric substance (EPS) (Costerton et al. 1999). Biofilm growth is an inherently tolerant lifestyle that is encountered in approximately 60-85 % of all infections (Coenye and Nelis 2010). The profound tolerance observed in biofilms has been partly attributed to persister cells that are markedly enriched in biofilms (Lewis 2001). Biofilms give them additional shielding against host defences among other things, which leads to the emergence of recalcitrant infections as the multidrug tolerant subcolony is left untouched to reinitiate the pathogen population.

The diverse and plentiful treatment-resistant biofilm infections cause a huge burden on healthcare systems (Parsek and Singh 2003; Bryers 2008). Since no available antimicrobial can effectively address persister bacteria regardless of the species and

genotypic susceptibility, the need for new generation anti-persister antimicrobials is imperious. Nevertheless, the drug discovery research available on biofilm infections let alone their persister involvement is still scarce, possibly owning to platform-related challenges and the lack of general acknowledgement of the phenomenon. The above-described scenario motivates this thesis, in which it is aimed to develop a drug discovery platform for anti-persister drugs and to discover relevant new activities in natural products.

2 LITERATURE REVIEW

2.1 Persisters

Persisters were first described by Hobby, Meyer, and Chaffee in 1942 and defined two years later by Joseph Bigger: the scientists were consistently left with a small subpopulation of surviving bacteria when experimenting with otherwise sufficient doses of penicillin (Hobby et al. 1942; Wood et al. 2013). The viable cell count would decline sharply after initiating treatment, but with some 1 % of the bacteria left alive the growth curve would become flattened to form a biphasic killing pattern. When the surrounding conditions returned favourable, the descendants of these surviving bacteria would again become susceptible to the previously used antibiotic upon starting to grow again without demonstrating any specific and heritable mechanism of antimicrobial resistance.

Even over 70 years after their initial discovery, the true nature of persisters remains elusive (Zhang 2014). In contrast to the genotype-associated antimicrobial resistance, persistence is a strictly phenotypic phenomenon, which makes it difficult to directly characterize. Complex reporter gene systems and microfluidic devices have been adopted for persister research, but the main methodologies are still unchanged from the 40’s; the susceptible population is eliminated and the remaining bacteria studied (Balaban et al. 2004; Lechner et al. 2012; Maisonneuve et al. 2013; Cañas-Duarte et al. 2014).

This type of an antimicrobial tolerance has been eventually linked to a special state of dormancy, in which bacteria or other microorganisms living under stressful conditions shut down major parts of their cellular machinery and stop multiplying (Lewis 2007). These metabolically inactive microbes are very tolerant to antimicrobials that target components of cellular division system or depend on active intake into the target cell. The persister phenotype is distinct from an endospore – a dormant structure produced by several Gram-positive bacteria in starvation – although the two share important functional similarities (Pommerville 2014). The goal of both is to ensure the survival of the organism over a rough patch of hardened conditions until the environment returns to a favourable state. Unlike persisters, however, endospores are morphologically divergent cells formed in an asymmetric division.

It has since been understood that persisters have an important role in the development of recalcitrant, treatment resistant infections (Lewis 2007). The successful chemotherapeutic eradication of a bacterial infection is a result of a synergistic interplay between antimicrobial drugs and host immune defences. Antimicrobials halt the division of bacteria (bacteriostatic drugs) or kill the susceptible majority (bactericidal drugs), while the immune system gets rid of the remaining non-growing bacteria and persisters that are defenceless against phagocytic activity. Therefore, for the infection to become chronic, it has to be able to evade the host defences. In chronic infections this is achieved by bacteria taking seat within a protective barrier of a biofilm matrix or in a granulomatous lesion (Tufariello et al. 2003; Thurlow et al. 2011).

2.1.1 Stringent response

To survive, bacteria must adapt to changes in their growing environment. For this purpose, a complex network of signalling pathways has evolved to translate the environmental conditions into biomolecular responses. Various environmental stressors including heat, acidity, oxidative stress and amino acid starvation lead to an optimization of cellular functions that is known as the stringent response (Poole 2012). The key mediators for this response are small nucleotide molecules, also known as alarmones, including guanosine tetraphosphate and pentaphosphate (ppGpp/pppGpp) that accumulate in cells undergoing starvation which leads to an activation of a plethora of different pathways and subsequently to a decrease in protein synthesis and reallocation of energy resources (Dalebroux and Swanson 2012). This prepares the bacteria to surviving in harsh conditions and may eventually cause cells to enter a non-replicative dormant state.

The alarmone (p)ppGpp is produced on nutrient depletion by several proteins dubbed Rel (Haseltine and Block 1973). In Gram-negative bacteria the enzyme mostly responsible for (p)ppGpp synthesis is named RelA, and it is distinct from the counteracting (p)ppGpp hydrolysing enzyme SpoT (Wexselblatt et al. 2012). In Gram-positive bacteria both functions are coupled in a single Rel/Spo enzyme, and the family of these stringent response proteins is often referred to as RelA/SpoT homologue proteins or RSHs. When a bacterium faces stress, its transcriptional machinery is introduced with an accumulation of uncharged transfer-RNA molecules that bind to the ribosome but do not carry their

amino acids. This stalls the ribosome and encourages the binding of Rel-proteins at the site, which upon activation and detachment of enzyme leads to the synthesis of a large number of (p)ppGpp and other downstream events culminating in the stringent response.

2.1.2 Toxin-antitoxin modules

The decrease in metabolic activity via the stringent response is closely related to persistence, and the underlying molecular mechanisms have been studied in parallel. A conserved key component between different persister species is toxin-antitoxin or TA modules that in fact function as the effectors in stringent response (Wang and Wood 2011). They are typically modules that consist of a stable toxin molecule that is constantly expressed and its labile antitoxin counterpart continuously broken down by several different protease enzymes (Unterholzner et al. 2013). Normally the expression of the labile antitoxin is sufficient to counterbalance the toxin, thereby rendering it safe. However, in the presence of certain signals more of the antitoxin is degraded than is being synthetized to replace it, which lets the toxin exert its effect – possibly leading to the stringent response and persistence (Figure 1).

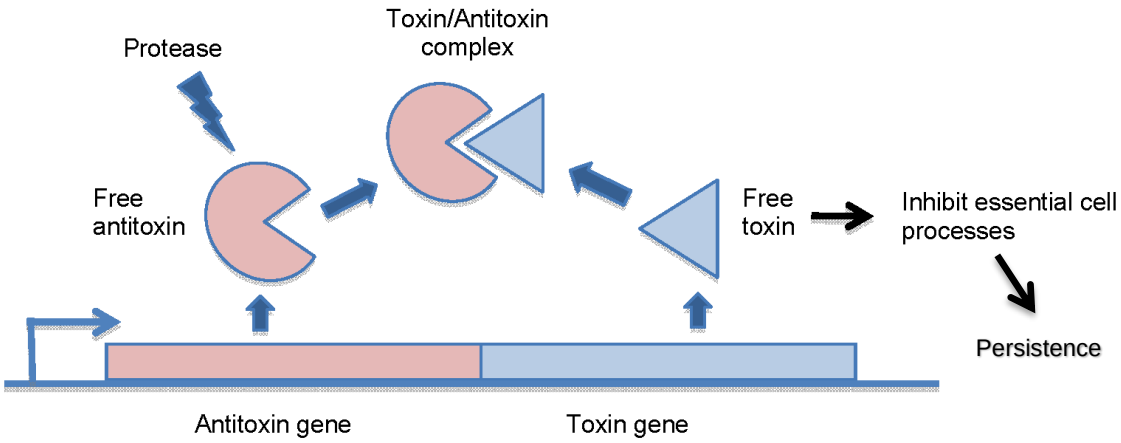


Figure 1. The typical toxin-antitoxin module (modified from Hong-Geller and Micheva-Viteva 2015). The labile antitoxin is normally produced in quantities sufficient for blocking the respective toxin. As a part of the bacterial stress response, the antitoxin becomes degraded by an increased protease activity. This leads to the accumulation of free toxin, since not enough antitoxin is available for countering toxin production. The imbalance may result in persistence.

Although universally found in bacteria, TA-modules have been studied in particular depth in *Escherichia coli*, in which a total of 11 of them are known to exist promoting persistence (Germain et al. 2015). In a majority of the TA-modules the toxin effector is by nature an mRNase – an endonuclease that cleaves messenger RNA or mRNA before it can be translated into a protein at a ribosome (Maisonneuve et al. 2011). This inhibits protein synthesis and leads to the reduction of metabolic activity, eventually causing the growth arrest associated with the stringent response and persistence (Keren et al. 2004a).

In mutation experiments carried out with *E. coli* a specific mutation was found to massively increase persister frequency and thus antimicrobial tolerance (Moyed and Bertrand 1983). The mutant was named *hipA7* after its characteristic high persistence. The associated gene *hipA* with its variably toxic transcription product of the same name were later discovered to be a part of a TA module *hipBA* with the autoregulating antitoxin counterpart of the module named HipB. *hipA7* was characterized as a gain-of-function mutant, in which the interaction between HipA toxin and HipB antitoxin and thus the autoregulation of the module is markedly diminished (Schumacher et al. 2009). Studies have since linked TA modules with the stringent response, *hipBA* being a crucial part of the above-described activation pathway (Germain et al. 2015). This was already pointed out by Korch et al. (2003), who demonstrated that *hipA7* mutants lose their outstanding ability to form persisters in high frequency when (p)ppGpp synthase RelA is rendered inactive.

Another example of such a regulatory TA-module found in *E. coli* is *IstR1-tisB* that promotes growth arrest upon SOS signalling – a pathway activated in bacteria by DNA damage (Vogel et al. 2004). IstR1 is an antitoxin that is able to neutralize the tisB toxin until it becomes downregulated as a part of the SOS-response. tisB is a membrane acting toxin that creates pores from inside the bacterial cell which leads to the disruption of the proton motive force as the ion gradient over the cell wall is lost (Gurnev et al. 2012). Since the synthesis of ATP depends on it, the cell enters growth arrest as ATP depletes.

The linkage to SOS response emphasizes the importance of *IstR1-tisB* from the perspective of antimicrobial treatment. Widely used broad spectrum antibiotics in the fluoroquinolone class exert their bactericidal effect by inhibiting the DNA-targeting enzymes gyrase and topoisomerase, which leads to DNA damage and thus provokes SOS

response (Phillips et al. 1987). Studies have confirmed that the fluoroquinolone antibiotic ciprofloxacin indeed induces persistence and makes the treated culture tolerant to further treatments with other antimicrobials as well (Dörr et al. 2010).

2.1.3 Dynamics

Although many factors predicting the metabolic state switch into persistent lifestyle have been described, the conversion is by nature a stochastic process (Balaban et al. 2004). The growth rate of microbial cells is inherently heterogeneous, arguably for the sole purpose to support the survival of the population as a whole by having a number of backup cells constantly in a tolerant phenotype.

From single-cell monitoring experiments with an ampicillin challenge Balaban et al. (2004) came to the conclusion that the studied *E. coli* populations had two distinct types of persisters: non-growing, slowly resuscitating cells that are introduced in the culture as carryovers from the stationary phase inoculum and slow-growing, quickly resuscitating cells that are stochastically induced also in an exponentially growing culture. Both withstood the exposure to ampicillin (an antibiotic targeting growing cells).

The alarmone (p)ppGpp has been proposed as the master regulator of phenotype switching (Maisonneuve et al. 2013). Using a fluorescent reporter in *E. coli* these authors demonstrated that cells with high level of the alarmone were non-growing and exceedingly tolerant to ampicillin. As described above, stochastic fluctuations in the level of the alarmone arising from the itself stochastic Rel/Spo activity have been shown to activate the persister-inducing TA modules, including HipBA that further elevates (p)ppGpp thus forming a feedback loop (Germain et al. 2015). The self-regulatory capabilities of HipBA have been accredited for the resuscitation of cells otherwise stuck in the inhibiting loop. The explanation is, however, challenged by the finding of Chowdhury et al. (2016a) that persisters are readily formed also without the presence of (p)ppGpp via different pathways.

2.1.4 Stationary phase bacteria

Studying the expression profiles of persisters in comparison with other forms of bacterial growth has given some insight into the nature of the dormant phenotype. Transcriptomic

comparisons have shown similarities between the expression profiles of persisters and stationary phase bacteria, although the opposite has also been demonstrated (Shah et al. 2006; Keren et al. 2011).

Microbial growth in a batch culture – the primary cultivating method in a laboratory – can be depicted as a curve that is divided into four distinguishable stages (Madigan et al. 2012) (Figure 2).

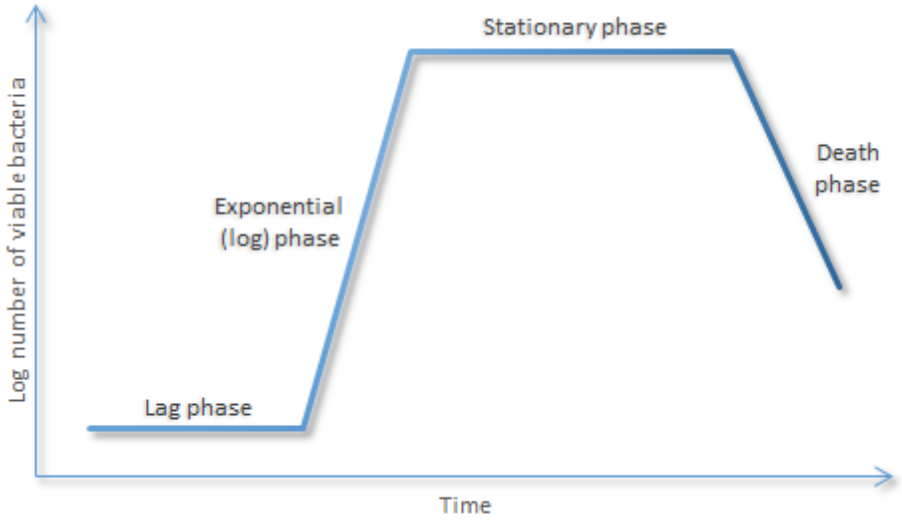


Figure 2. A typical bacterial growth curve. No growth is observed in the lag phase wherein bacteria are newly habituating to their environment. As the cells begin to multiply, the exponential phase sets in. As nutrients deplete and metabolic by-products accumulate, the exponential division is halted and the stationary phase starts. When enough toxic by-products have been produced and all available nutrient sources are exhausted, the bacteria begin dying rapidly, which is reflected in the downward trend in the death phase.

After the initial lag phase, in which the bacteria habituate into the growing conditions, follows the exponential phase (or log phase), where the bacteria divide without inhibition and grow exponentially (Madigan et al. 2012). Towards the end of the exponential phase the nutrients in the surrounding medium become scarce while growth-inhibiting metabolic products may be accumulated. This initiates the entry into the stationary phase, where observable growth stops as bacteria start dying at a rate that counters the already slowed-down division of cells.

Although stationary phase is not a defined physiological state but rather just descriptive term depicting a part of a typical growth pattern, it can be generalized that many characteristic changes occur as bacteria approach the environmental limit of exponential growth (Nyström 2004). Significant alterations in gene expression prepare the bacteria to survive in the aggravated conditions by radically changing the physiology and even morphology of the cells (Kolter et al. 1993). Bacterial cells may become smaller through reductive division to support the number of surviving cells without the nutrients required for proper growth. Cell walls may become thicker, more rigid, more adherent and less permeable. Overall protein synthesis is reduced, while the production of proteins essential for prolonged survival is up-regulated. In transition to stationary phase the bacteria become cross-tolerant to various environmental stressors such as heat, oxidative stress, osmotic challenge and toxic products (Nyström 2004).

Importantly, persister formation is strongly induced in mid-exponential phase and the fraction of persisters plateaus at roughly 1 % in the early stationary phase (Keren et al. 2004b). The expressional changes are complex, but a comprehensible connection between stationary phase bacteria and persisters can be made by comparing the behaviour of alarmones in both types of cells. The second messenger (p)ppGpp, along with cyclic di-adenosine monophosphate (c-di-AMP) – another small nucleotide messenger present in *Staphylococcus aureus* – are strongly present also when bacteria enter stationary phase (Chang et al. 2002; Corrigan et al. 2015). The alarmone signalling contributes to the induction of the stringent response via various transcriptional and translational regulators.

Stationary phase cultures are highly tolerant against antimicrobials, to which they again become susceptible upon metabolic activity induction for example by dilution in fresh medium (Mascio et al. 2007). This further demonstrates the increased presence of persisters. The connection between stationary phase bacteria and persister cells offers a fortunate advantage from an experimental point of view since it is straightforward to study simple planktonic bacteria in a liquid medium. It is, however, important to notice that non-growing stationary phase cells and persisters are distinguishable phenotypes (Lewis 2007). Not all cells in a static culture are in an actual state of persistent dormancy induced by toxin-antitoxin modules; another fraction has only ceased dividing and will quickly

begin to grow again when the environmental requirements are again met (Lewis 2007; Germain et al. 2015).

2.2 Biofilms

Although having been unwittingly experimented with for decades, biofilms were probably first characterized in detail in 1933 by Arthur Henrici, who discovered that a glass slide immersed in the water of an aquarium or a lily pond would in a few days be covered in a uniform layer of bacteria (Henrici 1933; reviewed by Dufour et al. 2010). In his article of freshwater bacteria Henrici describes a dense bacterial growth that, supported by a filamentous morphology and a "sheath of gum", attaches to glass slides tightly enough not to come loose under tap water rinsing (Henrici 1933). Even though the present terminology was born only later, it is quite evident that Henrici came to accurately depict biofilm growth complete with the microcolony morphology and the supportive matrix of extracellular polymeric substance.

In his paper Henrici went on to conclude that, contrary to earlier understanding, freshwater bacteria are mostly living in sessile colonies attached to an underwater surface and not as singular planktonic microorganisms freely floating in the water (Henrici 1933). This conclusion has since been extended to all natural habitats by a wealth of knowledge, and it is today estimated that 99 % of all bacteria live naturally in biofilms (Donné and Dewilde 2015).

Considered the founding father of modern biofilm research, Bill Costerton is responsible for the critically important induction in the 1970's that what is true for bacteria living in freshwater ponds or mountain streams, has to be true for bacteria colonising a host organism as well (Costerton et al. 1978; Lappin-Scott et al. 2014). Costerton made observations of the glycoprotein polysaccharide, glycocalyx, which facilitates the adhesion of bacteria onto surfaces. His pioneering work on many fronts granted the scientific community an exhaustive understanding of biofilm structure and the process of biofilm formation while elucidating the role of biofilm bacteria in recalcitrant infections and antimicrobial tolerance and resistance.

2.2.1 Structure and formation

A biofilm is created when bacteria adhere to a surface or to other microorganisms and form a microcolony covered in a viscous matrix of secreted extracellular polymeric substance (EPS) (Donné and Dewilde 2015). The extracellular matrix, taking up some 85 % of the total biofilm composition, consists mostly of polysaccharides, proteins and DNA.

