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Chapter

Bulgecins as β-Lactam Enhancers Against Multidrug Resistant (MDR) *Pseudomonas aeruginosa*

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Abstract

Antibiotic resistance in non-lactose fermenting pathogens such as *Pseudomonas aeruginosa* (*P. aeruginosa*) is increasing, making these clinical pathogens more difficult to treat. Multiple resistance mechanisms exist within *P. aeruginosa* that affect all classes of antibiotics used in the clinic. New strategies and treatment targets within these MDR pathogens must be exploited. One heretofore untapped target is the family of cell wall enzymes known as lytic transglycosylases (Lts). Lts work in concert with penicillin binding proteins (PBPs) and other cell wall proteins such as amidases and peptidoglycan hydrolases to affect normal cell division, and during stress and programmed cell death. Lts are inhibited by natural products called bulgecins, produced by non-pathogenic *Paraburkholderia* and *Burkholderia* spp. New research describing the ability of Lt inhibition to restore susceptibility to β-lactams in MDR *P. aeruginosa*, as well as the structural biologic basis for the activity of bulgecins will be reviewed. Other targets and applications of bulgecins will also be discussed.

Keywords: antibiotic resistance, *Pseudomonas aeruginosa*, metallo-β-lactamase, penicillin binding protein, lytic transglycosylase, bulgecin A

1. Introduction

This chapter will review our current state of the art knowledge about bulgecins, natural inhibitors of lytic transglycosylase cell wall enzymes, and their activity as β-lactam enhancers to inhibit growth of *P. aeruginosa*. Current known resistance mechanisms targeting β-lactams in *P. aeruginosa* be reviewed, followed by an introduction to the lytic transglycosylases of *P. aeruginosa*. The use of bulgecins as adjunctive agents with β-lactams will be described as well as the synthesis of Bulgecin A, the most active of these compounds.

2. β-Lactam resistance mechanisms in *Pseudomonas aeruginosa*

P. aeruginosa has intrinsically higher minimum inhibitory concentrations (MICs) against anti-pseudomonal β-lactams when compared to Enterobacteriaceae, even in the absence of specific resistance determinants. For example, typical MICs for the anti-pseudomonal cephalosporin, ceftazidime, for *Escherichia coli*

are 0.06–0.125 mg/L whereas for *P. aeruginosa* isolates, MICs are in the range of 1–2 mg/L. In the main, when *P. aeruginosa* is resistant to β-lactams, specific mechanisms are at play. These include downregulation of outer membrane porins, expression of intrinsic efflux mechanisms, acquisition of β-lactamase enzymes including carbapenamases such as IMP ("imipenemase"), VIM ("Verona imipenemase") and KPC ("Klebsiella pneumoniae carbapenemase"), hyperproduction of chromosomal β-lactamases ("AmpCs or Pseudomonas-Derived Cephalosporinases, "PDCs") and reduced penicillin binding protein affinity for β-lactams that are not considered "anti-pseudomonal" β-lactams. **Table 1** summarizes the mechanism and resistance determinants responsible (adapted from [1]).

Currently, in the clinical microbiology laboratory, susceptibilities are reported to particular antibiotics depending on the specific sample submitted, e.g., urine, blood, sterile body fluids (pleural, joint, cerebrospinal fluid). At least initially, genotypic testing to determine the presence of specific antibiotic resistance determinants is not performed, and it is left to the clinical infectious diseases expert to reason out the most likely resistance mechanisms based on susceptibility patterns, and to select the most appropriate antibiotic(s) for treatment.

2.1 Outer membrane porin loss (OprD)

The structure, function and regulation of *P. aeruginosa* porins is complex and has been recently reviewed [2]. Porins are involved in structural and signaling tasks in *P. aeruginosa*, as well as passage of nutrients. The Opr D family of porins is the largest and is subdivided into two groups OccD and OccK. These porins are each regulated through their own sigma factors. Porin loss can be related to formation of OprD containing outer membrane vesicles that are also found in high concentrations in biofilms. Resistance to carbapenems in biofilms may be related to this. Mutations in *P. aeruginosa* that cause oprD (occD1) to not be expressed are linked to imipenem resistance. Other occD1 mutations that do not effect transcription also lead to carbapenem resistance [3, 4]. OprD mutations or loss is often associated with overexpression of efflux pumps (see below) leading to high level resistance to carbapenems, other β-lactams and other classes of antibiotics such as aminoglycosides and fluoroquinolones [5].

