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Antibacterial and anti-biofilm activity of novel marine natural product mimics

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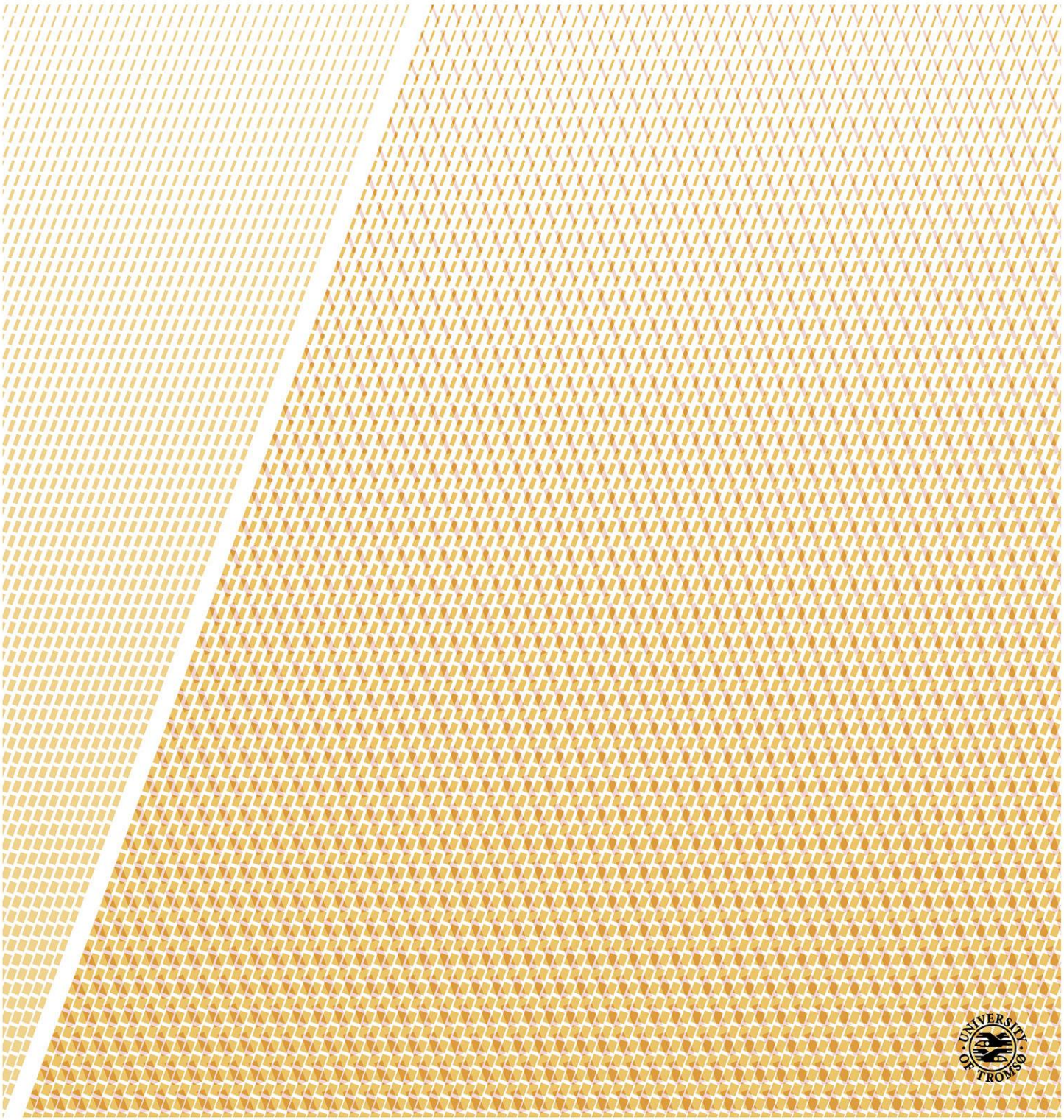

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Abstract

One of the current global healthcare challenges are re-emerging infectious diseases, such as healthcare-associated (HCAI) infections, often complicated by multidrug resistance and chronicity, and tolerance to conventional antibiotics. There is an obvious demand for the discovery and development of antibiotics with novel mechanisms of action (MOAs). Natural environments, including the largely unexplored marine locations are rich sources for promising novel natural products (NPs).

In this project, the antibacterial potential of a library of synthetic marine natural product mimics (MNPMs) was evaluated in collaboration with a PhD project in chemistry (both were parts of MabCent CRI, Centre for Research-based Innovation on Marine Bioactivities and Drug Discovery). The MNPM library was tested for antibacterial activity in a four-step screening workflow. The activity of selected compounds against the reference bacterial strains was verified in expanded screenings against random and multidrug-resistant clinical isolates, e.g. methicillin-resistant *Staphylococcus aureus* (MRSA).

[redacted]

MNPMs characterized in the current work, could become the leads for further development of bactericidal agents for treatment of chronic, including biofilm-associated, infections.

Резюме

Одной из актуальных глобальных проблем здравоохранения являются считавшиеся побежденными инфекционные заболевания, в частности инфекции, связанные с оказанием медицинской помощи (ИСМП). Борьба с такими инфекциями часто осложняется их хроническим характером и наличием мультирезистентности к традиционным антибиотикам. В связи с этим, очевидна необходимоть поиска и разработки антибиотиков с новыми механизмами действия (МД). Природня среда, в особенности, малоизученная морская среда является богатым источником потенциально новых природных соединений (ПС).

В рамках данного проекта были исследованы антибактериальные свойства ряда синтетических миметиков морских природных соединений (ММПС). Исследования проводились совместно с одним из Ph.d.- проектов по химии (оба проекта инициированы Центром научных инноваций в разработке морских биоактивных материалов и лекарств, MabCent CRI). Библиотека ММПС была протестирована на наличие антибактериальной активности в ходе четырех-этапного процесса скрининга. Активность отобранных кандидатов в отношении контрольных штаммов бактерий была верифицирована в процессе расширенного скрининга, где была использована случайная выборка клинических изолятов, а также мультирезистентные изоляты, в том числе, метициллинрезистентный золотистый стафилококк, *Staphylococcus aureus* (MPЗС).

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[redacted] Соединения-прототипы, охарактеризованные в рамках данного проекта, могут стать первым шагом в дальнейшем развитии бактерицидных агентов для лечения хронических инфекций, в том числе, связанных с биопленками.

Contents

Abbreviations

Aae	Autolysin/adhesin from S*. epidermidis*
Aap	Accumulation-associated protein
ABR	Antibiotic resistance
ADMETox	Adsorption, distribution, metabolism, excretion, toxicity
AMP	Antimicrobial peptide
AMR	Antimicrobial resistance
aps	Antimicrobial peptide sensor
AtlE	Autolysin E
Bap	Biofilm-associated protein
CAT	Chloramphenicol acetyltransferase
CLSM	Confocal laser scanning microscopy
CoNS	Coagulase-negative staphylococci
Embp	Extracellular matrix-binding protein
ESBL-CARBA	Extended spectrum β-lactamase - carbapenemase
FACS	Fluorescence-activated cell sorting
FC	Flow cytometry
GFP	Green fluorescent protein
HCAI	Healthcare-associated infection
HTS	High-throughput screening
IS	Insertion sequence
MIC	Minimal inhibitory concentration
MNMP	Marine Natural Product Mimic
MOA	Mechanism of action
MRSA	Methicillin-resistant *Staphylococcus aureus*
MRSE	Methicillin-resistant *Staphylococcus epidermidis*
MSCRAMM	Microbial surface components recognizing adhesive matrix molecules
NP	Natural product
PGA	Poly-γ-glutamic acid
PIA	Polysaccharide intercellular adhesin
PSM	Phenol-soluble modulin
SAR	Structure-activity relationship
SCV	Small colony variant
Sdr	Serine-aspartate repeat-containing protein
SSP	Surface-associated protein
SSSI	Skin and skin structure infection
TCP	Tissue culture plate
VBNC	Viable but nonculturable cell
VRE	Vancomycin-resistant enterococci

1. The challenge: bacterial infectious diseases

Nowadays, in the age of high technologies and achievements in medicine, many life-threatening infectious diseases of bacterial origin have been defeated. However, it seems to be too early to conclude that the fight is over. Indeed, infectious diseases (including parasitic diseases) killed 9.5 million people, corresponding to 17% of all deaths globally and remained the top cause of death in low-income countries in 2012 [1-3] and also in children under 5 years in 2015 [4] . Along with the emergence of new communicable diseases (AIDS, hepatitis C, dengue haemorrhagic fever), old infections are "re-emerging" with a new face (e.g., multidrug-resistant tuberculosis and infections caused by methicillin-resistant *Staphylococcus aureus, i.e.,* MRSA) [5]. Moreover, the importance of healthcare-associated infections (HCAI) is now increasing, becoming a great medical concern. Urinary tract infections, surgical site and medical device-associated infections play a significant role in HCAI morbidity [6]. In Europe alone HCAI lead to approximately 37 000 deaths and contribute to an additional 110 000 deaths annually [7].

