Engineered lysins as a solution for burn wound infections

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Abstract

The development of antimicrobial resistance (AMR) has rendered most of the (small-molecule) antibiotics ineffective against common infectious illnesses. Among these, burn injuries are commonly infected by (multi-)drug resistant pathogens, namely *Acinetobacter baumannii*. Additionally, these infections are often associated with biofilm formation which hinders the efficacy of the current treatments. However, with the discovery of the therapeutic potential of endolysins, a new antibacterial weapon has arisen: engineered lysins.

1. Antimicrobial resistance

Microbial organisms, such as bacteria, viruses and fungi, are a crucial part of the global ecosystem. The majority of these organisms are essential for the well-being of their hosts, with whom they establish important interactions. With the discovery and further development of antimicrobial agents and antiviral compounds, infections that used to be fatal could be overcome. However, several of these drugs have lost their potency due to resistance development [1].

Antimicrobial resistance has become one of the major worldwide health threats of the 21st century [1]. The World Health Organization (WHO) defines antimicrobial resistance (AMR) as the phenomenon that occurs when microorganisms such as bacteria, viruses and funghi evolve in ways that render the antibiotics used to cure the infections they cause ineffective. When resistance arises specifically in bacteria, it can be designated as antibacterial resistance (ABR) [2].

ABR - Reasons and Consequences

The reasons behind ABR development are multiversed, ranging from bacterium's evolution to human misconception of how to correctly use antibiotics [3]. The mode of operation of antibiotics relies on creating a selective pressure upon the bacterial population, preventing proliferation. However, a bacterium surviving this challenge becomes the dominant genotype amongst the population, spreading this characteristic among the future population [3]. Antibacterial resistance is acquired through genetic modification, which can be triggered by multiple factors, such as mutation and gene transfer. This leads to phenotypic changes, making bacteria resistant to antibacterials [4].

Aside from the bacterium's ability to adapt and evolve, human behaviour plays an essential role in this problem. Over the last decades, the world population has seen its numbers rise significantly, with the total world population doubling from 1950 to 1987 [5]. With the facilitation of transportation across the globe, it has never been easier for drug-resistant strains to spread faster and more widely [3]. Additionally, antibacterial drugs are frequently misused, both from the clinician's and the patient's perspective. For instance, the prescription of antibiotics without a thorough diagnosis is commonly used as a resort for a quick-fix solution [1, 6]. The agricultural sector has also been identified as a perfect reservoir for the development of resistance genes due to the excessive usage of antibiotics in crops for growth boosting and disease prevention [1, 7].

This imminent health threat has the potential to wreak havoc on global health, as infections with resistant bacteria lead to longer illnesses, increased mortality and prolongued stays in the hospital. Moreover, it can cause significant economic losses due to increased health expenses and decrease in productivity [7].

ABR - Solutions

With the increasing potential for new virulent and lethal pandemics, the collaboration between government agencies and national health/welfare

agencies has been fundamental in developing long-term plans to combat AMR. In particular, the World Health Organization (WHO) developed a Global Action Plan in 2014 which addresses AMR by following five strategic objectives in order to reduce mortality due to infectious diseases.

This problem is multi-faceted, which means it can be approached by multiple different routes. The first step in solving this crisis is to implement prevention measures such as educating the population about the situations in which antibiotics can be successfully used and encourage to follow the vaccination programs [8, 9]. In places with extensive use of antibiotics, such as hospitals, two of the strategies used to slow down the evolution of resistance comprise cycling and mixing antibiotics [10]. The former consists of using a specific class of antibiotic for a period of time, followed by a different class. The latter consists of using multiple distinct antibiotics in different patients to avoid spreading potentially resistant bacteria from patient to patient [10]. A study by McCaughey *et. al* (2013) showed that using a combination of fosfomycin and tobramycin against *Pseudomonas aeruginosa* and *Staphylococcus aureus* could prevent the development of resistance in a larger extent when compared to each of the antibiotics alone [11]. Nevertheless, it is still not clear whether this type of treatment is always beneficial.