In the clinical setting, OprD porin loss is often associated with a resistance phenotype in which one observes resistance to carbapenems including imipenem, but *in vitro* susceptibility to anti-pseudomonal pencillins and cephalosporins. In

	Resistance determinant	**Antibiotics affected**
Pseudomonas aeruginosa	OprD loss	Carbapenems, some cephalosporins, penicillins
	Efflux pumps (e.g., MexA-B/OprM)	Meropenem, some cephalosporins, penicillins
	Chromosomal Amp C of *P. aeruginosa*	Anti *P. aeruginosa* penicillins, anti *P. aeruginosa* cephalosporins except ceftolozane
	Acquired ESBLs (TEM, SHV,OXA, GES, VEB, CTX-M, PER)	anti *P. aeruginosa* cephalosporins except ceftolozane, cefepime
	Acquired carbapenemases (KPC, OXA, metallo-β-lactamases like NDM, VIM, IMP, SPM types)	anti *P. aeruginosa* pencillins, cephalosporins and carbapenems

OprD, outer membrane porin D; Mex-multidrug efflux; TEM, class A β-lactamase of E. coli, named for patient in which it was discovered; SHV, sulfhydryl variant of TEM; OXA, oxacillinase; GES, German extended spectrum β-lactamase; VEB, Verona extended spectrum; CTX-M, cefotaximase-München; PER, plasmidic extended spectrum β-lactamase; NDM, New Delhi metallo-β-lactamase; SPM, Sao Paolo metallo-β-lactamase.

Table 1.
β-Lactam resistance determinants in P. aeruginosa.

combination with other resistance mechanisms, such as over-expression of the chromosomal PDC enzymes, or presence of other acquired cephalosporinases such as TEM, SHV and OXA β-lactamases, higher level carbapenem resistance is observed as well as resistance to other classes of β-lactam antibiotics.

2.2 Efflux pumps in *P. aeruginosa*

As with porins, *P. aeruginosa* possesses a large variety of efflux pumps that perform different roles in the bacteria, but mainly function to extrude harmful substances from the cell. These pumps have been reviewed in [6]. Pumps of importance in carbapenem and other antibiotic efflux are in the resistance nodulation or RND type family and include the MexAB-OprM, MexCD-OprJ, MexEF-OprN and MexXY-OprM multidrug efflux pump systems [7]. Increased expression of these pumps leads to high level carbapenem resistance, often in association with OprD loss or modification. Notably imipenem is not a substrate of the multidrug efflux pumps of *P. aeruginosa* while meropenem is [7]. In the clinical setting, if one notes resistance to meropenem and other β-lactams, except for imipenem, then an efflux mechanism is at play. If resistance is noted to both meropenem and imipenem, but not to other β-lactams, OprD loss or modification is responsible. With resistance to carbapenems as well as other β-lactams, multiple resistance mechanisms can be at play including efflux, intrinsic and acquired β-lactamases and decreased permeability (porin loss).

2.3 Hyperproduction of PDC β-lactamases

As in other organisms, of which *Enterobacter cloacae* is the most well-known example, *P. aeruginosa* possesses a chromosomal AmpC β-lactamase also called PDC. Chromosomal β-lactamases likely play a role in cell wall maintenance, as well as degradation of β-lactam antibiotics. As characterized in *E. cloacae* [8], the AmpC cephalosporinases are under the regulation of *amp*R, a LysR type regulatory system [9]. Under normal circumstances, there is low level constitutive expression of the AmpC protein. Upon exposure to β-lactam antibiotics, muramyl pentapeptides are released that displace a repressor protein encoded by *amp*R from the promoter of AmpC. This leads to increased expression of AmpC cephalosporinase. The increased expression of AmpC can occur with exposure to cephamycins like cefoxitin for example. Increased expression of AmpC in *E. cloacae* occurs via a pathway involving NagZ, a N-acetyl-β-D-glucosamindase, or independent of NagZ [8]. The muramyl pentapeptides are also degraded by a cytosolic amidase, Amp D. This leads to re-association of the repressor to the promoter and resumption of normal levels of Amp C expression. There are also insertion sequence mutations in AmpR that can lead to increased expression of AmpC, as well as mutations in AmpD amidases that reduce degradation of muramyl pentapeptides. The regulation of Amp Cs differs somewhat in *P. aeruginosa*, involving 2 pathways that include the lytic transglycosylases Slt, SltB1, MltB and MltF, and PBP 4 in the generation of muramyl peptides [10]. Mutations in PBP4 are associated with higher levels of AmpC expression. Finally there are specific AmpC mutations that can lead to a carbapenemase phenotype in these enzymes, although the significance of this in terms of clinically relevant carbapenem resistance is unclear [11].

