

BACTERIOPHAGE-DERIVED ENDOLYSINS AS NEXT-GENERATION ANTIBACTERIALS: A COMPREHENSIVE REVIEW

Hafiz Anas Saeed^{*1}, Waqas Ahmed², Eisha Tur Raazia³, Marwah Shahryar⁴, Azeem Sharif⁵, Hajra Murtaza⁶, Zunaira Yasin⁷, Muhammad Din⁸, Muhammad Mohsin⁹, Amaila Qaisar¹⁰

^{*1}Center for Applied Molecular Biology, University of the Punjab, Lahore, Pakistan

²Department of Biochemistry- Hazara University, Mansehra, KPK, Pakistan

³Department of Microbiology, Government College University Lahore, Punjab, Pakistan

⁴Faculty of Veterinary and Animal Sciences, Pir Mehr Ali Shah Arid Agriculture University, Rawalpindi, Punjab, Pakistan

⁵Institute of Microbiology, University of Veterinary and Animal Sciences, Lahore, Punjab, Pakistan

^{6,7}Department of Life Sciences, University of Management and Technology, Lahore, Punjab, Pakistan

⁸Department of Microbiology, Abdul Wali Khan University Mardan, KPK, Pakistan

⁹Department of Pathology Lab Sciences, University of Sargodha (Sargodha Medical College, Sargodha), Punjab, Pakistan

¹⁰Faculty of Rehabilitation and Allied Health Sciences (FRAHS), Riphah International University, Islamabad, Pakistan

¹anassaheed04@gmail.com, ²drwaqasmultan@gmail.com, ³eishaturraazia19@gmail.com,

⁴marwahshahryar1@gmail.com, ⁵azeemsharif0067@gmail.com, ⁶hajram979@gmail.com,

⁷zuni.baloch2002@gmail.com, ⁸muhammaddinkhattak290@gmail.com, ⁹muhammadmohsin512@gmail.com, ¹⁰amaila.qaisar@riphah.edu.pk

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Corresponding Author: *

Hafiz Anas Saeed

Abstract

As antimicrobial resistance has become increasingly problematic, there is a growing need for new therapeutic approaches and bacteriophage-derived endolysins are a new generation of antibacterial agents. The peptidoglycan hydrolases have remarkable features such as potent bactericidal activity, high specificity of the target molecule and low tendency to induce bacterial resistance and have great potential for developing alternatives to antibiotics. Endolysins have a modular structure with catalytic domains that hydrolyse specific bonds of the bacterial cell wall and cell wall binding domains that provide target specificity to selectively lyse pathogenic bacteria whilst sparing beneficial commensal bacteria. Their action against biofilms and persister cells overcome important drawbacks of traditional antibiotics and the protein engineering methods such as domain shuffling, directed evolution and artificial intelligence-guided design have increased their therapeutic potential. Clinical development has made great strides and a number of candidates have advanced into clinical trials for infections from multi-drug-resistant pathogens. Although still a few hurdles remain, such as the barrier of the outer membrane in Gram-negative bacteria and the need for optimizing the system for delivery, endolysins offer a revolution in the field of antibacterial therapy. The present review discusses the biology, mechanisms, engineering strategies, clinical application, and future perspectives of bacteriophage-derived endolysins, as well as their potential to

1. INTRODUCTION

Antimicrobial resistance (AMR) has become a critical public health problem of the 21st century, impeding advances in human health, food security, and socio-economic development globally. With the undeterred emergence and spread of bacterial pathogens resistant to conventional antibiotics, the clinical usefulness of the traditional antibiotics is gradually decreasing, and many previously treatable infections are becoming more and more difficult or impossible to manage effectively. This is a massive crisis – in fact, antimicrobial resistance is believed to have caused 1.27 million deaths alone in 2012 and to be responsible for nearly 5 million deaths across the world, and these numbers are predicted to be on the rise in the next few decades if the situation continues unchecked[1]. AMR is expected to become a major cause of death, causing some 10 million deaths globally every year by 2050, above diabetes and cancer and to cost more than one hundred trillion US dollars in cumulative economic costs. The economic consequences of antimicrobial resistance go far beyond the direct cost of health care, including lost productivity, longer hospital stays with more complicated treatments, and the financial burden of more costly and invasive remedies – putting pressure on health care systems especially in the lower and middle income areas of the world where antimicrobial-resistant infections are at their peak and health care capacity is the thinnest[2].

Antibiotics are becoming less effective due to several factors, most importantly the widespread overuse and misuse of antibiotics in human, veterinary and agricultural medicine, leading to strong selective pressures for bacteria to evolve and disseminate resistance mechanisms. Bacteria have evolved an amazing ability to survive exposure to antibiotics by a variety of mechanisms: 1) drug inactivation by enzyme degradation or modification; 2) resistance to binding by DNA- or RNA-encoded changes in the target site; 3) resistance to uptake due to changes in the cell envelope; and 4) resistance to uptake through

active efflux pumps. These resistance determinants are often carried on mobile genetic elements (MGE) like plasmids and transposons, which allow for their rapid horizontal transfer between species of bacteria and between ecological niches, thereby helping to spread resistance determinants throughout the bacterial population[3]. The rise of multidrug-resistant and extensively drug resistant bacterial strains, such as methicillin-resistant *Staphylococcus aureus*, vancomycin-resistant enterococci, carbapenem-resistant enterobacteriaceae, multidrug-resistant *Pseudomonas* and *Acinetobacter baumannii*, has resulted in an increasing number of infections with no or limited effective antibiotic treatment. Even more alarming is the dearth of novel antibiotics entering the pipeline as the pharmaceutical industry has largely outsourced antibiotic development because of the difficulties in economic returns from such activity, and regulatory requirements have become increasingly difficult to overcome[4].

Multiple crises have led to a paradigm that is no longer sustainable – an antibiotics-only approach to dealing with bacterial infections – and the need for alternative methods of treatment is more urgent than ever. Resistance mechanisms are developing quickly and rapidly spreading around the world, more often than new antibiotics are being developed, with the result that there is a troubling unbalance in the therapeutic arsenal, which will only grow if we do nothing to change it. Intrinsic limitations of antibiotics such as broad-spectrum activity, which affects other congruous microbes, and the ability to become resistant through relatively simple genetic changes are reasons why agents with fundamentally different mechanisms that have higher evolutionary fitness barriers to resistance are desired[5]. In addition, economic disincentives and scientific challenges have halted antibiotic research and discovery, leading to a dearth of antibiotics in the pipeline to manage the range of resistant infections. Especially when caused by Gram-negative bacteria that have an outer membrane barrier that blocks antibiotic

entry and additionally express efflux pumps that further “pump” drugs out of the cell, making many antibiotics useless. Thus, alternative therapeutic strategies need to not only overcome existing resistance mechanisms, but also need to have a new mechanism of action, have a good safety profile, have favourable pharmacokinetics for clinical use and should be bactericidal[6].

The story of discovery and development of bacteriophage endolysins is an interesting one that goes from the early days of phage biology to the present day of protein engineering and therapeutic development. The most abundant biological entities on earth, the bacteriophages have evolved in sophisticated ways to attack and lyse bacteria including the production of specialized enzymes that are used to degrade the bacterial cell wall during the last step of the lytic reprogramming cycle. The first indication of the presence of these enzymes dates back to the discovery of bacteriophages between 1915 and 1926; however, their characterization was scanty for the next decades[7]. The successful preparation of highly purified lysin in 1971 led to the biochemical characterization of these enzymes in greater detail. In 2001, a research team was able to demonstrate the therapeutic activity of an endolysin against some respiratory streptococcal pathogens in vivo, thereby proving the concept for the use of endolysins as antimicrobial agents. The publication of this seminal work sparked a renewed interest in endolysin research that saw the first clinical trials with an endolysin-based drug (SAL-1) in 2013, with many more developments since then continuing to bring us closer to clinical translation. In the last twenty years, the amount of research on endolysins has exploded leading to a vast array of different endolysin structures, functions and host specificities, as well as demonstrating the high amenability of these enzymes to protein engineering to generate new therapeutic variants[8].

The aim of this comprehensive review is to offer a critical and integrated review on the biology, structure of diversity, mechanism of action, engineering, and clinical uses and perspectives of bacteriophage-derived endolysins as next-generation antibacterials. The review is organised

to lead the reader through basic science to translational applications with a focus on the unique properties that endolysins have compared to conventional antibiotics and other alternative approaches to antimicrobials[9]. This review aims to systematically describe the molecular architecture and catalytic diversity of endolysins, elucidate how they mediate rapid bactericidal activity, describe the endolysin classification and spectrum for clinically significant pathogens, critically evaluate the development of protein engineering and delivery strategies to augment their therapeutic potential, critically evaluate the current progress in clinical development and regulatory pathway, and report challenges and future research priorities that must be addressed in order to realize the full potential of endolysins as precision antibacterial therapeutics. This review aims to bring together the wealth of information on endolysins and provide readers with a snapshot of the current state of the field and what steps need to be taken to help translate endolysins from lab to clinic and beyond[10].

2. Biology of Bacteriophage Endolysins

2.1 Bacteriophage life cycle

The life cycle of bacteriophages, the viruses that invade and multiply within the bacterial host, represents the basic pattern of interaction between bacteriophages and bacteria and is the foundation of the biological function of endolysins. The lytic cycle, which is of primary relevance to endolysin biology, involves the primary interaction of the bacteriophage with specific receptors on the bacterial cell surface, the injection of the phage genome into the host bacterial cell, the synthesis and maturation of endolysin and any other viral proteins required, and the lysis of the bacterial cell to release the viral progeny. The phage genome then uses the cellular machinery of the bacterium to replicate (copy) the genetic content of the phage and to produce the structural proteins that are produced during the assembly of progeny virions[11]. This draws to a culmination when new phage is assembled within the infected bacteria, resulting in a large amount of pressure (as many virions are packaged). In the final step of the lytic cycle, the controlled destruction of the bacterial

cell wall allows the progeny virions to escape, but this process must be tightly regulated to ensure the efficient destruction of the wall at the right time so that virion production is not sacrificed by premature wall destruction. This is a critical step, realized through the concerted action of lysis proteins, that include endolysins, holins, and in Gram-negative phages, spanins, all of which contribute to the involution of the bacterial envelope and the release of newly assembled phages to the environment to initiate new infection cycles[12].

