From endolysins to Artilysin®s: novel enzyme-based approaches to kill drug-resistant bacteria

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Abstract

One of the last untapped reservoirs in nature for the identification of new anti-microbials is bacteriophages, the natural killers of bacteria. Lytic bacteriophages encode peptidoglycan (PG) lytic enzymes able to degrade the PG layer in different steps of their infection cycle. Endolysins degrade the bacterial cell wall at the end of the infection cycle, causing lysis of the host to release the viral progeny. Recombinant endolysins have been successfully applied as anti-bacterial agent against antibiotic-resistant Gram-positive pathogens. This has boosted the study of these enzymes as new anti-microbials in different fields (e.g. medical, food technology). A key example is the recent development of endolysin-based anti-bacterials against Gram-negative pathogens in which the exogenous application of endolysins is hindered by the outer membrane (OM). These novel anti-microbials, termed Artilysin®s, are able to pass through the OM and reach the PG where they exert their action. In addition, mycobacteria whose cell wall is structurally different from both Gram-positive and Gram-negative bacteria have also been reported to be inhibited by mycobacteriophage-encoded endolysins. Endolysins and endolysin-based anti-microbials can be considered as ideal candidates for an alternative to antibiotics for several reasons: (1) their unique mode of action and activity against bacterial persisters (independent of an active host metabolism), (2) their selective activity against both Gram-positive and Gram-negative pathogens (including antibiotic resistant strains) and mycobacteria, (3) the limited resistance development reported so far. The present review summarizes and discusses the potential applications of endolysins as new anti-microbials.

Introduction

Lytic (bacterio)phages are viruses that infect and hijack their bacterial hosts for their propagation. An estimated 10%–20% of the global bacterial population is infected and lysed every day [1]. In addition, bacterial cells are assumed to represent one of the most ancient cells; hence, there is no longer and no more intense co-evolution as between phages and their bacterial hosts. This co-evolution has shaped and fine-tuned phage-encoded enzymes with excellent properties that have attributed to major advances in molecular biology and biotechnology. Enzymes such as T4 DNA ligase, T7 RNA polymerase, ϕ29 DNA polymerase featuring high efficiency and processivity have since long proven their merits. Endolysins are yet another group of phage-encoded enzymes that have attracted much attention, especially since the start of the millennium and endolysin-focused research is still intensifying. Endolysins are essential enzymes produced by a bacteriophage at the end of its lytic replication cycle to lyse the infected host cell for release of the newly produced phage particles. Endolysins are peptidoglycan (PG) hydrolases that act as molecular scissors cutting the PG meshwork. PG or murein is the largest molecule in a bacterium that surrounds the bacterial cell. It maintains the cell shape and is responsible to withstand the internal osmotic pressure. This turgor pressure ranges from 5 atmospheres (1 atmosphere = 101.325 kPa) for Gram-negative bacteria to up to 50 atmospheres for Gram-positive bacteria. Consequently, impairing the PG results in an unsustainable internal pressure, causing osmotic lysis and the release of progeny virions [2,3].

Key words: anti-microbial, Artilysin(R), bacteriophage, endolysin, peptidoglycan.
Abbreviations: CBD, cell wall-binding domain; EAD, enzymatically-active domain; GH, glycoside hydrolase; LPS, lipopolysaccharide; OM, outer membrane; PG, peptidoglycan.
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Structural and biochemical diversity of endolysins

Although sharing a common biological function, i.e. lysis of the infected host cell, endolysins represent a class of enzymes with huge structural and biochemical diversity. Endolysins comprise dedicated modules for enzymatic catalysis [EAD (enzymatically-active domain)] and substrate binding [CBD (cell wall-binding domain)]. EADs show a large biochemical diversity and different chemical bonds of PG are targeted: the β(1,4) glycosidic bond between *N*-acetyl glucosamine

and *N*-acetyl muramic acid (glucosaminidases, EC 3.2.1.52), the β(1,4) glycosidic bond between *N*-acetyl muramic acid and *N*-acetyl-glucosamine (muramidases or lysozymes, EC 3.2.1.17; transglycosylases, E.C. 4.2.2.n1), the amide bond between the lactic acid group of *N*-acetyl muramic acid and the stem peptide (amidases, EC 3.5.1.28), the peptide bonds between amino acids of the stem peptide or the inter-peptide bridge (endopeptidases, different subcategories belonging to EC 3.4.-.-). Endolysins with a lysozyme activity have been classified in the CaZy database in glycoside hydrolase (GH) families GH19, GH24 (inverting mechanism), GH15 (retaining mechanism) and GH108 (unknown mechanism), whereas classified phage-encoded glucosaminidases and lytic transglycosylases belong to GH73 (unknown mechanism) and GH104 (retaining mechanism) respectively. Amidases and endopeptidase are not classified in the CaZy database as they act on the peptide stem or cross-link.