2.4 Acquired β-lactamases in *P. aeruginosa*

β-lactamases from all four Ambler classes have been described in *P. aeruginosa*, including Class A extended spectrum β-lactamases (ESBLs) of the TEM, SHV,

CTX-M, GES, PER and VEB types; Class A carbapenemases such as KPC variants; metallo-β-lactamases such as the VIM, IMP, NDM and SPM B1 di-Zn^{2+} enzymes: and OXA carbapenemases [9, 12]. Weak imipenemases in the so-called Class C AmpCs have already been discussed above. In combination with OprD loss and/or upregulation of MEX efflux pumps, high level carbapenem resistance can be seen in *P. aeruginosa* due to acquired β-lactamases. Traditional class A β-lactamase inhibitors such as clavulanic acid, sulbactam and tazobactam have *in vitro* activity versus the Class A ESBLs but not against other β-lactamases, e.g., the anti-pseudomonal combination ceftolozane-tazobactam is not effective against KPC, metallo-b-lactamases, or OXA enzymes [1]. New β-lactam-β-lactamase inhibitor combinations such as ceftazidime-avibactam and meropenem-vaborbactam will be active against isolates with KPC enzymes, and Class C β-lactamases, as long as they lack other resistance mechanisms that increase the β-lactam MIC beyond what is caused by the β-lactamase enzyme [13, 14].

2.5 Penicillin binding proteins (PBPs) of *P. aeruginosa*

PBPs of *P. aeruginosa* have high affinities for so called anti-pseudomonal β-lactams namely piperacillin, ticarcillin, ceftazidime, cefepime, ceftolozane, meropenem, imipenem, doripenem and aztreonam [1]. PBP 3 is the most important target of inhibition as it is essential for growth of the bacteria [15]. PBP3 is the primary target for ceftazidime whereas PBP2 is the target of carbapenems. Mutations in PBPs have not been described in *P. aeruginosa* leading to β-lactam resistance. The interactions of specific PBPs with specific lytic transglycosylases in the maintenance of cell wall will be further discussed below.

2.6 Current therapeutic strategies to treat infections with resistant *P. aeruginosa*

Given that 15–33% of *P. aeruginosa* isolates are multidrug resistant (have at least one resistance mechanism) [16, 17] and that resistance is associated with up to fivefold greater mortality [18, 19], choosing the right antibiotic combinations have a tremendous impact on patient outcomes. Advances in the rapid diagnosis of *P. aeruginosa*, and use of both rapid phenotypic tests such as CARBA NP [20] or rapid molecular diagnostics to identify specific ESBL and carbapenemase enzymes, have enhanced the clinician's ability to get patients on the right therapy sooner. Identification of patient risk factors, including prior antibiotic exposure, and knowledge of local trends in resistance patterns are useful in selection of empiric antibiotics, until antimicrobial susceptibilities and genotypic results are available for guidance. Carbapenems (meropenem or imipenem) and anti-pseudomonal cephalosporins in combination with colistin, an aminoglycoside or fosfomycin, versus ceftolozane/tazobactam or meropenem/vaborbactam or ceftazidime/avibactam are all good empiric choices for critically ill patients [16], provided multidrug resistance is not present. However, clearly more therapeutic options are needed for infections with extensively drug resistant and pan-resistant *P. aeruginosa*. Lytic transglycosylases represent a new target for bacterial inhibition.

3. Lytic transglycosylases of *P. aeruginosa*

Recently, lytic transglycosylases of *P. aeruginosa* have been extensively characterized [21–27]. These cell wall proteins are found in many other pathogenic bacteria and are classified according to amino acid sequence and function [28]. To date there are 11 *P. aeruginosa* lytic transglycosylases that have been described. Their functions

range from cell division to aiding in the insertion of secretion systems and two component regulatory systems. They are attractive drug targets to enhance the activity of our most commonly used and safest antibiotics, the β-lactam class (penicillins, cephalosporins, carbapenems and monobactams).

Lts in general catalyze a cleavage reaction that breaks the glycosidic bond between the peptidoglycan building blocks, MurNAc and GlcNAc (**Figure 1**).