2.2 Role of endolysins during phage replication

Bacteriophage replication requires endolysins which have an essential function during the productive cycle of the bacteriophage to lyse the bacterial cell wall and liberate progeny bacteriophages. In the late stages of phage replication these peptidoglycan hydrolases are synthesized and stored in the bacterium's cytoplasm until they are activated. The enzymatic activities of endolysins are targeted towards the peptidoglycan layer of the bacterial cell wall, which is a highly conserved and essential structure to maintain the integrity and shape of bacteria. Endolysins degrade specific bonds in the peptidoglycan matrix, destabilizing the cell wall and causing release of the contents of the cell because of the high osmotic pressure inside the cell, causing the cells to lyse and precipitate. This is called the lysis from within and is a very important adaptation that is necessary to get progeny virions out efficiently, which leads to a higher reproductive success of the bacteriophage. To prevent premature lysis, before enough virions have been assembled, endolysin activity must be strictly controlled, and this appears to be achieved by the coordinated action of other proteins involved in lysis that control the time and place of endolysin access to the peptidoglycan substrate[13], [14].

2.3 Holin–endolysin system

The holin–endolysin system is a complex regulation mechanism that allows the right timing of the bacterial cell lysis during the lytic cycle of the bacteriophage that infects it, which is the

primary reason why it has evolved and plays a crucial part in the process: it is important that the cell does not lyse too early, as this would mean losses of the progeny virions and the cell needs to lyse at the right time to ensure efficient release of the virions. The holins are membrane pore-forming proteins which are encoded by the bacteriophage and localize to the cytoplasmic membrane of the bacterium during infection, allowing the endolysin access to peptidoglycan layer. Holin-mediated permeabilization of the membrane is exquisitely timed, and will usually happen at a genetically programmed time after infection based upon the accumulation of holin molecules in the membrane. The endolysin can reach high levels within the cytoplasm during this time prior to lysis, because the enzyme cannot reach its substrate, peptidoglycan, until it is free of the cytoplasmic membrane. The holin induces membrane permeabilization, allowing the endolysin to reach the peptidoglycan layer where it rapidly degrades the cell wall leading to the osmotic killing of the bacterial cell. The holin–endolysin system thus acts as a molecular timer, coordinating the timing of cell lysis with completion of phage replication, so maximizing the number of progeny virions released from the infected cell, and the efficient propagation of the bacteriophage infection[15].

2.4 Types of bacteriophage lysis proteins

The bacteriophage lysis system is composed of several classes of proteins, which all act together in concert to coordinate the controlled degradation of the bacterial envelope; the endolysins, holins, spanins and signal-arrest-release endolysins are all involved in the lysis system and have all been shown to have distinct roles in the process. The main enzymatically active molecules that degrade cell walls are called endolysins and they break down particular bonds in the peptidoglycan layer to disrupt the integrity of the cell wall and cause the bacteria to get blown open by osmosis. The catalytic mechanisms, substrate specificity and structural organization of these enzymes are very varied and show evolutionary adaptation of the bacteriophages to their bacterial host and its niche. Holins are small membrane proteins located in the

bacterial cytoplasmic membrane that form pores at a programmed time to allow the endolysin access to its peptidoglycan substrate and thus start the process of lysis[16]. The holin-dependent regulation of endolysin activity ensures that lysis does not take place while phage replication and virion assembly is still ongoing because otherwise incomplete phage particles would be released. It is a group of lysis protein (spanin) that are present in Gram-negative bacteriophages to mediate the dissolution of the Gram-negative outer membrane, leading to disruption of the Gram-negative envelope and progeny virion release. One subclass of endolysins, called signal-arrest-release endolysins, has a signal that is targeted to the periplasm of Gram-negative bacteria and is removed from the endolysin during translocation to the periplasm, allowing these enzymes to act on the peptidoglycan from the outside of the cell[17].

2.5 Evolutionary diversity of endolysins

Given the high genetic and functional diversity of bacteriophages, the evolutionary diversity of bacteriophage endolysins is just as great, reflecting the constant coevolutionary race between phages and their hosts. Endolysins have evolved to specifically act on peptidoglycan, a highly conserved structural element of the bacterial cell wall, and are remarkably diverse in their catalytic mechanisms, substrate specificity and structural architecture. This diversity is presumably a reflection of evolutionary selection pressures imposed on the phages to maximize the efficiency of bacterial cell destruction and adapt to the range of structural variation in the peptidoglycan probably found among different bacterial species and under different growth conditions[18]. This modular design of endolysins (whereby the catalytic and cell wall binding domains can be separated) has allowed for the evolution of diversity in combinations of functional domains via domain shuffling and recombination, permitting rapid adaptation of endolysins to new bacterial hosts. Bacteriophages, and the resistance of their bacterial hosts, have also co-evolved, bacteria developing a repertoire of ways to thwart the action of endolysins and phages developing ways to counteract. This evolutionary pressure has

led to the evolution of a large diversity of endolysin sequences and structures, serving as a large resource of naturally occurring endolysin variants for therapeutic use and for which variants could be engineered for optimal antibacterial properties[14].

3. Structural Biology of Endolysins

3.1 Molecular architecture

Bacteriophage endolysins are, in general, modular proteins containing one or more enzymatically active domain(s) responsible for catalysis of a specific bond in the peptidoglycan network as well as a cell binding domain(s) responsible for target specificity and surface localization of the endolysin. This is especially true of the endolysins that act on Gram-positive bacteria, which often have a multidomain structure with the enzymatically active domain towards the N-terminus, followed by a flexible linker and the cell wall-binding domain towards the C-terminus. By contrast, endolysins which hydrolyse Gram-negative bacteria often have a much simpler globular structure, with a single catalytic domain of 15-20 kDa which is influenced by the different structural constraints of the Gram-negative cell envelope. The modular structure of endolysins has deep implications in their antibacterial action because the different parts of the protein can evolve and be optimized for catalytic efficiency and specificity separately and allow for rapid and easy engineering of new lysins by shuffling and recombination of domains. The knowledge of endolysin molecular structure is crucial for understanding the mechanism of action, prediction of its antibacterial range, and structural engineering of endolysin variants with improved properties for therapeutic applications[8].

3.2 Catalytic domains

The enzymatic activities of endolysins target specific bonds in the peptidoglycan network and are found in a diversity of endolysins as a reflection of the evolutionary adaptation of bacteriophages to take advantage of different weak points in the bacterial cell wall. The peptidoglycan layer is made from a glycan backbone of alternating N-acetylglucosamine and N-

acetylmuramic acid that is cross linked by peptide chains, which have multiple potential sites for enzymatic cleavage. Muramidases (also called 'lysozymes') lyse the glycan backbone by breaking the beta-1,4-glycosidic bond between N-acetylmuramic acid and N-acetylglucosamine, leading to a loss of structural integrity of the cell wall. There is an alternative mechanism for glycan backbone cleavage with glucosaminidases that break the beta-1,4-glycosidic bond between N-acetylglucosamine and N-acetylmuramic acid. Amidases break the amide bond between the N-acetylmuramic acid and the Alanine of the stem peptide, thus freeing the glycan strands from the peptide cross-links and destroying the network structure of the peptidoglycan. Endopeptidases act on the peptide cross-links and cut the bonds between amino acid residues of the peptide within peptide bonds between the adjacent glycan strands, resulting in the disintegration of the peptidoglycan network. Lytic transglycosylases are a unique group of catalytic domains that do not hydrolyse the glycosidic bond, but rather break the glycosidic bond and form a 1,6-anhydromuramoyl product, leading to degradation of a peptidoglycan by a different mechanism than classical hydrolysis[19].

3.3 Cell wall binding domains

The specificity of endolysins is provided by the cell wall binding domains which are essential functional modules of the endolysin that mediate its specific recognition and binding to its bacterial target, allowing endolysins to target bacterial pathogens whilst leaving beneficial commensals untouched. These domains recognise specific sites (epitopes) in the peptidoglycan layer or in other cell wall components like teichoic acids in Gram-positive bacteria and lipopolysaccharides in Gram-negative bacteria, allowing the targeting of the catalytic domain to the exact location of its substrate. The specificity of a cell wall binding domain will generally be genus- or species-specific as cell wall components display variability between the structures of different bacterial species, and bacteriophages have evolved with their host[20]. Structural studies with X-ray crystallography and cryo-electron microscopy enabled the solution of the molecular basis of this specificity; cell wall

binding domains will recognize unique structural features of the target cell wall through complementary shape and electrostatic interactions. Apart from its role in determining target specificity, the cell wall binding domain also helps to create a higher local concentration of the catalytic domain at the cell surface, and promotes the processive degradation of the peptidoglycan layer, thereby increasing the enzyme's efficiency. Due to their modularity, cell wall binding domains have been studied as targets for protein engineering as a way to create chimeric endolysins that have a different binding specificity and broader host range by domain swapping[21].

3.4 Modular versus globular endolysins

An endolysin is a structural dichotomy, with the modular design found in Gram-positive enzymes and the globular design found in Gram-negative enzymes, responding to the diverse structural problems of the two main groups of bacterial cell envelopes. Bacteriophages infecting Gram-positive bacteria have more often been found to have a modular endolysin, composed of two or more functional endolysin domains in sequence, one or more of which is enzymatically active, and a cell wall-binding domain at the C-terminus. This multidomain structure imparts a number of functional benefits such as independent optimization of catalytic activity and binding of substrates, cooperative recognition of complex cell wall epitopes by multiple binding domains, and allosteric regulation of enzymatic activity via interdomain communication[22]. The modular structure is also very suitable for protein engineering and the construction of chimeric endolysins by swapping and recombining the domains. By contrast, globular endolysins – typical for bacteriophages of Gram-negative host bacteria – often contain only one catalytic endolysin domain without an extended cell wall binding domain as the ability of the enzyme to reach the peptidoglycan is allowed through the outer membrane barrier. Many Gram-negative endolysins have a globular structure and their surface is typically positively charged, which allows them to interact with the negatively charged outer membrane and facilitates their passage through

the outer membrane, increasing their access to the peptidoglycan substrate[23].