Factors contributing to the emergence of infectious diseases in the modern world are:

- Extensive demographic changes (growing population, high mobility, urbanization);
- Antibiotic use and misuse (wrong doses, use in food and feed);
- Inappropriate hygiene standards and healthcare procedures, social inequality, mostly in developing countries;
- Immunocompromised patients (chemotherapy, post-transplantation and diabetes), mostly in industrialized countries;
- Others (climate change, wars etc.)

An infection is a bi-directional process, involving the interaction of a pathogen and a host organism (patient). The current challenging situation with the treatment of infectious diseases is thus a result of changes from both sides. This alteration is obviously a dynamic phenomenon and quite complex, especially on a global scale.

Undoubtedly, extensive research in this field is needed, including the search for new anti-infective agents, which could "fill in the gaps" in the currently available solutions and offer some alternatives for the future.

2. Objective of the study

The main aim of this MabCent PhD project was to investigate antibacterial and/or anti-biofilm activity as well as the mechanism of action (MOA) of compounds that are promising as potent anti-infective agents. These compounds were identified by selection from a library of synthetic marine natural product mimics (MNPMs) in close collaboration with another PhD project at MabCent.

In order to achieve the overall aim, the following specific aims were defined:

- Identify compounds with antimicrobial activity from a library consisting of two main groups of synthetic MNPMs by successive screenings against panels of relevant test bacteria;
- Determine the MOA of selected MNPMs by using optimized *in vitro* test systems;
- Evaluate the propensity of these MNPMs to induce antibiotic resistance (ABR) development;
- Identify MNPMs with anti-biofilm activity and characterize it, using two types of *S. epidermidis in vitro* model biofilms

3. Background

To set a background for the problems addressed in the current work, an overview of the challenges related to "re-emerging" infections will be given, with emphasis on bacterial HCAI, namely the issues of multidrug-resistant bacteria, persister cells, and microbial biofilms. Some aspects of treatment and prevention thereof will be presented accordingly.

3.1. The multifaceted problem

3.1.1. Drug resistance in bacteria

Being a part of antimicrobial resistance (AMR) in general, ABR is recognized as one of the main global problems associated with infectious diseases [8]. AMR leads to at least 50 000 deaths in the EU and US alone each year [9], and much more – worldwide (Fig. 1). More than 25 000 patients die in the EU from infections with multidrug-resistant bacteria annually. Overall, infections due to antibiotic-resistant bacteria result in extra healthcare cost and productivity losses of at least EUR 1.5 billion each year in the EU [10]. The current situation appears to anticipate the post-antibiotic era, when "common infections and minor injuries can kill" as predicted for the future [8]. Indeed, during the last decade the therapeutic options in treating community-acquired and HCAIs have been dramatically influenced by the changes in the susceptibility patterns [11]. The estimation is that in 2050 around 10 million deaths can be caused by AMR (Fig. 1).

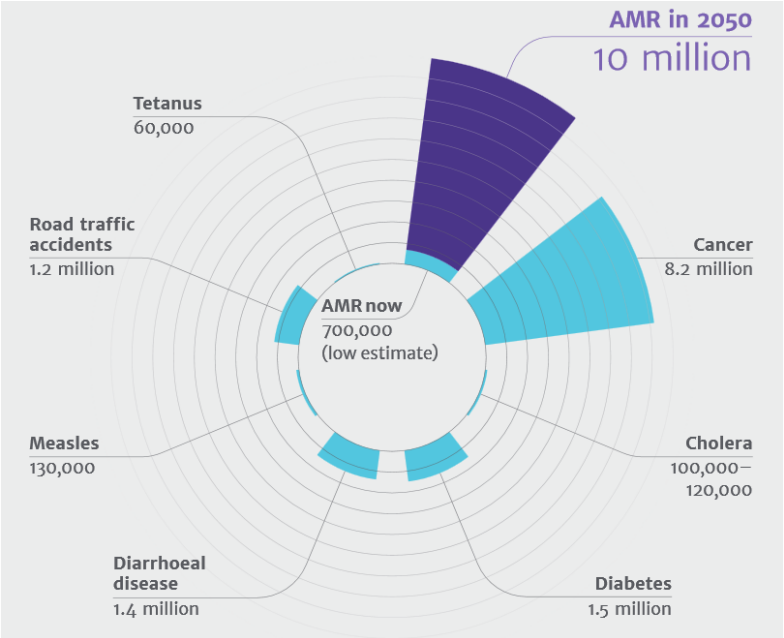

Figure 1. Annual deaths caused by AMR compared to other major causes of death. *Reprinted with permission from The Review on Antimicrobial Resistance, 2014* [9]*.*

The antibiotics era began with Paul Ehrlich's concept of a "magic bullet" [12]. Together with co-workers he tested hundreds of synthesized organoarsenic derivatives of a drug Atoxyl in syphilis-infected rabbits. In 1909 this screening resulted in compound 606, Salvarsan, which was successfully used for treatment. Then the approach of Paul Ehrlich's and co-workers led to a discovery of sulfa drugs. Bayer chemists, Josef Klarer and Fritz Mietzsch, synthesized sulfonamidochrysoidine Prontosil in 1932 (KI-730,) and Gerhard Domagk tested its antibacterial activity in a number of diseases [13]. The third notable event was the discovery of penicillin by Alexander Fleming in 1929 [14]. Interestingly, the first hospital use of a drug that could be called an antibiotic, Pyocyanase, presumably quorum sensing molecules preparation from *Pseudomonas aeruginosa,* was in 1899 [15].

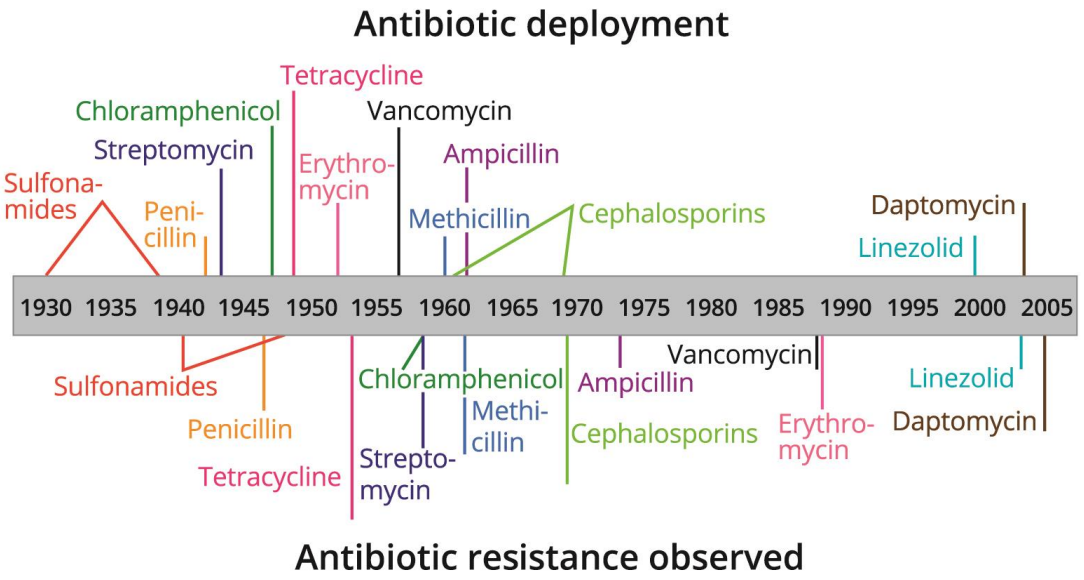

Figure 2. Co-evolution of antibiotic deployment and ABR development. *Modified from Clatworthy et al. [16].*

Unfortunately, ABR appeared quite early. For penicillin it was detected just after its' large scale usage in the 40's, and for sulfonamides it was recorded around the same time (Fig. 2). Many antibiotics from novel chemical classes were discovered between the 1950s and 1970s, and the challenge of resistance rose rapidly alongside (Fig. 2) [15]. Thus, the equilibrium in this continuing "arms race" seems to be easily broken. We should always consider that selective pressure can provoke diverse protective mechanisms in microbes [15]. The discovery of transferable resistance to sulfonamide [17] and fluoroquinolone antibiotics demonstrates that even the introduction of synthetic antimicrobials, which do not occur in nature, does not eradicate the risk of plasmid- or transposon-determined resistance [18].