Other forms of treatment have been developed, such as the use of monoclonal antibodies addressing toxins produced by certain bacteria [12], the use of bacteriophages and the development of vaccines [13]. In addition, the development of faster and more efficient diagnostic procedures could indirectly result in slowing down multi-drug resistance, since it would avoid treatments with inappropriate antibacterial drugs [9]. In this scope, the use of sequencing methods can help in identifying and profiling resistant microbes, which ultimately allows to choose the best way to treat infections [14].

Likewise, antimicrobial resistance is of significant importance in burn wound infections. Studies have shown that 42% to 65% of the total amount of deaths in burn victims are attributable to infections [15]. Thus, antibacterial resistance in burn injuries is likewise an urgent health concern that needs to be addressed.

2. Burn wounds - a global public health problem

Characterization of burn wounds

The skin is the largest organ of the human body, comprising 1,8 m^2 of surface area. Although colonised by a large variety of microorganisms, such as bacteria, its primary function is to protect the human body against foreign microorganisms [16, 17]. It is composed of the epidermis, dermis and a subcutaneous fatty region, as depicted in Figure 1. Each of these layers is enriched with multiple distinct structures, such as hair follicles, sweat glands, nerves, blood vessels and lymphatics [18]. Keratinocytes are the major cell type present in the epidermis and are responsible for creating a barrier against the entry of foreign microorganisms into the organism [17].

The immune response of the skin is fundamental upon wounding/infection. It also modulates the commensal microbiota that colonise the skin. In this scope, keratinocytes play a key role in the defense and detection of pathogens [19], as they express a number of immune receptors designated pattern recognition receptors (PRRs). These receptors sample skin bacteria and recognize pathogen-associated molecular patterns (PAMPs), such as lipopolyssacharides (LPS) from Gram-negative bacteria and lipoteichoic acids from Gram-positive bacteria [17]. Keratinocytes respond to microbes or tissue damage by releasing a broad range of inflammatory mediators, such as cytokines, chemokines and antimicrobial peptides [19]. Chemokines are a class of small proteins essential in recruiting T-cell and innate effectors to the site of infections, in a process called chemotaxis [20]. Cytokines direct the immune response to induce appropriate infection clearance mechanisms [21]. On the other hand, AMPs have the ability to directly kill bacteria, fungi and enveloped viruses. AMPs can also influence the immune response, further affecting inflammation [22].

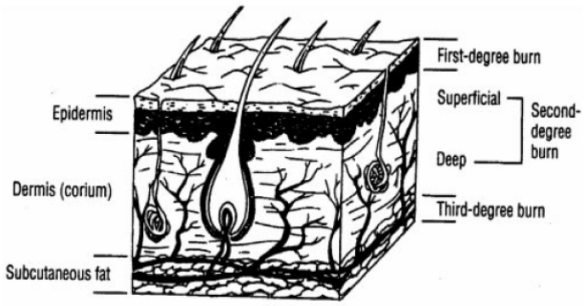

Figure 1: Schematic of skin anatomy and classification of burn injuries according to the depth of the injury. Adapted from [23].

Skin disruption can have multiple causes. Burn injuries is an example that results in the dysregulation of the host-skin microbiome and might lead to infections with opportunistic pathogens such as skin colonisers [24].

Annually, approximately 180 000 deaths are caused by burns, with the majority of them occur-

ring in low-income and middle-income countries [25]. Besides the high annual number of deaths caused by burns, wound infections is a leading cause of morbidity and mortality in burn patients [26].

Burn wounds breach the skin, causing the loss of the human's protective barrier against environmental microbes and the exposition of highly nutritive serum. A favourable environment for microbial growth and invasion is created, leaving burn patients more susceptible to local and even systemic invasion by opportunistic pathogens [26, 27].

A burn wound can get infected in multiple stages of the healing process. Accordingly, different microorganisms colonise the wound. *P. aeruginosa* is the most frequently found Gram-negative pathogen in a burn wound infection, but other species such as *A. baumannii*, *Escherichia coli* and *Klebsiella pneumoniae* are also found in established wound infections illustrating the polymicrobial nature of burn wound infections [26]. *Candida* species are the most prevalent fungi in burn wounds, although other fungi are emerging as well. Wound invasion is usually performed by fungi and drug-resistant bacteria, namely, multidrug-resistant *Pseudomonas* and *Acinetobacter* species as well as methicillin-resistant *S. aureus* [26].