3.5 Structural differences between Gram-positive and Gram-negative endolysins

The structural differences of endolysins that target Gram-positive versus Gram-negative bacteria are related to the basic differences in the architecture of the bacterial cell envelope and the different problems that each type poses for accessibility and activity of the enzymes. The thick, layered peptidoglycan of gram-positive bacteria is exposed on the cell surface and endolysins applied from the outside can readily act on it, thus allowing the rapid bactericidal activity that has made these enzymes especially interesting for therapeutic use. Typically, endolysins targeting Gram-positive bacteria have a modular structure, consisting of one or more enzymatically active domains and a cell wall-binding domain that is responsible for specific recognition of the target cell wall, allowing high affinity binding and efficient degradation of peptidoglycan[24]. The high concentration of endolysin molecules bound to the thick peptidoglycan layer may also explain why these endolysins exhibit very rapid bactericidal actions. On contrary, Gram-negative bacteria have a complex cell envelope, composed of an inner membrane, a thin peptidoglycan layer as target for the endolysin, and an outer membrane that is an extra permeability barrier highly limiting access of endolysins to the peptidoglycan target. Structures of endolysins against Gram-negative bacteria have, therefore, developed structural characteristics that enable translocation of the protein into the space between the outer and inner membranes, such as a small size and a positive surface charge that encourages interaction with the negatively charged LPS. The simple globular structure of these enzymes usually involves a single catalytic domain, while the binding of extended cell wall-binding domains is sterically blocked by the outer membrane barrier[25].

3.6 Protein folding and stability

The protein folding and stability of endolysins are key factors for the determination of the antibacterial activity, therapeutic efficacy and

manufacturability of endolysins, affecting enzyme activity, specificity of substrates, pharmacokinetics and immunogenicity. Under various conditions such as the high ionic strength of body fluids and the fluctuations of pH, as well as shear forces during manufacture and formulation and temperature changes in storage and transport, endolysins have to retain their native 3D structure. The folding stability of endolysins is regulated by the same rules for other proteins; hydrophobic interactions, hydrogen bonding and disulfide bonds are involved in stabilizing the native conformation[26]. Protein folding in some endolysins is complicated by the fact that many endolysins are made of several modular components that are independently folded but must assemble in the correct interdomain orientation and dynamics to form a functional enzyme. Protein engineering techniques have been used to increase the stability of endolysin, such as stabilizing mutations, optimizing the boundaries of the domains, and conjugating stabilizing groups like polyethylene glycol. Recent developments in the production and purification of proteins have also helped derive endolysins that have consistent folding and high specific activity that will allow the development of the enzyme as a therapeutic product. Stability of endolysins under certain applications, such as food preservation or topical therapy, has also been addressed by the formulation of the enzyme which protects it from degradation and retains its antibacterial activity[26].

4. Mechanisms of Antibacterial Action

4.1 Peptidoglycan degradation

Endolysins are also mainly known for their antibacterial activity, which is due to the enzymatic breakdown of the bacterial cell wall, the peptidoglycan. The peptidoglycan network is a complicated macromolecular network made up of glycan strands crosslinked by short peptides and which enclose the bacterial cell and withstand the high internal osmotic pressure. Endolysins lyse the peptidoglycan by cleaving specific bonds in this network, leading to the formation of localized defects in the cell wall and to the weakening of the cell wall and to the osmotic lysis of the bacterium.

Different endolysins cleave different bonds in the peptidoglycan structure, depending on the type of catalytic domain: muramidases, glucosaminidases, amidases and endopeptidases. The degradation of the peptidoglycan layer proceeds in a processive manner and the endolysin binds to its substrate and undergoes multiple rounds of bond breakage prior to dissociation for further maximum efficiency in cell wall degradation. Endolysins are also considered safe because they are highly specific to bacterial peptidoglycan, and will not affect the host tissues or eukaryotic cells since they lack peptidoglycan[27].

4.2 Osmotic lysis

The final effect of the endolysin-mediated peptidoglycan degradation is osmotic lysis, which means that when cell wall integrity is lost water rushes into the bacterial cell because of the high osmotic pressure that is maintained inside the cell. Bacteria have a very high solute concentration inside compared to outside of the cytoplasm, thus exerting a high osmotic pressure which is counterbalanced by the structural strength of the peptidoglycan layer. The degradation of peptidoglycan by endolysin activity leads to disruption of the integrity of the bacterial cell wall and entry of water into the bacterial cell, resulting in the swelling and eventual bursting of the cytoplasmic membrane. This osmotic lysis releases the protein, nucleic acids and metabolites inside the cell, and causes the irreversible loss of the viability of the bacteria. In particular, the osmotic lysis mechanism is very fast, normally endolysins are bactericidal within minutes of contact with the bacteria, which is much faster than conventional antibiotics, which inhibit the synthesis of macromolecules. One of the beneficial properties of endolysins is their rapid osmotic lysis, which prevents the bacteria from adaptive response or resistance mechanism[27].

4.3 Rapid bactericidal activity

A key distinguishing feature and salient clinical importance of endolysins is their rapid bactericidal activity that allows rapid clearance of bacterial pathogens, and reduces the risk of treatment failure or emergence of resistance. Some

endolysins have been shown to kill bacteria in minutes, with time-kill curves revealing a steep drop in cfu, a result of the direct and irreversible mode of action of peptidoglycan degradation. This bactericidal effect is very quick compared to the majority of traditional antibiotics that may take several hours or days for bacterial killing, especially for bacteriostatic antibiotics which only inhibit bacterial growth and don't kill the bacteria. The speed of the endolysin action may have important clinical implications because it could shorten the course of antibiotic therapy and decrease the chances of bacterial adaptation and the emergence of bacterial resistance. The fast bactericidal action of endolysins has been shown against many bacterial pathogens, such as multidrug-resistant Gram-negative bacteria, methicillin-resistant *Staphylococcus aureus*, and *Streptococcus pneumoniae* suggesting them as potential therapeutic agents for multiple infectious diseases[9].

4.4 Biofilm disruption

An interesting feature of endolysins is their ability to degrade bacterial biofilms, which are a major cause of the persistence of bacterial infections and resistance to traditional antibiotic treatments. Biofilms form when bacteria form structured communities within a self-made matrix of extracellular polymeric substances (EPS) composed of polysaccharides, proteins, and nucleic acids that shield the bacteria from the environment stresses, immune clearance, and antibiotic penetration. Endolysins can act via two mechanisms: they can degrade the peptidoglycan layer of the embedded bacteria and they can do the same with the extracellular polymeric substance matrix, which is made up of polysaccharides and proteins and may be susceptible to endolysin activity. Endolysins are able to penetrate the biofilm matrix because they are relatively small, and they may be able to break down the matrix components, allowing them to penetrate further. Endolysins have been found to disrupt biofilms of a wide range of clinically important pathogens, including *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Enterococcus faecalis* on varied surfaces including medical devices, wound

dressings and tissue surfaces. The dual mode of action of biofilm disruption and rapid bactericidal activity makes endolysins attractive therapeutics for treating infections involving biofilms, such as infective endocarditis, medical device-associated infections, and chronic wounds, which are resistant to antibiotics alone[28].

4.5 Activity against persister cells

Another important advantage of endolysins over conventional antibiotics is their activity against persister cells, a subpopulation of bacterial cells that enter a dormant state and are highly tolerant to antibiotic treatment. The persister cells are considered to have low metabolism and downregulate the targets of bactericidal antibiotics which depend on bacterial growth in order to exert their killing activity. The mechanism of endolysin action - direct enzymatic degradation of the peptidoglycan layer - does not require active processes that are inhibited in persister cells. This allows endolysins to target persister cells which are less susceptible to standard antibiotic treatment, one of the most common reasons for a failure to cure, and the return of bacterial infections. Endolysins have been shown to be active against persister cells both in planktonic and biofilm cells, indicating that endolysins may be able to kill the host of bacteria that form reservoirs leading to chronic and recurrent infections. Given the potential to kill persister cells together with their fast bactericidal activity and biofilm-disrupting property, endolysins have emerged as a potent weapon for treating persistent and recurrent bacterial infections that have proven to be resistant to conventional antibiotic drugs[10].

4.6 Synergy with immune responses

Antibacterial activities of endolysins can be boosted by synergy with host immune responses, in which the overall effect is greater than the sum of the individual effects of the enzyme and the immune system. Endolysins kill bacteria rapidly, lead to rapid clearance of bacterial pathogens, decrease the bacterial burden, and help to counteract the immunosuppressive effects that many bacteria have on host immune function. Endolysins may also degrade the bacterial cell wall,

releasing peptidoglycans fragments that stimulate immune receptors, stimulate the inflammatory process and allow influx of immune cells at the infection site and removal of bacterial wall. Endolysins have also been combined with other immune-active molecules like antimicrobial peptides or antibodies, which has been demonstrated to induce synergistic antibacterial activity suggesting that endolysins can complement and potentiate the function of host immunological molecules. This coupling between endolysins and immune responses has implications for the use of these enzymes in clinical patients, because their effects in vivo could be enhanced by including an immune cell response and they could be most useful when used in conjunction with immunomodulation[29].

5. Classification of Endolysins

5.1 Gram-positive endolysins

The antibacterial activity of endolysins can be increased by a synergy with the host immune response, generating an effect that is higher than the sum of the individual effects of the enzyme and the host immune response. The fast bactericidal effect of endolysins allows the clearance of bacterial pathogens, thereby lowering the bacterial load and at the same time mitigating some of the effects of bacterial pathogens on immune function, which are often immunosuppressive. Through endolysin degradation of the bacterial cell wall, immunostimulatory peptidoglycan fragments could also be released, which activate receptors of the immune system and amplify the inflammatory response, thus recruiting immune cells to the infection site and clearing up the bacterial residue. The use of endolysins in combination with immune-active compounds such as antimicrobial peptides or antibodies has been demonstrated to exhibit synergistic antibacterial activity, indicating that endolysins could be used together with, or as an enhancement to, the host's immune response. The combination of endolysins and immune response is important not only for the understanding of the mechanism of action of endolysins but also for the use of endolysins as clinically applied therapeutic enzymes since it indicates that, possibly, the activity of these

enzymes might be increased in immunocompetent patients as well as in combination with other modes of immunomodulatory therapy[10], [30].