The formation of a biofilm is a complex multistage process (Figure 3). It typically begins when planktonic bacteria transiently attach and adhere onto a surface – an event which itself is usually triggered by a change in the environment that calls for lifestyle conversion (O'Toole et al. 2000). The capabilities of different bacterial species to bind onto a surface differ, some being readily able to adhere to an unconditioned surface while others may need the assistance of the adhesion proteins of a pioneer colonizer.

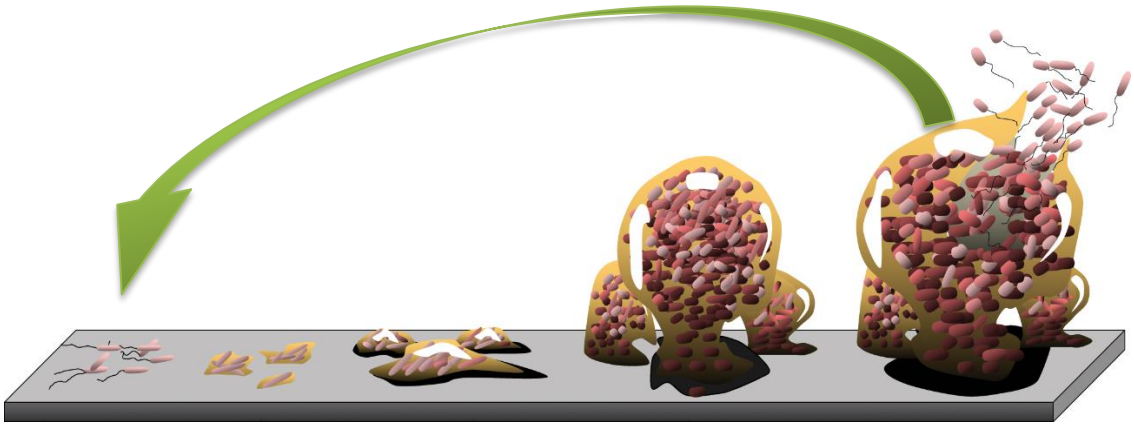


Figure 3. Biofilm formation scheme (modified from Monroe 2007). Planktonic bacteria adhere onto a surface and start developing into microcolonies. The production of EPS begins and the colonies become engulfed within the matrix, forming a biofilm. The biofilm then grows and maturates. From the mature biofilm planktonic bacteria and smaller microcolonies are again dispersed to complete the cycle.

Subsequently other nearby planktonic bacteria – either of the same species or a different one – will bind the surface itself or the prior adherer to form more stable cellular aggregate (O'Toole et al. 2000). Intricate networks of intra- and intercellular signalling become

stimulated as bacteria organize to form a biofilm. Changes can be observed in cellular morphology, physiology and communication. The up-regulated production of extracellular polymeric substance gives rise to the characteristic viscoelasticity and sliminess within the biofilm. From the maturated biofilm bacteria will again detach into the surrounding liquid as planktonic cells to complete the cycle of attachment and dispersal.

2.2.2 Medical challenge

Biofilm is a microbial lifestyle commonly associated with antimicrobial tolerance and resistance (Van Acker et al. 2014). Living as a community inside a sticky polymer matrix has various assets for bacteria colonising a host (Lewis 2005). When attached, the bacteria are not easily swept into the surrounding medium to be readily recognized and eliminated by the circulating components of the host immune system. The confinement within a protective layer of viscous matrix gives the bacteria some protection against phagocytic white blood cells in the event of an immune response (Thurlow et al. 2011). Additionally, bacteria in a biofilm community have capabilities to attenuate the pro-inflammatory response e.g. by modulating cytokines and affecting the activation of macrophages.

Conversely to the host defence components, the activity of antimicrobials is not strongly affected by the protective extracellular polymeric substance. Although some cationic antibiotics, such as aminoglycosides, may become trapped in a negatively charged matrix for example in the alginate rich biofilm produced by *Pseudomonas aeruginosa*, most are readily permeated inside the EPS (Hatch and Schiller 1998). Nevertheless, biofilms have proven to be extremely tolerant to antimicrobial treatment – a trait that makes them an important causative agent in various chronic infections (Lewis 2005). Naturally, many pathogenic bacteria have impressive arrays of antimicrobial drug resistance mechanisms that have evolved as a response to drug exposure, when the selection pressure caused by the antibiotic favours the survival of mutated strains with strategies to fight the treatment. The same is true with biofilms. Somewhat unexpectedly no specific and heritable mechanisms of drug resistance can often be found in biofilm bacteria that have regardless survived a treatment with supposedly effective concentrations of antimicrobials (Lewis 2005).

When combined with the perks of persistence, biofilm lifestyle is astonishingly resilient (Lewis 2005). Characteristic for biofilm lifestyle is a pronouncedly large subpopulation of persisters within the community. Even with prolonged courses of antimicrobial chemotherapy, these bacteria prevail to be regrown safe inside the biofilm after the environmental stressor has passed. Biofilm persisters give rise to recalcitrant infections that are very difficult to eradicate.

In persister bacteria no heritable mechanisms of antibiotic specific resistance have to be present. Still, the challenge of antimicrobial resistance cannot be omitted in this context. Bacteria constituting to relapsing biofilm infections may frequently become exposed to antimicrobial compounds in sub-inhibitory concentrations (Davies 2003). This can effectively breed resistant strains in an increased frequency. Additionally, the intra- or interspecies passage of mobile genetic material is rapid in a biofilm, where the highly heterogeneous microbial community demonstrates increased competence for transformation, i.e. the bacteria readily take in DNA from their environment (Madsen et al. 2012). Plasmid stability is also enhanced within a biofilm, which enables quick horizontal transfer of resistance genes. Finally, the frequency of transduction, i.e. the direct gene transfer between conjugated bacteria is increased in a closely associated population. In addition to the intrinsically tolerant nature of biofilms and the persisters harboured by them, antimicrobial drug resistance is another major challenge in biofilm infections. Targeting persisters is therefore a vital strategy to address both key challenges.

2.3 Biofilm and persister related diseases

Treatment-resistant infections that burden healthcare greatly are increasingly traced back to the presence of tolerant biofilms. Typical examples of such diseases include the lung disorder cystic fibrosis, the heart infection endocarditis, chronic urinary tract infections and chronic wounds (Costerton et al. 1999). Additionally, biofilm formation on indwelling medical devices is a common problem leading to costly reoperations where biofilm-colonized prostheses have to be replaced, for example in joints or heart valves.

2.3.1 Cystic fibrosis

Cystic fibrosis (CF) is a genetic disorder that stems from a mutation in the *CFTR* gene that leads to the expression of a faulty ion-channel by the same name – CFTR or cystic fibrosis transmembrane conductance regulator (Schwiebert et al. 1999). This causes the liquid covering airway surfaces to become dehydrated making it highly viscous and reducing its mobility, which severely impairs lung clearance. In addition to the weakened mucociliary clearance, the altered composition of the airway surface liquid also hampers other systems of innate immunity including white blood cells and cationic antimicrobial peptides (Döring and Gulbins 2009). Together these deficiencies expose the diseased lungs to a chronic polyspecies bacterial infection with a strong biofilm involvement.

From the viewpoint of antimicrobial chemotherapy, CF is a particularly challenging disease. The shortage of mechanical, cellular and chemical innate defences together with a niched environment with hypoxic and acidic conditions makes the disease-ridden airway epithelia favourable for biofilm and persister formation while significantly impairing drug delivery and efficacy (Høiby 2002). The prominent presence of dormant persister bacteria within the CF biofilm predisposes the lungs to remain infected even after a successful course of antimicrobial treatment (Mulcahy et al. 2010). The management of CF calls for repeated if not continuous antimicrobial treatments, and even then the recalcitrant infection is virtually ineradicable.

Presently the median life expectancy for CF patients is about 40 years and rising (MacKenzie et al. 2014). Along with the development of mucolytic agents and the more recent breakthroughs with CFTR-receptor correcting or potentiating drugs, the improving outcomes in CF treatment can be attributed to newly developed enhanced antimicrobial therapies. Together with various combinations of conventional antibiotics administered orally or intravenously, the recommended antimicrobials for CF treatment are inhalable formulations of the aminoglycoside tobramycin and the polymyxin antibiotic colistin (Sherrard et al. 2014). Up to a certain point the bacterial exacerbations can be managed with current tools. Nevertheless, the recalcitrant nature of the infection is still too big a challenge for antimicrobial chemotherapy and the infection will always relapse, even though novel treatments are able to deliver impressive concentrations of effective antimicrobials directly into the infection site. This is highly suggestive of persister

involvement in the infection dynamics of CF: the susceptible population is killed while a subpopulation of persisters stays alive in a dormant state to later cause the infection to relapse.

2.3.2 Tuberculosis

Tuberculosis, an infectious disease that usually affects the lungs, is a potentially life-threatening condition, which is typically treated with an extended course of multiple antimicrobials in combination (Liippo 2010). It is caused by species of the family *Mycobacterium*; most often the species *M. tuberculosis*. Based on its cell wall structure, the species belongs to a curious class in between Gram-negative and Gram-positive bacteria (Fu and Fu-Liu 2002). However, genetic analyses have shown that it shares more ancestry with Gram-negative bacteria demonstrating many characteristics of *Escherichia coli* and *Pseudomonas aeruginosa*.

The need for long-term treatment with disease-specific antimicrobials has been accounted to the persistent nature of the disease, which is relatively well understood and a subject of numerous studies (Zhang et al. 2012). Although most cases present themselves with a disease isolated in the lungs, tuberculosis may affect practically any organ system. Characteristic for tuberculosis is the presence of various secluded infection foci in e.g. granulomatous lesions or cavities, in which differing microenvironmental conditions (low pH, abnormally low or high oxygen content, and low level of nutrients) together induce persister conversion. In the diverse infection foci, the bacteria may exist in various states of dormancy, hiding out of reach of chemotherapeutics and host immune defences. This contributes to the often latent form of tuberculosis.

In a disease with such optimal conditions for enabling antimicrobial tolerance, relapses are common especially without the strict adherence to the treatment regime, which in turn provokes the breeding of strains with specific heritable mechanisms of antimicrobial drug resistance (Liippo 2010). Indeed, extendedly drug resistant forms of tuberculosis pose a serious risk for general health worldwide.

2.3.3 Lyme disease

Caused by the tick-borne bacterium *Borrelia burgdorferi*, Lyme disease (LD) is a multi-organ infection that typically first presents itself with a migrating erythema radiating from the tick-bite location (Hytönen et al. 2008). The infection spreads to different tissues readily via blood circulation. In addition to the early skin reactions, symptoms may classically emerge in the nervous system and joints, along with the significantly rarer manifestations for instance in the heart or in the eye. The typical symptoms of disseminated LD include inflammatory mono- or oligoarthritis, polyneuropathy and problems in the central nervous system, some of which may stay lingering for months or even years after the infection itself has been cured (Hytönen et al. 2008; Feng et al. 2015). However, in most cases the disease is caught and treated with antimicrobials – typically a few-week course of amoxicillin or doxycycline – in such an early phase that the consequences are limited to the initial skin rash and possibly some flu-like symptoms.

Still, even if treated accordingly, LD may in rare occasions become a chronic condition. This type of a chronic LD is becoming an increasingly popular subject of a fiery debate, in which the opposing sides are essentially arguing whether the lingering symptoms – usually of a highly subjective nature – are somatic and/or psychosomatic remnants of the since-cured destructive infection or a manifestation of a persistent infection that is somehow able to survive the intensive antimicrobial treatment and to avoid a perfectly competent host immune system (Wahlberg and Nyman 2009; Feng et al. 2015). Both arguments are probably correct, although it is very rare for the infection itself to become chronic (Wahlberg and Nyman 2009).

The implications of persistence in LD have warranted numerous publications. Persister bacteria have been managed to isolate from animal disease models and lab cultures of *B. burgdorferi* (Straubinger et al. 1997; Feng et al. 2015; Caskey and Embers 2015). An especially interesting finding concerns the morphology of the bacteria in different growth conditions (Feng et al. 2015). In its typical form in a normally growing culture, *B. burgdorferi* is a spirochete. However, when grown for many days as a stationary phase culture or in otherwise stressful conditions that generally induce persister formation in various species, the bacteria begin to increasingly convert into a round-bodied form. Additionally, aggregated microcolonies reminiscent of biofilm growth start to develop as

the culture is aging. In a 10-day old culture, the microcolony form is the dominant morphology.

The change in morphology is connected with a significant increase in antimicrobial tolerance and a decrease in metabolic activity (Feng et al. 2015). For all intents and purposes, the round-body and microcolony morphologies of *B. burgdorferi* are bacterial persisters. Moreover, when the surrounding conditions return to a favourable state the bacteria convert back to their spirochaetal form. The clinical significance arises from the notion that the aggregated microcolony forms abundantly present in a persister population of *B. burgdorferi* could indeed share some of the immunoevasive properties of a biofilm. This could explain the phenomenon of chronic LD infection while the non-infectious post-Lyme disease syndrome has been attributed to a prolonged autoimmune response triggered by a bacterial antigen.

2.3.4 Infections of indwelling medical devices

Because of the nature of microbial growth in liquid medium, biofilms tend to develop in the interfaces between the surface of an indwelling medical device, such as a catheter, and bodily fluids (Costerton et al. 1978; reviewed by Donlan and Costerton 2002; Percival et al. 2015). Many medical conditions necessitate the implantation of a medical device inside the body of a patient, where conditions are strongly favourable for biofilm formation on the non-living surface of the device. Indeed, indwelling medical devices give rise to a plethora of treatment-resistant nosocomial infections (Costerton et al. 1999; Percival et al. 2015).

Urinary catheterization is probably the most common source of these infections (Nickel and Costerton 1992; Bryers 2008). The mechanical irritation of the bladder lining caused by the catheter facilitates the infection from the biofilm colonization residing on the tubing. In addition to the obvious problems associated with cystitis, the catheter may become encrusted and blocked because of the crystallization of minerals within the biofilm in the presence of pH-increasing bacteria (Figure 4) (Jones et al. 2006). Antimicrobials are routinely needed to limit the biofilm growth and control the infection.

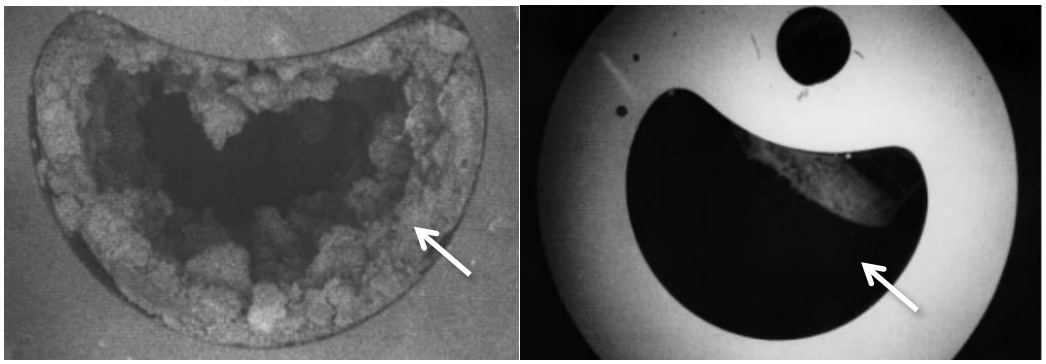


Figure 4. Biofilm encrustation in a silicone urinary catheter removed from *Proteus mirabilis* -infected bladder model on blockage (left). An antimicrobial treated test catheter removed after 7 days of incubation is free of encrustation (right). Modified from Jones et al. 2006.

Central venous catheters are another type of a tubular indwelling device prone to causing infections (Donlan and Costerton 2002; Percival et al. 2015). Because the catheter is in straight contact with bloodstream, host-derived proteins such as fibronectin and fibrinogen rapidly coat its surface, effectively facilitating bacterial adhesion and biofilm formation (Raad 1998). The colonization predisposes the catheterized patient to severe complications including embolization and sepsis.

Implanted prostheses, for example artificial joints and mechanical heart valves, are similarly vulnerable to biofilm development (Costerton et al. 1999). A biofilm growing on a prosthetic joint may cause a severe and painful infection while leading to the loosening of the prosthesis from the bone it is installed into (Barberán 2006). Biofilm infection of a prosthetic heart valve may lead to life-threatening endocarditis (Piper et al. 2001). Both manifestations of prosthetic biofilm infections are characteristically treatment-resistant and require aggressive antimicrobial therapy, possibly in combination with a surgical intervention and the replacement of the infected device (Piper et al. 2001; Barberán 2006).

2.3.5 Other biofilm-related diseases

Heart valves are susceptible to biofilm infection even without preceding surgical operation (Mills et al. 1984). Infective endocarditis is a severe recalcitrant condition characterized by microbial vegetation on heart endothelia and valves. Bacteria do not easily attach onto endothelial tissue, but the adherence is greatly facilitated by the production of a glycoprotein slime that eventually engulfs the growth into a biofilm that is responsible for the clinical manifestation of endocarditis (Scheld et al. 1978; Ramirez-Ronda 1978; Mills et al. 1984; Donlan and Costerton 2002). The disease damages the infected heart tissue and may cause infective emboli as microcolonies detaching from the biofilm shed to bloodstream (Donlan and Costerton 2002). Lodging of such an embolus inside a blood vessel can lead to serious consequences depending on the location of the blockade. Additionally, a persevered endocarditis can cause the infected valve to become calcified as minerals from the bloodstream crystallize within the biofilm (Poller et al. 1989). As with other biofilm diseases, infective endocarditis is often profoundly tolerant to antimicrobial treatment and may necessitate surgical intervention (Prendergast and Tornos 2010).

The microbes causing infective endocarditis typically originate in oral infection foci within the gingival tissue, from where they enter the bloodstream in the event of a disruption, for example a dental procedure (Parahitiyawa et al. 2009). Rather unsurprisingly, the microbial colonies in the oral environment are another example of an intricate biofilm system (Marsh 2006). Depending on the spectrum of species, oral biofilms may give rise to local infections, such as dental caries and periodontitis, the infection of the supportive tissue around teeth eventually leading to the destruction of the tissue and loosening and detachment of teeth (Sbordone and Bortolaia 2003).

2.4 Eradicating persisters

The previously described characteristics of persister bacteria make them a profoundly challenging target for any antimicrobial. However, a handful of compounds with some efficacy against persisters exist among the currently available antimicrobial drugs. Furthermore, persisters have become an important aspect of drug discovery, and a few

putative anti-persister agents have recently been described. Still, the scarcity of available and upcoming treatment options against persister diseases is worrisome.