Multi-drug resistant strains of bacteria have become more frequent and increasingly difficult to treat, causing an unanticipated rise in drug-resistant burn wound infections along with an increase in sepsis and associated deaths worldwide [26]. Although *P. aeruginosa* remains the main species to be responsible for sepsis and death related to burn infection, *A. baumannii* has been observed with increased frequency [28]. This pathogen can easily survive in environments with unfavourable conditions, such as hospitals. It also has the ability to colonise and form biofilms on both biotic and abiotic surfaces. Additionally, drug-resistant *A. baumannii* is often associated with biofilm formation in burn wounds, preventing antibacterial activity of topical agents used for burn treatment [29].

Biofilms in burn wounds

Biofilms are defined as microbial communities in which bacteria are embedded in a matrix. These communities can be attached to both a biotic or abiotic surface and can also be found in submerged or humidified conditions [30]. Biofilms can cause havoc in many different settings, ranging from industrial piping systems to medical devices, such as catheters and implants. The latter has become an emerging health concern, since medical devices colonised with biofilms often cause chronic infections [31].

A. baumannii is a Gram-negative, aerobic, opportunistic pathogen, responsible for a vast number of nosocomial infections due to its increased antibiotic resistance and virulence [32]. Its ability to colonise and form biofilms on both living and non-living objects remains one of the most relevant causes for chronic infections [33]. In fact, isolates of *A. baumannii* recovered from blood, urine, burned skin and catheters have been observed to form biofilms [34].

Biofilm composition highly varies from microorganism to microorganism. While bacteria account only for less than 10% of the dry mass, the matrix accounts for over 90%. The matrix is composed mainly of water and different types of biopolymers, known as extracellular polymeric substances (EPS), which include polyssacharides, structural proteins, enzymes, nucleic acids and lipids [35]. The self-produced EPS plays a fundamental role in shielding the bacteria from environmental threats, such as shear forces and host immune defenses [36]. Additionally, the matrix holds the bacteria together in a biofilm and retains water, resulting in organisms tolerant to drought. Another important function of the matrix is communication among microorganisms, which is facilitated by the close proximity existing in microorganisms that live in a biofilm. It also aids exchange of genetic information between biofilm cells [35].

Biofilm formation is mainly coordinated by quorum sensing (QS), bacterial cell-to-cell communication used to coordinate gene expression. This system is also involved in other processes such as symbiosis, virulence, conjugation and motility [37, 38]. QS monitors cell-population density by measuring the concentration of secreted signal molecules, termed autoinducers. When a threshold concentration of autoinducers is achieved, signal transduction cascades are triggered leading to alterations in gene expression and a consequent response in bacterial population [39].

The only QS system of *A. baumannii* is similar to the typical LuxI/LuxR system found in other Gram-negative bacteria, and can be observed in Figure 2. It is based on an acyl homoserine lactone (AHL) auto-inducer, comprising an enzyme (AbaI) synthesizing the auto-inducer and a receptor protein of the QS system (AbaR) [40]. The receptor protein binds to the AHL signal molecule, inducing a cascade of reactions. Although five minor AHLs have been detected in culture supernatants of an *A. baumannii* strain, the most predominant AHL molecule is N-(3-hydroxydodecanoil)-L-homoserine lactone (3-OH-C12-HSL) [41].

Biofilms are associated with a large number of human infections. According to the National Insti-

tute of Health and the Center for Disease and Prevention of the USA, it is estimated that 65% to 80% of human infections involve biofilm formation [32]. Some examples of diseases reported to be associated with bacterial biofilms include colitis, conjunctivitis and otitis [42]. Most importantly, bacterial biofilms are often involved in burn wound infection.