5.2 Gram-negative endolysins

Bacteriophage-derived enzyme lysozymes have been adapted to membrane-bound cellular envelopes of Gram-negative bacteria, which are structurally complex, and constitute a distinct class of gram-negative endolysins, where unique therapeutic development challenges and opportunities exist. Unlike Gram-positive endolysins, Gram-negative endolysins are often relatively small enzymes, ranging 15 to 20 kDa in mass, with a simple globular structure, so that these enzymes can penetrate and gain access to the peptidoglycan layer through the outer membrane barrier. In Gram-negative bacteria the outer membrane is a highly effective permeability barrier for many antibiotics and macromolecules including endolysins, and strategies must be developed to increase the penetration of these enzymes. Some Gram-negative endolysins also have an intrinsic ability to pass the outer membrane and this is frequently linked to a highly cationic surface that allows the enzyme to interact with the negatively charged lipopolysaccharide layer to allow the enzyme to pass through the outer membrane. The mechanism of action of Gram-negative endolysins involves an attack on the peptidoglycan layer, which is exposed in the periplasmic space after outer membrane permeabilization, allowing the rapid lysis of bacteria and release of intracellular contents[23], [31].

5.3 Chimeolysins

The chimeolysins are engineered endolysins, made by linking catalytic and wall binding domains of distinct parent endolysins in various combinations to generate new cocktails of functional modules with improved and extended antibacterial activity. The modular design of endolysins can be exploited to devise a protein engineering strategy based on shuffling domains, leading to the production of chimeric enzymes with the best of both worlds: high catalytic activity from one endolysin and specificity from another. This strategy has been

used to make chimeolysins with increased host range to include several species of bacteria, increased lytic activity to a particular target pathogen, or improved stability in harsh environments. Typically, chimeolysins are constructed by the identification of a suitable catalytic and cell wall-binding domains through bioinformatic analysis, and the design of new chimeric constructs that maintain the proper interactions and folding of the individual domains. Chimeolysins created as a result have been demonstrated to have antibacterial properties, which in some cases outperform the parent endolysins, thus showing promise of using domain shuffling as a strategy for improving endolysin properties for specific applications[32].

5.4 Artilysins

Several engineered versions of endolysins featuring antimicrobial peptides' property of membrane penetration have been created and termed artilysins, which are novel fusion proteins displaying potent antibacterial activity against Gram-negative bacteria. The outer membrane barrier of Gram-negative bacteria poses a significant challenge to the use of endolysins from the outside, so artilysins have been created that combine the endolysin with a polycationic peptide that can damage the outer membrane, allowing the enzyme in. Most artilysins have an antimicrobial peptide segment that is short, basic and has the ability to disrupt the outer membrane by binding the LPS (lipopolysaccharide) layer and disrupting the lipid membranes, allowing the endolysin to penetrate and degrade the peptidoglycan. Artilysins show strong antibacterial activity against a large number of Gram-negative pathogens, such as *Pseudomonas aeruginosa*, *Escherichia coli*, and *Acinetobacter baumannii*, with low micrograms per milliliter MIC values. Thanks to the modular design, the antibacterial activity of artilysins has been systematically optimized by variations of endolysin and antimicrobial peptide modules, resulting in the identification of candidates with high antibacterial activity and good safety characteristics[17].

5.5 Lysocins

Lysocins are a family of antibacterial proteins with the cell wall-binding specificity of bacteriophage endolysins, but the bactericidal effector domain is not necessarily a peptidoglycan hydrolase enzyme, thus developing novel antibacterial agents with new mechanisms of action. The specificity of the endolysin is provided by the cell wall-binding domain which allows the protein to be targeted to the cell surface of a bacterium and thus focuses the bactericidal activity onto the target. The effector domain of lysocins may contain different bactericidal moieties, such as antimicrobial peptides, membrane-disrupting proteins or enzymes capable of producing reactive oxygen species, thus creating a variety of killing mechanisms of the target bacterium. Modular architecture of endolysins has helped the construction of lysocins by the ability to easily interchange functional domains to produce new combinations of specificity and killing activity. Lysocins have demonstrated strong antibacterial activity, both against Gram-positive and Gram-negative bacteria, and the specificity of the cell wall-binding domain affects the spectrum of the antibacterial agent produced[17].

5.6 Engineered recombinant endolysins

The use of synthetic biology and protein engineering approaches has paved the way for the development of engineered recombinant endolysins that can be optimized for their properties for certain therapeutic applications, thus largely increasing the potential of these enzymes as antibacterial agents. Recombinant endolysins can be expressed in a heterologous expression system, usually *Escherichia coli*, to allow the large scale production of these enzymes for research and therapeutic purposes. Because of their recombinant production, it is possible to introduce mutations and modifications to improve the endolysin's anti-bacterial activity, stability or its pharmacokinetic properties, using methods like directed evolution, site-directed mutagenesis, and domain swapping. Recombinant engineering has resulted in the development of variants with broadened host ranges, increased lyse potency, physiological stability and decreased

immunogenicity, which overcome many of the constraints that have hampered the therapeutic use of wild-type enzymes. Engineered recombinant endolysins with improved therapeutic characteristics have been developed through the use of advanced protein engineering approaches such as rational design, computational protein modeling and high throughput screening, which have further helped to speed up the process of endolysin engineering[8], [10], [33].

6. Protein Engineering and Synthetic Biology

6.1 Domain shuffling

Because of the modular structure of endolysins, domain shuffling is a powerful approach to the engineering of endolysins to achieve enhanced or expanded antibacterial properties; functional modules of endolysins which are concatenated can have altered host range, catalytic activity or stability properties. In most cases, the domain shuffling procedure involves identifying appropriate catalytic and cell wall binding domains from distinct parent endolysins, and then making a chimeric gene with the desired domain combination. Chimeolysins may have antibacterial activity against bacterial species that do not belong to the spectrum of activity of either parent endolysin, thus creating an extended host range, which can prove useful for treating polymicrobial infections. Another approach to optimizing the catalytic activity of endolysins is through domain shuffling, in which a combination of a cell wall-binding domain, which efficiently binds its target substrate, with a catalytic domain, which has a high enzymatic turnover, will synergistically improve antibacterial activity. Using domain shuffling has been made possible by identifying conserved structural motifs and functional relationships helping the design of functional chimeric enzymes and allowing the rational design of endolysins with predictable properties[14], [32].

6.2 Directed evolution

A great protein engineering tool that mimics the process of natural selection, and can be used to create endolysins with improved properties like antibacterial activity, stability or substrate

specificity, even when no structural information is available. It comprises forming a library of endolysin variants by random mutagenesis or random recombination of the endolysin gene; and screening or selecting the library of endolysin variants for endolysin variants that have the desired property. Then the selected variants are remutagenated and rescreened, further enriching the population for variants retaining further improvements of properties. By using directed evolution, endolysin variants with enhanced catalytic activity, host range, thermostability, and/or lowered immunogenicity have been created, highlighting the potential of this strategy for endolysin optimization. The use of endolysins for directed evolution has been aided by the development of high throughput screening techniques which allow fast evaluation of large collections of different variants and fast discovery of better enzymes. Endolysin properties have been further optimized for therapeutic applications through the combination with other protein engineering strategies such as rational design and domain shuffling together with directed evolution[34].

6.3 Fusion proteins

The generation of fusion proteins with endolysins and other functional domains has become an effective method for optimising their antibacterial action and augmenting their therapeutic efficacy to control the action of multiple activities in a single polypeptide. The primary challenge for the use of endolysins for external use against gram-negative bacteria is the need for the endolysin fusion protein to be able to penetrate the outer membrane, and this can be accomplished through the inclusion of an outer membrane-penetrating domain, e.g., antimicrobial peptides or membrane-disrupting sequences. Fusion proteins can also contain domains which promote the binding of the endolysin either to particular surfaces or to delivery vehicles that would channel the enzyme delivery system to an infection site. The endolysin protein has been fluorescently or affinitively tagged to help track and immobilise the enzyme for use in endolysin biology studies and applications to develop biosensors. Incorporation

of endolysins into other antimicrobial proteins (e.g., antimicrobial peptides or bacteriocins) has demonstrated synergic antibacterial activity exceeding the sum of the activity of each component. The recent advances in recombinant DNA technology and protein expression have helped in the development of endolysin fusion proteins, which can easily be constructed and produced[35].

6.4 PEGylation

Covalent attachment of PEG polymers to endolysins (PEGylation) is a proven method to improve the pharmacokinetic characteristics of protein therapeutics such as extended circulation half-life, lower immunogenicity, and greater stability. The presence of polyethylene glycol chains on the surface of the endolysin provides a protective coating to reduce its recognition by the immune system and slow its clearance from the blood, allowing the enzyme to stay in the circulation longer for exposure to the antibacterial agent. PEGylation can also increase the stability of the endolysins against proteolytic degradation and aggregation, and improve their shelf life and function in a physiological environment. Modification of endolysins can usually be done by chemical modification of the lysine residues or by site specific conjugations that retain activity of the protein. The PEGylation level and location has to be maximized to fully leverage the maximum benefits of PEGylation on the endolysin's pharmacokinetics, otherwise too much PEGylation can lead to reduced catalytic activity, or the PEGylation can hinder cell wall binding. Systemically administered PEGylated endolysins have been shown to be therapeutically effective in preclinical models reflecting and supporting the PEGylation approach for endolysins[36].