This reaction does not involve a water molecule but rather, an active site Glu or Asp residue functions as a general acid, donating a proton to the oxygen in the β-1,4 glycosidic linkage. Then the deprotonated active site residue acts a general base as a nucleophile to break the glycosidic bond. The result is a 1,6-anhydroMurNAc containing final peptide product. This unique cap on the muramyl peptide is a signal and a way for the cell wall peptidoglycan cleavage products to be trafficked for recycling [26]. The reaction shown in **Figure 1** is within the strand or "endolytic". Some Lts also catalyze an end of strand or "exolytic" cleavage.

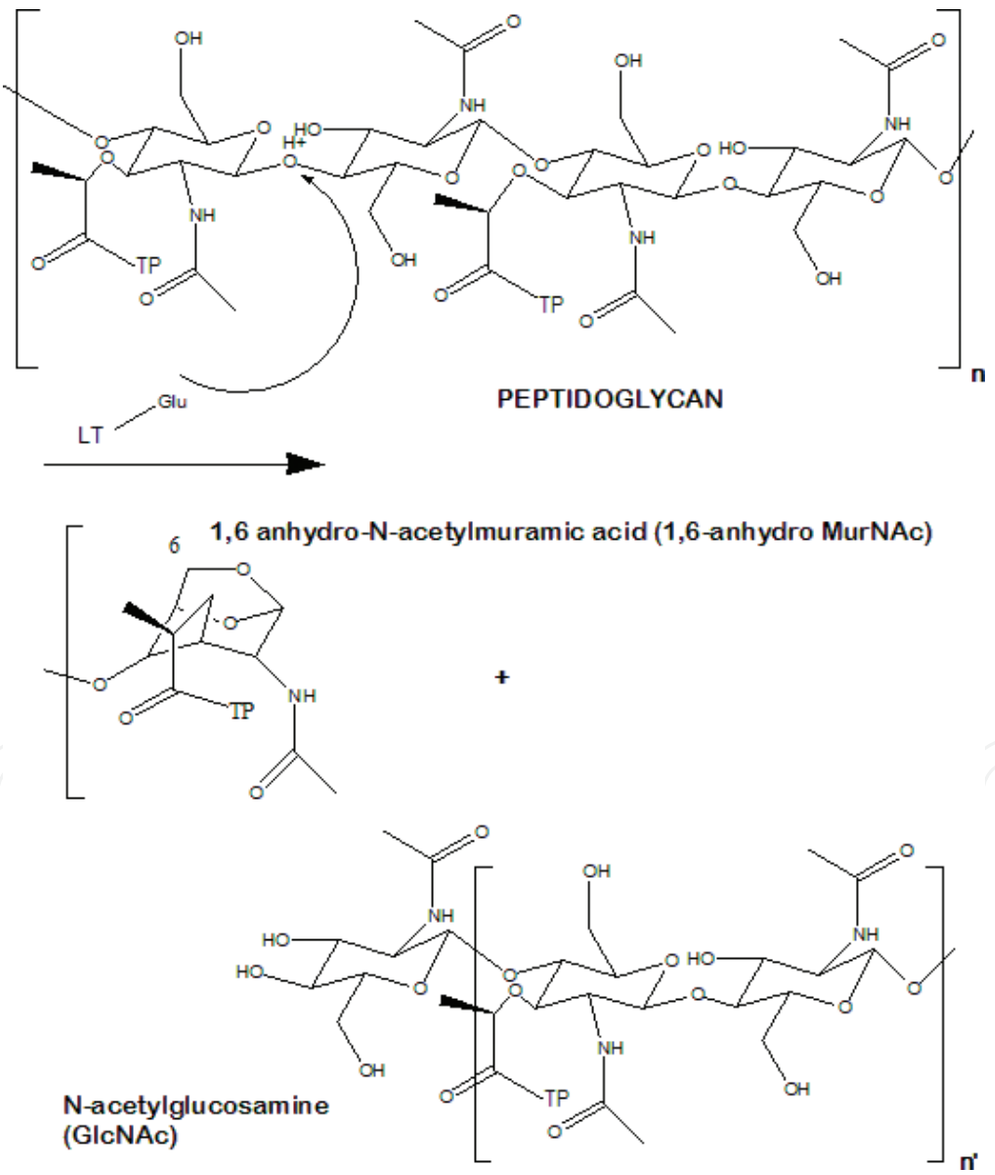

Figure 1.
Lt reaction in cell wall remodeling in Pseudomonas aeruginosa. When the transpeptidase (crosslinking function) of a PBP is inhibited by a β-lactam, the tranglycosylase function of the PBP continues to produce strands of uncrosslinked peptidoglycan (PG). The soluble Lt in the periplasm of Gram negative bacteria initiates recycling and cleavage of PG via endolytic (within strand) reaction. Once this first cleavage reaction occurs, the 1,6-anhydroMurNAC-GlcNAC containing fragments are cleaved and released. In P. aeruginosa, these 1,6-anhydromuramylpeptide fragments affect regulation of Amp C β-lactamase production. TP designates tetrapeptide.

Lts are classified according to amino acid motifs and function, into 6 distinct families. Even within a family, there is little sequence homology; however, the proteins in families do appear to share distinct folds (**Figure 2**). Lts are also divided into membrane (designated