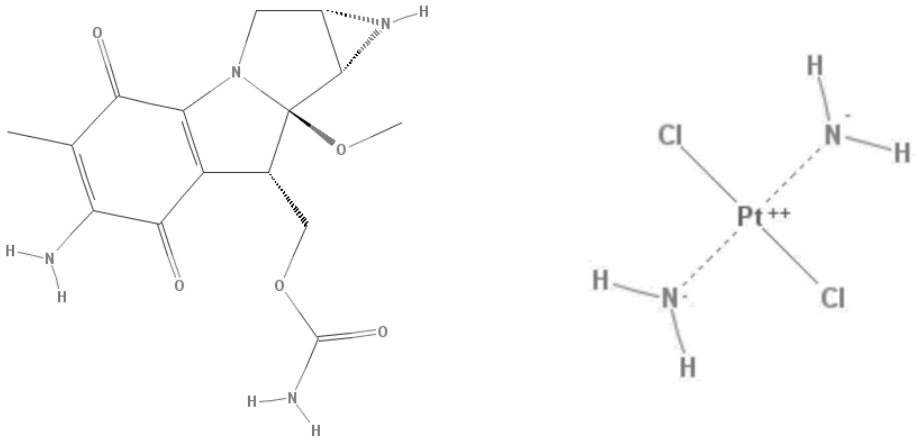
2.4.1 Conventional antibiotics

Although most antibiotics are practically useless against persister bacteria, some have demonstrated limited efficacy (Keren et al. 2012). Long courses of high-dose rifampicin, an RNA polymerase inhibitor, have been shown to eradicate persister cultures in *Mycobacterium tuberculosis* (Hu et al. 2015). The proposed mechanism is that even though rifampicin works as a bacteriostatic antibiotic, a long time inhibition of protein synthesis could lead to irreversible dormancy through the disruption of the numerous TA-modules needed for metabolic state control in *M. tuberculosis* (Keren et al. 2012). Interestingly, rifampicin is often successfully used in combination with other antibiotics to treat biofilm-related conditions (Jacqueline and Caillon 2014; Gbejuade et al. 2015). This could be due to its ability to affect the persister subpopulation within the biofilm.

Additionally, aminoglycoside tobramycin and fluoroquinolone ofloxacin have been successfully used in eradicating *Pseudomonas aeruginosa* persisters when applied in high concentrations and for an extended time period (Keren et al. 2012). This type of treatment is especially relevant in the prevention and treatment of CF exacerbations: courses of inhalable aminoglycosides have been widely adopted as a treatment protocol (Sherrard et al. 2014).

2.4.2 DNA crosslinkers

Mitomycin C (MMC) (Figure 5a), an anti-cancer drug isolated from the soil bacterium *Streptomyces caespitosus*, is a compound in which anti-persister activity has been demonstrated both *in vitro* and *in vivo* (Kwan et al. 2015). It is a prodrug that acts as a DNA-crosslinking agent upon activation by enzymatic reduction, a reaction also present in metabolically inactive cells.



a **b**

Figure 5. The structures of MMC (a) and cisplatin (b) (modified from PubChem).

By its destructive mechanism of effect, MMC does not rely on the division or metabolic activity of the target bacteria. Therefore, it can rapidly eradicate persisters in various species. However, as the current indication of the drug demonstrates, it is generally highly toxic to mammalian cells as well. The pronounced off-target toxicity prevents it from being a candidate drug for antimicrobial therapy. MMC is still highly suitable for experimental use, as it is very effective and fast-acting against both Gram-positive and Gram-negative bacteria. Additionally, it does not interfere with assays relevant to drug discovery, and it is even used in the validation of such assays (Invitrogen 2012).

Based on the successful experimentation with MMC, research has been extended to cisplatin (Figure 4b), another FDA-approved anticancer agent with similar DNA-crosslinking capabilities (Chowdhury et al. 2016b). As hypothesized, it proved to be effective against a broad spectrum of bacteria – even more active than MMC itself. However, the above-described limitations concern cisplatin too, since it is non-specifically toxic towards host cells.

2.4.3 Resuscitation

As the mechanisms of persister multi-tolerance are unspecific and do not rely on the destruction of the antimicrobial compound or keeping it from binding by altering the conformation of the target site, traditional antimicrobials could be used successfully if the persisters were resuscitated back to their metabolically active and reproductive state (Zhang 2014). Strategies for achieving this have been also described.

Making the environment of the microorganisms favourable again leads to the awakening and metabolic activation of the persisters (Joers et al. 2010). *In vitro* this is easily done by diluting the static persister culture into fresh medium. A respective possibility in *in vivo* treatment could be to reintroduce a carbon source into the dormant culture, as demonstrated by Allison et al. (2011) in a mouse urinary tract model of infection. The group used metabolites such as glucose, mannitol and fructose to successfully re-sensitize *E. coli* persisters against aminoglycoside antibiotics. Indeed, inhaled mannitol has been successfully experimented with in the treatment of CF, a chronic lung disease, which is strongly affected by the presence of *Pseudomonas aeruginosa* persisters. Although its effectiveness in CF has been originally attributed to osmotic enhancement of liquid transport, it seems that mannitol is also improving the sensitivity of *P. aeruginosa* against aminoglycosides by stimulating proton motive force over the bacterial cell wall and increasing the metabolic activity of the bacteria (Barraud et al. 2013). However, the antibiotic sensitizing effect could not be replicated in a CF-lung derived epithelial cell line – a model more representative of *in vivo* conditions (Price et al. 2015).

Persisters' ability to wake up upon the improvement of the surrounding conditions has yet to be elucidated on most species. It has probably been best documented in high guanosine-cytosine -content actinobacteria, from which a first common resuscitation promoting factor (Rpf) protein was discovered by Mukamolova et al. (1998). This group found the protein from *Micrococcus luteus* and demonstrated its ability to resuscitate dormant bacteria of the same species, along with various *Mycobacterium* species. Other resuscitation factors have since been found to function in different mycobacteria (Mukamolova et al. 2002). Hints of a similar resuscitating moiety existing outside actinobacteria were recently observed by Pascoe et al. (2014). The group discovered that spent medium supernatant from *Staphylococcus aureus* culture could reactivate dormant

cells. The effect was greatly diminished by heat denaturation or trypsin digestion of the supernatant, which points towards the existence of a resuscitation promoting protein akin to those reported earlier. A small-molecule compound with the ability to resuscitate and resensitize *E. coli* persister cells has additionally been described (Kim et al. 2011). A novel moiety only known as C10 was demonstrably able to speed up persister regrowth and re-enable norfloxacin killing.

2.4.4 Relacin, a ppGpp analogue

A new type of an anti-persister compound recently discovered is relacin (Figure 6), a ppGpp analogue that inhibits (p)ppGpp synthetizing Rel enzymes (Wexselblatt et al. 2012). According to *in silico* modelling, relacin tightly occupies the active site of Rel/Spo type of a (p)ppGpp synthetase via several bonding interactions. This inhibition leads to the disruption of the stringent response, which deprives the target bacteria of survival mechanisms such as stationary phase transition in *S. aureus* and sporulation in *Bacillus subtilis*. Biofilm formation was also shown to be greatly reduced.

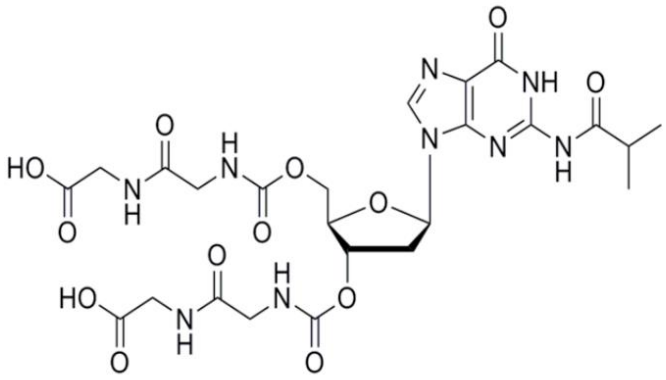


Figure 6. The structure of relacin, a ppGpp analogue (Wexselblatt et al. 2012).

As the enzymes in the RelA/SpoT homologue family are highly conserved, the novel antibacterial compound can be expected to target an impressive array of different species (Wexselblatt et al. 2012). Indeed, the inhibition of both Gram-positive and Gram-negative Rel enzymes was confirmed *in vitro*. However, the group only managed to demonstrate

the activity of the compound against Gram-positive bacteria and proposed that the inability to affect Gram-negative species was probably caused by challenges in permeation trough the thicker cellular membrane.

2.4.5 ADEP4

Another recent finding for an anti-persister compound is the acyldepsipeptide ADEP4 (Figure 7), a molecule capable of activating a bacterial proteolytic enzyme ClpP so that it starts functioning unspecifically (Conlon et al. 2013). In its normal state, ClpP degrades misfolded proteins ATP-dependently. However, once hyperactivated by ADEP4 that decouples the enzyme from ATP, it begins to attack a myriad of other, physiological proteins (Kirstein et al. 2009). This forces the cell to self-digest, which eventually leads to its death. Together with rifampicin ADEP4 was shown to be readily able to eradicate a deep-seated *Staphylococcus aureus* persister infection in mouse-thigh model of a chronic infection (Conlon et al. 2013). The model is designed to mimic the conditions in treatment-resistant recalcitrant infections in human.

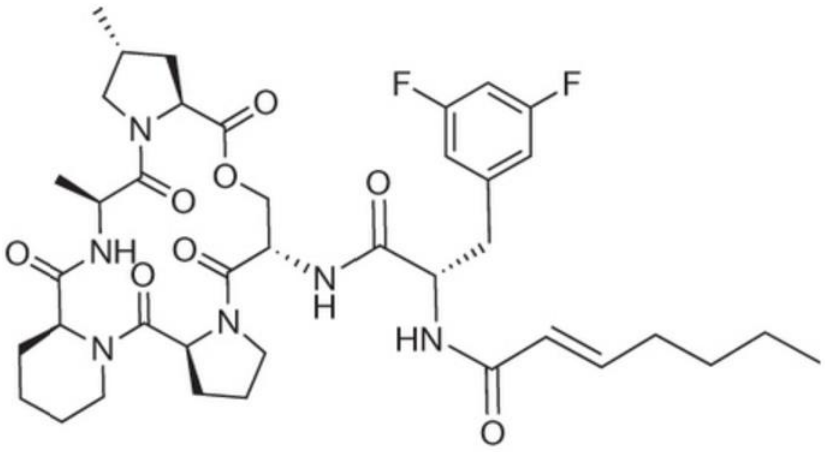


Figure 7. The structure of ADEP4 (Conlon et al. 2013).

2.5 Antimicrobial drug discovery

Despite the recent advancements described above, the arsenal of effective anti-persister strategies is limited. The problem is common to the field of antimicrobial drug discovery

in general: antimicrobial resistance in bacteria keeps advancing while the discovery of novel compounds has been stagnant for decades.

Most of the antimicrobial drugs in use today arise in some from mid-20th century, a period frequently referred to as the golden era of antibiotics (Lewis 2012). Numerous novel compounds with diverse mechanisms of action were discovered over a relatively short time-span. Since then, the discovery work seems to mainly have been about refining the already existing classes of antimicrobials by making most of them via chemical modifications.

Most of the success of the golden age can be accounted to Selman Waksman, who screened the abundant and diverse soil bacteria in search of the secondary metabolites with which the bacteria battle each other (Lewis 2012). Different species of *Streptomyces* bacteria proved to be a particularly fruitful source of antibiotic compounds, many of which are still in use as such or as (semi)synthetic derivatives with streptomycin being the archetypical example. Still, new antimicrobial properties are being discovered in the already isolated secondary metabolites of *Streptomyces* and other soil bacteria.

The stagnation of antimicrobial drug discovery since the mid-nineteenth century, also known as the discovery void, arises from a couple of central challenges. One basic problem has been that of the non-culturable bacteria. It is estimated that only 1 % of all bacterial species can readily be cultured in laboratory conditions (Rappé and Giovannoni 2003). It can be expected that many new classes of natural antibiotics become available for studying as the spectrum of explorable microbiomes extends. Another key challenge concerns the limitations of the discovery platforms currently in use (Lewis 2012). Different screening assays used in high-throughput screening campaigns tend to ignore e.g. compounds that do not penetrate the bacterial cell membranes and inactive pro-drugs with reactive, enzyme activated metabolites. The latter type of an antimicrobial drug is especially useful with persister bacteria, in which the limited metabolic activity is still sufficient to convert a harmless pro-drug into a reactive compound that non-specifically and permanently attacks a multitude of targets such as DNA within the bacterium.

Fortunately, a recent breakthrough in cultivation techniques has brought major advancement to the screening of the previously non-culturable bacteria. Nichols et al.

(2010) developed a platform that allows the initial cultivation of soil-isolated bacteria to go through at their collection site to support further culturing in laboratory environment. Bacteria collected from an environmental site were planted in a microchamber array chip and sealed between semi-permeable membranes. The chip, coded as isolation chip or iChip, was then brought back to the original environment to cultivate the bacteria in the presence of their required growth factors to form initial colonies that were later studied in a laboratory. Indeed, using iChip, a new species temporarily named *Elefteria terrae* was discovered, and along it a novel depsipeptide antibiotic teixobactin produced by the newfound species (Figure 8) (Ling et al. 2015). Teixobactin proved to be effective against several multi-resistant strains of Gram-positive (but not Gram-negative) bacteria and – more importantly – no development of resistance was observed against the multi-targeted antibiotic with a unique pharmacodynamical profile.

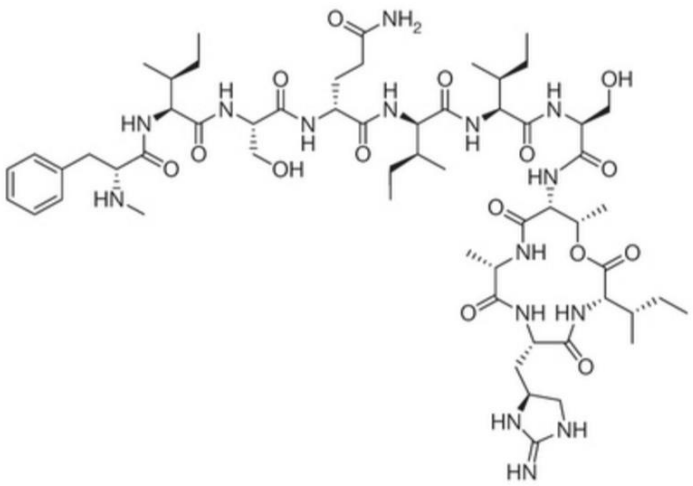


Figure 8. The structure of the depsipeptide antibiotic teixobactin (Ling et al. 2015).

Natural products are a fundamentally attractive source for new antimicrobials (Newman and Cragg 2016). Over time, different organisms have developed intricate methods of self-defence. This development is particularly rapid and uninhibited in microbes that need to constantly evolve new ways to occupy their territory midst countless competing species. Moreover, as biofilm growth is the predominant microbial lifestyle encountered in the nature, antibiotics of natural origin have potentially evolved to be effective against

biofilms of the competitor species (Donné and Dewilde 2015). Additionally, many secondary metabolites of plants have properties that are interesting in the viewpoint of antimicrobial drug discovery (Bazargani and Rohloff 2016).

No means of combinatorial chemistry can match the sometimes incomprehensible complexity of the various compounds created in such conditions, which makes the investigation of natural products still a viable option for drug discovery in parallel to the high-throughput screening of billion-molecule house libraries of compounds of synthetic origin.

3 AIMS OF THE STUDY

The significance of biofilms and persisters in antimicrobial drug discovery is not yet widely recognized, and therefore very few platforms exist for such research. The aim of the first part of the experimental work was to establish a stable persister model to be used in the following experiments. The model development was supported with thorough validation. Assay methods for persister viability were additionally explored along with the validation effort to enable miniaturization and automation for the subsequent screening.

The second part of the study consisted of a small-scale pilot screening with the aim to discover novel anti-persister agents. Diverse natural product -derived compounds were screened for activity against the newly-established persister model.

For the third and final part of the study a proof-of-concept experiment was designed and carried out to demonstrate the effect of anti-persister compounds on biofilm persisters. The aim of the concluding experiment was to link the established anti-persister activity to the ability to prevent regrowth from a mature biofilm – an endpoint property desirable for a clinically successful anti-persister drug. The study aims are summarized in Figure 9.

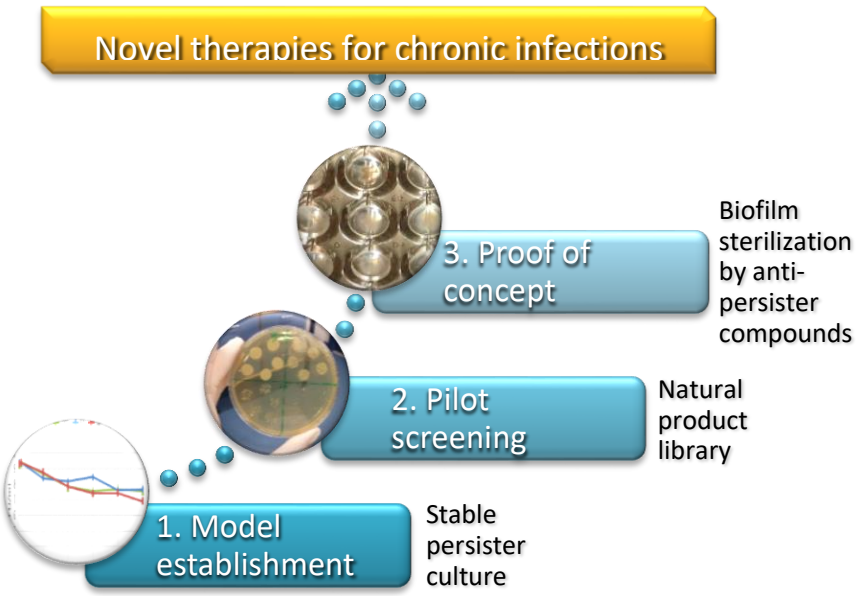


Figure 9. Aims of the study.

4 MATERIALS AND METHODS

4.1 Materials and bacterial strain

4.1.1 Materials and reagents

Penicillin G sodium salt, vancomycin hydrochloride hydrate, glycerol, resazurin sodium salt, Müeller-Hinton broth (MHB) and tryptic soy broth (TSB) were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). Tryptic soy agar (TSA) was also purchased from Sigma-Aldrich and additionally from Lab M (Lancashire, UK). Phosphate buffered saline (PBS) was purchased from Lonza (Basel, Switzerland). The SCREEN-WELL® Natural Product library of 502 compounds was purchased from Enzo Life Sciences (Farmingdale, New York, USA). Additional MMC was purchased from Cayman Chemical Company (Ann Arbor, Michigan, USA) to supplement the SCREEN-WELL® library. Dehydroabietic acid was obtained from Wako Pure Chemical Industries (Osaka, Japan). Bovine serum albumin (BSA) was purchased from Sigma-Aldrch and additionally from Amresco (Solon, Ohio, USA). Colorimetric BrdU ELISA cell proliferation assay kit was purchased from Roche Diagnostics (Indianapolis, Indiana, USA). Nunclon™ Delta Surface polystyrene flat-bottom 96- microwell plates were purchased from Thermo Fisher Scientific (Nunc; Roskilde, Denmark). Absorbance and fluorescence measurements were carried out with Thermo Fisher Varioskan LUX plate reader (Vantaa, Finland).