6.5 Nanoparticle conjugation

Taking advantage of special properties of nanomaterials for therapeutic use, endolysin conjugation to nanoparticles has become an interesting strategy for both increasing their antibacterial activity and directing them to sites of infection. Nanoparticles can provide a vehicle for the endolysins to protect them from degradation,

deliver them across biological barriers and release them at the target site of infection. Conjugation of endolysins to nanoparticles can also boost the antibacterial activity of these biocarriers by raising the concentration of the enzyme on the surface of the bacteria or by combining with the activity of the nanoparticles, which themselves may have antibacterial activity. Since endolysins are normally being delivered to various types of bacteria, their functionalisation with targeting ligands may improve the specificity of their delivery to bacterial pathogens with minimal off-target effects, which will improve the selectivity of antibacterial therapy. Pre-clinical studies have been carried out with nanoparticle-based endolysin delivery systems across several applications, such as the treatment of wound infections, biofilm-associated infections, and systemic bacterial infections. With improvements in particle synthesis and characterisation, endolysin-nanoparticle conjugates have been developed which will allow systematic optimisation of delivery systems for specific therapeutic applications[35].

6.6 AI-assisted protein engineering

The incorporation of artificial intelligence and machine learning techniques in the protein engineering workflow has transformed how optimized endolysins can be developed, with the ability to predict structure-function relationships quickly, create better variants and fasten the discovery process. Different types of AI tools, such as deep learning and protein language models, have been used to predict the 3-D structure of endolysins based on their amino acid sequence, which has given insights into the structure that underlie their catalytic activity and target specificity. Machine learning algorithms are trained using datasets of endolysin sequences and their antibacterial activities and used to predict the activity of new variants and design new and better enzymes. By combining the use of generative models with endolysin engineering, new areas of sequence space have been explored to uncover novel variants with improved properties that would be hard to obtain using directed evolution on their own[37]. AI also allows for the fusion of

computational prediction and experimental validation, creating an effective feedback mechanism for endolysin engineering. The use of artificial intelligence in conjunction with experimental screening strategies has further opened up an effective feedback loop for endolysin engineering, allowing for the iterative optimisation of enzyme properties. By applying AI-assisted protein engineering to endolysins, the time and cost needed to identify and optimise lead candidates could be significantly reduced, potentially hastening the development of these enzymes as therapeutics[8].

6.7 CRISPR-assisted optimization

With the development of CRISPR-based technologies, endolysin engineering has extended its possibility to optimise the endolysin quickly and precisely by utilising the excellent genome editing function of CRISPR systems for protein engineering and bacterial host engineering. CRISPR-mediated optimisation of endolysins can be done by introducing site-specific mutations into the endolysin gene that increase the catalytic activity, stability, or host range. CRISPR methods can also be used to engineer the bacterial host in which the endolysin is expressed to enhance the enzyme yield of production and assist in purification and characterisation[38]. Through the use of CRISPR to study endolysin function, systematic studies of structure-function relationships have been carried out, with the identification of important residues and domains that define the properties of the enzymes and provide a rational basis for protein engineering. The combination of genome editing using CRISPR technology with high-throughput screening has facilitated a rapid generation and assessment of endolysin variant libraries, speeding up the process of finding optimised enzymes. CRISPR technologies can be used to engineer endolysins and is an emerging field likely to have a profound influence on the development of these enzymes as therapeutic agents, as it will allow them to be precisely and quickly optimised for clinical use[39].

7. Delivery Strategies

7.1 Topical delivery

Topical delivery is one of the simplest and most advanced ways to deliver endolysins, as it allows the use of high concentrations of the enzyme directly on the site of infection, giving fast bactericidal activity. The topical use of endolysin has been much explored and involves the use of the enzyme in creams, ointments or gels which are applied directly on the skin or soft tissue where the infection is happening, including MRSA infections. The benefits of topical delivery are that it will avoid systemic exposure, thus reducing the risk of immunogenicity and off-target effects; and that it will provide a high local concentration which will provide a rapid clearance of any bacterial pathogens. Endolysin formulations have been tested in clinical trials for topical use on atopic dermatitis, such as endolysin SA. 100 has been shown to be safe and well tolerated in patients with mild to moderate atopic dermatitis. Formulation strategies designed to protect and enhance the stability of the enzyme, encapsulation of the enzyme in lipid-based delivery systems, and use of penetration enhancers can help to achieve better efficacy with topical delivery of endolysin. The relative ease of manufacturing and formulating topical products has allowed the efficient translation of preclinical leads to clinical studies, which have been helpful for the development of topical endolysin products[40].

7.2 Intravenous administration

The administration of endolysins intravenously is the route of choice for treatment of systemic bacterial infections, as therapeutic concentrations of the enzyme would be rapidly delivered to the bloodstream, and clearance of the bacterial pathogens from the circulation and/or from other tissues could be expected. Clinical trials have tested intravenous use of endolysins for treating bacteremia and infective endocarditis, and endolysin SAL200 has a good safety profile and pharmacokinetics following intravenous administration in healthy volunteers. However, the delivery of endolysins systemically is fraught with issues that must be resolved for their clinical use: the short half-life of endolysins in the

circulation, the potential for them to be immunogenic and the need to obtain therapeutic levels at the site of infection. In vivo, endolysins are subject to the same pharmacokinetics as other therapeutic protein products, including the effect of clearance mechanisms (e.g., renal filtration and reticuloendothelial uptake) and protein engineering (e.g., PEGylation) to extend the half-life in circulation. The safety of intravenous endolysin administration has been proved in preclinical and clinical studies where the enzymes were well tolerated and without any significant indication of toxicity or adverse effect[41].

7.3 Pulmonary delivery

The use of pulmonary delivery for endolysins provides a direct pathway for the delivery of the enzyme directly to the site of infection in the lungs and airways. Bacterial pathogens like *Streptococcus pneumoniae*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* cause respiratory infections, which are a major cause of morbidity and mortality, especially in vulnerable individuals like the elderly, immunocompromised individuals and those with underlying lung disease. Endolysins can be aerosolised using nebulisers or dry powder inhalers for pulmonary delivery and deposition of therapeutic levels in the airway to clear bacterial pathogens. Pulmonary delivery has several benefits, such as the avoidance of systemic exposure and high concentrations locally at the site of infection, preventing immunogenicity. Due to the many advances in aerosol delivery technology and formulation science, stable and respirable enzyme formulations have been made possible, which has helped the development of pulmonary endolysin formulations. Pulmonary endolysin has been shown to be effective in preclinical models of respiratory infection and could be used to treat bacterial pneumonia and other respiratory infections[40].

7.4 Oral delivery

The delivery of endolysins via the oral route is challenging because of the degradation of the enzyme and its low bioavailability in the harsh conditions of the gastrointestinal tract, including the acidic stomach pH, presence of

gastrointestinal proteolytic enzymes, and gastrointestinal microbial community. Despite these challenges, there have been attempts to use endolysins as oral drugs, such as in gastrointestinal infections, where endolysin is applied in the intestinal lumen, where the infection is present. Different strategies have been put into practice for the development of oral endolysin formulations, which include enteric coating, encapsulation in suitable protecting matrices, and carrying oral endolysin in a suitable delivery vehicle that is capable of enhancing the enzyme stability and release in the gastrointestinal tract. Oral delivery of oral endolysin has been proved to be effective in preclinical models of *Clostridioides difficile* infection, where the enzyme was shown to reduce the colonisation of the bacteria and alleviate symptoms of the infection. Oral endolysin delivery has also been extended to the treatment of other gastrointestinal infections and for the modulation of the gut microbiome, taking advantage of the specificity of endolysins to only target pathogenic bacteria and not harmful commensals[42].

7.5 Liposomes

Liposomes are a proven drug delivery platform and have been used for the encapsulation and targeted delivery of endolysins, protecting the enzyme from degradation and achieving targeted release at the site of infection. Liposomes are spherical vesicles made of lipid bilayers which are able to enclose molecules that are water-soluble in their aqueous interior, thereby protecting them from environmental influences and guiding them toward target cells or tissues, such as endolysins. Encapsulation of endolysins in liposomes could be demonstrated to increase the stability of the endolysin at physiological conditions, extend its circulation half-life and lower the immunogenicity, thereby increasing its therapeutic potential for systemic administration. To improve the targeting selectivity and minimise the off-target effects, endolysins can be functionalised with targeting ligands such as antibodies or peptides by conjugating them to liposomes. Liposome-based delivery systems for endolysins have been shown in preclinical models of bacterial infection to have an improved antibacterial activity when delivered

in liposome form compared to when they were delivered as free enzymes. Advances in liposome preparation and characterisation have made the development of liposomal endolysin formulations possible, making it possible to systematically optimise the delivery systems for specific therapeutic applications[43].

7.6 Hydrogels

Hydrogels are a versatile type of delivery systems that can be used for endolysins, as they are hydrated three-dimensional polymer systems able to encapsulate the enzyme and help it release slowly at the site of infection. In the case of topical or wound applications, hydrogels can be directly applied to the area of interest and will maintain the wound site moist which facilitates wound healing while also releasing enzyme to the wound site at therapeutic levels. The inclusion of endolysins in hydrogels provide protection against enzyme degradation and allow for sustained and controlled release over longer time periods, leading to less frequent dosing and better patient compliance. Endolysins have been incorporated into hydrogels, which has been reported to improve their antibacterial effectiveness against wound pathogens and endolysins have been observed to remain active in the gel, mediated a sustained wound pathogen clearance. There are different natural and synthetic polymers that can be used to make the hydrogels, which can be tailored to specific applications by changing the mechanical properties and the release characteristics. Progress in polymer chemistry and the fabrication of hydrogels has helped to create endolysin-loaded hydrogels that can be tailored to have optimal properties for wound healing and infection control[44].