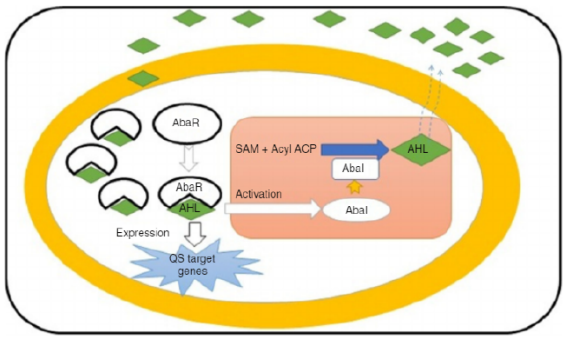

Figure 2: Illustration of the quorum-sensing mechanism in *A. baumannii*. In the auto-inducer synthesis process, AbaI uses S-adenosyl methionine, which binds to the Acyl group of the acyl-carrier protein, leading to the production of the AHL signal molecule. AHL will bind to the receptor protein, AbaR, triggering a series of reactions, controlled by QS target genes. Adapted from [41].

It has been established that biofilms show elevated tolerance against a significant number of antibacterial agents, compared to the bacteria in a planktonic culture. This is explained by the number of mechanisms bacteria have developed, conferring them with antibacterial resistance and/or tolerance [30, 43].

Bacterial resistance and bacterial tolerance have been introduced as different concepts over the last years: bacteria are tolerant when they are able to survive in the presence of antimicrobial agent, yet incapable of proliferating; resistance, however, relates to the bacteria's capacity to proliferate under the same conditions [44]. An example of a mechanism that contributes to antibacterial tolerance is the biofilm's role as a penetration barrier, delaying antibiotic diffusion [43]. This mechanism (represented in Figure 3) can happen due to chemical reactions between the antibiotic and the extracellular matrix or sequestration of the antibiotic by binding to polyssacharides [43, 45]. It has been reported that antibiotic penetration is hindered only for some antibiotics such as vancomycin (in *S. aureus* biofilms) and chloramphenicol (in all *E. coli* biofilms) [46].

Another tolerance mechanism is characterized by a slow bacterial growth rate, since conventional small-molecule antibiotics are most effective against metabolically active cells [47]. When a bacterial cell culture becomes starved for a particular nutrient, a cellular stress response is induced, characterized by repression of growth and division [45]. Thus, since some biofilms experience reduced metabolic activity, it might justify the enhanced tolerance to treatments with antibiotics that typically target growth factors in planktonic bacteria [48]. A study by Tanaka *et. al* (1999) evaluated the impact of growth rate in antibiotic treatment of *P. aeruginosa* biofilm. When testing β-lactams and fluoroquinones, it was revealed that the former had weaker bactericidal activity to biofilm cells and displayed greater activity in younger, growing biofilm cells [49].

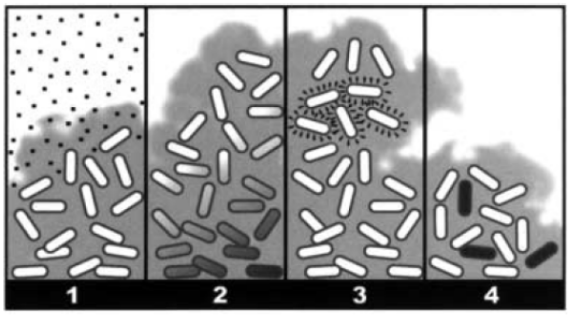

Figure 3: Biofilm resistance and/or tolerance mechanisms. 1 - Slow or incomplete antibiotic penetration. 2 - Shaded cells represent zones of slow or non-growing bacteria: nutrient limitation induces cellular growth repression. 3 - Marked cells represent adaptative stress response. 4 - Dark cells represent persister cells. Adapted from [43].

Antibiotic treatment of microbial populations is also hampered by the existence of a persister phenotype among them, confering temporary tolerance for antimicrobials [45]. Persisters are (multi-)drug tolerant cells which adopt a slow or non-growing rate, by transforming into a dormant state or selectively inactivating biological processes typically targeted by antibiotics. This type of cells has no acquired resistance through genetic modification, demonstrating the stochasticity of this event in microbial populations [50]. Once antibiotic pressure starts to drop, the surviving persister cells will create a population that is as susceptible as the original cell population, with a similar proportion of persisters [50]. This new population can then cause a relapsing infection [45].