3.1.2. Persisters

Another type of challenge in the treatment of bacterial infectious diseases is associated with persistence. This phenomenon has been discussed in the context of chronic infections for a long time. Although discovered already in 1942 [19], it still appears to be still incompletely understood.

Physiological states associated with persistence include:

- Cellular invasion and intracellular persistence. Some bacteria responsible for chronic (persistent) infections are obligate intracellular pathogens (*Rickettsia*, *Chlamydia*). Other bacteria can adapt to the intracellular environment, like *Staphylococcus and Streptococcus* [20, 21].
- Naturally occurring electron transport deficiency, auxotrophy for thymidine (and several other traits) manifested as the "small colony variant" (SCV) phenotype, a slow-growing bacterial resistant subpopulation [22]. The aforementioned intracellular persistence of staphylococci has been shown to be associated with SCVs [23].
- Dormancy, when cells are non-growing and have reduced metabolism [24]. Dormant physiological state of bacteria was shown to be associated with host immune evasion, decreased antibiotic susceptibility and therefore, prolonged survival duration [25]. Importantly, persister cells and dormant cells are different phenomena, although being dormant might preclude becoming a persister cell.

In the strict sense, a persister cell is defined as a member of a specific "dormant" subpopulation randomly formed in a microbial population which can easily survive the antibiotic treatment while the majority of the population is killed/eradicated [26]. The mechanisms behind switching to a persister state and backwards can be different, and overall "the persister" phenomenon appears to be a collective of several traits/features. Recently it was stated that the phenomenon of persistence might not be always stochastic, but also induced [27, 28].

A hypothesis about the role of persisters in a bacterial population was proposed by Spoering and Lewis [29]. They inferred that upon antibiotic treatment, the majority of cells in a bacterial culture is killed by a mechanism of programmed cell death [30]. The culture survival is provided by a small persister subpopulation, in which this mechanism is inactivated. Experimental data confirmed that assumption: biofilms and stationary phase cultures, both containing persister subpopulations, had strongly reduced susceptibility to antibiotics, compared to exponentially growing cells [29].

Overall, the formation of persistent subpopulations and their survival at fluctuating environmental conditions are believed to be regulated by a complex of several mechanisms, including toxin-antitoxin modules, alternative energy production, SOS response, enhanced efflux activity, etc. Importantly, under certain conditions the persister cells revert to "normal" growing forms [31]. Thus, persistence can be characterized as adaptive resistance connected to phenotypic variation [32].

3.1.3. Biofilms and associated infections

HCAIs were associated with the use of medical devices in 60-70 % of the cases already in 2008 [33]. Device-related infections like catheter-associated urinary tract infections, central line-associated bloodstream infections and ventilator-associated pneumonia [34, 35], together with tissue-based infections associated with cystic fibrosis [36] and wounds (surgical [37]and diabetic [38]), are probably the major infections believed to be linked to microbial biofilms [39]. The reason why biofilms successfully colonize these niches lies in the nature of the biofilm life style, which is reviewed in the next section.

Implants, catheters and prostheses are successfully colonized by bacteria and offer favorable conditions for biofilm development. For example, within the implant environment, penetration of host defense mechanisms can be impaired [40] and surface association of bacterial cells with implant material is facilitated by bacterial adhesins and surface preconditioning [41]. As displayed in *Table 1*, there are few groups of microorganisms that commonly cause infections, and the leading position belongs to the group of Coagulase negative Staphylococci (CoNS) [42, 43]. Concerning the current development of the medical devices sector, the clinical relevance of CoNS will increase.

Table 1. Medical device-associated infections

Medical device	**Causative microorganism**	**Ref.[b]**
Ventricular assist devices and shunts	***Staphylococcus* spp.**[a], *Candida* spp., *Streptococcus* spp., *Pseudomonas* spp., *Corynebacterium* spp.	44
Central venous catheter	***Staphylococcus* spp.** (**CoNS** and *S. aureus*), Gram-negative bacteria (*Klebsiella pneumoniae, Pseudomonas aeruginosa*), *Candida* spp., *Enterococcus* spp.	44-46
Fracture-fixation devices	***Staphylococcus* spp.**, *Propionibacterium* spp., *Corynebacterium* spp.	44
Artificial heart valves	***Staphylococcus* spp. (CoNS** and *S. aureus***)**, *Streptococcus* spp., Gram-negative bacteria, diphteroids, *Enterococcus* spp., *Candida* spp.	44-46
Endovascular grafts	Enteric Gram-negative bacteria, *Pseudomonas* spp., *Staphylococcus* spp**.** (CoNS and S. aureus), *Streptococcus* spp.,	44, 46
Orthopedic devices (artificial joints, hips etc.)	***Staphylococcus* spp.,** (CoNs and *S. aureus*), *Streptococcus* spp., Gram-negative bacteria (*P. aeruginosa*), *Enterococcus* spp.	44-46
Urinary catheters	***E. coli***, *Enterococcus* spp., *Candida* spp., *K. pneumoniae, CoNS*	44-46
Others: breast implants, artificial voice prosthesis, intrauterine device	***Staphylococcus* spp.,** *E. coli, Streptococcus spp*., *Candida* spp., *Lactobacillus* spp.	44, 45
Contact lenses	*Pseudomonas* spp., *Staphylococcus* spp. (CoNS), Gram-positive cocci, *Actinomyces* sp., *Candida albicans*	45

[a] Bold font indicates the primary causative infectious agent;
[b] Based on Parra-Ruiz et al. , Khan et al. and Thomas et al. [44, 45, 47] and references therein.

One should keep in mind that the association of biofilms with foreign-material infections demonstrated by direct microscopy [48] and animal models [5, 49, 50], may not be that clear-cut [1]. The key role of adherence by slime (biofilm) production was proposed for *S. epidermidis* device-related

infections already in 1982 [51]. However, later experiments with animal models suggested the complexity of the pathogenesis of such infections, where a biofilm might be just one of the factors involved [52, 53]. This conclusion was also in line with data from analysis of clinical specimen [54].

Biofilm formation and composition

Presumably, the first documented evidence of microbial biofilms was given by Antonie van Leuwenhoek in the late 17th century when he examined dental plaque under the home-made microscope [55]. The term "biofilm", however, was introduced later. Probably, it was used in a publication for the first time by Mack et al. in 1975 [56]. Since that time, the "biofilm" definition has been changing and updating continuously. The current definitions state that a biofilm is a complex community of microorganisms embedded in a matrix made of self-produced and external substances, which is floating or attached to a surface [57, 58].

The currently accepted general model of a biofilm life cycle, as exemplified by *S. epidermidis* biofilm, is depicted in Fig. 3. Biofilms are often (but not always) sessile communities. The attachment is a complex process that involves both physical and chemical interactions between the bacteria, the surface and molecules that are present on the surface [59]. After the attachment, cell division and matrix production eventually results in a mature biofilm with specific architecture. Common structural components of the biofilm extracellular matrix are:

- Polysaccharides (e.g., Polysaccharide intercellular adhesin, PIA);
- Proteins (incl. enzymes);
- Extracellular DNA (eDNA);
- Teichoic acid.

