Acyldepsipeptide Analogues for Tuberculosis Treatment

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Contributor: Sinazo Z. Z. Cobongela, Maya M. Makatini, Phumlane S. Mdluli, Nicole R. S. Sibuyi

Acyldepsipeptides (ADEPs) are a new class of emerging antimicrobial peptides (AMPs), which are currently explored for treatment of pathogenic infections, including tuberculosis (TB). These cyclic hydrophobic peptides have a unique bacterial target to the conventional anti-TB drugs, and present a therapeutic window to overcome *Mycobacterium Tuberculosis* (*M. tb*) drug resistance. ADEPs exerts their antibacterial activity on *M. tb* strains through activation of the protein homeostatic regulatory protease, the caseinolytic protease (ClpP1P2). ClpP1P2 is normally regulated and activated by the ClpP-ATPases to degrade misfolded and toxic peptides and/or short proteins. ADEPs bind and dysregulate all the homeostatic capabilities of ClpP1P2 while inducing non-selective proteolysis. The uncontrolled proteolysis leads to *M. tb* cell death within the host. ADEPs analogues that have been tested possess cytotoxicity and poor pharmacokinetic and pharmacodynamic properties

Keywords: acyldepsipeptides ; antimicrobial peptides ; caseinolytic protease ; Mycobacterium tuberculosis ; antimicrobial ; tuberculosis ; multi-drug resistance bacteria

1. Antimicrobial Peptides (AMPs) as Future Antibiotics

AMPs, are host defense peptides that are endogenously produced by organisms for protection against pathogens [1]. AMPs are generally cationic with less than 50 amino acid residues [2]. They are part of innate immune response produced by eukaryotes and prokaryotes [3]. Cationic peptides exhibit diverse antimicrobial activities by enhancing bacterial agglutination, metal ion chelators, peroxidase activity, proteolytic inhibitors and impair cell wall activity [4]. These peptides have been proven to be active in vivo [5][6], effective against broad spectrum microbes [7][8][9] with reduced bacterial resistance [10] and side effects [11]. The antimicrobial activity of various AMPs have been reported against Gram-positive bacteria [12][13], Gram-negative bacteria [14][15], and fungi [16]. In addition, synthetic analogues of these peptides have demonstrated similar or enhanced activities compared to the natural AMPs. The synthetic peptides also have low minimum inhibitory concentration (MIC) [7], neutralizes the outer membrane lipopolysaccharides of Gram-negative bacteria [17], promote wound healing [18], and show synergistic activity with conventional antibiotics [19].

AMP Mode of Action

When AMPs are introduced to living microorganisms, they trigger anatomical/functional changes at cellular and molecular levels. So far, disruption of membrane integrity and inhibition of intracellular activity have been identified as the two main mechanisms of action for AMPs [2]. In order to achieve this, AMPs must gain access to the cell by penetrating or interacting with the cell wall and/or cell membrane. Most of the AMPs have a cationic net charge due to their high content of the positively charged amino acid residues such as arginine, lysine, and histidine [20]. Due to the presence of lipopolysaccharides or teichoic acid, prokaryotes have a negative charge on their outer layer. In Gram-negative and Gram-positive bacteria, the cationic charge facilitates the accumulation of the AMP on the negatively charged surface of their outer membrane and cell wall, respectively [21]. The amphiphilic interaction between the AMPs and phospholipid bilayer of the bacterial cell wall/membrane is favorable in prokaryotes, this interaction encourages transmembrane pores which in turn disrupts the phospholipid bilayer and thus causing rapid cell lysis and eventually cell death [20]. Bacterial death by AMPs occur through similar mode of actions to those of antibiotics, by inhibition of DNA, RNA, and protein synthesis.