While resistance is commonly attributed to genetic factors, resistance towards certain classes of antibiotics may also be intrinsic and dependent on innate characteristics of the cell. In Gram-negative bacteria, one of the most conventional examples is

the presence of an outer membrane which is impermeable to many molecules, and the expression of MDR efflux pumps that act by reducing the intracellular concentrations of the drug [51]. Another common escape mechanism from antibiotics are efflux pumps: transmembrane proteins whose role is to remove specific compounds, such as antibiotics, toxins and waste metabolites, from within the bacterial cell into the external environment [52]. Efflux pumps have a multifunctional role in biofilm formation. Generally, they can contribute to the efflux of EPS and QS molecules to facilitate matrix formation and regulate QS. *A. baumannii*, which has been characterized by contemplating three efflux systems belonging to the RND superfamily (AdeABC, AdeFGH and AdeIJK), has shown that it requires a certain expression profile of efflux pumps to initiate and maintain biofilm formation [53]. For example, it has been shown that overproduction of AdeABC and AdeIJK alters membrane composition, leading to decreased biofilm formation due to the underexpression of proteins belonging to chaperone-usher pilus assembly systems [54]. These are known to play a major role in the initial stages of biofilm formation, by promoting initial adhesion and surface colonisation but also formation of microcolonies.

Additionally for *A. baumannii*, the pathogen contains a multitude of virulence genes contributing to biofilm formation and pathogenicity [40]. Such genetic elements are controlled by complex regulatory networks, based on the presence of antibiotic resistance genes, environmental conditions or cell density [40]. Genes associated with biofilm formation are the *csu* operon, the *pga* locus, *ompA* and *bap* [55].

The *pga* locus, for instance, encodes genes for poly-N-acetyl glucosamine (PNAG) synthesis [40]. PNAG is one of the most relevant polysaccharides in biofilm formation in both Gram-positive and Gram-negative microorganisms. This has been proved by the creation of a knockout: a deletion mutant of *pgaABCD* in an *A. baumannii* S1 strain resulted in the loss of a strong biofilm phenotype, which was restored after complementation [56].

OmpA is a prominent porin in Gram-negatives and thus *A. baumannii*, which contributes to passive drug extrusion across the outer membrane, revealing its role in antimicrobial tolerance. This porin couples with inner membrane efflux systems, such as efflux pumps [33]. Furthermore, OmpA targets mitochondria upon binding to host epithelial cells, leading to the release of proapoptotic molecules and consequent induction of apoptosis [57]. It is still not clear whether OmpA plays a direct or indirect role in bacterial attachment and biofilm formation. Nonetheless, OmpA inactivation leads to alterations in the bacterial cell wall, significantly decreasing the minimal inhibitory concentration (MIC) of some antibiotics such as chloramphenicol, most likely due to the destabilization of the outer membrane [33].

Other factors contribute to pathogenicity in *A. baumannii*, such as LPS and capsular polysaccharides. In fact, a study by Geisinger *et. al* (2015) showed that capsular polysaccharides increase the antimicrobial tolerance in *A. baumannii*: mutants deficient in the production of these polysaccharides have lower intrinsic resistance to peptide antibiotics, such as colistin, erythromycin and rifampicin. In the presence of chloramphenicol and erythromycin, hyperproduction of capsular polysaccharides is triggered as well [58].

Thus, *A. baumannii* has become one of the top priority pathogens to which new antibiotics must be developed, according to the WHO [59]. *A. baumannii* has acquired resistance against several classes of antibiotics, with carbapenem-resistance being currently the most alarming threat [60]. Carbapenems are a class of β-lactam antibiotics and one of the most commonly used antibiotics for multi-drug resistant infections [59]. *A. baumannii* possesses a number of resistance determinants such as β-lactamases and aminoglycoside-modifying enzymes, which confer resistance against β-lactams and aminoglycosides, respectively [60].