M in their nomenclature) and soluble (S) forms. It is hypothesized that these proteins are associated with numerous other cell wall proteins such as PBPs so that even the soluble Lts might be physically associated with the inner membrane of bacteria. Some Lts are also associated with the outer membrane, e.g., RlpA (see below) and likely have distinctive roles [29].

Lts serve many cellular functions including cell wall recycling, cellular division, insertion into cell wall of important structures like secretion systems and flagellar apparati. Lt redundancy is similar to that of the PBPs, and studies looking at gene knockouts of these proteins show that in *P. aeruginosa*, only loss of the RlpA LT is associated with a change in bacterial morphology [29]. Attempts to prepare multiple Lt knockouts were unsuccessful.

Recently significant research has been conducted on the Lts of *P. aeruginosa*, including structural and kinetic studies defining structure function relations in these varied proteins (reviewed in [26]). These studies are summarized next.

3.1 Kinetic studies of *P. aeruginosa* lytic transglycosylases

As previously indicated, *P. aeruginosa* possesses 11Lts: MltA, MltB/Slt35, MltD, MltF, MltF2, MltG, RlpA, Slt, SltB1 (SltB), SltB2 (SltG), and SltB3 (SltH).

In a tour-de-force of biochemical characterization, including synthesis, purification and characterization of the reaction of soluble forms of all 11 *P. aeruginosa* Lts with 4 synthetic substrates and *P. aeruginosa* sacculus to yield 31 distinct peptidoglycan (PG) products, Lee et al. [25] have thoroughly described the structure