It is vital that AMPs gain access to the intracellular targets through the cell wall and/or cell membrane. The cell wall of Gram-positive bacteria embodies a porous (40–80 nm) thick mash where AMPs can pass through to the cell cytoplasm [22]. Alternatively, the cationic AMPs can also manipulate the charge exchange mechanism for transmembrane on Gram-negative bacteria where it competes with the Ca^{2+} and Mg^{2+} bound to lipopolysaccharide of the outer membrane [23]. Proline-rich peptides are one of the AMPs that target the intracellular processes to inhibit bacterial growth [24]. Once inside

the cell, the AMPs may inhibit vital biological processes such as enzyme activity, nucleic acid synthesis, and protein folding or synthesis [21][25][26].

One of the examples of the AMPs that uses these properties to translocate to the cytoplasm is indocilin [27], investigations showed that it inhibits DNA synthesis thus inhibiting bacterial growth [28]. There are some AMPs that bind to both RNA and DNA such as buforin II causing rapid cell death [29]. PR-39 is a proline-arginine-rich peptide that was found to inhibit the growth of MDR *M. tb* by binding to DNA and inhibiting intracellular protein synthesis [30][31]. Though there are a number of intracellular AMP targets, bacterial proteases are one of the underexploited for new AMPs. There is a number of bacterial proteases such as ATP-dependent zinc metalloprotease complex such as caseinolytic protease (ClpP) complex that can be targeted as therapeutic targets [32].

2. ClpP as a Putative Bacterial Therapeutic Target

ClpP is a serine peptidase that plays a crucial role in general protein quality control by degrading misfolded or aggregated proteins [33]. The ClpP belongs to a family of AAA+ ATPases and it is important for protein turnover and homeostasis in order to maintain vital cellular functions particularly under stress conditions. This protease is ATP dependent and referred to as proteolytic core [34][35]. It is made up of fourteen ClpP protomers that form a tetradecameric barrel-shape with two heptameric rings [36]. The catalytic residues are embedded within the barrel with seven hydrophobic pockets for interaction with the Clp-ATPase [37]. Clp-ATPase supports the refolding of proteins independent of ClpP, failure to achieve this, Clp-ATPase directs the misfolded or aggregated proteins to the proteolytic core where it is unfolded and degraded [38]. The misfolded proteins have a degron tag following the post-translation modifications [39]. The degron tag then binds to the adaptor protein which activates AAA+ ATPases to bind to the inactive ClpP [40].

The role and importance of ClpP on the bacterial cell viability can then be explored as a target for antibacterial abilities. Currently, there are three mechanisms to deregulate ClpP i.e., disruption of the AAA+ ATPases coupling to the ClpP, the inhibition, and activation of the ClpP proteolysis chamber [32]. As mentioned above, ClpP is dependent on ATPases to select and unfold aggregated or misfolded proteins, disruption of this partnership deregulates the proteolytic activity of ClpP. Similarly, lassomycin and ecumicin have been proven to bind to the Clp-ATPase N-terminal domain and activate ATPase activity [41][42].

2.1. ADEPs Competes with Clp-ATPases to Deactivate ClpP

ADEPs, also known as cyclic ADEPs, are a class of AMPs that bind and deregulates bacterial ClpP [43]. They are naturally produced by *Strepomyces hawaiiensis* [44] and are most active against Gram-positive bacteria [32]. ADEPs bind to the cavities formed by two of ClpP monomers. ClpP/ADEP complex adopts a proteolytic active conformation in the absence of Clp-ATPase [43][45]. ADEPs mimic the Clp-ATPases activity and competitively binds to the hydrophobic pockets. This event inhibits interaction between ClpP and Clp-ATPases, and therefore eliminates all natural functions of the Clp-protease that require Clp-ATPase-mediated degradation [46][47][48].

The catalytic core of the ClpP has three amino acid residues, Serine98, Histidine123, and Aspartic acid172, that form a catalytic triad [49]. On the active conformation of ClpP, Serine98 undergoes a nucleophilic attack on the electron deficient carbonyl group of the peptide bond. The imidazole ring on Histidine123 abstracts a proton from the serine hydroxyl group and the resultant positively charged histidine imidazole ring is stabilized by the carboxyl function of the aspartic acid172. The aspartate acyl-ester undergoes hydrolysis regenerating the serine for the next catalytic cycle [36].