CBDs bind a specific ligand in the cell wall with high affinity (reported affinities vary between $K_a = 10^{-6}$ and 10^{-9} M [4–6]), conferring a varying degree of specificity to the endolysin: some CBDs restrict the specificity to the serovar level (e.g., the CBD of PlyP35 specifically recognizes terminal *N*-acetyl glucosamine moieties of the teichoic acids of *Listeria monocytogenes* serovars 1/2a, 3a and 4a [7]), some CBDs are specific for a certain species (e.g., pneumococcal phage CBDs target choline, which is only present and indispensable in teichoic acids from *Streptococcus pneumoniae* [8]); CBDs that directly bind the PG layer chemotype A1γ have the broadest spectrum as this chemotype is present in all Gram-negative species and some Gram-positive species (although de-acetylation of the *N*-acetyl glucosamine moiety, which takes place in some Gram-positive species with chemotype A1γ, excludes these species from the spectrum) [9]. The most prevalent CBDs in endolysins are LysM (classified as CBM50 in the CaZy database), pfam01471 (PB_binding_1), SRC homology 3 domain (SH3) and Cpl-7 [10].

Oliveira et al. [10] performed comparative genomics and identified 723 endolysins in public databases. Among these endolysins, 24 different EADs and 13 CBD types were found. The EADs and CBDs can be arranged in 89 different architectural organizations. The large structural variations are strongly related to differences in cell wall structure of Gram-positive and Gram-negative bacteria [2,10]. Endolysins encoded by phages infecting Gram-positive species and mycobacteria have a modular composition with one or two N-terminal EADs and a C-terminal CBD; however, a central CBD squeezed between two EADs has been reported as well [2,11]. These modules are typically connected by a flexible inter-domain linker sequence, which can vary in size and provides the space so that the composing modules can function autonomously. In contrast, the majority of endolysins produced by phages infecting Gram-negative host cells only comprise one EAD and no CBD (coined globular endolysins). Nevertheless, several endolysins with an N-terminal CBD and a C-terminal EAD [6,9] and the opposite arrangement have been described [12]. Such modular endolysins generally excel globular endolysins in terms of enzymatic activity [13]. However, it has been demonstrated that an N-terminal fusion of a CBD specific for A1γ PG to an EAD encoded by a Gram-negative infecting phage triples its enzymatic activity [5]. Exchange of a CBD can also shift the activity spectrum from the original spectrum to the spectrum of the fused CBD moiety [14–16]. Duplication of a CBD results in an increased affinity for the cell wall [14]. Thermophilic properties of an EAD can be transferred to a new chimeric fusion with a CBD that also confers a changed anti-bacterial spectrum [17]. Linker composition between the different modules may play a pivotal role in successful module shuffling as shown by the different effects of charged and uncharged linkers on the interaction between CBD and EAD modules of the mycobacteriophage D29 endolysin [18].