The ratio between the matrix components is species- and conditions- specific [60]. The same is true for the biofilm architecture, as it depends on the environmental factors, such as nutrition, surface, oxygen and shear forces [61]. Biofilm maturation and dispersal that together ensure the populations well-being and spreading to colonize new habitats, are governed by both mechanical and chemical mechanisms (e.g., phenol-soluble modulins, PSMs, Fig. 3) [62]. Adaptation to heterogeneous environmental factors leads to the formation of physiologically diverse subpopulations within biofilms. For example, oxygen gradients lead to differentiation into aerobic or fermentation metabolism and, together with nutrient gradients, lead to subdivision into active and dormant cell subpopulations [63, 64].

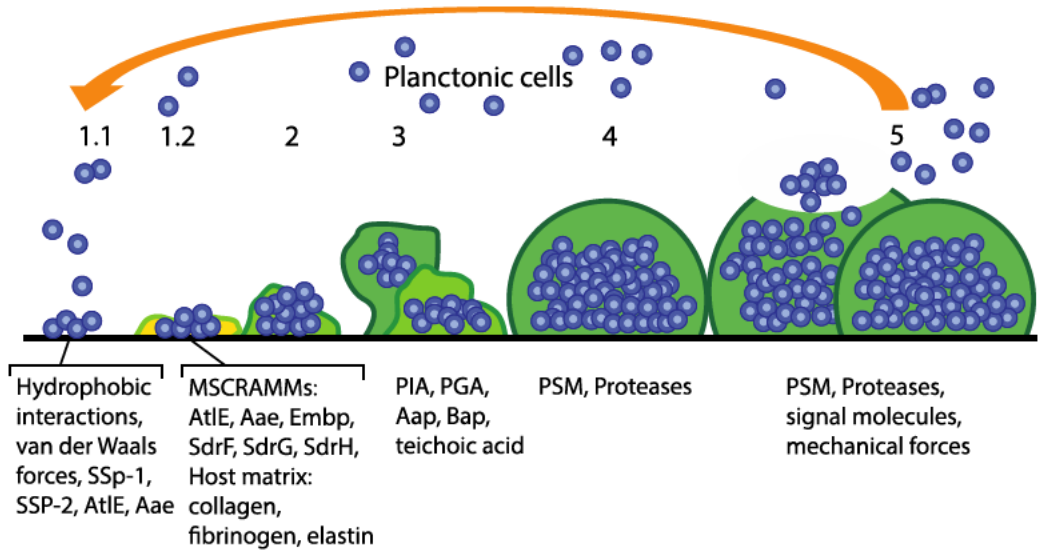

Figure 3. The life cycle of a* S. epidermidis *biofilm. *1.1) attachment to non-modified surface, 1.2) attachment to host matrix molecules, 2) irreversible attachment, 3) biofilm growth, 4) mature biofilm, 5) biofilm dispersal. Factors involved in the establishment of respective steps are presented under the line indicating a surface. These factors are also considered as the virulence factors of* S. epidermidis. *Modified from Granslo et al. and Otto* [62, 65, 66].

These features allow bacteria in biofilms to function as a complex system, to optimally use the environmental conditions (space, nutrients, etc.), be flexible and be able to adapt to changes, be protected, and eventually ensure the survival of the population. Unsurprisingly, around 99% of bacteria in natural habitats are considered to live in biofilms [67], and 65 to 80 % of human infections are related to biofilms [68, 69]. To make it even more complex, most of the naturally occurring biofilms are multispecies communities [70, 71]. Everyday examples are dental and wound biofilms [72, 73], soil and aquatic biofilms[74, 75], which also include eukaryotic species [56].

Biofilm-related resistance mechanisms

When it comes to antibiotic treatment of infections, the "bullet-target" concept is not always applicable to biofilm communities. Indeed, if a biofilm is regarded as a complex community with different subpopulations as its members, it is not just an assembly of parts, but rather a system. Therefore an effective treatment against a biofilm has to act on a system level [15].

There are several resistance mechanisms specific to biofilms [76]:

- Physical resistance – decreased penetration of antimicrobials. For example, the matrix polysaccharides (alginate, Psl and Pel in *P. aeruginosa*) can bind cationic antimicrobials (aminoglycosides), and eDNA can provide defense by chelating cations [77, 78].
- Physiological status of cells in different microenvironments, such as dormant cells [79].

- Tolerance mechanisms induced by the presence of antimicrobials. They are not strictly biofilm-specific, but are performed differently within biofilms, for example, biofilm-specific upregulation of efflux pumps [61, 78], degradation of antibiotics [80, 81], stress-induced mutagenesis [82].
- Quorum sensing [83]. Not being a resistance mechanism per se, it plays a key role in the biofilm community organization and functioning, supporting the community-level protective mechanisms [84].

In biofilms, actively growing cells can be located close to non-growing cells, which can be persisters [85] that considerably contribute to biofilm tolerance [86]. Following the antibiotic treatment and killing the active cells, the dormant cells/persisters can repopulate the biofilm [64] (Fig. 4). A well-known example of such recalcitrance are biofilm-associated infections in cystic fibrosis patients [87, 88]. Among Gram-positive pathogens, the importance of persisters for the tolerance to antibiotics was demonstrated in a model *S. epidermidis* biofilm [89, 90].

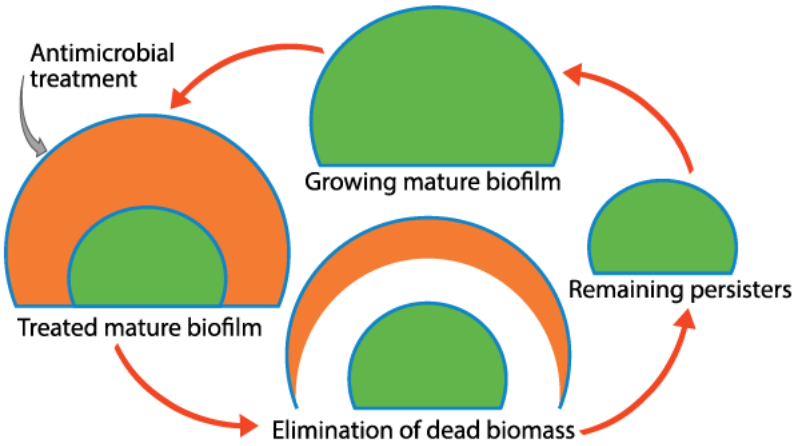

Figure 4. The recurrence of a biofilm infection caused by the persister phenomenon. *After the treatment, the susceptible part of a biofilm is killed and/or eradicated, while a small subpopulation of persistent cells remains unaffected; these cells repopulate the biofilm under more favorable conditions*

The exposure to sublethal concentrations of antimicrobials can provoke the development of multidrug resistance in bacteria, which might be even more relevant within biofilms since there are gradients of diffusion, meaning that in some microenvironments the antimicrobial concentration will be lower than in others [78]. Stress response induced by antimicrobial treatment can increase the mutability within biofilms, which under sublethal concentrations can favor novel resistant genotypes. Additionally, the proximity of cells in biofilms might facilitate horizontal gene transfer of e.g. AMR genes [61]. In a multispecies biofilm, additional protection is offered by members of the community [91].

CoNS are opportunistic pathogens

In the late 1990s-early 2000s, the majority of the infection-causing CoNS were antibiotic resistant isolates [54, 92]. *S. epidermidis* is the predominant member of the group encountered in infections [47, 93]. For example, it accounted for 34.7% of the bacteremia cases in a study conducted among 1760 patients of a tertiary care hospital over a period of 10 months [94].

Commensal CoNS, given a chance, can use their colonization abilities as virulence factors [1]. These staphylococci have also an arsenal of mechanisms protecting them from host immune responses, such as antimicrobial peptides (AMPs). *S. epidermidis* is, for instance, able to modify its cell surface charge when the *aps* (antimicrobial peptide sensor) defense system is activated in response to positively charged AMPs [95]. The putative transporter systems, VraG and VraF, probably are able to remove AMPs from the bacterial surface [95, 96].