4.1.2 Bacterial strain

Staphylococcus aureus strain ATCC 25923 was obtained from the HAMBI collection (University of Helsinki, Faculty of Agriculture and Forestry, Division of Microbiology and Biotechnology). The bacteria were stored in -80 °C as cryo-stocks suspended in a mixture of TSB with 20 % glycerol.

4.2 Methods

4.2.1 Cultivating the bacteria

The bacteria were grown in plastic centrifuge tubes of 15 or 50 ml volume depending on the desired final suspension volume. TSB medium was inoculated with a pipetted aliquot

of the -80 °C glycerol stock in a 3:1000 ratio and incubated with 220 rpm aeration in 37 °C. The cultures were let grow for a minimum of 18 hours to achieve static conditions. For viable count determination the bacteria were plated on TSA plates.

4.2.2 Viable count determination

The sampled bacteria were centrifuged for one minute in 10 000 rpm or roughly at 8000 g and resuspended in sterile PBS after decanting and aspirating out the supernatant. The centrifugation pellets were not washed to avoid losing cells into the washing liquid. The resuspended bacteria were made into 10-fold serial dilutions in TSB and the dilutions were plated in 5 drops of 10 µl each on TSA plates. The plates were let dry with the lids off after which they were incubated upside down overnight in 37 °C for colony counting.

Colonies were counted from each of the 5 drops of the countable dilutions. Viable count from a single drop was calculated using the formula below:

Viable count = (Colony count × Dilution factor) / Drop volume.

A mean CFU/ml value from the 5 drops was calculated for each dilution. The number of colony forming units per 1 ml was calculated from two adjacent dilutions with a countable amount of colonies. A mean value of the dilutions was used as the final concentration. Viable counts are later presented as common logarithmic units ($\log_{10}$). The calculation method gives rise to a detection limit of 2 units. An example of a plating result is shown below in Figure 10.

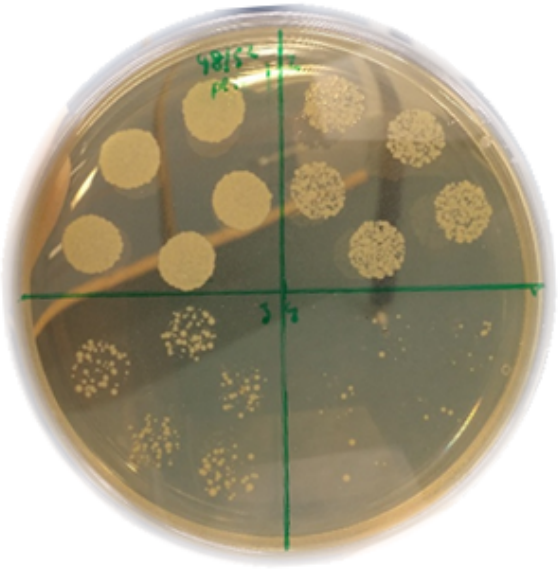


Figure 10. An overnight-incubated agar plate cultivated with 5 drops of 10 µl from subsequent 10-fold dilutions.

4.2.3 Antibiotic susceptibility testing

Minimal inhibitory concentration values (MIC) were established by an antibiotic dilution test. MHB stock solutions of each antibiotic with a final well concentration of 1.024 mg/ml were made into 2-fold dilution series on a microplate ($1.024 – 4.88 \times 10^{-7}$ mg/ml). Overnight-grown pre-cultures of the bacteria were diluted 1000X and grown for 4h (at 37 °C and 220 rpm) to achieve an exponentially growing culture. After the 4-hour incubation the diluted culture suspension was further diluted 100-fold to achieve a concentration of 10^6 CFU/ml and transferred into the microplate with the antibiotics to a total volume of 200 µl per well. The plates were incubated for 24 hours in 37 °C with aeration (200 rpm). After the incubation the plates were first inspected visually to find the wells corresponding to MIC concentrations. The result was then confirmed by an optical density measurement, which was repeated after the planktonic bacteria had been transferred into a clean microplate. Absorbance from two replicate wells of each antibiotic concentration was measured at 595 nm. Subsequently a resazurin staining was carried out to define metabolic activity and establish minimum bactericidal concentrations (MBC).

An inhibition percentage was calculated using the formula below:

$$Inh\text{-}\% = 100 \times (Signal_{control,\ mean} - Signal_{sample}) / (Signal_{control,\ mean} - Signal_{medium,\ mean}).$$

Percentages of over 90 % were considered inhibitory. Similarly, a minimum bactericidal concentration was established for each antibiotic by calculating the inhibition percentages from the resazurin assay results.

4.2.4 Validation of the persister model

The persister model was validated by susceptibility testing using 10 × MIC concentrations of penicillin G and vancomycin. Stock solutions of the antibiotics were prepared in MHB. The antibiotics were added into 18-hour stationary phase culture tubes, and the cultures were sampled for viability counting at 0, 1, 3, 24, 48, 72, 96 and 120 hours of exposure as described above.

A growth curve was established from the viability results. Logarithmic reduction of growth (LogR) values were additionally calculated for each antibiotic using the formula

$$LogR = Log_{10}\ (CFU/ml_{control}) - Log_{10}\ (CFU/ml_{treated})$$

4.2.5 Killing with mitomycin C

Killing curves were established for MMC concentrations of 20 µM and 100 µM. MMC DMSO stock was added into 10 ml stationary phase cultures grown in plastic tubes for 68 hours, while plain DMSO was added into a control tube. Incubation was carried out at 37 °C with 220 rpm aeration. All of the tubes were sampled for viable counts (as described above) at 0, 5, 24, 48 and 72 h time points and additional 1 and 3 h samples were withdrawn from the MMC-treated tubes. Killing kinetics were studied from the viable counts of the sampled points.

Additional comparative testing was carried out with an overnight-grown stationary phase culture. MMC DMSO stock was added into 2 ml aliquot to reach a 20 µM concentration

and incubated alongside a control in 37 °C with 220 rpm aeration. The tubes were sampled as previously after a 24-hour incubation.

Finally, the efficacy of MMC (at 100 µM) was compared to that of penicillin G (1 mg/ml i.e. 8000 × MIC) against stationary phase *S. aureus*. DMSO stocks of the compounds were added into 3 ml aliquots of a 5-day old stationary phase culture to reach the desired concentrations. Tubes were then incubated for 24 hours in 37 °C with 220 rpm aeration.

4.2.6 Metabolic activity induction

A stationary phase *S. aureus* culture grown for 18 hours was diluted 1:1000 with fresh TSB and incubated in 37 °C with 220 rpm aeration. Penicillin G was added at 10 × MIC at 0 or 3 hours of growth. Samples of 1 ml were withdrawn from the treated tubes hourly for 5 hours and at 0 and 4 h time points from an untreated control tube as described above.

4.2.7 Anti-persisters screening

A screening of 26 natural compounds was carried out utilizing the Enzo SCREEN-WELL® Natural Product library of natural products. 2 mg/ml DMSO stocks of the selected library compounds were pipetted into tubes with 3 ml (or, in the case of mithramycin A, echinomycin, chromomycin A3, coumermycin A1 and valinomycin, 2 ml) of *S. aureus* stationary phase cultures to achieve a final concentration of 100 µM of each compound. The cultures were pre-grown for at least two days prior to experimentation. DMSO was added to standardize the vehicle quantity, also in a negative control tube. 100 µM MMC was used as a positive control. The tubes were then further incubated for 24 hours, after which viable counts were determined as described earlier.

4.2.8 Biofilm regrowth assay

S. aureus ATCC 25923 pre-cultures grown for 3 days were diluted 1:1000 with fresh TSB medium and incubated in 37 °C with 220 rpm aeration for approximately 6 hours until an optical density of 0.3. The bacteria were then again diluted 1:100 and 200 µl of suspension per well was transferred into a 96-microplate. The plate was further incubated for 18 hours to establish mature biofilms. 50 × stock solutions of the test compounds penicillin G, DHA and MMC were prepared in DMSO to a final plate concentration of 400 µM. After

the incubation the planktonic solution was aspirated off and 4 µl of the test compounds were added with 196 µl of fresh medium. The biofilms were treated for 24 hours and washed once with PBS after discarding the planktonic solution. 200 µl per well of fresh TSB was again added and the plate was incubated for 4 hours to assess relapse from the biofilm. Three replicate wells from each treatment group were then vigorously scraped to disperse the biofilms into the medium. The scraped wells were subsequently made into 10^{-1} to 10^{-8} dilution series and plated in 10 µl drops on TSA to assess viability as described above. Another experiment was carried out with a 24-hour regrowth incubation time.

4.2.9 Measuring metabolic activity

4.2.9.1 Resazurin staining

Staining with the redox dye resazurin was carried out according to Skogman et al. (2012) to detect and measure metabolic activity in the studied bacteria. 10 µl of resazurin 1 mg/ml stock was added into each microplate well containing 200 µl of bacteria or medium control. The plate was then incubated with shaking (250 rpm) in darkness for 5 minutes to achieve a readily detectable concentration of the reduction product resorufin, which in most cases can be visually verified from the change in colour (from resazurin's blue to resorufin's pink). Fluorescence was measured with Varioskan LUX at the excitation wavelength of 560 nm and emission wavelength of 590 nm. A photograph of a stained plate is presented below in Figure 11.

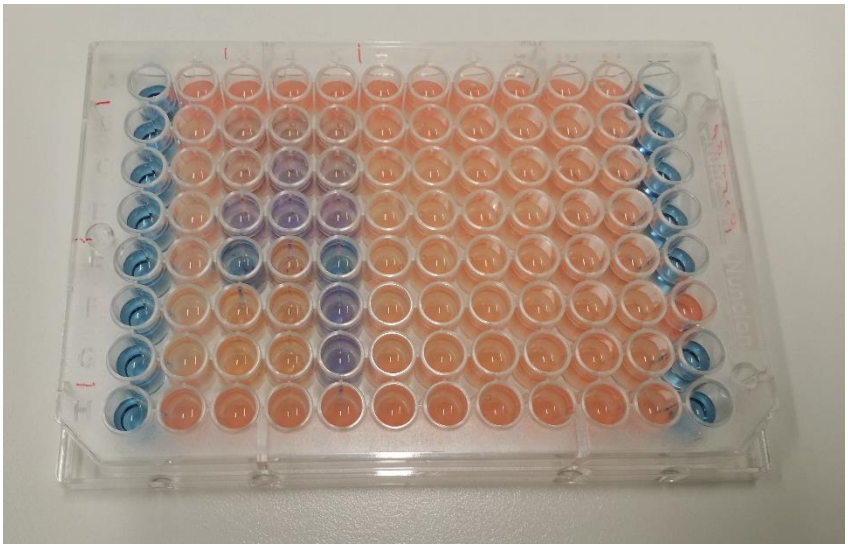


Figure 11. A 96-microwell plate with bacterial suspension and test compounds after having been stained with 10 µl/well resazurin 1 mg/ml solution and incubated in room temperature. The outermost blue wells (columns 1 and 12) represent the medium controls, wherein very little reductive colour change is observed with the exception of one contaminated well. Bacterial suspension was added to other wells that readily turned pink apart from those treated with bactericidal concentrations of the test compounds (columns 3-5).

4.2.9.2 BrdU ELISA

The assay was carried out following the manufacturer's instructions. To obtain a stationary phase culture, 10 ml of TSB was inoculated with 30 µl of *S. aureus* ATCC 25923 glycerol stock and incubated for 3 days in 37 °C with 220 rpm aeration. To achieve an exponential phase culture 3 ml of TSB was inoculated with 10 µl of glycerol stock and incubated for 18 hours. This pre-culture was diluted 1:100 with TSB and further incubated for 2,5 hours until an optical density of ca. 0.1.

The test cultures were incubated with BrdU labeling reagent for 2 hours (37 °C) in three replicates of 100 µl. For a blank, 100 µl of growth medium with BrdU labeling reagent was used. To obtain a background value, 100 µl of bacterial suspension without the BrdU labelling reagent was incubated with the samples. Blank and background samples were made in 3 replicates as well.

The cells were then centrifuged (300 g, 10 minutes) and the supernatant discarded. The bacteria were dried in 60 °C for 1 hour. DNA was fixed and denatured using a supplied

ethanol-based reagent. An optional blocking treatment was then carried out by adding 200 µl of 1 or 5 % BSA dissolved in sterile PBS in the wells followed by a 30-minute incubation before continuing to antibody treatment.

An anti-BrdU-peroxidase antibody conjugate was added and let bind into the denaturated DNA. After repeated washing the bound antibody was detected by TMD, a peroxidase substrate that gains a readily detectable blue colour upon donating hydrogen atoms for the peroxide reduction. The colorimetric measurement was carried out at 370 nm. A mean absorbance was calculated for the three replicates of each sample type with standard deviations. Blank values were subtracted from the background and sample measurements, and the results were compared. A photograph of a positive result is presented below (Figure 12).

Figure 12. A close-up photograph from a 96-microtiter well plate after carrying out the BrdU ELISA protocol in whole. The strong colouration is produced by metabolically active bacteria from an exponential phase culture.

5 RESULTS AND DISCUSSION

No standard tools or techniques exist for anti-persister drug discovery, and therefore the experimental part of this thesis starts from developing and validating a persister cell model. Results relevant for model development are presented and discussed first. The establishment of a stable persister model is followed by the experimentation with persister viability assays, one of which is selected for the subsequent pilot screening. The screening results are then presented and the hit compounds discussed in depth. The experiments are concluded by the proof-of-concept biofilm assay. Finally, recent findings and future prospects are discussed.

5.1 Establishment of a stable persister cell model using stationary phase *Staphylococcus aureus* cultures

5.1.1 Available persister isolation techniques

There are only two general ways to establish a persister culture documented in the literature. Neither is typically associated with drug discovery, as instead the methods are mostly utilized in the research of persister physiology. The first one is a method utilizing fluorescence-assisted cell sorting (FACS) to isolate dormant cells according to their phenotype that was described by Shah et al. (2006). Bacteria are first genetically modified to produce unstable green fluorescent protein (GFP) alongside a ribosomal promoter, so that the level of GFP produced correlates to transcriptional activity. The cells are then run through a specialized flow cytometer equipment and sorted by the amount of emitted green fluorescence. The scientists demonstrated that the cells with dimmer fluorescence had a unique expression profile associated with the persister state and were highly tolerant to test antibiotics. Another, significantly more basic way to grow a persister-rich culture is to cultivate bacteria until they reach stationary phase (Figure 1). Persisters can then be isolated from the metabolically active bacteria by simply killing off the susceptible population, which is usually achieved by treating the culture with antimicrobials. Although prone to variation, this method has been successfully implemented in persister studies (Keren et al. 2004b; Lechner et al. 2012).

The FACS method made it possible for the first time to accurately isolate persister cells without the interfering use of antibiotics (Shah et al. 2006). However, it is relatively laborious and requires both specialized equipment and a modified bacterial strain. The stationary phase culture method can be easily adopted to any basic laboratory environment without mentionable expenditures. The agent used to eliminate the replicating bacteria may, however, pose a challenge for further experiments. Using an antimicrobial drug can significantly affect the remaining bacteria by activating various stress responses and alter the number of persisters (especially so with the SOS-response triggering fluoroquinolones), as is pointed out by Cañas-Duarte et al. (2014), who presented an alternative take on the method based on alkaline and enzymatic cell lysis of bacteria.

5.1.2 Untreated stationary phase model

In antimicrobial drug discovery this challenge may not be as pronounced yet. We reasoned that it is not always necessary to wipe out the non-persisters before the treatment experiments as long as antimicrobial resistance is ruled out as the causative agent in survival by prior experimentation. Thus, the model may be simplified by removing the pre-treatment step. If the relative size of the susceptible population within a persister model culture is established, the anti-persister effect of test compounds can be distinguished from general antibacterial activity without the use of interfering antimicrobial agents. Based on its stability and convenience, the untreated, simplified stationary phase model was chosen for further experimentation. A summary of the persister models discussed above is presented here as a comparison (Table 1).

Table 1. A comparison of the methods of persister obtainment (Shah et al. 2006; Lechner et al. 2012; Cañas-Duarte et al. 2014).

	FACS	**Treated stationary phase model**	**Untreated stationary phase model**
Strain pre-requirements	Yes (reporter gene transfection)	No	No
Equipment needed	Flow cytometer  *Wikimedia Commons, released for public domain*	Basic labware	Basic labware
Workflow	1. Cultivation 2. Flow cytometry cell sorting 3. Experimentation	1. Cultivation 2. Pre-treatment 3. (Purification) 4. Experimentation	1. Cultivation 2. Experimentation
Final isolate composition	Pure isolate; cells of only the desired phenotype included	Stationary phase culture with trace pre-treatment agents; susceptible cells excluded	Stationary phase culture consisting largely of persisters; susceptible cells included
Final isolate properties	Stable isolate; expression profile unaltered, growth stage controllable	Initial cell viability and expression profiles possibly affected by the preceding treatment; potentially labile culture	Stable culture; distinct phenotypes of stationary phase bacteria and persisters present

5.1.3 Response of the persister cell model to conventional antibiotics

The studies with the persister model began with the testing of antibiotic susceptibility. Minimum inhibitory concentrations (MIC) for vancomycin and penicillin G were established in a susceptible exponential phase culture to define suitable test concentrations for the persister model validation. MICs for vancomycin and penicillin G were determined to be 0.016 mg/ml and 0.125 µg/ml respectively. An 18-hour old stationary culture was exposed to 10 × MIC concentrations of the antibiotics to assess antibiotic susceptibility. The results of the 5-day exposure are presented below in Figure 13.