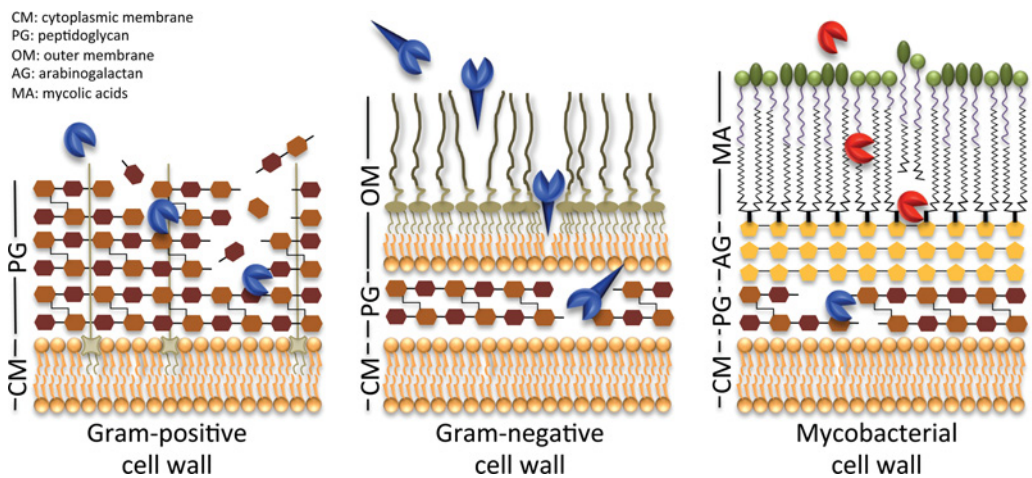
Endolysins as a novel class of anti-bacterial proteins against Gram-positive pathogens

The increasing interest in endolysins came with the development of endolysins as a novel class of anti-bacterials. Indeed, exogenous addition of purified endolysin to a Gram-positive bacterium results in cell lysis upon contact and an immediate cell death, irrelative of the presence of existing drug mechanisms (Figure 1). This anti-bacterial activity was initially described by Gasson [19] for the use of endolysins to eliminate food-borne pathogens such as *L. monocytogenes* and *Clostridium tyrobutyricum*. However, extensive experimental validation took only off from 2001 [20], concomitantly with the emerging antibiotic resistance and increasing need for novel classes of anti-bacterials. These studies were initially focused on pathogenic Gram-positive bacteria such as *Streptococcus* sp., *Bacillus anthracis*, *Enterococcus* sp. and *Staphylococcus aureus* [21–24]. This focus was evident given the immediate accessibility of the thick PG of Gram-positive bacteria, comprising up to 40 layers, to exogenously added endolysins.

Such anti-bacterial properties have been described and exploited in the past for PG degrading enzymes such as vertebrate lysozymes, hexosaminidases and lytic transglycosylases (extensively reviewed by Masschalck and Michiels [25]). However, different remarkable assets featuring endolysins as anti-bacterials render them more appropriate PG-degrading enzymes to use as anti-bacterial. The high specificity of the CBD and the specificity of some EADs (e.g., endopeptidases that cleave the species-specific pentaglycine bridge in *S. aureus*) generally confer a narrow-spectrum to endolysins, leaving beneficial flora unaffected. Nevertheless, the modularity principle of endolysins allows creating narrow-spectrum endolysins against virtually any Gram-positive pathogen. Indeed, bacteriophages are the most abundant biological entity on earth, outcompeting the number of bacteria with one order of magnitude and phages; thus, endolysins can, in principle, be identified against any cultivable bacterial species. The active mode-of-action, i.e. osmotic lysis through enzymatic degradation, is much faster than the majority of antibiotics. The latter often rely on the

Figure 1 | **Endolysin and endolysin-based anti-bacterials against different cell wall types**

The Gram-positive bacterial cell wall (left panel) consists of a CM and a thick PG layer (up to 40 layers), which is directly accessible to exogenously added endolysins (blue). Endolysins degrade the PG layer upon contact, followed by osmotic lysis and cell death. The Gram-negative bacterial cell wall (middle panel) has a thin PG layer (1–2 layers) but an additional protective OM. The LPS-destabilizing peptide of an Artilysin® acts as a wedge through the LPS-layer and enables passage of the endolysin moiety through the OM. Thereafter, Artilysin® acts in a similar way as native endolysin. The mycobacterial cell wall (right panel) has a mycolyl–arabinogalactan–PG complex. Mycobacteriophages produce a PG hydrolase (LysA, blue) and a lipase (LysB, red) that both have growth inhibiting activity when added exogenously.



inhibition of an essential metabolic step, which leads to a slow, gradual deterioration of the cell condition, ultimately leading to cell death. Gram-positive species are killed within seconds or minutes, depending on the concentrations [2].

Endolysins are considered to have a good safety profile. Phages are an integral part of the human microbiome. Thus, humans have continuously been exposed to phages and their released endolysins since long without negative consequences [26]. In addition, endolysins are highly specific for the pathogen targeting unique and highly conserved bacterial structures that are absent from mammalian cells [27].