In addition to the aforementioned cellular resistance mechanisms, the ability to adapt to diverse conditions within the host organism is provided by bacterial community-related mechanisms. Biofilm production is considered as a key virulence factor for *S. epidermidis* [97-99]. Other CoNS presumably also rely on their colonization mechanisms during the establishment of infection [43]. The gene products of the *icaADBC* operon (found in *S. epidermidis* and other CoNS) are responsible for synthesis of PIA [100, 101], which is the main extracellular matrix component of *S. epidermidis* biofilm. Additionally, PIA-independent biofilm formation has been described in *S. epidermidis*, e.g. Aap (accumulation-associated protein)- or EmbP (extracellular matrix-binding protein)- dependent mechanisms [102, 103]. The matrix components provide a protection from phagocytosis by host immune cells [104]. An exopolymer poly-γ-glutamic acid (PGA) of CoNS was not shown to be associated with biofilm formation, however, the corresponding gene locus is upregulated in biofilms [96]. PGA repels host antimicrobial molecules and prevents phagocytosis [105].

The complex of these and other features of CoNS generates a diversity of phenotypes within a given population, which facilitates the adaptation to the changing environmental conditions [106].

3.2. The search for solutions: discovery and study of novel antimicrobials

3.2.1. Drug discovery and development process

The challenge of treating infectious diseases triggers the search of novel antimicrobials, i.e. the drug discovery and development. This process is commonly organized in a "pipeline" of successive tasks and procedures as illustrated in Fig. 5. Although a considerable portion of all drugs, i.e. about 30 %, have been developed "purely" chemically [107], here we focus primarily on the discovery of drugs with the natural origin.

The process starts with the biomaterial collection, preparation of extracts and fractions and screening for bioactivity, which results in isolation/detection of "hits" (Fig. 5, red section), that exceed

certain activity threshold values [108]. From the "hits", the "leads" are selected, i.e. the molecules with pharmacological or biochemically relevant activity and selectivity [108]. "Leads" are developed by structure-activity relationship (SAR) investigation and rational drug design (Fig. 5, green section) together with MOA studies and disease models. This results in a "candidate", a drug-like molecule or biological therapeutic that may progress into further development [109]. The next step involves pre-clinical investigation (and optimization) of the pharmacodynamic and pharmacokinetic properties and evaluation of toxicity. Finally, clinical trials in an increasing number of human volunteers are done (phases I, II and III) and sales start, accompanied by postmarketing safety studies during phase IV (Fig. 5, green section). The whole process takes at least 10 years, and chances of a candidate to become a drug are not high. For MRSA, for example, from 13 compounds entering preclinical trials, only one has ended up as a launched drug [110]. This limited "success" rate necessitates the extensive research in the field and involvement of commercial partners [111].

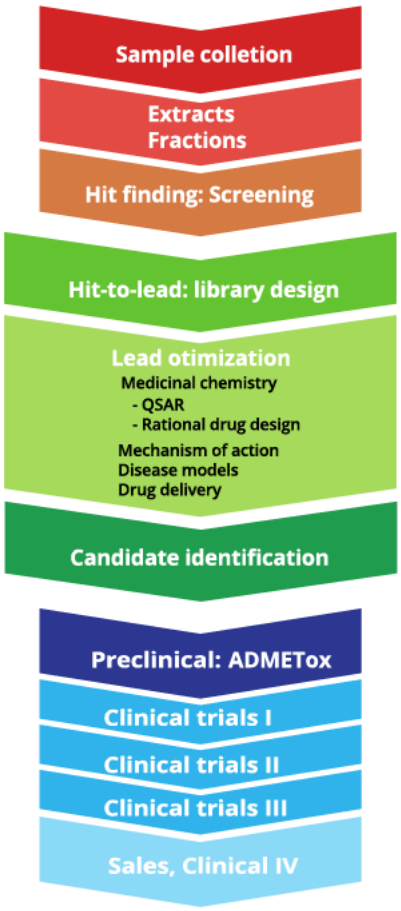

Figure 5. Example of a drug discovery and development pipeline based on bioassay-guided purification as a starting point. *The upper (red) and the middle (green) sections correspond to the drug discovery phase and the hit-to-lead-to-candidate development, while the lower (blue) section corresponds to the phase of drug(s) development. Based on Kore et al., Trosset et al., Abida et al. and Ashburn et al.* [112-115].

Historically, antibiotic drug discovery has been tightly linked to molecules produced by organisms in nature, called "natural products" (NPs), as sources of novel chemical structures [116, 117]. Although humans have been searching for and using naturally occurring bioactive substances since ancient times, this search shaped a research field, "bioprospecting", during the 20th century. According to the World Health Organization, bioprospecting is the systematic search for and development of new economically valuable products from nature. Or, briefly, bioprospecting is "looking for ways to commercialize biodiversity" [2]. The current bioprospecting concept includes several approaches that comprise the complex of activities leading to a commercially interesting candidate (e.g., a compound) with a potential to be developed into a final "product". For example, at the "starting point", living organisms can either be explored by genomic tools for the potential to produce interesting compounds, or used directly for such a production through extraction/isolation. In the latter case, the isolation and characterization of these compounds can be determined either by their novel chemistry, or their bioactivity (bioassay-guided purification) [118]. To date, drug discovery seems to be one of the main directions of bioprospecting. Fig. 5 illustrates the drug discovery and development pipeline based on the bioassay-guided bioprospecting approach. An example of the implementation of this approach is a multidisciplinary pipeline, MabCent CRI (Centre for Research-based Innovation on marine bioactivities and drug discovery, Tromsø, Norway, 2007-2015). Focused on the identification of commercially interesting bioactive substances, it coordinated the work of the national marine biobank (Marbank) and the screening platform (Marbio) between 2007 and 2015 [119].

Advances in screening techniques make it possible to establish high-throughput drug discovery platforms. Unfortunately, these platforms do not always give high yields of positive hits. For example, out of 13 000 plant NPs screened for anti-biofilm activity, only one active compound was identified [120]. Testing of a library of 4 509 compounds against *P. aeruginosa* biofilms resulted in one candidate after the second screening round [121]. In another study, out of the 66 095 compounds, 61 exhibited anti-biofilm activity [122]. In another study, 42 865 compounds included in the screening against *S. epidermidis* biofilms yielded 352 hits selected for further studies [123]. An alternative to the screening of huge numbers of "random" extracts or synthetic libraries could be the rational search, i.e. selection and/or design of compounds to be screened. For instance, looking for anti-biofilm compounds in natural environments based on antifouling observations, specific features of secondary metabolites or using the combinatorial chemistry approach [107].

3.2.2. Bioactivity testing approaches

The complexity of living organisms makes it difficult if not impossible to perform bioactivity studies *in situ*. Therefore, it is necessary to make simplified models of the biological systems and/or their components. At the same time, as "all models are wrong" [124], one should, first, make sure that a

model is appropriate for the purpose and, secondly, carefully interpret the data obtained. This certainly applies to the construction of the bioactivity testing systems and the inferences based on the results.

Antimicrobial bioactivity testing can be target-based and phenotype-based (Fig. 6) [125-127]. Target-based screening is performed by biochemical assays with purified single protein targets defined and selected in advance (Fig. 6A). The phenotypic approach uses whole-cell assays, and, therefore, reveals the activity in more biologically relevant contexts, which "pre-validates" the test compounds and their targets while performing the screens (Fig. 6B) [125, 126]. Indeed, the activity against pre-determined single protein targets does not necessarily mean the ability to affect whole cells [18, 126], which may have networks of multiple targets rather than single ones [128]. Moreover, since the observed phenotypic effect may arise from the multitargeted activity, there is a possibility to discover new therapeutic targets in subsequent target identification studies [125].

The upper section of Fig. 6 illustrates that the constant feedback loop between the pool of the test compounds and the screening results, either biochemical or whole-cell-based, directs the process from "hits" to candidate identification (compare with the respective steps in Fig. 5). The lower section of Fig. 6 gives an expanded view of the ongoing activities during this process, with the focus on the phenotype-based screening approach.