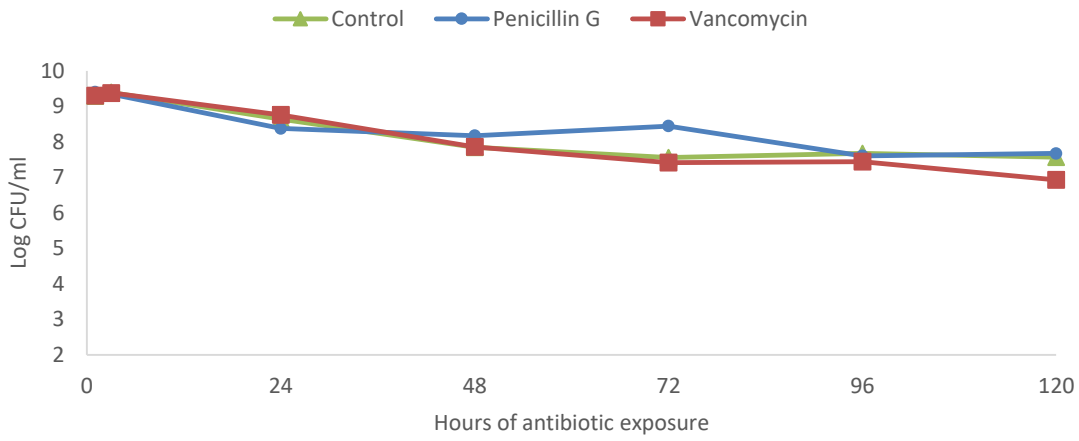


Figure 13. The antibiotic susceptibility of stationary phase *Staphylococcus aureus* culture treated with 10 × MIC concentrations of penicillin (circles) or vancomycin (squares). The untreated control culture is marked with triangles. Only average results are presented for simplicity, although the results were confirmed in parallel experiments.

Although the biphasic killing pattern is not clearly distinguishable, our results are quite in line with those reported previously in persister experiments (Conlon et al. 2013). At the end of the experiment the concentration of vancomycin-treated bacteria is slightly (less than 1 logarithmic unit) lower than the control culture, whereas the culture treated with penicillin G is indistinguishable from the control. The stationary *Staphylococcus aureus* culture seems to be tolerant to conventional antimicrobials.

5.1.4 Reverting tolerance by inducing metabolic activity

The ability of the persistent cultures to resuscitate was then confirmed by dilution experiments. Additionally, the change in antimicrobial susceptibility upon resuscitation was observed to confirm the transient nature of the multi-drug tolerance in our persister model. First, the resuscitation was observed in differently-aged stationary phase cultures (Figure 14).

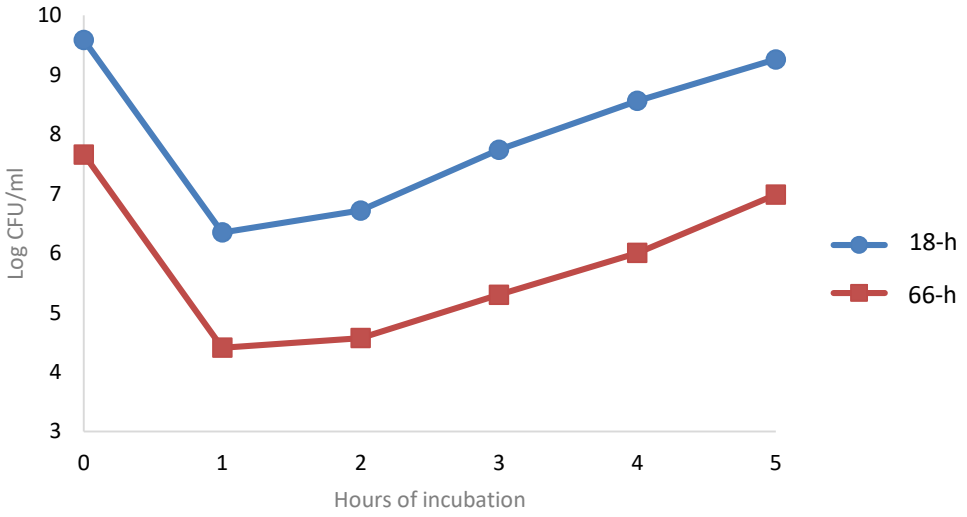


Figure 14. The regrowth of *Staphylococcus aureus* after a 1000X dilution made from an 18-hour old culture (circles) or a 66-hour old culture (squares).

The culture age difference does not impact the regrowth profile or the slope of the growth curve. However, since the same inoculum volume was used for both cultures, the starting concentrations differ leading to lower viable counts with the older culture throughout the observed time points.

A determining factor of the persister phenotype is its multi-drug tolerance without hereditary mechanisms of antimicrobial resistance (Keren et al. 2004b). Therefore, the bacteria should become susceptible upon resuscitation. This was confirmed by treating a diluted culture with penicillin G (Figure 15).

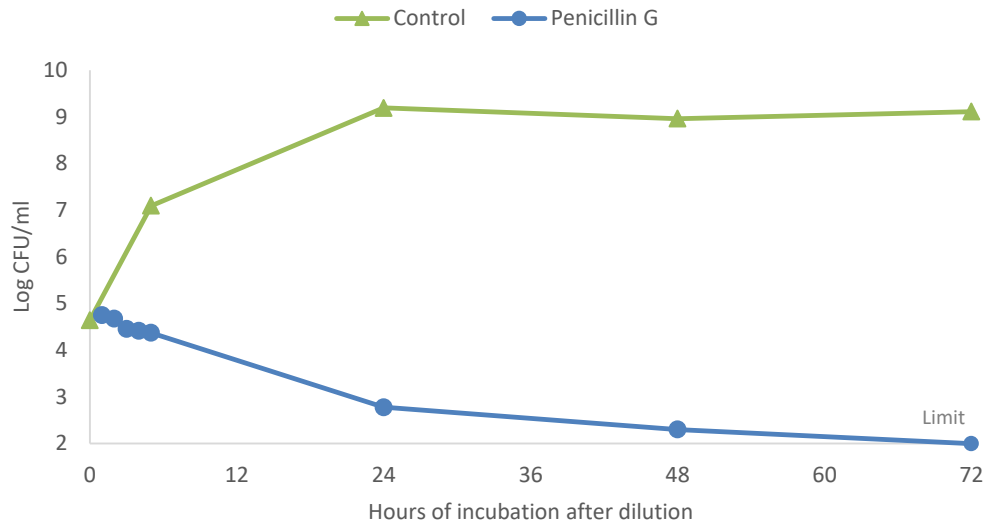


Figure 15. The regrowth of *Staphylococcus aureus* diluted 1000X with 10 × MIC penicillin G added at dilution (circles). An untreated control was similarly diluted (triangles).

When administered at the time of the dilution, penicillin G prevents regrowth and eradicates the bacteria to the detection limit within 72 hours. Still, after only one such passage, tolerant bacteria are likely to exist within the diluted culture as carryovers from the inoculum and as newly induced persisters (Balaban et al. 2004).

When the bacteria are allowed to reach exponential state before starting the antibiotic treatment, the killing effect is not as pronounced (Figure 16). Yet, a drop in viability is immediately observed, and after 48 hours of exposure the bacterial counts are reduced significantly more than what is seen in stationary phase cultures. It can be concluded that dilution in fresh medium facilitates resuscitation in the stationary phase persister cultures and restores antimicrobial susceptibility, as expected.

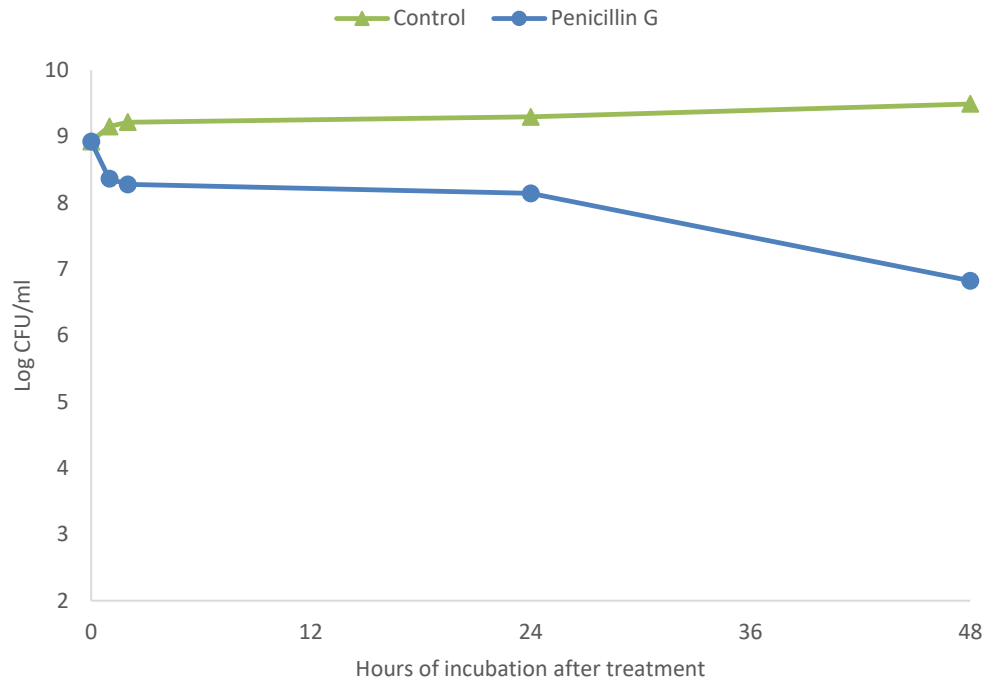


Figure 16. The effect of penicillin G on *Staphylococcus aureus* culture regrown for 4 hours after a 1000X dilution (circles). An untreated control is marked with triangles.

5.1.5 Validation of the stable persister cell model with mitomycin C

Mitomycin C (MMC) is a compound with known activity against persister bacteria owning to its ability to damage bacterial DNA (Kwan et al. 2015). The persistent nature of the stationary phase model culture was validated by comparing the efficacy of MMC (33 µg/ml; 100 µM) to that of a high concentration (1 mg/ml; 2800 µM) of penicillin G, a conventional antibiotic (Figure 17).

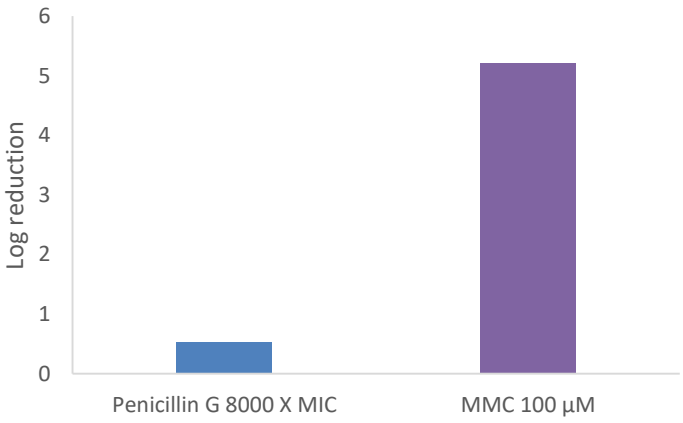


Figure 17. The effect of penicillin G and MMC on stationary phase *Staphylococcus aureus*.

The anti-persister activity of mitomycin C is preserved even in a 5-day old stationary phase culture, whereas penicillin G has no effect despite the extremely high concentration tested. The results give confirmation to those published earlier by Kwan et al. (2015). The stationary culture is highly tolerant against a conventional antibiotic whereas the anti-persister compound MMC retains its significant activity, reinforcing the notion that the stationary phase *S. aureus* culture is a valid persister model for further experiments.

Next, to obtain a time-kill curve, the persistent stationary phase cultures were challenged to two MMC concentrations (Figure 18).

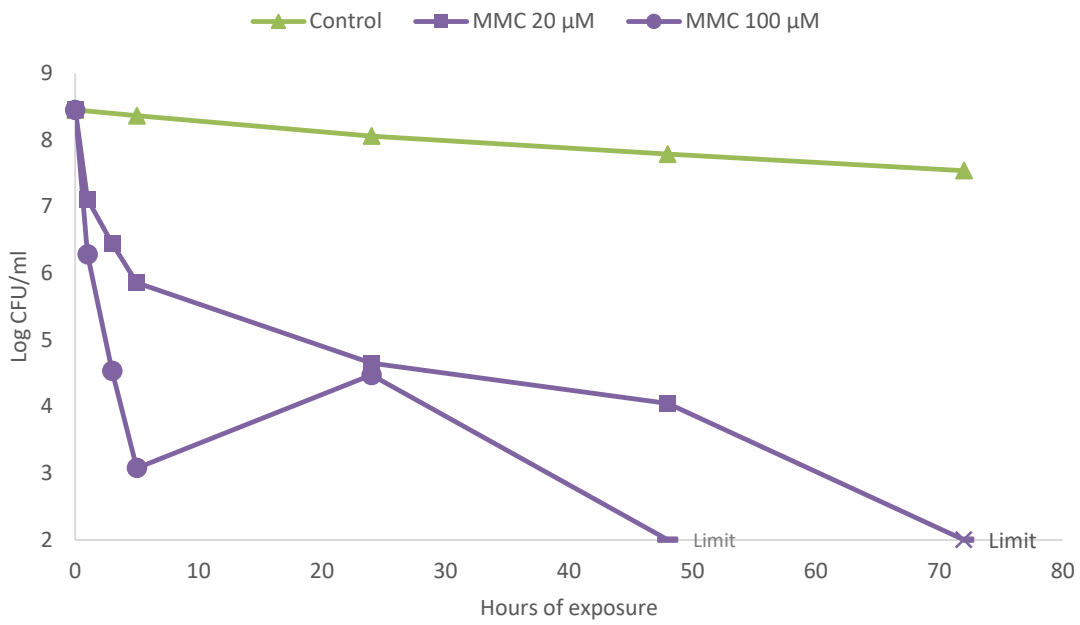


Figure 18. A time-kill curve of MMC 20 µM (squares) and 100 µM (circles). An untreated control is presented alongside (triangles).

Both of the tested MMC concentrations eradicated the bacteria to the detection limit within the 3-day experiment. A 100 µM concentration resulted in a more rapid eradication especially during the first hours, and the detection limit was reached earlier. Based on the efficacy of MMC in these initial experiments it was selected as the positive control against which an anti-persister activity pilot screening of natural products was carried out.

As observed in the regrowth experiments, the age of a stationary phase culture can affect its behaviour. Practical challenges of the work gave rise to the need for using differently aged static cultures. To further confirm the validity of the method, cultures grown overnight (18 hours) and for 3 days were challenged with the same concentration of MMC (Figure 19).

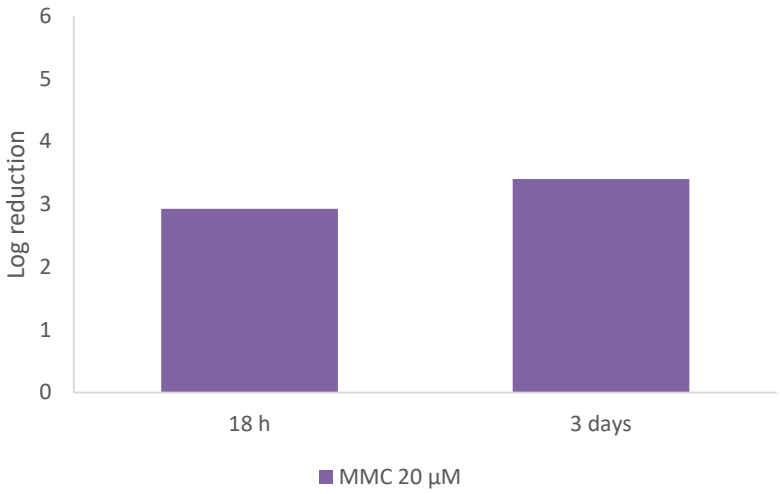


Figure 19. MMC activity is conserved in differently aged cultures. Cultures were grown for 18 hours or 3 days prior to the 24-hour exposure.

The activity of MMC is similar in the differently aged cultures, which suggests that the persister model is stable after the initial incubation. Based on the initial antibiotic susceptibility results (Figure 13) and the validation made with MMC it can be concluded that a stationary phase *S. aureus* culture is a suitable model for anti-persister pilot screening. A time of 2 days was set as the minimum cultivation time prior to experimentation to ensure stability.

5.2 Assay miniaturization

One of the aims of the study was to establish a miniaturized assay for the screening of potential anti-persister compounds. An ideal method for such a screening should be rapid (to enable high throughput and to minimize state transitions in the bacteria studied), specific (to only return hits with actual persister-killing capabilities as opposed to e.g. agents with growth-hindering properties) and sensitive (to report a decrease in the already low concentration of persisters). Furthermore, it should support microwell-plate format to allow miniaturization.

5.2.1 Measuring metabolic activity

In persister experiments the measuring of metabolic activity is useful for two different purposes. Firstly, metabolic activity is a useful determinant of total viability (Sandberg et al. 2009). A reduction in the overall cellular metabolism within a culture may indicate a drop in the viable count. It is noteworthy, that this is not observable by standard optical density measurement: the cellular debris from dead bacteria will stay in the suspension, so no meaningful difference in absorbance values can be observed even at dramatic fluctuations in bacterial viability.

Secondly, as metabolic activity is one key determinant of persistent lifestyle, it is beneficial to characterize its level to become aware of the possible changes in the bacterial state. Before experimentation it is reasonable to verify that the working culture is indeed in a persister-like state of dormancy. The metabolic response for the experimental treatment is another subject of interest: will the bacteria be resuscitated as the population becomes cut down? Thus, the persister viewpoint actually introduces an unfortunate ambiguity for metabolic activity monitoring: an active compound may spur measurable metabolism within the dormant culture, or further reduce it from the already low basal level, which would make detection a serious methodological challenge.

5.2.1.1 Resazurin staining

Resazurin, a blue redox dye that gains measurable fluorescence only after being converted by bacterial enzymes to a pink reduction product resorufin, can be used in the determination of metabolically active bacteria in both planktonic and biofilm lifestyles (Guerin et al. 2001; Sandberg et al. 2009). Resazurin staining assay is relatively easy, effortless and quick to carry out, and the dye itself is safe to handle. Furthermore, resazurin does not severely interfere with the bacteria metabolizing it making the assay non-destructive.

Here, it was attempted to develop a resazurin-based persister viability assay to establish a screening method. Resazurin staining was carried out alongside most experiments, including the antibiotic susceptibility testing of stationary phase cultures (Figure 20).

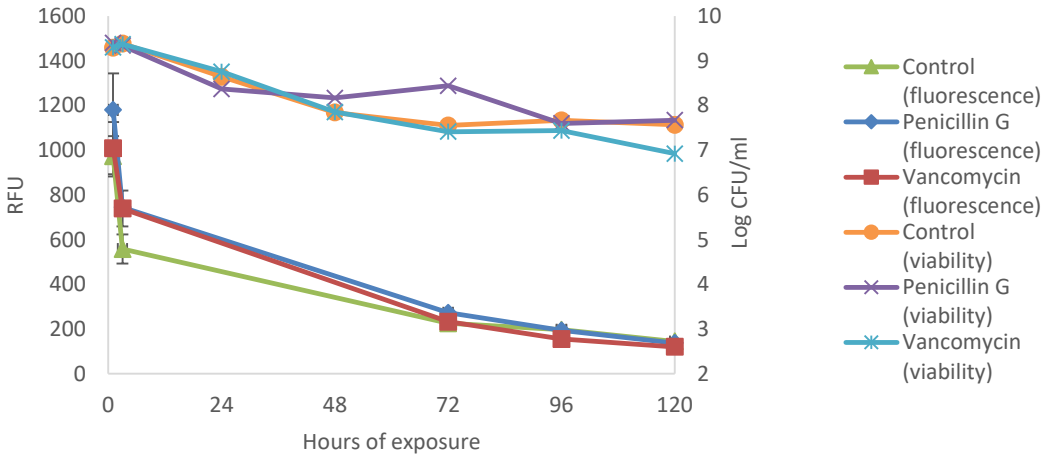


Figure 20. The development of metabolic activity of stationary phase *Staphylococcus aureus* cultures as determined by resazurin staining, presented with the respective viable counts. The cultures were treated with 10 × MIC concentrations of penicillin G and vancomycin. The error bars in the fluorescence measurements represent the standard deviation of three replicate readings.