Notwithstanding, several *A. baumannii* isolates have been reported to be resistant to several other classes of available antibiotics, such as aminoglycosides, fluoroquinones and polymyxins [40, 60]. Likewise, drug-resistant *A. baumannii* has been associated with biofilm formation in burn wounds, hindering the antibacterial action of topical agents used for treatment [29].

Among the solutions designed to address this challenge, an alternative was designed based on a promising novel class of antibacterials, with a unique mode of action: endolysins.

3. Endolysins - A Solution to Antibacterial Resistance

Endolysins are lytic enzymes produced by bacteria-invading bacteriophages which contribute to the degradation of the peptidoglycan from within the host cell, leading to cell lysis [61]. More specifically, these enzymes degrade the peptidoglycan layer of the host after translocation over the inner membrane through small hydrophobic proteins: holins. Once a critical concentration is reached, holes are created through the cytoplasmic membrane by oligomerization, allowing the endolysins to access the peptidoglycan layer [62].

A key difference between Gram-negative and

Gram-positive bacteria is the composition of the cell wall (Figure 4). Both Gram-positive and Gram-negative bacteria have peptidoglycan as a common polymer in the cell wall. However, whereas peptidoglycan comprises 30% to 70% of the cell wall of Gram-positives, this is only 10% in Gram-negatives [63]. Moreover, Gram-negative bacteria cell walls also contain lipopolysaccharide (LPS) and proteins, whereas Gram-positive microorganisms have teichoic acids [63].

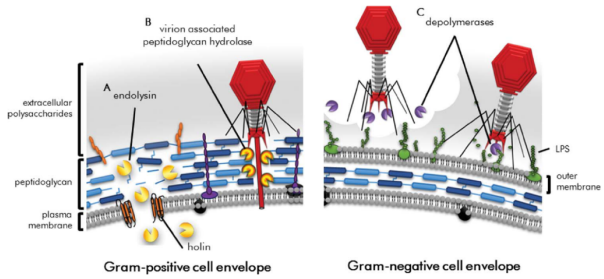

Figure 4: Schematic of Gram-positive bacterial cell envelope and Gram-negative bacterial cell envelope. Adapted from [64].

Additionally, one of the main differences is the presence of an outer membrane (OM) in Gram-negative bacteria, contrary to Gram-positive. The OM is a lipid bilayer with an inner leaflet composed of phospholipids and an outer leaflet with phospholipids anchored to LPS. The phosphate groups and acidic sugars of the LPS molecules provide the cell surface with a negative charge. Divalent cations (Mg^{2+}, Ca^{2+}) stabilize the OM through ionic interactions with the phosphate groups of adjacent LPS molecules. The peptidoglycan layer of Gram-negative organisms resides subjacent to the OM. Likewise, the surface proteins and carbohydrates usually found in the peptidoglycan layer will be present in the OM. This structure conveys the outer membrane with high asymmetry and, consequently, exceptional impermeability [65].

Regarding the composition of the peptidoglycan layer, it consists of linear strands of alternating N-acetylmuramic acid (MurNAc) and N-acetylglucosamine (GlcNAc) residues, coupled by β(1-4) linkages, which altogether comprise the glycan polymer in the peptidoglycan. This polymer is covalently linked to a short stem peptide through an amide bond between MurNAc and an L-alanine, the first amino acid of the peptide component. The remainder of the stem peptide is composed of alternating L- and D-form amino acids. These are well conserved in Gram-negative bacteria but have variable composition in Gram-positive bacteria [66]. For numerous Gram-positive bacteria, the third residue of the stem peptide is L-lysine, which is respectively linked to an opposing stem peptide on a separate glycan polymer through an interpeptide bridge. However, Gram-negative bacteria usually contain a mesodiaminopimelic acid (mDAP) residue at position three instead of L-lysins. In this case, mDAP residue cross-links to the terminal D-alanine of the opposite stem peptide, without establishing an interpeptide bridge [66].