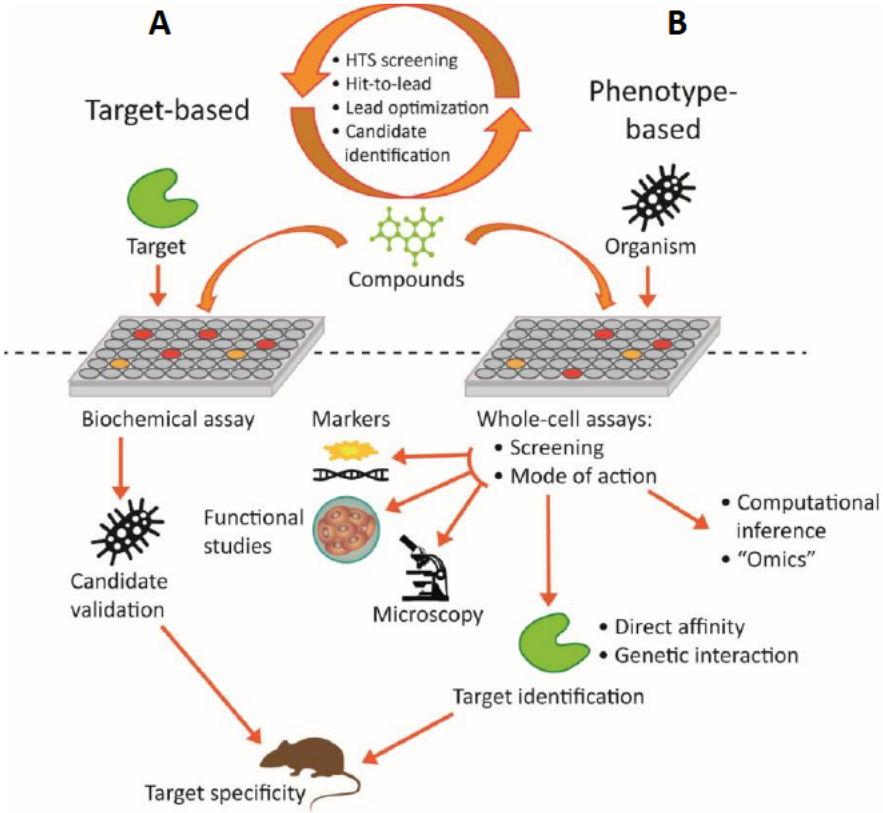

Figure 6. Bioactivity testing approaches for drug discovery and MOA studies. *(A) Target-based approach. (B) phenotype-based approach. Based on Schenone et al., Fischer et al., Kohanski et al. and Stockwell* [125, 126, 128, 129].

Initial antimicrobial testing is generally performed with planktonic bacteria using the "standard" broth microdilution inhibitory assay [130], which reveals the minimum inhibitory concentration (MIC) of the test compound. However, additional assays employing direct microscopic observation [131] or biomarker signals [132] for detection of antibacterial effects were proposed (Fig. 6). The following critical task is to infer the MOA of the leads. Common tools for the whole-cell bioactivity testing presented in Fig. 6, can be used both for the initial screenings and for the MOA studies. The "omics" techniques, such as metabolomics, transcriptomics and proteomics [133, 134] are increasingly popular tools that help to extract valuable information from high-content data sets. Computational inference tools give additional support to the information obtained empirically [125]. Examples are bioactivity profiling by comparison of test molecules with the existing databases [135, 136] or direct computer simulation [137]. Combined and integrated analysis of the data will aid the formulation of a hypothesis of the MOA of the leads. However, identification of the exact molecular targets requires subsequent experimental confirmation [125]. In the context of antibiotic drug discovery, it can be useful to indicate early, whether the compound displays bacteriostatic (inhibitory) or cidal (killing) properties. The latter often results from membrane-disruptive activity, which can be tested before attempting to elucidate intracellular targets [138]. However, antibiotic activity can be complex and involve multiple targets in addition to the cell envelope [125, 129, 139].

Molecular antibiotic targets can be revealed by direct biochemical methods (e.g., affinity purification) together with genetic and genomic methods (e.g., microarrays)[125, 129]. Overall, the integration of several aforementioned tools seems to be the most beneficial approach [125].

3.2.3. Tools for MOA studies

Some of the tools used for the screening and MOA studies within the phenotypic approach, as exemplified in Fig. 6, are described below.

Biosensors and reporter assays

Biosensor is a device (biological system) that can recognize input (environmental) signals and report it as a transduced output signal detectable by an instrument and proportional to the input signal intensity [140, 141]. Today, biosensors are ubiquitously used in life science, especially the ones based on fusions of reporter genes with regulatory elements, such as promotors [142-144]. *Table 2* gives examples of commonly used reporter genes, i. e., genes with easily detectable products. The advantages of light emission-based reporters are the possibility for kinetic measurements within the same sample and the automated signal detection, which increases the inter-laboratory comparability.

Obviously, microbial biosensors that specifically recognize certain treatments, have a potential of application in drug discovery, both in bioscreening and MOA studies. In whole-cell biosensors based

on promoter-reporter constructs, measuring the abundance of a specific marker gene or protein, gives an indication of a certain cellular phenotype [129] as the response to antimicrobial treatment. The stress response mechanisms are associated with strong induction of "pathway-specific stress promoters", which can be exploited as such markers. To identify suitable antibiotic markers, "reference compendia" based on stress-induced gene expression profiles are employed [126]. The resulting cellular biosensors can be used for categorization of unknown antibacterial agents according to their MOAs [126, 145].

Table 2. Examples of reporter genes

Product	Gene	Principle	Reference
Chloramphenicol acetyltransferase (CAT)	*cat*	Chromatographic detection of CAT reaction products in transfected cells; selectable marker	[146]
β-galactosidase	*lacZ*	Spectrophotometric/fluorometric/visual detection of substrate analogues conversion;	[147]
Green fluorescent protein (GFP)	*gfp*	Fluorescence in expressing cells when excited with UV/blue light; no external substrate required	[148]
Luciferase	*lucGR*	Fluorescence in expressing cells in presence of substrate D-luciferin, excitation not required	[149, 150]
	luxABCDE	Fluorescence in expressing cells, no external substrate and excitation required	[151]

Imaging and flow cytometry

Light signals produced by biosensors as well as light emission by various cellular structures bound to specific fluorescent labels, are commonly analyzed by microscopy and flow cytometry (FC). These tools allow researchers to track biological processes at the single cell level, including visualization and quantification of the treatment effect on microbes. Importantly, the antimicrobial test compounds can also be labelled, and a number of fluorescent drug analogues exists [84, 152, 153]. Fluorescent labelling, being relatively safe and convenient, has become more popular than radioisotope labelling. However, isotope labelling is still used, for example, in ADMETox (for "Adsorption, distribution, metabolism, excretion, toxicity") studies [154]. Imaging, especially fluorescent microscopy, is one of the promising approaches in current drug discovery, offering an arsenal of tools to choose according to the researcher's goals [155] and proven to be suitable for work with NPs [156]. Modern microscopic techniques allow for obtaining high-quality images with spatiotemporal resolution and are compatible with high-throughput screening (HTS), both *in vitro* and *in vivo* [152, 157]. Single-cell imaging followed by cytological profiling allows to identify the cellular pathways affected by test compounds [131]. Emerging label-free imaging techniques might be more important in the years to come. One example is the Raman spectroscopy used to classify antimicrobials according to their MOA [158]. It has been suggested for phenotype characterization at the single-cell level [159]. Another example is X-ray spectromicroscopy [160].

FC is another technique that has a potential in antimicrobial studies [161, 162]. It is better suited for quantitative analysis (statistics) than microscopy while giving data at the individual cell-level, but it also requires comparatively expensive equipment and trained personnel [162]. A combination of light (forward and side) scattering and DNA content measurements in bacteria in response to antibiotics of different classes, allowed to distinguish between the resistant and susceptible strains within a short time period in a dose- and time-dependent manner [163]. Furthermore, a combination of fluorescent probes allows to perform multiparametric analysis of cell response to treatment, although discrepancies between the staining techniques have been reported [161, 162]. With the advantages of being rapid and accurate, FC is a useful tool to study the heterogeneous response of bacterial subpopulations to a stress (antimicrobial treatment). Moreover, if FC is combined with fluorescence-activated cell sorting (FACS), these different subpopulations can be subsequently analyzed, for example, by proteomics/transcriptomics techniques, or in conventional growth based assays [162]. The latter can be used to isolate and characterize, e.g., viable but nonculturable cells (VBNCs) [164] and persister cells [165].

Although apparently FC is better suited for studies of planktonic cultures, while microscopy for studies of biofilms, the latter were also successfully studied using FC [166]. The combination of microscopy and FC made it possible to quantify at the single-cell level and characterize the spatial distribution of bacterial subpopulations in biofilms [162].