The measured fluorescence decreases over the 5-day experiment in all of the cultures including the untreated control. No meaningful difference can be seen between the different treatments, as is expected knowing that the bacterial counts were also very similar. The decline in the fluorescence is steep within the first hours. As the test cultures were only let saturate overnight (compared to the 2-3-day preceding incubations in the latter experiments), the metabolic activity is still diminishing in the control culture at the beginning. The fluorescence values were plotted logarithmically against the defined total viable counts to study the correlation (Figure 21).

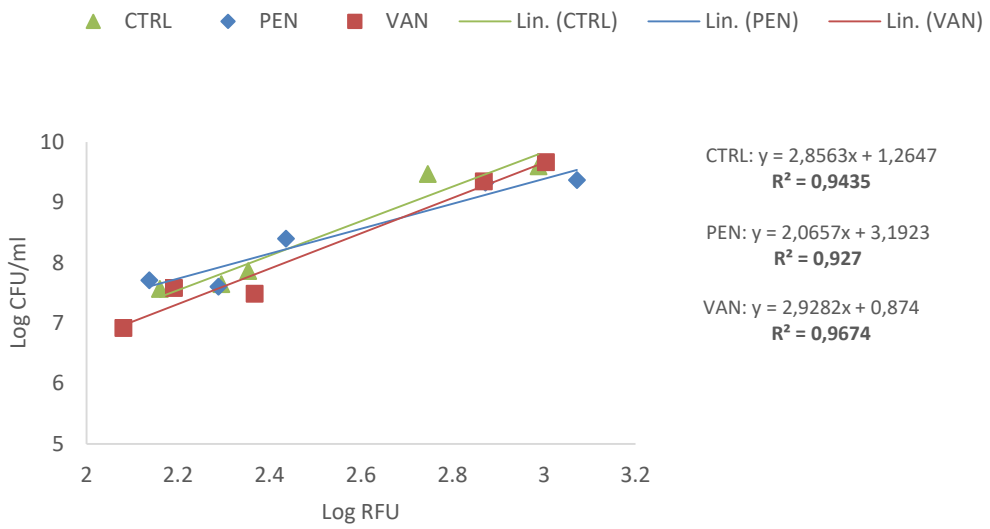


Figure 21. The logarithmic bacterial concentrations of stationary phase *Staphylococcus aureus* cultures treated with 10 × MIC concentrations of penicillin G (PEN; circles) and vancomycin (VAN; squares) presented as a function of log relative fluorescence. An untreated control (CTRL; triangles) is included. Regression lines (with the least squares fitting), line equations and the coefficients of determination (R^2 values) are presented for each series.

The coefficient of determination (R^2) is over 0.90 in all treatments indicating an adequate correlation between resazurin fluorescence and viable count. However, as earlier pointed out by Sandberg et al. (2009), this assay requires a relatively concentrated bacterial culture (and sufficient metabolic activity therein) to detect significant differences. This becomes evident when resazurin staining is carried out with the MMC-challenged cultures, where the viable count variation is more dramatic (Figure 22).

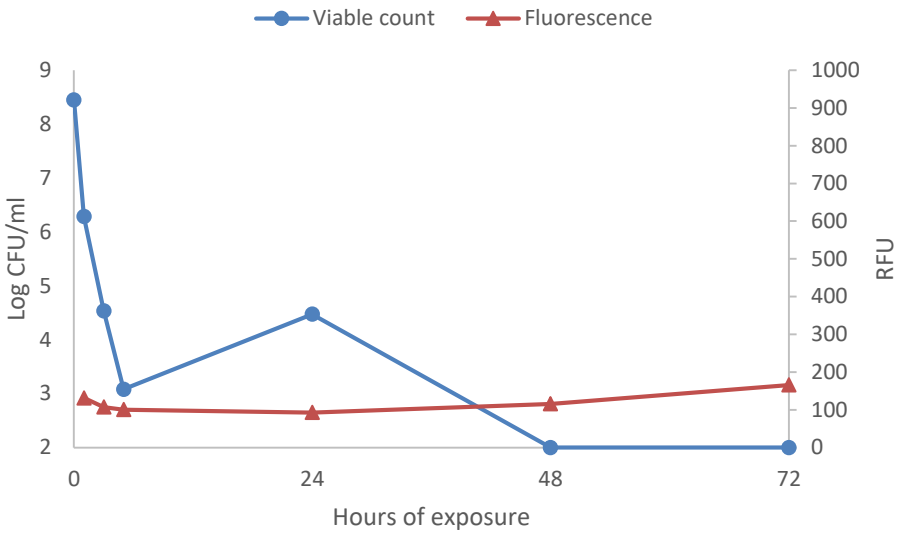


Figure 22. The development of resazurin fluorescence (triangles) in a stationary phase *Staphylococcus aureus* culture treated with 100 µM MMC as compared to the viable count established by plating (circles).

It is apparent that the initial metabolic activity is lower because of the longer saturation period pre-experimentation. The fluorescence intensity stays barely over the background level of ca. 100 relative units throughout the test, and no fluctuation can be perceived despite the harsh variations in the viable count.

The main challenge with resazurin staining is its relatively low sensitivity (Sandberg et al. 2009). Even though the method is linear when applied to concentrated cultures, its reliability may decline sharply with the decreasing number of bacteria. The intrinsically low metabolic activity of persister bacteria calls for linearity that extends to lower cell counts. However, resazurin has been successfully used in experiments with dormant mycobacteria, which makes it potentially usable for anti-persister activity assays at least with some species (Taneja and Tyagi 2007).

5.2.1.2 BrdU ELISA

Monitoring the incorporation of the thymidine analogue BrdU (5-bromo-2'-deoxyuridine) into newly synthetized DNA by enzyme-linked immunosorbent assay or ELISA is a method typically used for mammalian cell proliferation studies that was recently reported to have been successfully used in an antibacterial assay as well (Bao et al. 2015). In the

assay bacteria are incubated in the presence of BrdU to label the DNA synthetized over the incubation. The incorporated BrdU is then detected with its specific peroxidase-conjugated antibody that upon addition of a chromogenic substrate gives rise to a measurable colour change relative to the degree of proliferation within the bacteria.

As a highly sensitive, albeit a relatively labour-intensive assay BrdU ELISA could be a useful tool in the determination of changes in the metabolic state of non-replicative persister cells. With some modifications – for instance, by replacing the chromogenic enzyme conjugate with an existing fluorescent probe detection system – the BrdU assay could be made compatible with many microscopic and cytometric applications and even single-cell studies.

The BrdU ELISA assay was another method applied here, in order to explore alternative analytical solutions for persister research. It was hoped that the method would give information about the minute changes in persister metabolism and viability, thus serving as an alternative for the labour-intensive viable count plating. It was first confirmed that the assay could distinguish metabolically active, exponential phase culture from a non-growing stationary phase culture by testing the assay on two differently aged cultures with similar bacterial concentrations (log CFU/ml 8.24 and 7.50 for the stationary and exponential phase cultures, respectively) (Figure 23).

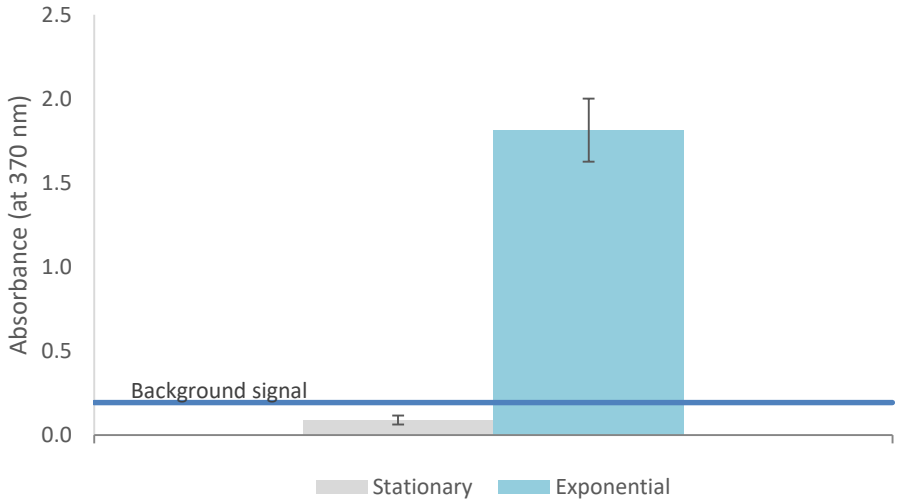


Figure 23. The BrdU ELISA absorbance measurements from a stationary phase culture (left) and exponential phase culture (right). The blank signals are subtracted. The horizontal line represents the background signal from stationary phase bacteria incubated without BrdU. The error bars depict the standard deviations of 3 (for exponential phase bacteria) or 2 (for stationary phase bacteria) replicate measurements.

As it is evident from Figure 23, the assay can readily identify a metabolically active culture. The colour development by the detection reaction was immediately visible upon adding the substrate into the exponential phase wells. The clear distinction gives further validation to the persister model itself, since the difference in replicative activity is confirmed. Instead, the stationary phase sample only produced a slight colour within the incubation. Moreover, the possible signal from the non-growing sample was masked by the relatively high level of unspecific binding demonstrated by the background control in which the bacteria were incubated without BrdU.

The probable cause for the pronounced background signal is the high number of cells within the mixture. The number of cells in the bacterial test suspensions is several magnitudes higher than that typically seen in a mammalian cell culture. With such high cell counts there is more area available for the unspecific (non-BrdU-dependent) binding of the antibody probe. Complicating things further, the cell counts cannot be manipulated prior to experimentation: working with dormant cultures brings the singular restriction that the bacteria cannot be diluted long before the testing, since it would cause them to resuscitate. This would lead to the induction of metabolic activity rendering the

measurement fundamentally meaningless. Since the basal activity level in persistent cultures is low by definition, the lack of sensitivity caused by the high noise is a major challenge to overcome before the assay can be used for persister detection.

On the hopes to reduce the background signal, several optimization steps were considered. For example, BSA was added (at concentrations of 1 and 5 %) before applying the detection antibody to block the sites of unspecific binding. As a result, all signals increased, but the background signal was still equal or higher compared to that of the stationary phase sample (results not presented). Another option considered for improving the signal-to-noise ratio in the BrdU assay was the purification of the bacterial DNA by salt precipitation before the ELISA assay. With the bacteria and cellular debris excluded from the measurement there would be considerably fewer sites available for the unspecific binding of the probe. This could dramatically enhance the sensitivity of the test. For this work, however, the option of adding a DNA extraction step to the already labour-intensive assay was rejected for being too costly time-wise.

At least without further adjustments, the BrdU proliferation assay is not suitable for the described kind of persister analysis. Still, the clear distinction between the different culture types is a valuable piece of information as such.

5.2.2 Viable plate count

The superior sensitivity of viable count plating over e.g. fluorescence-based bioassays is well recognized (Mariscal et al. 2009). With different dilutions the plating method can be adjusted to suit any bacterial concentration. Being based on colony growth, plating also makes an obvious differentiation between live and dead cells (Hazan et al. 2012). However, it is a markedly labour-intensive method, and the results take at least some 20 hours to become readable, thus making the method unsuitable for purposes that necessitate a quick reaction to the current bacterial concentration. An example of such an experiment is the above-described monitoring of MMC killing, where the correctly timed administration of a follow-up treatment upon resuscitation would require knowledge of the present cell counts.

An additional although unrelated concern with plating methods is that they may disregard another phenotype of dormancy, the viable but non-culturable bacteria (VBNC) (Li et al.

2014). VBNCs are viable – judging by their cellular integrity, metabolism and gene expression – but dormant bacteria that cannot be cultivated by routine plating methods. The described lifestyle bears much resemblance to the persister phenotype, but the two are considered distinct and coexisting based simply on their differing ability to grow upon plating without an additional lengthy process of resuscitation (Ayrapetyan et al. 2015). Because of this basic limitation the VBNC bacteria are difficult to observe and take into account, which may be problematic in persister studies, since the abundance of VBNCs can greatly surpass that of persisters (Orman and Brynildsen 2013). Still, persisters resuscitate and grow readily on standard agar, so the method is well suitable for the purpose of solely observing persister viability.

Summarizing the pros and cons of the tested methods it becomes evident that although viable count plating is not ideally suited for screening, it is the best method available to meet the requirements of this study. It robustly provides information of differently aged and varyingly active bacterial cultures with significantly fluctuating cell counts, which is key in anti-persister compound discovery.

5.3 Pilot screening for anti-persister activity using the stable persister model

As pointed out in the first part of the thesis, natural products are an abundant source for potential new antimicrobials (Newman and Cragg 2016). Compounds for the pilot screening were selected from an in-house library of purified natural products with elucidated structures. The diverse composition of the library includes compounds from different classes of natural or semisynthetic products. Alkaloids, coumarins, flavonoids, macrolides, terpenoids and peptolides are all represented in the pilot screening along with additional compounds outside these main classes. The persister model cultures were challenged with 100 µM concentrations of the selected library compounds.

5.3.1 Hit selection and the identified hits

The screening results are presented below in Figure 24. According to the validation results the hit limit was set to 2 logs of growth reduction to exclude the possible remaining susceptible subpopulation.

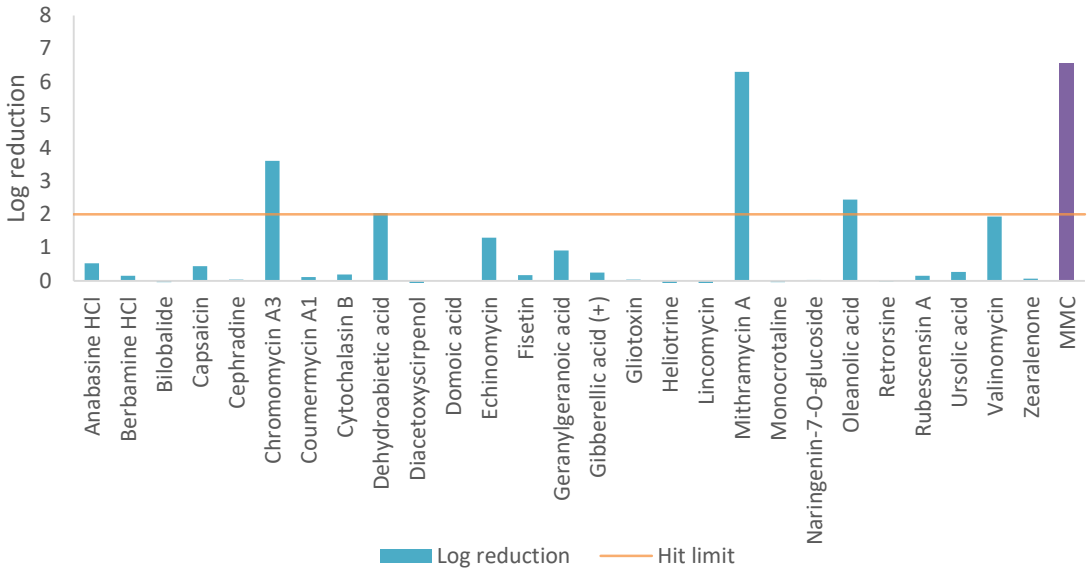
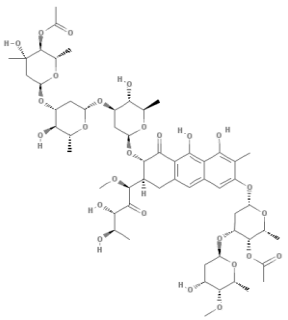
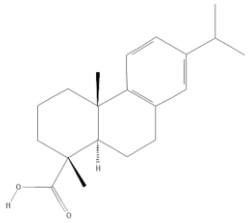
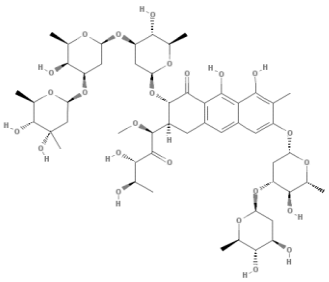
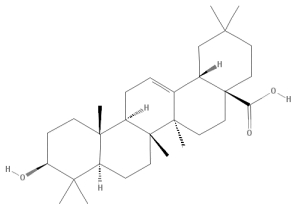


Figure 24. The pilot screening results are presented as logarithmic reductions of viability. The culture viability and the detection limit of 2 logs determine the maximum observable reduction. For the positive control MMC the highest observable log reduction value is presented.

A total of 4 out of the 27 tested compounds were able to reduce the viability of persister model cultures enough to be classified as hits (Table 2).

Table 2. Hit compounds. Log reductions are presented together with the mean reduction and standard deviation of all respective MMC controls. Structures (modified from PubChem) and weights (MW) are additionally listed for the screened natural products.

Compound	**Log reduction**	**Structure**	**MW (g/mol)**
Chromomycin A3	3.62		1183.25
Dehydroabietic acid	2.04		300.44
Mithramycin A	6.30		1085.15
Oleanolic acid	2.45		456.70
MMC	5.49 ± 0.96		

5.3.1.1 Aureolic acids

The pilot screening campaign included the antitumor antibiotics chromomycin A3 and mithramycin A from the aureolic acid class (Table 2) (O'Connor 2004). Chromomycin A3 and mithramycin A were originally isolated from *Streptomyces griseus* and *plicatus* respectively (Slavik and Carter 1975). Both compounds inhibit RNA synthesis by DNA intercalation (the insertion of the compound between DNA base pairs) leading to remarkable antibacterial (Gram-positive bacteria) and antitumor activity (O'Connor 2004). Of the two, only mithramycin A has been approved to limited clinical use in cancer treatment. To the best of our knowledge, no studies of the anti-persister properties of aureolic acids have been conducted prior to the work described in this thesis.

Both compounds demonstrate activity against the persister model cultures (Figure 24). Chromomycin A3 reduces the viable count of the treated bacteria by more than 3 logarithmic units indicating a significant efficacy. Mithramycin A is even more effective, readily eradicating the bacteria down to the detection limit. In fact, its efficacy surpasses that of the positive control MMC in its respective round of screening. These findings combined with the earlier work of others on DNA-targeting compounds supports a generalization that bacterial DNA is a prospective target for anti-persister antimicrobials (Kwan et al. 2015; Chowdhury et al. 2016b).