Endolysins are, like other proteins, considered non-corrosive and biodegradable; but due to this proteinaceous nature, they could induce an allergic reaction, although such cases have not been reported yet. An adverse immune response would result in the generation of neutralizing antibodies. This antibody response could interfere with the treatment, leading to the neutralization of the endolysin *in vivo* after repeated exposure. Indeed, serum antibodies could be raised against endolysins from diverse origins. *In vitro* mixing of antibodies with endolysins slowed down the anti-bacterial effect, but could not eliminate the effect. These observations were confirmed *in vivo* with equal rescue of infected mice that were previously immunized or still native [28]. Different studies, using different endolysins and pathogens, confirmed these results [29,30]. The reasons for these results are not yet fully understood. The binding affinity of some CBDs for the bacterial cell wall may exceed the affinity of endolysin-specific antibodies and thus outcompete neutralization by antibodies [4]. Another hypothesis that has been offered is the fast kinetics of bacterial killing by endolysins (upon contact). Killing may take place prior to elimination of the endolysins by circulating antibodies [29].

Generally, endolysins are expected to have a short half-life, but this should not compromise the anti-bacterial activity since endolysins act fast upon contact with the bug. Nevertheless, endolysins need to be administered in a repeated way to maintain a sufficiently high serum concentration [28]. Systematic administration of endolysins could induce the sudden release of pro-inflammatory cellular debris of bacteria. This bacterial debris may cause an increase in cytokine production and host inflammatory response [31]. In this regard, it has been found that administration in 12-h intervals reduces cytokine concentrations compared with untreated, infected animals, whereas continuous systematic administration results in an increased cytokine production. The latter has been explained due to an increased fragmentation of the bacterial cell wall upon continuous exposure to a high endolysin concentration, stimulating cytokine release. Optimization of the dosing regime is thus essential [32,33].

Despite several attempts to generate strains resistant to endolysins, no cases of such resistant strains have been reported to date [22,28,34]. This might be the result of the long-term co-evolution between phages and bacteria, where endolysins target the 'Achilles heel' of the bacterium, conserved components in the cell wall. It should be mentioned that, despite these hopeful results, some bacteria are resistant to other types of PG hydrolases, especially exolysins.

Examples are resistance to human lysozyme and to the bacteriocin lysotaphin, which were achieved by a variety of modifications in the PG layer to shield the recognition site from the enzyme [35,36].

Artilysin®s provide a solution against Gram-negative pathogens

Gram-negative bacteria, although having a much thinner PG (only 1–3 layers) compared with Gram-positive bacteria, have a protective outer membrane (OM) with a lipopolysaccharide (LPS) layer, which is only permeable for compounds smaller than 600 Da through OM embedded porins [37]. Diverse alternative approaches for endolysins to overcome the OM layer have been demonstrated [13]. Some endolysins have an innate anti-bacterial activity against Gram-negative species. Especially *Acinetobacter baumannii* appears to be susceptible for such endolysins with some reported cases (LysAB series [38,39]; <1 log reduction); PlyF series [12] (between ∼1 and >5 log cells depending on conditions such as pH, salt concentration, growth stage). This effect is observed when high doses are used (100–1000 μg/ml) and may be attributed to a positively charged N- or C-terminal domain that interferes with the negatively charged LPS layer [38,39]. Lai et al. [39] describe for LysAB3 that enzymatic activity is not required for anti-bacterial activity, since the N-terminal domain comprising the catalytic residues can be completely deleted without loss of anti-bacterial activity. Moreover, a synthetic peptide corresponding to the C-terminal cationic helix has comparable bactericidal activity. Compounds that destabilize the OM either by chelation of the stabilizing divalent cations (EDTA, weak organic acids), by competitive displacement of the stabilizing cations (polycationic agents) or by acidification (weak organic acids) have been shown to facilitate endolysin access to the PG layer [40–42]. Also carvacrol, an aromatic essential oil that is known for its disintegration of the OM by inducing LPS release, sensitizes *Escherichia coli* and *Pseudomonas putida* for the engineered pneumococcal endolysin Cpl-7S [43]. High hydrostatic pressure transiently permeabilizes the OM for endolysins, resulting in an efficient elimination of *Pseudomonas aeruginosa*, but this approach may only find application as a food conservation technology [44]. T4 lysozyme fused to the N-terminal domain of pesticin is taken up in a receptor-mediated process by *E. coli* strains that harbour the FyuA receptor, followed by cell death by PG degradation. FyuA is a major virulence factor and the engineered T4 lysozyme specifically targets virulent pathogens [45,46].