7.7 Polymeric nanoparticles

Polymeric nanoparticles are a very versatile delivery system of endolysins, a platform that allows endolysins to be encapsulated and delivered with targeting tunable release kinetics and surface properties. Polymeric nanoparticles are usually made from a biodegradable polymer and can be designed to load and release endolysins at the site of infection and to protect the enzyme from

degradation and to enable the display of the enzyme in a controlled manner to the bacteria. Polymeric nanoparticles can be modified with targeting ligands that promote specific targeting of the particles to the bacteria, thus leading to more selective endolysin therapy and minimised off-target effects. Endolysin can be encapsulated in polymeric nanoparticles that have been demonstrated to increase both enzyme stability and antibacterial activity, and the functionality of the nanoparticles containing endolysins was found to be superior to the free one. Targeted antibacterial treatment based on polymeric nanoparticle-based delivery systems for endolysins has been demonstrated in the preclinical models of bacterial infection. In the effort to develop endolysin-loaded polymeric nanoparticles, the advances of the nanoparticles' fabrication and characterisation have proved useful in order to formulate nanoparticles with the desired properties for therapeutic applications[45].

7.8 Smart delivery systems

A new possibility for endolysin delivery is the development of smart delivery systems that will respond to specific stimuli at the site of infection, allowing the deliberate release of endolysin in response to bacterial signals or environmental stimuli. This can be accomplished by designing smart delivery systems that release the endolysin only if the pH changes, the temperature changes or when bacterial enzymes are present, thereby delivering the enzyme specifically to the site of infection and when desired in response to the presence of bacterial pathogens. The use of stimuli-responsive materials as delivery systems has led to the design of endolysin formulations that remain intact in the bloodstream and are activated in response to the bacterial infection, thereby reducing side effects and enhancing therapeutic effects. Alternatively, smart delivery systems could be designed to activate the release of endolysins in the presence of specific bacterial pathogens, using the specificity of bacterial signalling molecules or virulence factors to release endolysins. Smart delivery systems for endolysins have been developed thanks to progress in both the fields of materials science and drug delivery, where

formulations with complex responsiveness to biological stimuli can be fabricated[46].

8. Clinical Applications

8.1 Methicillin-resistant *Staphylococcus aureus*

One of the most important targets for endolysin therapy is methicillin-resistant *Staphylococcus aureus*, which is a major cause of healthcare-associated infections and resistant to a number of classes of traditional antibiotics. *Staphylococcus aureus* is a versatile opportunistic pathogen which colonises the nasal mucosa and skin of a considerable proportion of the human population and can cause a wide spectrum of infections ranging from mild skin and soft tissue infections to serious, life threatening conditions such as bacteremia, infective endocarditis, osteomyelitis, meningitis and pneumonia. The rise of methicillin-resistant *Staphylococcus aureus* has drastically reduced the number of therapeutic options available to combat these infections and there is a need for new antibacterial agents to treat them. A number of endolysins displaying activity against *Staphylococcus aureus* (SA) have been extensively characterized and are developed for clinical use with several clinical trial candidates. The endolysin SAL200 (tonabacase) was successfully completed in healthy volunteers and a Phase II trial in *S. aureus* bacteremia patients, and was safe and tolerated in both trials. Microes' chimeric endolysin XZ.700, which has been designed to specifically target *Staphylococcus aureus* but spare commensal, good bacteria on the skin, is currently under investigation in clinical trials for topical treatment of atopic dermatitis (AD). Endolysin CF-301/exebacase had also been shown to have strong activity against various strains of staphylococci and streptococci and was studied in phase III clinical trials for bacteremia and right-sided endocarditis, but was abandoned due to lack of statistical power. The substantial clinical development work focused on endolysins for MRSA highlights the therapeutic potential of endolysins for this pathogen, and the realization of the medical need of MRSA infections[43], [47].

8.2 *Streptococcus pneumoniae*

Streptococcus pneumoniae is a prominent respiratory pathogen responsible for several infections, such as community-acquired pneumonia, meningitis and otitis media, and is a major cause of morbidity and mortality around the world, especially in young children and elderly people. With the appearance of penicillin-resistant and multidrug-resistant *Streptococcus pneumoniae* infections, therapeutic choices for infections due to this organism have been reduced, and alternative agents against the bacterium are needed. The endolysins with activity against *Streptococcus pneumoniae* have been identified and studied for their antibacterial and therapeutic properties, and some of the candidates have shown good activity, including against drug-resistant strains. The specificity of pneumococcal endolysins has been used in the development of diagnostic applications, where the enzymes have been used to rapidly detect and identify *Streptococcus pneumoniae* in clinical samples. The route of delivery for endolysins in pneumococcal infections has been the development of endolysins that are aerosolised and delivered directly to the respiratory tract. Endolysins have also been considered for use in the prevention of pneumococcal colonisation; here the enzyme is delivered via the nose to clear the carrier state and decrease the likelihood of an invasive pneumococcal disease[48].

8.3 *Enterococcus faecalis*

Enterococcus faecalis is an opportunistic pathogenic bacterium that causes a diverse range of healthcare-associated infections (HAIs), such as urinary tract infections (UTIs), bacteraemia, wound infections, and infective endocarditis, and has become a major public health threat as it has intrinsic resistance and acquired resistance to many antibiotic agents. Recently, the development of vancomycin-resistant *Enterococcus faecalis* strains has further reduced the therapeutic options for infections caused by this pathogen, leaving a need for alternative antibacterial agents. Endolysins lytic to *Enterococcus faecalis* have been characterised and shown to exhibit antibacterial activity against both antibiotic-susceptible and

vancomycin-resistant *E. faecalis*, suggesting a potential application of these enzymes to treat enterococcal infections. The endolysins (Ls) of *Enterococcus* phage PEF1, LysPEF1-1 and LysPEF1-2, demonstrated strong activity against multidrug-resistant *Enterococcus faecalis* strains and their biofilm, with the mixture of both Ls showing better antibacterial activity. Interest in the enterococcal endolysins' activity against biofilms is particularly relevant for the treatment of so-called 'persister' infections, including biofilm-associated infectious diseases such as infective endocarditis or urinary tract infections, for which conventional antibiotic therapy fails[49].

8.4 *Clostridioides difficile*

Clostridioides difficile (*C. difficile*) is a Gram-positive, spore-forming anaerobic bacterium that is a leading cause of healthcare-associated infection (HAI) and antibiotic-associated colitis (AAC) and recently has seen the emergence of hypervirulent and antibiotic-resistant strains that have increased the clinical impact of this infection. The pathogenesis of *C. difficile* infection includes the production of toxins that mediate the inflammatory response and damage to the intestinal epithelium, and difficulties in managing this infection include the persistence of infecting spores which are resistant to antibiotic treatment and can result in recurrent CDI. With the specificity of endolysins being able to kill the vegetative form of the pathogen while maintaining beneficial gut microbes, endolysins which target *C. difficile* have been developed for the treatment of this infection. The enzyme, called endolysin LMN-201 by its developer, Lumen Bioscience, is being tested in a Phase II clinical trial for the treatment of *Clostridioides difficile* (*C. diff*) infection (CDI) recurrence, where it is taken orally to directly interact with the pathogen in the gastrointestinal (gut) tract. Endolysins have also been tested against *Clostridioides difficile*, where the enzymes were tested for their ability to decrease the number of spores and suppress intestinal colonisation[50].

8.5 *Listeria monocytogenes*

Listeria monocytogenes is a foodborne pathogen and the causative agent of listeriosis, a life-

threatening infection that occurs mainly in pregnant women, elderly people and immunocompromised individuals and, despite correct antibiotic treatment, has a high case fatality rate. *Listeria monocytogenes*' ability to grow at refrigeration temperature and to form biofilms on food processing surfaces makes it a major concern for food safety and requires new approaches for controlling this pathogen in the food industry. Endolysins against *Listeria monocytogenes* have been characterised for their antibacterial activity against planktonic cells and biofilms with potential applications for food safety and infection control. Endolysin applications to control *Listeria monocytogenes* in food processing environments have been investigated, and enzymes have been formulated as biocontrol agents to decrease the contamination of food pathogens and to prolong the shelf life of the food products. Listerial endolysins have been used in species-specific diagnostic applications because of their specificity for their target species, with the possibility of rapid detection and identification of *Listeria monocytogenes* in food and environmental samples[51].

8.6 *Bacillus anthracis*

Rapid and effective countermeasures are needed to prevent and treat anthrax infection, because *Bacillus anthracis* is the causative agent of anthrax, a zoonotic disease that has significant potential as a bioterrorism agent and is a threat to public health. The endolysins of *Bacillus anthracis* have been characterised for their antibacterial and therapeutic potential, and the endolysins were shown to be very active against the vegetative form of the pathogen. Endolysins for anthrax treatment have been investigated for use both as therapeutics and as prophylactics when the enzymes are being used to kill the organism and stop the disease from progressing. The specificity of anthrax endolysins for their target has been exploited for the development of diagnostic applications, and it is possible to use rapid detection and identification of *Bacillus anthracis* in clinical and environmental samples. The public health importance of anthrax and the availability of suitable animal models have

spurred the development of endolysins for the treatment of this disease[52].

9. Endolysins Against Biofilms

9.1 Biofilm biology

Biofilms are communities of living bacteria in the form of the biofilm, which is a structured community of bacteria encapsulated in a self-produced matrix of extracellular polymeric substances, polysaccharides, proteins and nucleic acids that attach to surfaces and are highly organised with spatial and functional properties. Biofilm formation involves several stages: initial attachment, formation of an extracellular polymeric substance matrix, maturation of the biofilm to a three-dimensional structure, and eventual dispersion of cells to colonise new surfaces. The benefits of the biofilm mode of growth over planktonic growth are many and include improved resilience of the bacteria to environmental stresses, greater antibiotic resistance and the promotion of horizontal gene transfer between the bacteria. Bacterial infection persists in humans by the formation of biofilms, and it has been estimated that up to eighty per cent of bacterial infections in humans are associated with biofilms, such as chronic wound infection, medical device-related infection, respiratory tract infection and gastrointestinal tract infection. Biofilms that develop on medical devices like catheters, prosthetic joints, heart valves, etc. are a special problem in controlling infections because the surface is not accessible to conventional antibiotics or host immune defence[53].