Endolysins can be classified according to their catalytic activity site in the peptidoglycan layer, as depicted in Figure 5 [64]. They can have glycosidase, amidase, endopeptidase or lytic transglycosylase activity. A glycosidase cuts between glycan residues, whereas an amidase hydrolyses the amide bond between the glycan moiety (MurNAc) and the peptide moiety (L-alanine). Endopeptidases degrade peptide bonds between two amino acids, and a transglycosylase degrades the β(1-4) linkage between MurNAc and GlcNAc [64, 66]. Glycosidases are further categorized as N-acetylmuramidases that cleave the glycan component of the peptidoglycan on the reducing side of MurNAc, or as N-acetylglucosaminidases that cleave the glycan component of the peptidoglycan on the reducing side of GlcNAc [64, 66].

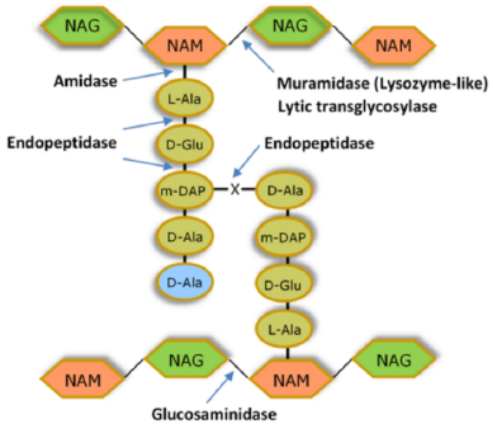

Figure 5: Basic structure of the bacterial cell wall peptidoglycan and representation of cleavage sites by endolysins. Adapted from [67].

Applications of endolysins as antibacterials was initially limited to Gram-positive organisms, since their cell wall is not protected by an outer membrane [61]. However, different endolysins have been reported to have an intrinsic antibacterial activity against Gram-negative pathogens: *in vivo* studies with *A. baumannii* ATCC 17978 reported over 99% of antibacterial rate following incubation, for 1 hour, with endolysins LysAB3 and LysAB4 [68]; 0.5 mg/mL of endolysin LysPA26 is able to kill up to 4 log units of *P. aeruginosa* D204 in 30 minutes, when incubated with 10^8 exponential cells of

host bacteria, in the absence of EDTA. The bacterial cells were more sensitive when treated with 1mM EDTA, which resulted in the chelation of the divalent cations that stabilize the outer membrane, causing its disruption [69].

Endolysins can be classified as modular or globular endolysins, according to their structure. Modular endolysins are composed of an enzymatically active domain (EAD) and a cell wall binding domain (CBD) attached to a short linker region that connects the EAD to the CBD. Globular endolysins, on the other hand, are composed of a unique EAD. The EAD acts by breaking a specific bond in the peptidoglycan structure, while the CBD targets the EAD to its substrate by binding peptidoglycan or another cell wall component [65].

Generally, the vast majority of endolysins derived from Gram-positive bacteria are modular with an N-terminal EAD and a C-terminal CBD, whereas endolysins derived from Gram-negative organisms are usually single-domain, globular proteins and lack CBDs [64, 70]. These endolysins will typically consist of a single catalytic domain and have a mass of 15 to 20 kDa [66]. It has been speculated that the presence of a CBD in endolysins from Gram-positive infecting phages but not in Gram-negative equivalent is justified by the high affinity of a CBD for its ligand, with the CBD keeping the endolysin tightly bound to cell debris after cell lysis [71]. This way, new potential host cells are prevented from lysis before being infected by the phage virions. The presence of an OM in Gram-negative bacteria eliminates this risk, rendering a CBD unnecessary in the composition of endolysins from Gram-negative infecting phages [65].

Although most have a globular structure, some endolysins derived from Gram-negative infecting phages have been reported to contain a modular structure, with a C-terminal EAD and an N-terminal CBD. The first two endolysins found with this composition in a Gram-negative microorganism derived from *P. aeruginosa* infecting-phages, named KZ144 and EL188 [72].