Artilysin®s are engineered endolysins to obtain high bactericidal activity against Gram-negative bacteria. A LPS-destabilizing peptide is fused to either the N- or the C-terminus of endolysins, without affecting the secondary and tertiary structure of the endolysin. This peptide with amphipathic or polycationic properties locally punctures the LPS layer through interference with its stabilizing ionic and hydrophobic forces, leading the more bulky endolysin moiety as a wedge through the OM. A high and quick bactericidal activity results from active PG degradation and osmotic lysis (Figure 1). The synergy with OM permeabilizers, such as EDTA is conserved in Artilysin®s, enabling complete sterilization of a bacterial culture (>5 log) in 30 min. Artilysin®s are featured by many anti-bacterial properties described above for endolysins killing Gram-positive pathogens. Resistance development through genetic alterations under highly selective pressure could not be selected. They show no cross-resistance with existing antibiotic resistance mechanisms and thus kill multidrug-resistant isolates. The unique mode-of-action based on enzymatic PG degradation and osmotic lysis does not require an active metabolism, consequently Artilysin®s can also completely eliminate the persister fraction of a bacterial culture in contrast with traditional antibiotics. In a skin infection model, LoGT-022 could protect keratinocytes from an otherwise lethal *P. aeruginosa* infection. Two dog otitis case studies with Artilysin®s were reported. Otitis caused by *P. aeruginosa* strains could not be healed with standard antibiotic treatment over a prolonged time. Repeated exposure to Art-085 resulted in a quick improvement of the inflammation symptoms and complete elimination of the infections without reported relapses [47,48].

A cocktail of LysA and LysB enzymes to kill mycobacteria

Mycobacteria pose other challenges to the application of endolysins as anti-bacterials. Their cell wall is structurally different from both Gram-positive and Gram-negative bacteria, with a consequent inconclusive Gram-staining of the cell wall. The mycobacterial cell wall consists of a mycolyl–arabinogalactan–PG complex: the PG is covalently attached to arabinogalactan, which is in turn esterified with long α-branched, β-hydroxy fatty acids (mycolic acids), the latter representing a truly lipid barrier [49]. Therefore, mycobacterium phages generally produce two lytic enzymes, LysA and LysB. LysA is a PG hydrolase, whereas LysB cleaves the linkage of mycolic acids to the arabinogalactan layer and has lipolytic activity [11,50]. Surprisingly, the *lysA* gene of phage Ms6 encodes a shorter, embedded gene in the same reading frame that encodes a second functional PG hydrolase. A drop assay with crude *E. coli* extracts that contain one of these two LysA PG hydrolases inhibited the growth of Gram-positive bacteria and unexpectedly also of mycobacteria. It is suggested that the mycolic-acid-rich OM may be bypassed during septal PG synthesis and cell division [51]. Grover et al. [52] have shown that LysB of two different mycobacterial phages also exerts a bacteriostatic effect on *Mycobacterium smegmatis* (as a non-pathogenic surrogate for *Mycobacterium tuberculosis*) but only in presence of surfactants. This effect may be explained by the hydrolysis of the outer layer of mycolic acids, weakening the cell wall. This bacteriostatic effect is most pronounced in presence of Tween80, as LysB also releases oleic acid from Tween80, exerting an additional anti-bacterial effect [52]. It has been suggested that exposure of mycobacteria to LysB could

sensitize them more for LysA, but this potential synergy is yet to be tested [51] (Figure 1).

Conclusion

The unique mode-of-action, the bactericidal activity against multidrug-resistant strains and persisters and the low probability of resistance development are appealing features of endolysins for their development as anti-bacterials. Whereas endolysins were initially considered to be only useful against Gram-positive bacteria, Gram-negative bacteria have now come into reach as well. Mycobacteria will be the next hurdle to take. Combinations of LysA and LysB may bring solution here.

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