Obviously, any compound with unspecific activity on DNA is potentially very hazardous for the host as well, as can be derived from the anti-cancer treatment indication held by many such agents including the FDA-approved MMC and mithramycin A (Taylor et al. 2011; Kwan et al. 2015). Directing the effect more specifically towards bacterial cells would probably require developing a pro-drug that remains inactive until bacterial intake and subsequent activation. A valuable example of such a drug is metronidazole, which becomes destructive only after a nitroreductase-mediated activation by anaerobic bacteria living in reductive niches (Knox et al. 1983). Discovering this type of an exploit with a more widely present, bacteria-specific activator and coupling it with the like of an anti-persister compound discussed above could ultimately give rise to very successful new antimicrobials. MMC fulfils this requirement partly by being converted into its active form by enzymatic reduction, but the low specificity and high abundance of suitable enzymes also in human host takes out the advantage (Paz 2009).

5.3.1.2 Oleanolic acid

Oleanolic acid (Table 2) is a pentacyclic triterpenoid compound that is found, among other medicinal plants, in the extracts of *Salvia officinalis* or sage (Horiuchi et al. 2007). In our experiments oleanolic acid demonstrated significant activity against the persistent cultures (Figure 24). It is a compound with established activity against Gram-positive bacteria including drug-resistant strains of *S. aureus* and *Enterococcus faecalis* (Horiuchi et al. 2007; Kim et al. 2015). It has additionally been reported to be effective against the intracellular pathogen *M. tuberculosis* both *in vitro* and *in vivo* (Jimenez-Arellanes et al. 2013).

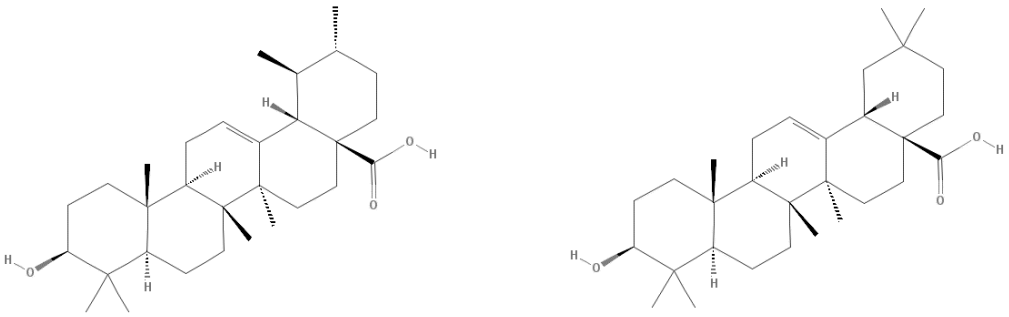
Over a 24-hour treatment oleanolic acid reduces the viability of the test culture more than 2 logarithmic units. The result is noteworthy, since the amount of reduction is higher than what was previously reported with conventional antibiotics. Moreover, based on previous experiments the proportion of susceptible bacteria within the culture is likely lower than the amount of bacteria killed, which indicates at least some level of anti-persister efficacy in addition to general antibacterial activity.

The antibacterial properties of oleanolic acid seem to stem from its ability to destroy bacterial cell membranes with peptidoglycan hydrolase enzymes being the proposed target (Kurek et al. 2010; Kim et al. 2015). However, the profound effect of oleanolic acid against the persister-rich and non-growing stationary phase culture as seen in our experiments is somewhat conflicting to this hypothesis, as antimicrobials targeting peptidoglycan production primarily affect replicating cells with more rapid peptidoglycan turnover.

The cytotoxicity of oleanolic acid has previously been assessed (Kim et al. 2015). It demonstrated relatively low toxicity against HEp-2 cell lines derived from human larynx. Moreover, due to its other medicinal properties outside the antimicrobial activity including anti-inflammatory, anti-hyperlipidaemic and hepatoprotective effects, it has been used in complementary medicine especially in Asia (Liu 1995). The tolerability of over 3-month term treatment further reinforces the safety of oleanolic acid for human use.

Motivated by the positive results obtained with oleanolic acid, ursolic acid, its closely related structural isomer was additionally screened (Figure 25a). Ursolic acid has been

reported to share similar antibacterial and anti-biofilm effects with oleanolic acid (Wolska et al. 2010; Zhou et al. 2013; Jimenez-Arellanes et al. 2013).



a **b**

Figure 25. The structure of ursolic acid (a) compared to its isomer oleanolic acid (b) (modified from PubChem).

In our experiments ursolic acid lacks the marked effect seen with oleanolic acid (Figure 24). The pharmacological profile of ursolic acid has previously been determined to be very similar to that of oleanolic acid's, which makes the established difference curious (Liu 1995; Wolska et al. 2010; Kurek et al. 2010; Zhou et al. 2013; Jimenez-Arellanes et al. 2013; Jesus et al. 2015). Their structures only differ by one methyl group (Figure 25b).

5.3.1.3 Dehydroabietic acid

The oleoresin-derived diterpenoid DHA (Table 2) was identified as another small-molecule hit. It is a constituent of coniferous resin with antibacterial and anti-biofilm properties (Fallarero et al. 2013). In the resin it acts as a defensive agent protecting the plant from pathogenic processes. In the pilot screening it demonstrates significant activity against the model culture reducing its viability for 2 log units (Figure 24).

The newly-established anti-persister properties complement the compound's impressive activity against *S. aureus* biofilm previously reported by Fallarero et al. (2013). The group additionally characterized the non-specific mammalian cytotoxicity of DHA in parallel to the biofilm inhibition testing. Tested against three different types of mammalian cells,

the concentrations sufficient to kill half of the cells over the 24 h treatment period range between approximately 100 and 180 µM depending on the cell line used. A biocompatibility index – a value that relates the concentration sufficient to reduce bacterial growth for three logarithmic units to the half-lethal concentration for mammalian cells – was defined. The biocompatibility index of DHA was found out to be around 1 with two out of the three cell lines, which nevertheless indicates a favourable balance between the antibacterial efficacy and non-specific toxicity.

To proportion the anti-persister activity of DHA to the formerly determined anti-biofilm properties, additional testing with more concentrated DHA was carried out (Table 3).

Table 3. The activity of 2 different DHA concentrations against the persister model culture.

DHA concentration	**Log reduction**
400 µM	4.30
100 µM	2.04

Since the 3-log-reducing DHA concentration in these persister experiments can be reasoned to fall between the tested 100 and 400 µM, the corresponding biocompatibility index can be expected to be similar to that reported earlier (Fallarero et al. 2013). In contrast to the highly toxic aureolic acids and MMC, DHA is plausible in terms of biocompatibility making it a potential antibacterial lead compound, paving the venues for further exploration.

5.3.2 Evaluation of the pilot screening campaign

The pilot screening was able to provide several interesting hits despite its limited scale. In addition to identifying the profound anti-persister properties of DNA-intercalating aureolic acids, the screening produced two distinct hits, DHA and oleanolic acid, with relatively low non-specific cytotoxicity. Differing mechanisms of effect are demonstrably not omitted, which is an additional benefit of the chosen method.

The stationary phase model was stable enough to enable timely flexible workflow, although further normalization of the pre-growth time could aid in improving the moderate variability that is seen with the positive controls. The persister model culture was easily adapted for the screening use, since no additional treatments were required.

The most important drawback of the pilot screening was the lack of miniaturization and automatization options. Plating and colony counting is labour-intensive manual work. This severely limits the throughput of the screening. Establishing a microplate-based bioassay with an endpoint that is measurable with an automated plate reader is essential for further screening campaigns.

5.4 Biofilm regrowth inhibition

To assess the main cause of the medical challenge associated with persistence, an assay was developed to test the ability of a range of natural products to reach and kill the persister bacteria residing within the confines of a biofilm. The matured 18-hour old biofilms were treated and the growth medium changed to see whether the films now ideally devoid of any living bacteria could seed new growth in the fresh broth. In a way the experiment simulates the conditions in a deep-seated infection, where antimicrobial treatments one after another wipe out the susceptible population just for the surviving persisters to recolonize the infection site every time.

For the proof-of-concept experiments MMC, penicillin G and the screening hit DHA were tested for the activity to prevent regrowth. The viable counts obtained by scraping the biofilms to loosen the bacteria within the incubated medium are presented below in Figure 26.

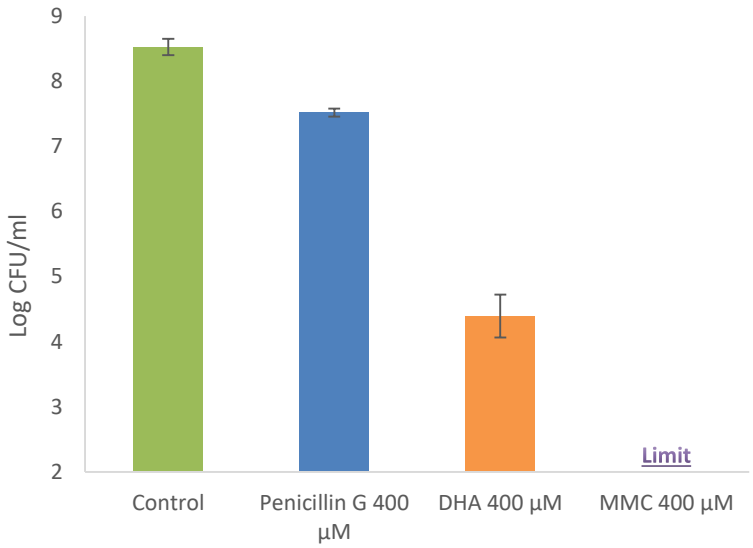


Figure 26. The 4-hour regrowth of *Staphylococcus aureus* from treated biofilms. From left to right: the untreated control, penicillin G 400 µM, DHA 400 µM and MMC 400 µM. The error bars represent the standard deviations of three wells plated in replicate.

As expected, penicillin G has very limited capability to prevent regrowth from the biofilms with only a 1-log reduction in the viable count compared to DMSO control. On the contrary, DHA is able to cause an impressive 4-log reduction that is in line with the previously presented results obtained from the stationary phase culture experiments and the earlier reports of the compound's anti-biofilm properties (Fallarero et al. 2013). Still, the observable amount of regrowth indicates that some persisters survived the treatment within the biofilm. Instead, the anti-persister model compound MMC seems to have eradicated persister bacteria and thus prevented regrowth completely. The expectedly opposing results with penicillin G and MMC serve as a validation for the assay.

The results obtained from this experiment suggest applicable validity, and as a proof-of-concept assay for overcoming biofilm tolerance the regrowth test is successful. However, this biofilm regrowth assay is not without drawbacks. It was observed that unspecific growth became observable within a 24-hour incubation. Additionally, the washing of the wells before replacing the medium over the treated biofilms was not sufficient to remove all of the test compound from the wells. The retention was evident in the MMC-treated wells, as the strongly coloured compound left visible deposits within the biofilm that were not removable by washing. A photograph of the washed biofilms with MMC deposits is

presented below in Figure 27. Compound carryover from the treatment can attribute to some of the growth inhibition in the final regrowth step of the assay, thus masking the real persister-eliminating effect and giving rise to possible false positives. In summary, the biofilm regrowth assay is a clinically relevant platform capable for recognizing true anti-persister compounds, but further optimization is required before moving on to routine screening.

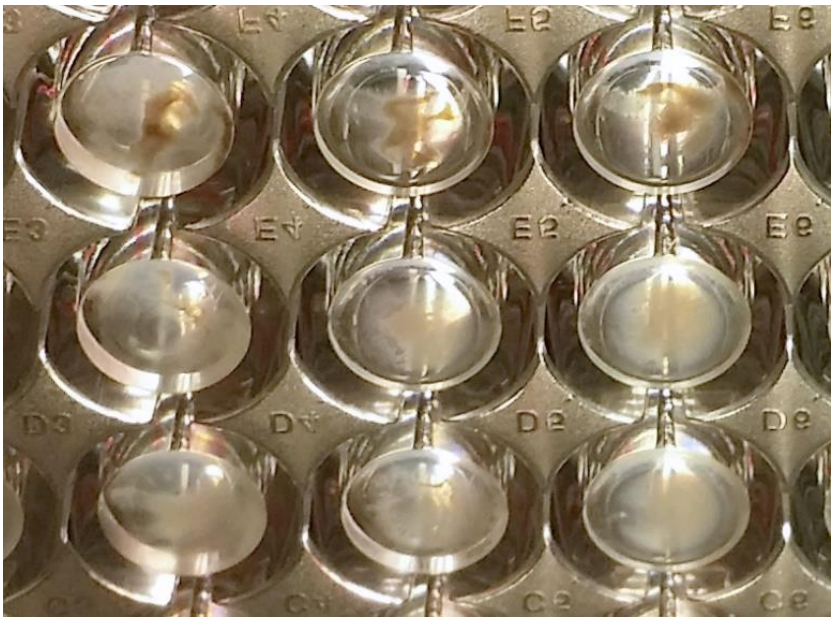


Figure 27. A close-up photograph taken of the bottom of a 96-microwell plate. The biofilms on the bottom of the wells have been washed with phosphate buffered saline after a 24-hour treatment. Coloured MMC deposits can be observed within the biofilms.

5.5 Future prospects of anti-persister drug development

5.5.1 Evaluation of recent discoveries

5.5.1.1 ADEP4

ClpP as a novel and unique target for antimicrobial activity makes ADEP4 a particularly interesting finding (Conlon et al. 2013). However, the ADEP4 molecule is too large to penetrate the outer membrane of Gram-negative bacteria, which significantly limits its spectrum (Cain 2013). Another, more definitive challenge possibly reducing the therapeutic potential of ClpP as a drug target, is that the enzyme is conserved from bacteria to mammalian cells (Gispert et al. 2013). In eukaryotic cells ClpP is a mitochondrial peptidase with important regulatory functions. An NCBI VAST+ analysis suggests a significant structural similarity between the human ClpP and the homologous counterpart of *S. aureus* NCTC 8325, with a 0.77 Å root-mean-square deviation of atomic positions and 1225 spatially aligned residues with 53 % sequence identity (Panchenko and Madej 2004; Madej et al. 2014). This raises the question of possible adverse effects in the form of off-target toxicity. The superposition of human and *S. aureus* ClpP domains is presented below (Figure 28).

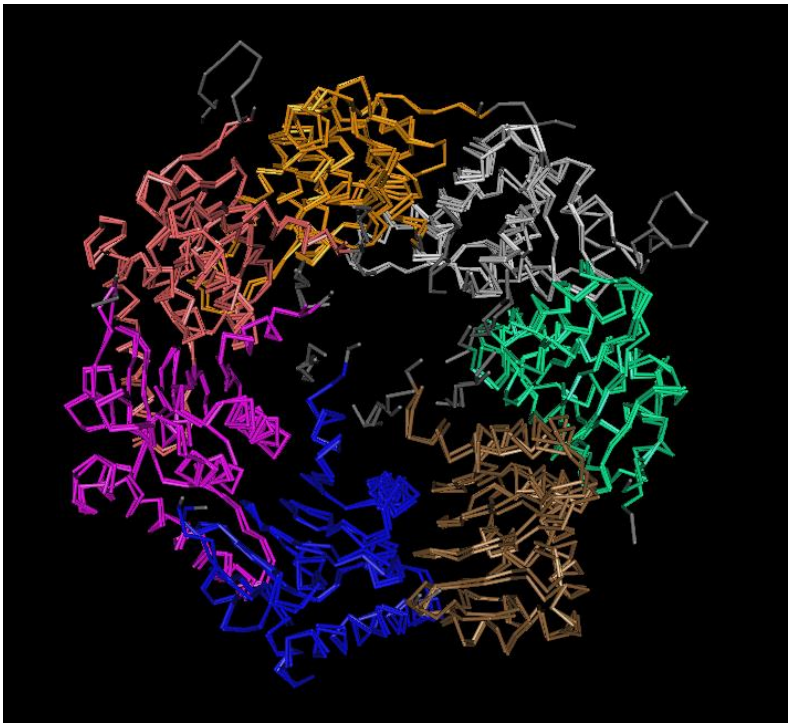


Figure 28. A 3D superposition of the common domains of human and *Staphylococcus aureus* ClpP enzyme. The result of an NCBI VAST+ similarity analysis (Madej et al. 2014) as visualized in the Cn3D viewer (Wang et al. 2000).

Furthermore, the peptide structure of ADEP4 makes it problematic in the formulation point of view. The possibilities of oral administration are highly limited, since digestive enzymes will disintegrate the therapeutic molecule quickly as it enters the stomach. As a therapeutic agent ADEP4 would have to be given parenterally. Topical usage is still highly relevant in treating wound infections, and the possible side-effects can be mitigated if systemic absorption is low.

The anti-persister activity of ADEP4 is nevertheless an immensely promising discovery, as chronic, increasingly drug-resistant infections caused by *S. aureus* are a serious threat frequently encountered in hospital environment. Being a multi-targeted protease in its decoupled form, ClpP is in itself an interesting target for anti-persister drug action.

5.5.1.2 Relacin

The ppGpp analogue relacin does not share the probable caveat of off-target toxicity, as RSH proteins have no homologous counterparts in mammals (Wexselblatt et al. 2012).

Additionally, its mechanism of effect does not exclude Gram-negative bacteria, although the permeation through the Gram-negative outer membrane remains a challenge. The bacteria-specific but potentially broad-spectrum activity of the ppGpp analogue is a desirable property, as many of the most treatment-resistant and severe nosocomial infections are caused by Gram-negative bacteria (Kang et al. 2005). Furthermore, the relatively small size of the relacin molecule grants a good starting point for drug formulation, although it has several easily hydrolysable functionalities. In summary, relacin is a plausible drug candidate to begin with.

5.5.1.3 Resuscitation

It is possible that the breakthrough in combatting persisters is not another antimicrobial compound but instead an agent capable of resuscitating the dormant, tolerant bacteria. Upon the induction of metabolic activity within the persistent culture the actual eradication treatment could be picked from the spectrum of existing antimicrobials according to the genotypic susceptibility of the strain. The preliminary success of persister resensitization by reintroducing a carbon source is promising, although the results have been inconsistent depending on the type of model used (Allison et al. 2011; Barraud et al. 2013; Price et al. 2015).

Similarly inspiring is the discovery of C10, a selectively acting small-molecule stimulant of persister reactivation (Kim et al. 2011). Interestingly, C10 was found to first reduce the viable count in the persister culture before inducing resuscitation and enabling norfloxacin killing. Therefore, another interpretation of the presented results is possible. Since C10 considerably thinned out the persister population before enacting the observed reactivation and the associated sensitization, the resuscitation could have been purely due to the change in the population dynamic. The killing of a substantial fraction of the cells would reintroduce carbon source into the culture upon the collective bacterial lysis, and this release of nutrients could potentially be enough to bring upon resuscitation.