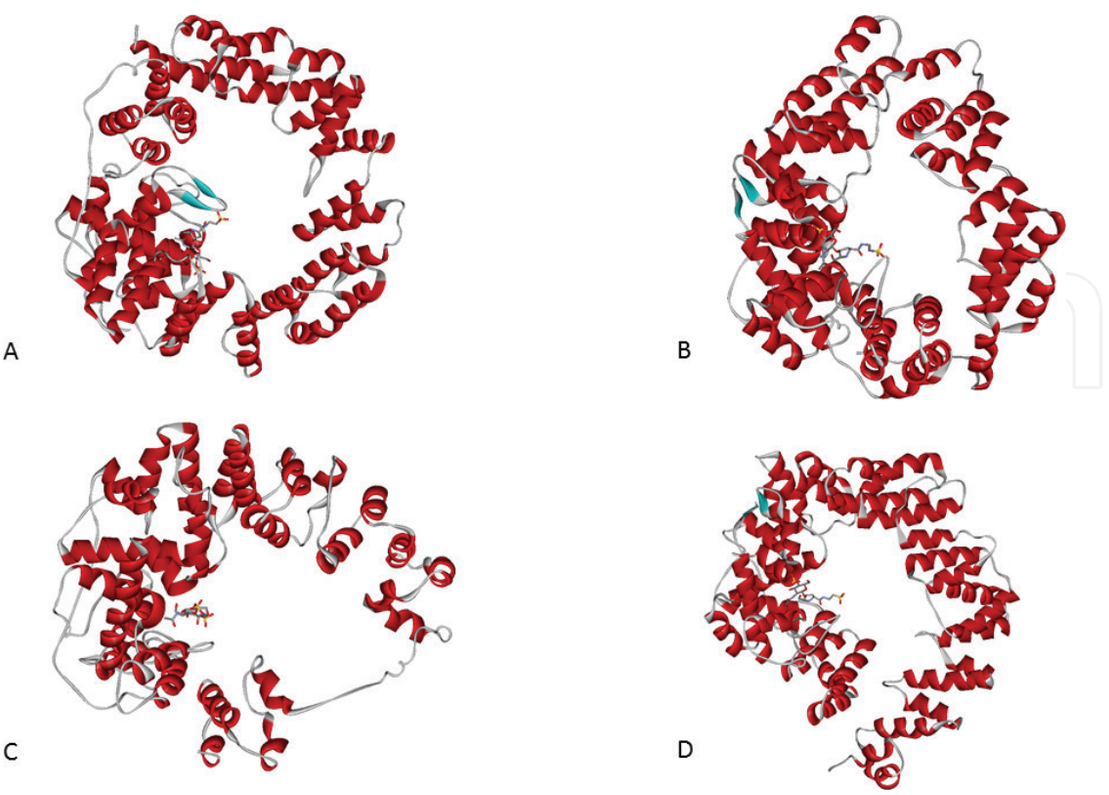

Figure 2.
(A) Slt70 of E. coli in complex with Bulgecin A. (B) Lt of Neisseria meningitidis in complex with Bulgecin A. (C) Lt Cj0843 of Campylobacter jejuni in complex with Bulgecin A. (D) Slt inactive mutant E503Q from Pseudomonas aeruginosa in complex with Bulgecin A.

function relationships for *P. aeruginosa* Lts. Of interest is that each solubilized Lt enzyme could perform both endolytic and exolytic reactions on the PG substrates.

Using the simplest synthetic substrate, NAG-NAM(pentapeptide)-NAG-NAM(pentapeptide), Lee et al. found that only MltB and the SltB1–3 Lts could recognize this substrate. A second substrate, a NAG-NAM(tetrapeptide)-NAG-anhydroNAM(tetrapeptide), incorporated the anhydroNAM that is likely recognized better by the exolytic Lts. For this substrate, MltA as well as MltB and the SltB1–3 Lts were able to react to convert 100% of the substrate to NAG-anhdroNAMtetrapeptide product. The soluble Lts, SltB1–3 of *P. aeruginosa* show the greatest activity in assays designed to study soluble proteins, as compared to solubilized membrane Lts [25]. These Lts were able to cleave NAG-NAM(pentapeptide)-NAG-NAM(pentapeptide) with specific activities of 0.4, 0.4 and 0.3 nanomoles of product/min/mg of protein respectively. Slt, the structural homolog of *E. coli* Slt70, showed no reaction with the synthetic peptidoglycan and slower turnover with the tetrapeptide substrate: 0.1 nmoles/min/mg.

3.2 Structural studies of the soluble Lts, Slt, SLtB1 and SltB3 of *P. aeruginosa*

X-ray crystal structures of Slt in its apo form as well as in complex with various synthetic PG substrates and reaction products demonstrated that this Lt has both exolytic and endolytic activity [23]. It is a donut shaped protein like Slt of *E. coli*. Notably, it is only after the binding of substrates that contain pentapeptide stems that it can exhibit endolytic activity due to a conformational change of the protein on substrate binding. A movie of this rearrangement is available in the supplementary material of reference [23]. Additional studies suggest protein–protein interactions with inner membrane PBPs are also important [26].

SltB1 [22] and SltB3 [24] have also been studied using x-ray crystallography. SLtB1 protein structures suggest that the protein forms a so-called "catenane" homodimeric structure in which the active sites face one another and are thus completely occluded. It is speculated that this soluble dimer may represent a form of activity regulation [22]. SltB3 is an exolytic enzyme with four distinct enzymatic domains within the donut shaped annular protein [24]. SltB3 can recognize PG substrates that are 4–20 sugars in length. These PG chains thread through the annular structure during catalysis.

3.3 Structural studies of the endolytic Lt, MltF

X-ray crystal structures of a solubilized MltF [21] show that this Lt binds a tetrapeptide stem of the substrate in an allosteric domain. Binding causes a large conformational change that leads to enzyme activation. In the kinetic studies, this solubilized membrane had very low activity with any of the 4 synthetic substrates or the *P. aeruginosa* sacculus. This raises some questions regarding the actual role of this Lt and whether the conformational changes are relevant when the protein is membrane bound.