The presence of the OM in the cell wall of the Gram-negative bacteria makes the exogenous addition of an endolysin insufficient to obtain access to the peptidoglycan without a mechanism to translocate the protein across the OM [66]. However, the integrity of the outer membrane can be disturbed by certain agents that weaken the stabilizing interactions between OM components: outer-membrane permeabilizers (OMPs) [73]. OMPs can be of physical, chemical or biological origin, according to the type of OM permeabilization. Considering OMPs of chemical origin, two classes can be considered: polycationic agents, which act by competing with the stabilizing divalent cations of the outer membrane for the negatively charged LPS. The cations are consequently displaced leading the disarrangement of the OM [74]; the other class is represented by chelators with EDTA as a commonly present compound. EDTA removes by chelation the stabilizing divalent cations from their binding site in LPS, resulting in the release of a significant proportion of LPS from the cells, and hence OM disruption [74].

Regarding OMPs of biological origin, Artilysins are a versatile approach based on a novel type of protein-engineered endolysins. The principle of Artilysins is centered on the fusion of highly-active bacteriophage-encoded endolysins to outer membrane-permeabilizing peptides.

Artilysins - An answer to Gram-negative pathogens

Artilysins are a new class of antibacterials, with the capacity to penetrate the outer membrane. These enzymes covalently combine highly active endolysins with outer membrane-permeabilizing peptides, which can be introduced in the form of antimicrobial peptides (AMP) [65]. Several AMPs possess outer membrane destabilizing properties, which accounts for the potential of Artilysins [65]. AMPs are produced by a wide variety of organisms and have quite diverse amino acid sequences. Typically, outer membrane destabilizing peptides possess an amphipathic conformation. However, the overall positive charge of the peptides allows them to accumulate at the polyanionic cell surface of the bacterium, which corresponds to the LPS of the outer envelope in Gram-negative bacteria. Therefore, Artilysins can be designated as engineered endolysins with LPS-destabilizing properties.

Artilysins do not need an active bacterial metabolism to employ their bactericidal effect, given that they actively degrade the peptidoglycan layer, resulting in immediate osmotic lysis [75]. Additionally, an interesting study by Briers *et. al* (2014) reported even that the activity of a series of Artilysins (LoGT-001 to LoGT-014) was enhanced by the presence of a linker of increasing length, which suggested that linker length may influence the Artilysin antibacterial activity [76].

Art175 is an example of an efficient Artilysin against *P. aeruginosa*, consisting of a SMAP-29 peptide (sheep myeloid antimicrobial peptide) comprising 29 amino acids fused to the KZ144 endolysins. Art175 has a superior bactericidal effect against persister cells [75]. Remarkably, a more recent study proved that Art175 is equally effective in killing both stationary-phase cells and persister cells of multidrug-resistant *A. baumannii*. Moreover, killing could be enhanced with the addition of 0.5 mM EDTA [77].

In skin infections, Artilysin LoGT-008 demonstrated strong antibacterial activity against both *P.aeruginosa* and *A. baumannii*, with MICs of 4 and 8 μg/mL, respectively [76].

Endolysins: sustainable solution to AMR?

An important advantage for endolysins as a solution against AMR is that the development of resistance seems unlikely. Multiple reasons seem to support this idea, such as the continuous coevolution observed among phages and their respective host bacteria. Several studies have been executed to assess potential bacterial resistance against endolysins: for instance, a study with cells of *Streptococcus pneumoniae* repeatedly exposed to the Pal endolysin did not contribute to the development of resistant phenotypes [78].

Additionally, endolysins are highly specific and recognize highly conserved structures in the cell wall for cleavage [79]. The fact that several of the endolysins possess two EADs that hydrolise different bonds in the peptidoglycan is also believed to reduce chances for acquiring resistance [80].

More importantly, endolysins can be used combined with antibiotics to treat infections, resulting in a synergistic effect effective against bacterial infections [80]. All in all, endolysins appear to not be significantly susceptible to bacterial resistance strategies.

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Note to the reader

A Confidentiality Disclosure Agreement (CDA) has been signed by the Katholieke Universiteit Leuven and Instituto Superior Técnico - University of Lisbon. Therefore, the main goal of the project and the results obtained are confidential (and protected by the CDA) and are not be presented in this document.

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