The aspartic acid and threonine side chains create a polar environment whereas the aliphatic side chain and benzene ring of the phenylalanine are buried deep into the hydrophobic pocket of ClpP [46]. The aliphatic side chain and N-acylphenylalanine moiety closely resembles the isoleucine/leucine-glycine-phenylalanine structure of one of Clp-ATPase (ClpX) hence this is a minimum structural requirement for ADEP activity [50]. Moreover, Tyrosine62 residue form two hydrogen bonds with N-group of phenylalanine e and a carboxylic group of alanine thus increasing rigidity between ADEP and ClpP [46]. The ADEP1 Factor A (A54556) is one of the ADEPs to be discovered [43], and has been reported to have antibacterial activity against Gram-positive bacteria [51] and Gram-negative bacteria [52]. ADEP1 was also reported to be bactericidal against a number of antibiotic-resistant Gram-positive bacteria such as penicillin-resistant *S. pneumoniae*, vancomycin-resistant *Enterococcus faecium*, and methicillin-resistant *Staphylococcus aureus* [51].

2.2. ADEP1 Analogues

There is a number of modification that have been done on the original ADEPs to yield better pharmacokinetics and pharmacodynamics. ADEP1 Factor A is cyclic peptide composed of four natural amino acids (proline, alanine, serine, and

phenylalanine), two methylated amino acids (4-methyl proline and N-methylated alanine) and octa-2,4,6-trienoic acid. ADEP1 Factor A is active against various microorganisms including *Bacillus subtilis*, *S. pneumoniae*, *Streptococcus pyogenes*, *Enterococcus faecalis*, *E. faecium*, *S. aureus* [43][52], etc. The very first modification was the removal of the methyl group from proline (ADEP1 Factor B) which is active against *S. aureus*, *E. faecalis*, and *S. pneumoniae* [52][53]. The removal of the methyl proline in Factor B led to a decrease in antibacterial activity in *S. aureus* [52][53]. Other ADEP1 analogues have been used in different types of enoic acids (ADEP1 Factor D) to reduce the number of double bonds in octa-2,4,6-trienoic acid for thermal stability [52]. This analogue was active against a number of Gram-positive (Methicillin-resistant *S. Aureus*, vancomycin-resistant *enterococcus*, and penicillin-resistant *S. pneumoniae*) and Gram-negative pathogenic bacteria (*Neisseria meningitidis* and *Neisseria gonorrheae*) [51][54]. The use of fluorinated *bis*-fluorophenylalanine side chain in a place of phenylalanine has shown enhanced antibacterial activity of ADEP2, ADEP4, and ADEP5 on *S. aureus* [43]. Modifications have also aimed in rigidifying the macrolactone core.

2.3. ADEPs Activity on M. tb ClpP1P2

ADEPs have particularly been of interest in *M. tb* targeting due to its distinct mode of action on ClpP [43]. The *M. tb* ClpP not only maintains intracellular protein homeostasis, it also contributes to the mycobacterium virulence and helps with the dormancy of the *M. tb* within the host [55][56][57]. Unlike other bacteria with a single gene for ClpP, *M. tb* has two genes encoding for ClpP1 and ClpP2 proteolytic subunits [58]. Like other ClpP, ClpP1 and ClpP2 heptamer rings are inactive until they associate to form a 300 kDa ClpP1P2 tetradecamer [55][59]. Upon binding together, both these rings influence each other's conformation [59][60]. In vitro, ADEPs exclusively bind to the *M. tb* ClpP2 which in turn changes the whole ClpP1P2 tetradecamer conformation to open both heptamer axial pores thus activating the ClpP1P2 [60]. This phenomenon is naturally controlled by ATPases in response to increased concentration of substrates. In low concentrations of substrates, ATPase-bound ClpP1P2 remains inactive. However, ClpP1P2 activation by ADEPs is independent of the amount of substrate present. The anti-mycobacterium activity of ADEPs and the exact mechanism of how they bind thus causing bacterial growth inhibition through ClpP1P2 is poorly understood.