Such a resuscitation-by-killing treatment was experimented with in this study by combining an initial MMC exposure to a follow-up treatment with penicillin G. MMC was able to stimulate moderate regrowth (Figure 18) and penicillin G was administered, but all bacteria were eliminated to the detection limit regardless of whether follow-up

treatment was carried out (results not presented). The experimentation with a combination treatment was complicated by the lack of tools for the real-time determination of viability; by the time the regrowth was observed and penicillin G administered, MMC had already reverted the regrowth and killed all bacteria. The combination treatment is nevertheless a potential approach, which deserves further research as soon as a quicker viability assay becomes available.

The increasing understanding of the mechanisms governing resuscitation in bacteria gives another approach to persister resensitization. Resuscitation factors have been discovered and characterized in depth in actinobacteria (Mukamolova et al. 1998; Mukamolova et al. 2002). Indications of similar proteins have been observed in *Staphylococcus aureus* as well (Pascoe et al. 2014). If such a resuscitation promoter could be utilized in a drug discovery perspective, it could offer new ways to tackle persisters. For example, inducing the resuscitation protein could revert the tolerance in persister subpopulation. However, the described tactics of resensitization through resuscitation have the inherent hazard of being potentially able of waking up latent pathogens unintentionally. A safer alternative course of action could be to inhibit such resuscitation promoting factors instead (Kaprelyants et al. 2012). Keeping the persister pathogens from waking up for long enough could turn the advantage around and lead to eradication. This mechanism of action is already implied in long-course rifampicin, which supposedly causes irreversible dormancy by disrupting regulatory TA modules (Keren et al. 2012).

5.5.1.4 Teixobactin and the unculturables

Many of the most successful antimicrobials are derived from soil microbiota, and there is no reason to believe that the resource had become exhausted (Rappé and Giovannoni 2003; Lewis 2012). The iChip method for one can be expected to produce more of interesting lead compounds after teixobactin, its proof-of-concept realization (Ling et al. 2015).

As a novel, multi-targeted antibiotic teixobactin is an inspiring finding, although no studies have been conducted on its activity against persister bacteria (Ling et al. 2015). Similarly to ADEP4, teixobactin also lacks the activity against Gram-negative bacteria, which is a major limitation. In any case, it can be expected that the associated

breakthrough in antimicrobial drug discovery platforms will benefit the battle against persisters tremendously.

5.5.2 Cationic antimicrobial peptide derivatives

Another class of compounds that may well become essential in tackling the persister challenge are the cationic antimicrobial peptides and their peptidomimetic derivatives. Antimicrobial peptides are a diverse group of oligo- and polypeptides that are encountered in all organisms as a vital and conserved part of their innate immunity (Hancock 2001). Cationic antimicrobial peptides target cellular membrane integrity and, upon permeation, various intracellular targets, while also interacting with other components of the host immune system. They are being studied as potential new antimicrobial compounds because of their many useful activities.

The key mechanism of the bactericidal effect of cationic peptides is the permeabilization of bacterial membranes (Hancock 1997). The peptides, amphipathic by nature, target the negatively charged cell membranes of bacteria with their positively charged residues, which leads to pore formation and the subsequent, rapid death of the target organism. Conversely to conventional antimicrobials targeting the peptidoglycan metabolism within the cell walls of metabolically active bacteria, the membrane-disrupting effect of cationic antimicrobial peptides does not rely on peptidoglycan metabolism. Their activity should therefore be sustained against persister bacteria as well. Indeed, cationic antimicrobial peptides were demonstrated to quickly kill persister bacteria in both planktonic cultures and biofilms while potentiating the effects of conventional antibiotics (Chen et al. 2011). Fortunately, the powerful mechanism is quite specific towards bacteria, as eukaryotic cell membranes have lower potential and lack the profound negative charge (Hancock 1997).

As discussed previously with regard to peptides as antimicrobial drugs, they have some important drawbacks. Peptides are readily degraded by digestive enzymes in the gastrointestinal tract, and the manufacture of such large and intricate molecules is additionally both challenging and costly (Hansen et al. 2010). To address this challenge, Hansen et al. (2010) developed an assortment of peptidomimetics – small-molecule equivalents of the minimal pharmacophore of select short antimicrobial peptides. The newly synthetized peptidomimetics demonstrated significantly improved enzymatic

stability and low toxicity against human erythrocytes while maintaining impressive antibacterial activity against both Gram-positive and negative bacteria (*S. aureus* and *E. coli* respectively). Antimicrobial peptides show great promise in battling treatment-resistant infections, and their putative anti-persister qualities in particular warrant further research.

5.5.3 Dehydroabietic acid

Combined, the newly discovered anti-persister properties and the previously established anti-biofilm activity of DHA together with its relatively low toxicity bring some interesting implications. Coniferous resin, of which DHA is one main constituent, has been a subject of medical research based on its traditional usage in folk medicine to heal wounds and skin infections (Rautio et al. 2007; Sipponen et al. 2008; Sipponen et al. 2012). Resin salve from Norway spruce (*Picea abies*) was shown to be effective in the treatment of infected surgical wounds and pressure ulcers, both of which are characterized by the presence of a recalcitrant biofilm infection (Sipponen et al. 2008; Sipponen et al. 2012; Trøstrup et al. 2013). The efficacy of resin salve against wound infections that are typically tolerant to more conventional antimicrobial interventions hints towards its ability to overcome the biofilm persister challenge.

This adds to the value and credibility of the results obtained in the pilot screening. Furthermore, the approval of coniferous resin salve for medicinal use and the success of the product speaks for not just the activity but a favourable tolerability as well. The topical administration further limits the potential toxic effects of resin constituents with symptoms of resin hypersensitivity being the only side-effect reported in the product leaflet of a commercial product derived from the experimental salve (Repolar Oy 2013). An important drawback seen with DHA as well is its lack of activity against Gram-negative bacteria, which limits its spectrum (Manner et al. 2015).

5.5.4 Novel screening methods

Judging by the lack of success with the described bioassays outside the golden standard of viable count plating, it is evident that the need for more suitable screening methods persists. Fortunately, the spectrum of available putative methods is not limited to the ones

tested at this time. A successful high-throughput screening of anti-persister antimicrobials may only depend on finding a proper assay.

5.5.4.1 Thioflavin T

Used characteristically for the detection of amyloid fibrils, the fluorescent probe thioflavin T has recently been ingeniously adopted for the detection and quantification of transcriptional activity (Sugimoto et al. 2015). Based on the discovery that, in addition to amyloid structures, thioflavin T upon binding fluoresces with nucleic acids – especially with RNA – the group proposed a simple and versatile method for observing mRNA synthesis and monitoring metabolic activity even in real-time. By incubating the cells with thioflavin T and subsequently carrying out a fluorescence measurement, it was possible to quantitatively detect RNA, as the fluorescence intensity is relative to its amount. The method was further validated for studying persister-state transformations with a reporter *E. coli* strain and tested for general growth phase detection for various other bacteria both Gram-negative and Gram-positive.

Since the fluorescence intensity of thioflavin T depends on its binding to an RNA molecule, the method does not require washing steps before making the measurements, and it is compatible for microscopic single-cell studies as such (Sugimoto et al. 2015). Additionally, the reagent itself is economical to obtain. Based on the preliminary findings thioflavin T seems to be a promising addition for the metabolic state determination and quantification of persister bacteria.

5.5.4.2 Start growth time

Trying to develop a viability determination method applicable for high-throughput screening, Hazan et al. (2012) developed an assay based on the notion that the time it takes for bacteria to reach a set threshold amount of growth is proportional to the initial viable count in the inoculum. Monitoring this time by optical density measurement and comparing it to a previously established calibration data made it possible to accurately predict the sample viable count in a couple of hours and, more importantly, on a 96-microplate format.

The described start growth time assay supposedly takes persister bacteria into account and is suitable for viable count determination regardless of the bacterial concentration (Hazan et al. 2012). However, the method may be limited by the intrinsically varying characteristic regrowth delays of persisters and stationary phase bacteria (Balaban et al. 2004). In a persister-rich culture this might have a significant effect on the start growth time. Still, the simple and elegant method seems highly promising when used in appropriate conditions.

6 CONCLUSIONS

In the thesis it was demonstrated that stationary phase *Staphylococcus aureus* is a suitable model culture for anti-persister drug screening. The culture stays sufficiently stable after the initial incubation and demonstrates metabolic inactivity and antimicrobial tolerance – the key properties of persistent phenotype – consistently. As expected, both features are revertible by dilution in fresh medium. Despite rapidly developing tolerance against conventional antibiotics, the stationary phase culture stays susceptible to mitomycin C (MMC), an established anti-persister model compound.

New compounds were identified as potential anti-persister agents by a pilot screening. Chromomycin A3 and mithramycin A exhibit significant activity against the model culture, the latter actually matching or even surpassing that of MMC the model compound. All three share a similar DNA-damaging mechanism that is responsible for the general cytotoxicity of the compounds. Whereas these novel findings are not any more suitable antimicrobial drug candidates than MMC owing to their harmful off-target effects, their activity supports the notion that bacterial DNA is a putative target for anti-persister drug action. Further research is necessary to find ways to overcome the mammalian cytotoxicity while exploiting this mechanism. On the contrary, the plant-derived screening hits dehydroabietic acid (DHA) and oleanolic acid show more promise from drug discovery perspective as such, despite their lower activity. Based on earlier reports, both are relatively well tolerated and have been used in traditional medicine. Moreover, the small-molecule compounds are flexible starting points for chemical derivatization and formulation.

We have additionally designed a proof-of-concept trial to piece together the anti-persister activity of a compound and its ability to sterilize a biofilm. Based on bacterial regrowth from a treated biofilm, the assay mimics the conditions seen in a relapsing treatment-resistant infection. Whereas a conventional antibiotic expectedly fails to affect a mature biofilm, DHA significantly reduces regrowth in a given time frame and MMC prevents it completely. On the basis of what is known of biofilm tolerance, the previously established anti-biofilm properties of DHA may partly be due to its newly identified anti-persister properties.

Streamlining the measurement of persister viability is a challenge yet to be addressed. Resazurin staining and BrdU ELISA are techniques worth further exploration, but at the time being neither is suitable for persister assays. Viable count plating is a robust method that lacks the automation and miniaturization options that are prerequisite for a larger-scale screening campaign. Promising new assay methods are nevertheless being introduced as persister research is gaining momentum. Based on the success of even the limited scale pilot-screening, building an effective screening campaign for natural products on a novel assay could give rise to numerous new anti-persister leads.

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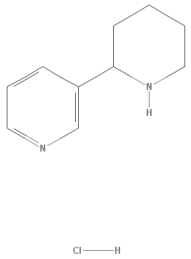
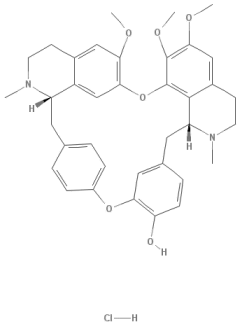
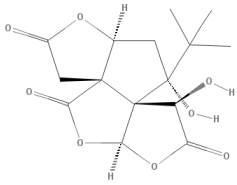
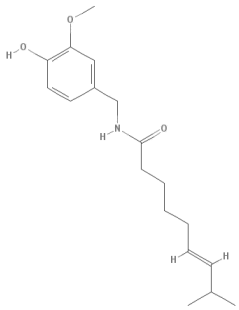
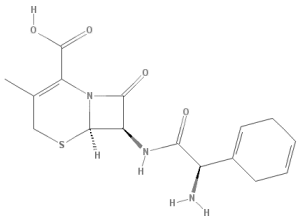
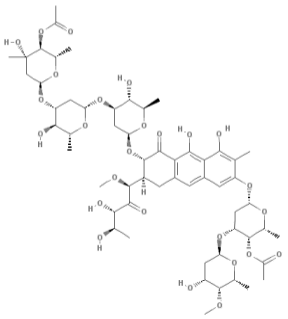
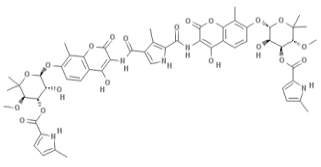
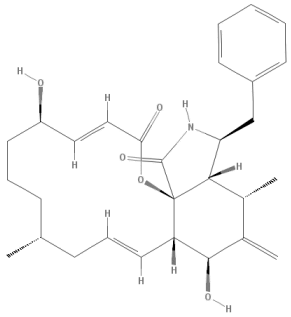
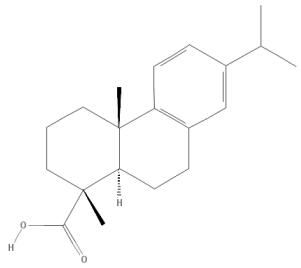
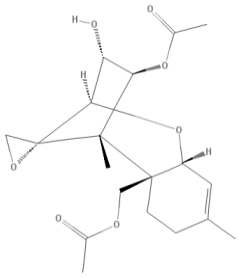
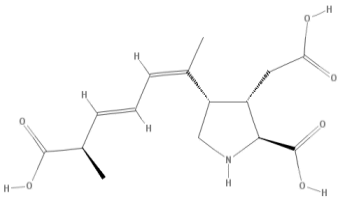
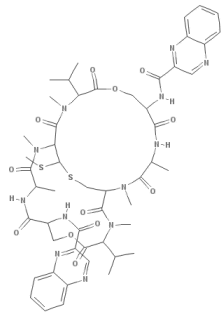
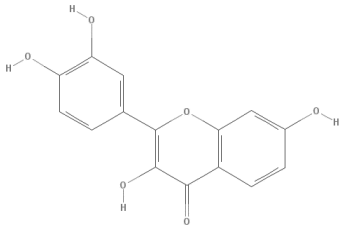
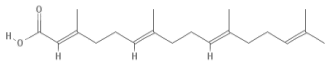
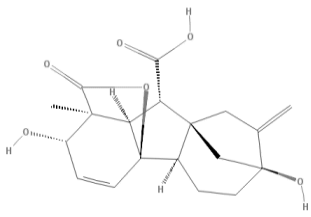
8 ANNEX 1

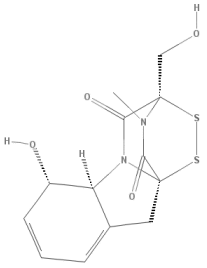
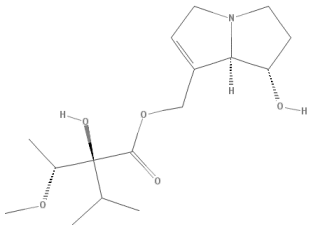
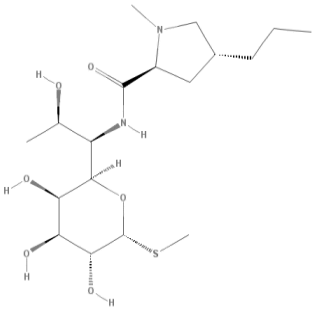
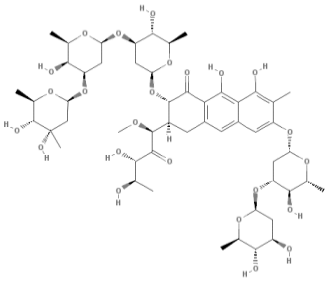
Table 1. The screened compounds (all structures modified from PubChem).

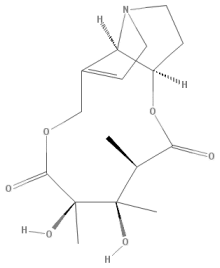
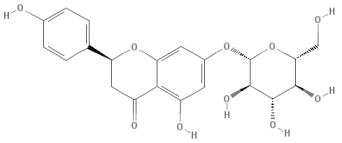
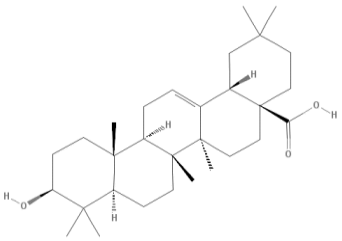
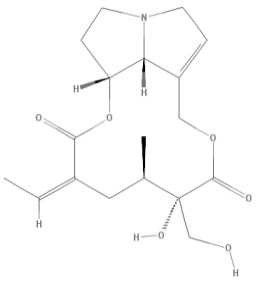
Compound name	**Molecular weight (g/mol)**	**Structure**	**Log reduction**
Anabasine HCl	198.69		0.53
Berbamine 2 HCl	645.18		0.15
Bilobalide	326.30		-0.04

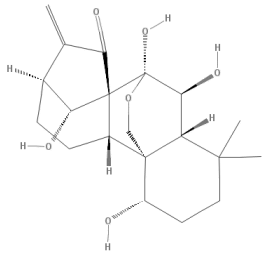
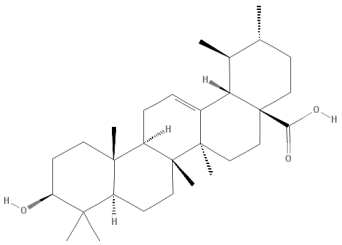
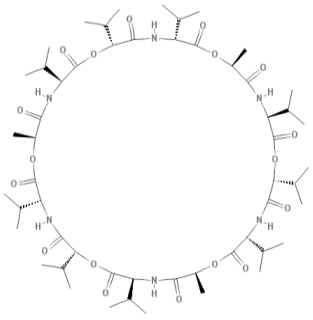
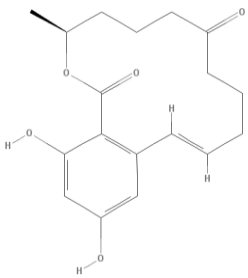
Compound name	Molecular weight (g/mol)	Structure	Log reduction
Capsaicin	305.41		0.44
Cephradine	349.40		0.03
Chromomycin A3	1183.25		3.62
Coumermycin A1	1110.08		0.11

Compound name	Molecular weight (g/mol)	Structure	Log reduction
Cytochalasin B	479.61		0.19
Dehydroabietic acid	300.44		2.04
Diacetoxyscirpenol	366.41		-0.11
Domoic acid	311.33		0.02

Compound name	Molecular weight (g/mol)	Structure	Log reduction
Echinomycin	1101.26		1.30
Fisetin	286.24		0.17
Geranylgeranoic acid	304.47		0.91
Gibberellic acid (+)	346.37		0.25

Compound name	Molecular weight (g/mol)	Structure	Log reduction
Gliotoxin	326.39		0.03
Heliotrine	313.39		-0.10
Lincomycin	406.54		-0.14
Mithramycin A	1085.15		6.30

Compound name	Molecular weight (g/mol)	Structure	Log reduction
Monocrotaline	325.36		-0.04
Naringenin-7-O-glucoside	434.39		0.03
Oleanolic acid	456.70		2.45
Retrorsine	351.39		-0.02

Compound name	Molecular weight (g/mol)	Structure	Log reduction
Rubescensin A	364.43		0.15
Ursolic acid	456.70		0.26
Valinomycin	1111.32		1.93
Zearalenone	318.36		0.06