3.2.4. Further studies

It is extremely important to evaluate the applicability of identified candidates for the future clinical use before actual clinical trials are initiated (Fig. 5, blue section). Apart from the preliminary assessment of the potential to develop AMR, the candidates are tested in terms of pharmacokinetics. Biocompatibility is explored by imitating physiological conditions as closely as possible (in presence of buffers, serum, plasma, etc.) and followed by *in vivo* assays. These activities compose the complex of ADMETox studies (Fig. 5, blue section). If reduced susceptibility or toxic effects are revealed at this step, the ways to overcome these challenges/adverse effects are explored as well. The “omics” approaches used during the pre-clinical evaluation of potential adverse effects may help to reduce the failure rates during clinical studies of drug candidates [167].

3.2.5. Biofilm *in vitro* models and model bacteria

Technological challenges

To our knowledge, here are no universal “standard” guidelines for activity tests against biofilms and no reliable tests for biofilm susceptibility to treatment[168]. This is in contrast to liquid planktonic cultures, that are screened in broth micro- or macrodilution inhibitory assays [130]. A wide range of different

biofilm-screening techniques exists. Often biofilm test systems are based on static biofilm models, also called tissue culture plate (TCP) assays. This is similar to standard minimal inhibitory concentration (MIC) assays [130] with regards to studying growth inhibition, but biofilm eradication is assayed on an established biofilm [52].

TCP assay in particular, and biofilm assays in general, require adjustments to different species/strains and several replicates due to the high variability [169]. In addition, the plate materials and surface treatments, such as the *Nunclon™ Delta* (Thermo Scientific) treatment used for cell attachment, should be considered as they can cause the attachment of the test substances as well. A modification of the TCP assay where a biofilm is formed on the pegs fitted into a standard microplate-format tray under shear conditions, is the Calgary Biofilm Device (Innovotech, Canada) [170]. This device was approved in 2008 by Health Canada as a clinical diagnostic tool for *P. aeruginosa* infections [171].

Both biofilm models can be used for a wide range of subsequent analyses [172, 173], although certain limitations should be considered. For example, while the TCP-grown biofilms can be directly observed under the confocal microscope [174], the peg-attached bioflms of the Calgary Device have to be removed for the microscopy, making further incubation impossible [175]. At the same time, the latter allows to avoid harsh washing steps and minimize the manual handling of the samples.

"Closed" biofilm systems [176] such as the TCP-based static model, are useful for high throughput screening. They benefit from simplicity and inexpensiveness, are less susceptible to contamination and can easily be scaled up/down [177].

There are also variable continuous flow biofilm models, such as different flow cells [178], the *CDC* (*Center for Disease* Control) reactor [179], the rotating disk reactor [180, 181] and the drip flow reactor [182]. They share some common components, i. e., a pumping system, nutrient medium supply and waste collector. These systems allow for dynamic adjustments of culture conditions and better mimicking the natural environment, obtaining large amounts of biomass and direct and continuous monitoring instead of endpoint measurements. In the anti-biofilm research context, such models are preferable for follow-up and more detailed studies of few selected candidates. The benefits of a flow system can now be experienced in a microplate format, for example in a microfluidic system [183].

Assuming the overall complexity of biofilm matrix composition in addition to its organism- and conditions-specific variation, it is obviously challenging to directly compare the results obtained by different techniques. One of the possible ways to improve the comparability of the anti-biofilm studies is to perform quantitative evaluation of biofilm structures using a range of selected biofilm parameters, for example, on the basis of confocal laser scanning microscopy (CLSM) image stacks analysis [184].

Depending on the test system used, the anti-biofilm activity can be assessed in different ways:

- Based on the total biomass assessment - for evaluation of eradication activity [52];
- Based on the viability- for evaluation of killing activity:

- Metabolism (MTT [185], Resazurin [186], fluorescent tagging [184]);
- Cell integrity (SYTOX [187], propidium iodide combined with Syto9 [188]);
- Ability to grow on artificial media (CFU counts for the minimum biofilm eradication concentration [189]).

When performing anti-biofilm activity screening, the combined assessment of both killing and eradication seems to be beneficial [173], like in a system with the parallel assessment of the biomass (by crystal violet), the viability (by Resazurin) and the matrix (by wheat germ agglutinin-Alexa Fluor 488) in biofilms [173]. For more in-depth studies, like the investigation of the mechanisms of biofilm assembly-dissassembly and intercellular signaling, artificial colloidal biofilm mimics could be used [190, 191].

Biological challenges

Being a part of the biofilm community [213], the persister subpopulations mentioned in section 3.1.2 by definition contribute to the "biological challenges" associated with anti-biofilm studies.

3.3. The solutions: traditional and novel treatment strategies

3.3.1. NPs as antibacterial agents

The global challenge of infectious diseases has been addressed at different levels and possible strategies have been formulated [111]. For example, an interdisciplinary European initiative, the *Action on Antibiotic Resistance (ReAct)*, was established to develop "an independent global network for concerted action on antibiotic resistance" [214]. Another global initiative is the *Antibiotic Action* (founded by the British Society for Antimicrobial Chemotherapy), a forum that aims to promote the discovery and development of "antibiotic agents of the future" by rising awareness of the ABR problem [215]. The *NewDrugs4BadBugs* project launched by the Innovative Medicines Initiative (IMI,[216]), is focused on private-public collaboration for research on fighting the infections caused by Gram-negative bacteria [217].

The aforementioned initiatives consider the use of commercial antibiotics, which are the "classical" and currently the major agents to fight infectious diseases. In general, an antibiotic is an antimicrobial agent that can kill or inhibit the growth of bacteria. Most of the antibiotics are or originate from NPs. As the resistance development against all antibiotics introduced to the market seems to be inevitable (Fig. 2), novel synthetic or semi-synthetic antibiotic analogues are designed and developed. Antibiotics belong to diverse chemical classes and cover the major biosynthetic pathways of bacteria as their targets (MOAs), as shown in Fig. 7.

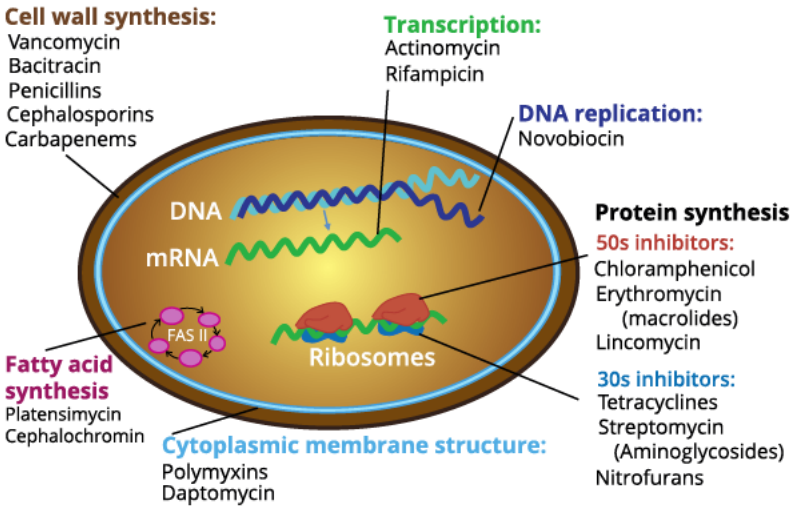

Figure 7. Classes of antibacterial agents according to their targets (or MOAs) in bacteria. *The antibiotics presented in the figure are NPs or derived thereof. However, all of these classes also include synthetic representatives. Modified from Madigan et al. and Zhang et al.* [218, 219].

In light of the risks associated with conventional antibiotics inefficiency, a number of alternatives are gaining more and more attention. So called "biologicals" (combinations of monoclonal antibodies and antibiotics, immune modulators such as cationic peptides, probiotics, vaccines, phages) are on top of the list, with some representatives being currently in clinical development [220]. Additional alternative approaches include antibiotic adjuvants, such as β-lactamase inhibitors, and various anti-virulence strategies (targeting quorum sensing, type 3 secretion systems, toxins). Such treatments could be used in combination with traditional drugs or in cocktails [15, 220].