It has been previously reported that the anti-mycobacterium activity of ADEPs is through nonselective protein degradation by *M. tb* ClpP1P2, and/or allosterically binding to ClpP1P2 to prevent physiological activity of the ATPase [60]. Anti-mycobacterium activity of ADEPs is also through ClpP1P2 inability to degrade and eliminate toxic proteins [61]. The ADEPs and analogues bind to leucine-glycine-phenylalanine hydrophobic pockets on the ClpP2 which is the same binding site for the ATPases [62][63]. A list of ADEP analogues (ADEP2, ADEP3, ADEP4, IDR-10001, and IDR-10011) were found to be active against *M. tb* with an MIC range between 25 and 100 µg/mL [64]. Although the ADEP binding to the ClpP1P2 is similar to other bacterial ClpP complexes, the maximum effect of ADEPs on ClpP1P2 require activators such as dipeptide benzyloxycarbonyl-leucyl-leucine [61]. ADEPs were also shown to have improved activity with addition of efflux pump inhibitors which prevents export of intracellular ADEPs [64][65] and therefore, have potential to be effective where the current anti-TB drugs have failed.

2.4. Limitation of ADEPs

The problems mainly associated with novel therapeutic AMPs, including ADEPs are their poor pharmacokinetic properties such as susceptibility to enzyme degradation, short plasma half-life (leading to frequent invasive administration), lack of oral availability, difficulty in membrane permeability, lack of selectivity thus causing toxicity to normal cells [66][67]. These complications raise a need to improve the natural peptides in drug like molecules with less toxicity, high selectivity, good solubility, increased proteolytic stability, and membrane permeability. This in turn will increase drug bioavailability and consequently increasing their therapeutic activity. Therefore, modification and modulation of conformational dynamics of these AMPs and designing of similar systems that mimic the potential therapeutic peptides will be the first step toward improving the pharmacokinetic properties of the AMPs. There are several ways that can be employed to improve the pharmacokinetic properties of novel peptides. Peptide modification and use of drug carriers are some of the strategies that have been successful in enhancing the bioactivity of AMPs and their analogues [12][23][24][68].

2.4.1. Potential Strategies to Improve on ADEP's Pharmacokinetics and Pharmacodynamics

ADEPs are produced by natural sources such as plants and microorganisms, often in minute quantities and majority of them are less soluble, which presents a huge challenge for their application in therapeutics. Drug modifications has been successful in improving the membrane permeability, drug stability, and controlled delivery at the target site. Some of the strategies that can be employed to prevent ADEPs biodegradation or interaction with biomolecules include chemical modification of the drugs, substitution of amino acids [12][23][24][68], and use of drug delivery agents.

Chemical Modification

Chemical modification and amino acid substitution in lead drugs, no matter how small it is, have significant impact on their bioactivity; a lesson learnt from nature, where a slight change in a structure can completely alter its function, target, mode of action and improve drug selectivity [69]. Changing the drug structure conformation through cyclization is another way of increasing their metabolic stability compared to linear analogues. Cyclization can be formed through chemically stable bonds such as ether, disulphide, lactone etc., and the most common technique used in peptide chemistry [70].