9.2 Why antibiotics fail

The inability of conventional antibiotics in the treatment of an infection associated with the growth of a biofilm is the result of several factors, which give the bacteria a protected place not to grow in the presence of antibiotics. Biofilms form a physical barrier to antibiotics through the extracellular polymeric substance matrix, which prevents penetration to the bacteria within the biofilm, especially for those antibiotics that are either large or very hydrophobic. Within the biofilm, there are oxygen and nutrient gradients that cause metabolic heterogeneity and lead to

reduced metabolic rates and growth rates of cells in the deeper layers of the biofilm, making them less susceptible to antibiotic action that targets actively growing cells. Persistent cells within biofilms also help explain the antibiotic tolerance; such cells are dormant and thus resistant to antibiotics that kill via cell activity. The close association of cells in biofilms allows the horizontal transfer of determinants of resistance, which allows the rapid spread of resistance traits among the cells of the biofilm. These factors render a microenvironment that allows the bacteria to persist and incubate even in the presence of antibiotics, causing antibiotic failure and re-infections associated with the biofilm[54].

9.3 Anti-biofilm mechanisms

Because conventional antibiotics are limited to their ability to inhibit the growth of embedded bacteria, endolysins have a two-pronged strategy to attack biofilms: they generally both break down the extracellular polymeric substance matrix and lyse the peptidoglycan layer of bacteria. An endolysin's ability to degrade the extracellular polymeric substance matrix, which is commonly made of polysaccharides and proteins that may be degraded by endolysin, can disrupt the biofilm and free bacteria trapped in the protection matrix. Relatively small endolysins and their enzymatic activity that can break down the matrix and form channels for deeper penetration facilitates penetration into the biofilm matrix. After the endolysins cross the biofilm and enter the embedded bacteria, they break down the peptidoglycan and the bacteria lyse due to osmosis. These enzymes have been shown to be active against a range of clinically relevant pathogens on different surfaces, and their combined action on both the biofilm matrix and the bacteria has been shown to effectively eradicate the biofilm. Endolysins have been shown to act against *Staphylococcus aureus* biofilms on polystyrene, glass, surgical mesh and catheters and in human synovial fluid and against *Pseudomonas aeruginosa* and *Enterococcus faecalis* biofilms[55].

10. Endolysin Resistance

10.1 Can bacteria develop resistance?

A key issue for the clinical development of these enzymes is whether or not they can confer resistance to bacteria, because this would limit their therapeutic use. As of yet, no study has seen the development of bacterial resistance to the endolysin when used at the same concentration and dosage as other bacteria-resistant antibiotics. This extraordinary lack of resistance development is believed to be due to the mechanism of endolysin action, which is towards the peptidoglycan layer, a highly conserved, essential structural component of the bacterial cell wall. Co-evolution with their host bacteria has minimised the possibility of the emergence of resistance since the phages have been selected to target bonds in the peptidoglycan – which are ones that are vital for the bacteria. The non-development of resistance has been seen with many endolysins which are acting on Gram-positive and Gram-negative bacteria, making this observation generalisable. The advantage of endolysins is that they do not have a high resistance induction potential, unlike conventional antibiotics that tend to select for resistant mutants in a short time[56].

10.2 Cell wall modifications

Resistance to endolysins has not been seen in the laboratory or clinical environment, but the potential for the emergence of resistance by manipulation of the bacterial cell wall has been discussed. For example, bacteria could alter their peptidoglycan structure so as to lessen the binding or activity of the endolysins, either by changing the target bond or by masking the target site. Bacteria may also change their cell wall so that the peptidoglycan is no longer accessible to the endolysin, e.g., by simply making more cell wall components which act as a physical barrier to the action of the enzyme. But this acquisition of such modifications would be limited by the need to maintain bacterial cell integrity, since any change that would affect the structural integrity of the cell wall would prove lethal for the bacterium. The conservation of the peptidoglycan structure warrants that such resistance development is even

more limited among bacterial species, because mutations that change target sites of endolysins would also be expected to have pleiotropic effects and would therefore affect bacterial fitness. The resistance mechanisms that have been theorised for endolysins have never been detected in practice, indicating that endolysins have a low tendency to induce resistance[47].

10.3 Evolutionary constraints

Evolutionary constraints that hinder the ability of bacteria to develop resistance to endolysins include the critical importance of the maintenance of the peptidoglycan layer in the survival of the bacteria and the conservation of the target bonds that are recognized by endolysins. Peptidoglycan is the most important component of the bacterial cell wall, which provides cell shape and resistance to the high osmotic pressure. Any change to an otherwise intact peptidoglycan layer would likely kill the bacterium since it is the main component of the bacterial cell wall and maintains cell shape and protects against the high osmotic pressure. The bonds endolysins cleave are within the peptidoglycan structure and are essential for its function, because they are the ones that provide the cross-links between the glycan strands and the scaffold of a cell wall in the form of a mesh. Mutations that change these target bonds would be limited because any such mutation that lowers the strength or integrity of the cell wall would be detrimental to the fitness of the bacteria. Further, the peptidoglycan structure is highly conserved among all bacterial species so that the target bonds are the same in every bacterium and the development of resistance would require the co-ordinated modification of several genes. Another benefit of endolysins is the resistance to these enzymes cannot be acquired in the same way as resistance to the more widely used conventional antibiotics that target more variable and less critical parts of the bacterial structure[57].

10.4 Comparison with antibiotic resistance

Comparing resistance development of endolysins and conventional antibiotics gives a fundamental understanding of the differences in the evolutionary process and mechanisms of how

resistance is developed and the benefit of endolysins. Resistance to antibiotics can arise in a number of different ways, such as by a change in the antibiotic target, by degrading or modifying the antibiotic, by actively pumping it out of the cell, or by changing the membrane permeability, many of which can be acquired by relatively simple genetic modification. Typically when acquiring antibiotic resistance, there is little fitness cost to the bacterium—the bacterium is capable of surviving and multiplying when the antibiotic is present without being significantly less virulent or slow-growing. Antibiotic resistance is a problem because antibiotics are broadly used in medicine and agriculture, and this provides significant selective pressures, selecting for antibiotic resistance factors. In contrast, resistance to endolysins would have to involve changes that would affect the fundamental peptidoglycan structure and thus be of high fitness cost, making it difficult to develop resistance. Additionally, resistance to endolysins is not seen even after prolonged use and exposure, which is in stark contrast to the quick emergence of resistance to conventional antibiotics, which gives endolysins a strategic advantage in their use in antibacterial therapy[56].

10.5 Strategies to minimize resistance

The low occurrence of resistance development by endolysins can be further strengthened by implementing strategies which reduce opportunities for resistance development and thus the long-term efficacy of the enzymes. When combined with traditional antibiotics, endolysins have demonstrated synergistic antibacterial activity, and resistance was unlikely to occur because the bacteria would have to withstand two mechanisms of action at once. Endolysins in combination with other antimicrobial compounds (e.g., bacteriophages and antimicrobial peptides) have also been demonstrated to exhibit synergy and decrease the likelihood of resistance development. Engineered endolysins with more than one catalytic domain, such as those that possess two domains targeting multiple bonds in the peptidoglycan (glycan backbone and peptide cross-links), are even less susceptible to resistance

because the bacteria have to modify more than one target site to resist the enzyme. Optimised formulations or delivery systems that cause endolysins to act quickly on bacteria will decrease the time for development of adaptive responses and selection for resistance. Furthermore, by implementing antimicrobial stewardship practices that discourage the overuse of endolysins and encourage their judicious use for infections where they may be most beneficial, their effectiveness and the likelihood of resistance development can be further preserved[58].

11. Combination Therapies

11.1 Endolysins plus antibiotics

The use of endolysins in combination with conventional antibiotics has become a new approach to treating bacterial infections that could be beneficial due to the synergistic effects of the combined agents, which would improve the antibacterial activity and decrease the development of resistance and clinical outcomes for the patient. Endolysins have been tested clinically for the treatment of systemic infection as adjuncts to standard-of-care antibiotics, and they are co-administered with the antibiotic to boost antibacterial killing and cure rates. Endolysins have been demonstrated to have synergistic effects on the antibacterial properties with antibiotics. Synergistic effects have been seen with combinations of endolysins and antibiotics acting on the bacterial cell wall or membrane, such as beta-lactams, lipopeptides and glycopeptides, with the ability to decrease the minimal inhibitory concentrations of the antibiotics and restore susceptibility in otherwise resistant bacteria. The combination of endolysin with antibiotics has been shown to be synergistic against many different bacterial pathogens, such as *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and methicillin-resistant *Staphylococcus aureus*. The use of endolysins with antibiotics may also limit the development of resistance; bacteria would need to break two mechanisms of killing to survive[43].

11.2 Endolysins plus bacteriophages

Endolysins combined with bacteriophages is a strategy that takes advantage of complementary mechanisms of action of these agents: the endolysin possesses fast bactericidal activity against the vegetative form of the pathogen, and the bacteriophage has replication-dependent activity, which can amplify its antibacterial effect. The endolysins and bacteriophages synergistically interact with one another, indicating that the activity of the two agents, when used together, is greater than the sum of the activities of each agent. The bacteriophage can lyse the cell and release progeny phages to infect and kill further bacteria, but the endolysin can kill bacteria that are not infected with the phage or have become resistant to the phage infection. The use of endolysins coupled with bacteriophages has the potential to make the emergence of resistance even more difficult, because the bacteria need to withstand the infection of a phage and the destruction of the cell wall by endolysin. The synergy of endolysin-bacteriophage combinations has been shown against a wide range of bacterial pathogens such as *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Acinetobacter baumannii*. Endolysin has been tested in combination with the bacteriophages in preclinical infection models and shown to increase clearance of the bacteria and lead to better survival rates than either agent alone[59].

11.3 Endolysins plus antimicrobial peptides

The pairing of endolysin with antimicrobial peptides is a tactic used to push endolysin closer to the peptidoglycan layer by breaking down the membrane of the cell with the antimicrobial peptide, especially for the Gram-negative bacteria, which have an outer membrane which is a formidable barrier for endolysin. Antimicrobial peptides are naturally occurring molecules with a wide spectrum of antimicrobial activity which can also disrupt bacterial membranes to provide access to other antimicrobial agents. Endolysins have been shown to act synergistically with antimicrobial peptides (AMP) to kill Gram-negative bacteria: AMP permeabilising the outer membrane and endolysin degrading the peptidoglycan. Endolysins have been combined

with antimicrobial peptides (AMPs), and the synergy between the combination and the antimicrobial peptides has been shown against a number of Gram-negative pathogens, such as *Pseudomonas aeruginosa*, *Escherichia coli* and *Acinetobacter baumannii*. showing that the combination is more active against the pathogens than either alone. Engineered strategies involve the construction of artilysins: the fusion of the endolysin to a polycationic peptide which permeabilises the outer membrane, thus creating synergistic effects of endolysin-antimicrobial peptide combinations within a single molecule[35].