At the same time, it is well recognized now that antibiotic discovery based on the chemical diversity of NPs, has not been exhausted, which is marked by the "renaissance" of this traditional approach in the modern context [15], meaning the flourishing of bioprospecting activities. To date, more than a million NPs have been characterized [84]. From nineteen antibiotic candidates undergoing clinical trials in 2013, eleven were derivatives of NPs [221]. The use of synthetic chemical libraries with focus on specific targets failed when applied to antibiotic research because of the incomplete understanding of complex antimicrobial targets [128]. NPs provide a rich and largely unexplored source of chemical scaffolds with antibiotic potential [15]. The exploration of the unculturable majority of microbiota has started with the recent development of genomic and metagenomic techniques [15, 222, 223]. Another approach is *in situ* cultivation which allows growing previously uncultured bacteria. This technique has already resulted in the discovery of a promising AMP Teixobactin [224].

One of the relatively novel trends is the exploration, in addition to traditional terrestrial sources, of new ecological niches and less accessible and more extreme environments. Thus, the

marine environment has been shown to be a rich source of NPs [15, 225]. Marine NPs benefit from the novelty of scaffolds compared to terrestrial ones [226]. According to an optimistic prognosis [227], marine NPs can become "a new wave of drugs". Indeed, by the year 2014, eight marine NPs were approved as drugs [228, 229]. However, there are still no antibacterials among the approved marine NP-derived drugs, to the best of our knowledge, underscoring the uncovered potential of the ocean.

Another trend in NP-related research is the combining of knowledge about NPs and opportunities of synthetic chemistry for "mimicking" the NPs. Indeed, understanding the fundamental principles underlying the biosynthesis of NPs, knowledge about the building blocks and scaffolds can be a basis for the design and development of novel synthetic compounds [222]. Application of methods for structure optimization provided by combinatorial chemistry, has resulted in the optimization of several recently approved drugs [15, 107]. NPs are still successfully used as sources of novel structures [107]. Moreover, not only structures *per se*, but also the properties resulting from such structures can be mimicked.

One example is peptidomimetics, unnatural oligomeric sequences designed to mimic biophysical and functional characteristics of AMPs [230]. Such compounds have the "necessary minimum" of chemical features which are responsible for the bioactivity in AMPs [231]. AMPs [232] are NPs with a broad-spectrum activity due to their unique MOA, i.e. they can have multiple targets usually in addition to the cell membrane, being so called "dirty drugs" [233]. At the same time, AMPs may suffer from certain drawbacks, such as toxicity, possible resistance development, limited bioavailability and structure complexity, the latter leading to high production expenses [230, 233, 234]. To overcome all these AMP-associated challenges, peptidomimetics with improved pharmaceutic characteristics are designed.

3.3.2. Biofilm treatment strategies

Another crucial aspect related to infectious diseases is the problem of chronic and recurrent infections. Being often hospital-acquired infections, they can be complicated by ABR, as the clinical environment with high selective pressure promotes resistance development and spread [235]. Chronic infections are believed to be mostly associated with biofilms and require specific treatment approaches [236] [76], as conventional treatment may fail to eradicate a biofilm, leading to a recurrent infection.

Therefore, the therapeutic regimens are optimized specifically for biofilm-associated infections [86]. Systemic antibiotic prophylaxis [237] and antibiotic lock therapy [238] applied to lower the risk of contamination and to sterilize the infection site are some examples. However, quite often the excision of infected tissue and removal of the colonized device is still the best solution [86].

According to Miquel et al., the term anti-biofilm stands for "a natural or induced process, leading to reduction of bacterial biomass through the alteration of biofilm formation, integrity and/or

quality" [239]. The wide range of anti-biofilm strategies that have been or are being developed reflects the extensive research in this area. Anti-biofilm strategies can be categorized based on the mechanism (targeting bacteria/targeting biofilm), the nature of treatment (physical/chemical), the target stage of the biofilm life cycle (attachment/maturation/dispersal), etc. These strategies can give synergistic effects when used as combinations [240] [241, 242]. A brief and non-comprehensive summary is presented in *Table 3*.

The occurrence of persisters is an important recalcitrance determinant in biofilm-related chronic infections. An antimicrobial agent that can disable the formation of persisters and thus make conventional antibiotics effective, would be a promising solution for anti-biofilm combination treatment [26, 243, 244]. Several compounds targeting persisters have been reported [245-247]. Targeting the cell membrane, which is essential in cells independently of their metabolic state is also a promising approach [24, 85].

Table 3. Anti-biofilm strategies [a].

Treatment/strategy	Mechanism	Examples
Chemical (biochemical)		
Prevention	Anti-adhesion	Coating of surfaces: Chlorhexidine-silver sulphadiazine, minocycline-rifampicin, organoselenium, triclosan, AMPs
		Targeting attachment appendages: Mannosides, pilicides, curlicides
		Antibodies neutralizing attachment molecules: Vaccine based on *S. aureus* antigens, anti-*Pseudomonas* immunoglobulin Y
	Anti-matrix	Enzymes: DNAse, Dispepsin B, lysostaphin
		Chelating agents: Sodium citrate, minocycline-EDTA
		Matrix biosynthesis inhibitors: Allicin
	Signalling inhibitors	Halogenated furanones
	Complex effect (signal interference, anti-adhesion, etc.)	Polysaccharides: Pel, Psl from *P. aeruginosa*
Weakening/Eradication	Antibacterial	Silver nanoparticles
		AMPs: cathelicidines, colistin, daptomycin
		Phenolic and quaternary ammonium cation compounds: ageloxime D, ellagic acid, berberine
		Terpene-based NPs
		Conventional antibiotics: linezolid, rifampicin, fluoroquinolones
	Anti-matrix	Enzymes: DNAse, dispepsin B, alginate lyase
		Chelating agents (in combinations): Metals, tetrasodium-EDTA
	Anti-virulence	Neutralizing antibodies: β- lactamase-specific antibodies
	Signalling inhibitors	Affecting dispersing signals, quorum sensing and c-di-GMP (cyclic diguanylate): Azythromycin, ajoene, D-amino acids, norspermidine, furanones, RNA III inhibiting peptide and hamamelitannin
Physical (biophysical)		
Prevention	Non-invasive sterilization	Ultraviolet C treatment of surfaces
	Modified surface topography	Attachment-repelling anodic nanoporous surfaces, “sharklet” micropattern
	Repulsion of initial attachment	Low-energy surface acoustic waves
Eradication	Physical excision	Surgical
	Destruction by microbubbles	Ultrasound
	media electrolysis/ improved antibiotic binding/ increased matrix permeability	Electric field: Alternating, direct currents and superimposed potentials
Biological		
Prevention		Probiotics: *Lactobacillus*, *Bifidobacteria*
Eradication		Bacteriophages in combination therapy

[a] Based on Bjarnsholt et al., Miquel, Harvey and Kostakioti [239] [61, 221, 248] and references therein.

4. Summary of the main results

Paper I

Synthesis and antimicrobial activity of small cationic amphipathic aminobenzamide marine natural product mimics and evaluation of relevance against clinical isolates including ESBL–CARBA producing multi-resistant bacteria

A library of small synthetic MNPM aminobenzamide derivatives was constructed and tested against relevant bacterial panels in MIC assays, to select the lead molecules based on antimicrobial activity profiles.

- Several compounds were potent against Gram-positive bacterial reference strains; the most potent compound **E23** displayed the MICs of 0.5-2 µg/ml (1.1-4.2 µM) and a good selectivity towards bacteria (selectivity index, SI, of 37).
- The potency of nine selected structurally diverse MNPMs was confirmed in tests with 25 clinical isolates of common human pathogenic bacteria; the activity of two of these MNPMs, i.e. **D19** and **E23**, was further verified by screening against 250 more isolates.
- Clinical isolates of MRSA and vancomycin-resistant enterococci (VRE) were susceptible to **D19** and **E23** as well, while the extended spectrum β-lactamase - carbapenemase (ESBL-CARBA) producing Gram-negative isolates were slightly less susceptible (MICs ≥16 µg/ml, ≥33.7 µM).
- An *in vitro* luciferase assay with several derivatives from the library revealed that the MOA resembled that of membrane-targeting antimicrobials.

- *Overall conclusion: Structural motifs found in marine natural antimicrobials can be a valuable source for making novel antimicrobial lead-compounds, such as **E23**, as verified by the expanded in vitro screenings against clinical isolates.*

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Paper I

Paper II

Paper III

Paper IV