Amino Acid Substitution

Protease stability is also vital in the development of peptide-based drugs, the incorporation of unnatural amino acids is one of the approaches used to increase their stability and bioavailability. There is quite a number of processes to achieve these unnatural amino acid analogues such as, substitution with either cationic [71], D-amino acids or N-methylated amino acids [72], can help improve on the metabolic stability and potency of peptide-based therapies. For instance, cationic peptides exhibit more bactericidal effects than their anionic counterparts. Therefore, replacing negatively charged amino acids (aspartic acid and glutamic acid) with positively charged ones (lysine and arginine) increases the positive net charge of the peptides. Moreover, lysine and/or arginine rich peptides easily interact with the negatively charged cell membrane and are used to shuttle biomolecules across the cell membrane [71]. The cationic peptides will therefore serve dual functions as cell-penetrating peptides and antimicrobial agents [73][74]. Additionally, substituting the natural L-amino acids with D-amino acids may be used to increase proteolytic stability of the peptide drugs. The L-amino are easily metabolized by the body and are susceptible to protease degradation. D-amino acids on the other hand, have similar activity to L-amino acids but are not susceptible to degradation, thus improving their bioavailability and drug activity [75][76]. N-methylation and fluorination of the amino acids also improve pharmacokinetic characteristics, activity, selectivity, and delivery of peptides [72]. N-methylation also improved metabolic stability and intestinal permeability of peptides that were highly active but with poor bioavailability [77]. Nature has also employed N-methylation of peptides on the ADEP1 to improve its biological functions and mode of survival by inhibiting enzymatic degradation [52]. Multiple N-methylation also increased selectivity of a cyclic hexapeptide integrin antagonist toward different integrin subtypes [72][78]. Attention has also been given to fluorination to modulate physicochemical properties of proteins, especially of hydrophobic amino acids (phenylalanine, isoleucine and others), to increase peptide stability and prevent proteolytic degradation [79].

Lipophilic Molecules

Lipophilic molecules were also used to improve and facilitate the cellular uptake of AMPs or ADEPs as their mode of action is targeted on intracellular proteins. Lipophilic molecules such as linoleic, oleic, and palmitic acids are often conjugated to the drugs to increase membrane permeability of peptide-based drugs. They are mostly used in pharmaceuticals to enhance uptake of chemicals [80]. The conjugation of lipids to drugs increases their lipophilicity which enhances their interaction with the cell membrane thus increasing cellular uptake. Amongst other advantages of lipid-drug conjugates, they also improve the oral bioavailability and decrease toxicity of the drug molecule [81].

Nano-Carriers

The urgent need for development of new drug delivery systems with improved properties to achieve desirable therapeutic efficacy is driven by the toxicity and side effects of drugs. Nanomaterials have a wide range of applications in various fields. In the pharmaceutical industry, they are receiving significant interest and are being investigated for drug formulation and delivery (nanomedicine) [82]. Nanomaterials are very small in size, usually ranging from 1 to 100 nm, and yet have a larger surface area that can be easily manipulated by conjugating compounds of interest [83]. Because these particles are too small, they easily diffuse through cell membrane pores and ion channels, and their target specificity can be further improved when the targeting ligands are attached to nanomaterials [84]. Nanomaterials as drug carriers can also help in increasing drug solubility therefore increasing bioavailability of drugs [85]. Additionally, they can reduce toxicity and side effects by increasing selectivity and can improve transfer across membranes including the blood–brain barrier [86] without the aid of targeting moieties. These conjugates also decrease enzyme susceptibility of unstable drugs at physiological conditions [87]. There are several organic and inorganic nanomaterials that are promising drug delivery agents. Inorganic or metal nanoparticles such as zinc, copper, and iron oxides nanoparticles have been explored for this application, mostly due to their antibacterial activity.

The nanomaterials have been shown to exhibit antimicrobial properties against a wide range of pathogens including drug-resistant strains. For example, copper oxide (CuO) nanoparticles were effective against methicillin-resistant *S. aureus* and *E. coli* [88][89]. When loaded with drugs, they can translocate their cargoes across the cell membrane. Iron oxide (FeO) nanoparticles loaded with doxorubicin could transport doxorubicin across cellular membranes without any targeting moiety and accumulate in the nucleus [90]. The nanoparticles increased the selectivity and bioavailability of the drugs to the target cells and at the same time reduce the side effects. Nanomaterials such as zinc oxide (ZnO) nanoparticles were also

shown to possess anti-TB activities, and could be used as both antimicrobial as well as drug delivery agents. Interestingly, bimetallic NPs, herein mixing ZnO with silver nanoparticles improved their biocompatibility and reduced the toxicity resulting from individual metal nanoparticles in *M. tb* [91]. Thus, NPs alone or in combination with targeting molecules such as antibodies or targeting ligands can help improve the potency and bioavailability of ADEPs.

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