11.4 Endolysins plus nanoparticles

Overall, the use of nanoparticles with endolysins is a promising approach that harnesses the distinct characteristics of nanoparticles to deliver and enhance the antibacterial activity of the enzyme itself, while also imparting additional antibacterial properties to the nanoparticles. The use of nanoparticles as vehicles for endolysins could help to preserve the activity of the enzyme, help it to enter biological barriers like the gastrointestinal tract, and ensure that it is released at the site of infection. The activity of endolysins can be further boosted by conjugating them to nanoparticles, so that the enzyme is present in larger amounts at the bacterial surface or because nanoparticles may also have antibacterial activity of their own which work synergistically with the endolysin. Targeted delivery of endolysin to bacterial pathogens using nanoparticles could be achieved by functionalizing the NP with targeting molecules, which would also help to minimize the potential for off-target side effects and increase the specificity of an antibacterial treatment. The preclinical models of infection have shown the combination of endolysins and nanoparticles to have superior antibacterial activity and therapeutic efficacy than the free enzyme without the nanoparticle. The use of endolysins as nanoparticles has also been studied for the treatment of infections caused by biofilms, and the nanoparticles have been able to penetrate the enzyme to the bacterial biofilm and eliminate the biofilm[60].

11.5 Endolysins with probiotics

Overall, the use of endolysins in conjunction with probiotics could be a synergistic approach to achieve the benefits of both types of agents, potentially restoring the healthy microbial balance and preventing the onset of bacterial infections, especially in the gastrointestinal tract. Probiotics are living bacteria that are claimed to have beneficial effects on the host when consumed in sufficient quantities and have been used in the prevention and treatment of various conditions such as antibiotic-associated diarrhoea, gastrointestinal infections and inflammatory bowel disease. The use of the endolysins in conjunction with the probiotics would allow targeted killing of pathogenic bacteria and simultaneously allow probiotics to colonise and survive and thereby restore a healthy microbial community and avoid the colonisation of pathogenic bacteria. Endolysins' specificity for the targeted pathogens allows for their use to selectively eliminate pathogens while maintaining the beneficial bacteria required for gut health. The use of endolysins in conjunction with probiotics has been investigated in the prevention of the *Clostridioides difficile* infection, where the endolysin would decrease the pathogen load and the probiotics would re-establish a healthy microbial community. Endolysin-probiotic combinations, especially with regard to gastrointestinal health, are an emerging field of research with potential for developing new therapeutic strategies in a variety of diseases[61].

12. Emerging Technologies

12.1 AI-guided endolysin discovery

The use of AI in the discovery of new endolysins is a paradigm-shifting strategy that utilises computational techniques to streamline the identification and characterisation process from the wealth of available genomic and metagenomic data. AI-based methods such as machine learning and deep learning can be used to detect gene candidates of endolysins in bacteriophage genomic sequences and metagenomic datasets and for using sequence features to predict their enzymatic activity and specificity. Utilising AI in the discovery of endolysins can facilitate the

analysis of a variety of environmental and extreme environments that might harbour endolysins with unique properties. Combined with experimental validation strategies, this AI-powered discovery pipeline allows for the identification and characterisation of potential endolysin candidates for therapeutic development in a much faster and streamlined manner. AI technologies for endolysin discovery could significantly speed up the discovery of therapeutic lead endolysins, cutting down time and cost for identification[62].

12.2 Machine learning

Machine learning methods have been used to predict endolysin activity, stability, and target specificity; these can help quickly assess candidate enzymes and optimise their properties for therapeutic use. The machine learning algorithms can be trained with sets of endolysin sequences and their associated antibacterial activities, thus predicting the activity of newly discovered endolysins and identifying sequence properties that associate with optimal activities. Several endolysins have been modified through machine learning to create, through their engineering, different enzymes that have increased antibacterial activity and stability, reducing the time required to optimise the lead candidates for therapeutic development. Machine learning methods can also be used to foresee the immunogenicity of endolysins, permitting variants to be created that have lower immunogenic potential and more favourable safety attributes. The combination of machine learning methods and experimental validation can form a strong feedback loop for endolysin engineering, allowing the properties of endolysins to be optimised iteratively based on predictions made by the models and experimental tests conducted[8].

12.3 Metagenomics

To date, metagenomic techniques have been used to detect novel endolysins from environmental sources by tapping into the great genetic diversity of bacteriophages found in nature; this allows the detection of enzymes with unusual properties or new enzymatic activities. Bacteriophages, which cannot be cultured in the lab, can be directly

analysed using metagenomic sequencing, and this approach can help discover endolysins with novel catalytic activities and substrate specificities. Using metagenomics to search for endolysins has yielded enzymes active against a number of bacterial pathogens, including those resistant to traditional antibiotics. Characterisation of metagenomic endolysins has identified novel features of endolysin structure and catalysis, allowing for broadening the understanding of endolysin biology and discovery of new therapeutic opportunities. Using metagenomics and bioinformatic tools to find endolysins can lead to the rapid identification and characterisation of the most promising enzyme, speeding up the process of finding therapeutic enzymes[63].

12.4 Structural bioinformatics

Based on the structure of the endolysin, structural bioinformatics methods have been used to predict the enzyme structure and to analyse the structure-function relationship, which in turn have helped to inform the design of improved enzymes and understanding of enzyme mode of action. The use of protein structure prediction methods such as homology modelling and ab initio prediction has allowed the generation of structural models of endolysins which could be used to rationally design improved variants. The structural studies on endolysins have now shed light on the molecular mechanism of their catalytic activity and specificity, thus offering a template for the development of endolysins with improved properties. Using molecular dynamics simulations, the dynamics of endolysins have been studied, and the conformational changes that are relevant for their activity have been identified, providing a guide for the design of stabilising mutations. The combination of structural bioinformatics and experimental methods has now made it possible to systematically optimise the endolysin properties for therapeutic use[8].

12.5 Protein language models

Protein language models, which are deep learning models trained on massive protein sequence datasets, have become a powerful tool to predict protein properties and to design new proteins that

function as needed. Protein language models could be used to predict endolysin activity, stability, and target specificity since they capture the statistical patterns of amino acid sequences, which reflect their evolutionary and structural relationships that underpin their functions. By leveraging protein LM, endolysin engineering has allowed prediction of the impact of mutations on enzyme behaviour, which in turn facilitates the design of new variants that are more effective and stable against bacteria. Protein language models can also be used for the de novo design of endolysins, where novel sequences are generated that are predicted to have desired properties and a diverse set of enzymes is created. Combining protein language models with experimental validation techniques provides an exciting platform for endolysin engineering, allowing the efficient design and optimisation of lead candidates for therapeutic development[64].

13. One Health perspective

The One Health approach acknowledges the shared aspects of human, animal and environmental health; the use of endolysins in this context highlights the need for holistic solutions to antibacterial therapy to tackle the common problem of antimicrobial resistance. In human, veterinary and agricultural medicine, using endolysins should be weighted in the context of the general antimicrobial resistance problem to ensure that the usefulness of these enzymes is retained and any development of resistance is minimised. Application of endolysins in the fields of veterinary medicine and agriculture may help control the use of antibiotics in these fields, which may lessen the selective pressure for resistance and help maintain antibiotic activity in human medicine. Monitoring of antimicrobial resistance in the environment and implementation of a surveillance system to monitor AMR trait emergence and dissemination among sectors is also of major importance in the One Health approach[65].

Conclusions

The detailed examination of bacteriophage endolysins as novel antibacterial agents points to a class of enzymes with extraordinary features that

make them promising agents to solve the global antimicrobial resistance problem. Extensive progress in the fields of endolysin biology, structural biology and protein engineering has led to the development of powerful antibacterial enzymes that can combat a diverse array of bacterial pathogens, including antibiotic-resistant ones. The enzymatic degradation of the highly conserved peptidoglycan layer is a unique mechanism of action that endolysins possess, which confers several advantages over conventional antibiotics, such as rapid bactericidal activity, high target specificity and low tendency to induce bacterial resistance. Distinguished from antibiotics, endolysins can lyse both biofilms and persister cells, which are two of the major hurdles in antibiotic treatment and recurrence of bacterial infections. The clinical work on endolysins has advanced markedly, and a few clinical candidates have been taken to the clinic, and one endolysin has been marketed for topical use. The inclusion of new technologies such as artificial intelligence, machine learning, metagenomics, structural bioinformatics, and proteins language models can speed up the discovery and optimization of endolysins, thereby generating enzymes with improved therapeutic attributes. However, remaining translational barriers have to be overcome to fully exploit the potential of endolysins as antibacterial therapeutics. The outer membrane barrier of Gram-negative bacteria is still a problem, and strategies need to be developed for getting these enzymes to their peptidoglycan substrate. Successful clinical translation of these enzymes requires optimization of delivery systems as well as clinical trial evidence of safety and efficacy and regulatory pathways for endolysin therapeutics. Continued discovery and characterization of new endolysins, optimization of endolysin properties using protein engineering/synthetic biology, development of effective methods/vehicles for systemic and local administration, and evaluation of endolysins in combination with conventional antibiotics and other antimicrobial agents are important areas for future research. The future of endolysins as the next generation of antibacterial drugs seems bright; they provide a paradigm shift in the treatment of

bacterial diseases and in combating antimicrobial resistance. Endolysins, coupled with antimicrobial stewardship and the creation of other non-antimicrobial methods of combating bacterial infections, may hold the key to maintaining the effectiveness of our current antibacterial armamentarium and our continued ability to treat bacterial infections in the midst of the escalating resistance crisis.

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