4. Bulgecins as Lt inhibitors

Bulgecins were first described by Imato et al. in the 1980s [30, 31]. These natural analogs of GlcNAC-MurNAC are produced by various bacterial species including *Burkholderia mesoacidophila* and *Paraburkholderia acidophila* [32, 33], part of the *B. cepacia* complex. Bulgecins are produced together with sulfazecin, a monobactam antibiotic. Three different bulgecins are produced by these bacteria. Bulgecin A is produced in the highest amount and is the most active inhibitor of Lts (**Figure 3**, Bulgecin A).

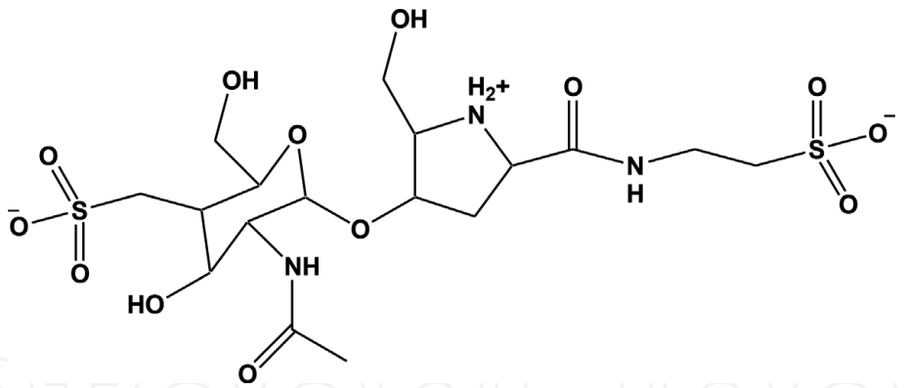

Figure 3.
Bulgecin A, the most active of the bulgecins of Paraburkholderia acidophila and Burkholderia mesoacidophila. The pyrrolidine ring (right side of the molecule) and the N-acetylglucosamine potion (GlcNAC) (left side other molecule) are features of Bulgecin A transition state structure.

Early research by Takeda Pharmaceuticals Japan led to the natural product isolation and purification of the bulgecins [30, 31]. It was discovered that when Bulgecin A was paired with a third generation cephalosporin, cefmenoxime, which targets PBP 3 of Enterobacteriaceae, large bulges were formed in the bacterial cell wall leading to osmotic lysis of the bacteria [30, 31]. Subsequently, investigators discovered the soluble Lt of *E. coli* and solved crystal structures of SltE in complex with Bulgecin A [34]. Through kinetic experiments, it was determined that Bulgecin A was a noncompetitive inhibitor of SltE with an IC_{50} of 0.5 μM. [35]. While Bulgecin A appeared to be a potent inhibitor of Lts in pathogenic Enterobacteriaceae and led to bacterial killing when paired with β-lactams affecting PBP3 particularly, development of the drug was halted for unknown reasons. Over the next decade, more advanced generation cephalosporins, as well as β-lactam-β-lactamase inhibitor combinations, carbapenems and fluoroquinolones were introduced into the clinic to address the growing problem of Gram negative resistance. Recently a natural product synthesis of the bulgecins was reported for the first time by Tomoshige et al. [36] prompting renewed interest in the use of Bulgecin A as an antimicrobial adjuvant, and possible drug optimization via medicinal chemistry.

Since the original discovery of the bulgecins and Slt in *E coli*, Lts have been characterized in many additional bacteria including *P. aeruginosa, Acinetobacter baumannii, Helicobacter pylori, Neisseria meningitides* and *Campylobacter jejuni* [21–25, 28, 34, 37–50]. In general, these organisms have many Lt enzymes with different functions, including endolytic (within strand) and exolytic (end of strand) cleavage of peptidoglycan. Many of these enzymes including those of *P. aeruginosa* have been expressed for biochemical assays of function, and inhibition by Bulgecin A. Crystal structures of many of the Lts of these organisms have also been obtained, some with substrates or Bulgecin A in the active site (**Figure 2**).

A recent publication shows that while *P. aeruginosa* possesses 11 known Lts, three appear to be the main targets of inhibition by Bulgecin A [27]. This work is discussed further below.

5. Microbiological effects of Bulgecin A

Bulgecin A in combination with cefmenoxime and other β-lactams has been studied against Enterobacteriaceae and reported in the original studies by Takeda Pharamceuticals [30, 31]. Later investigators studied Bulgecin A in combination with ampicillin in mouse models of *Helicobacter pylori* infection and found that the combination was effective in eradicating the organism, and Bulgecin A did not

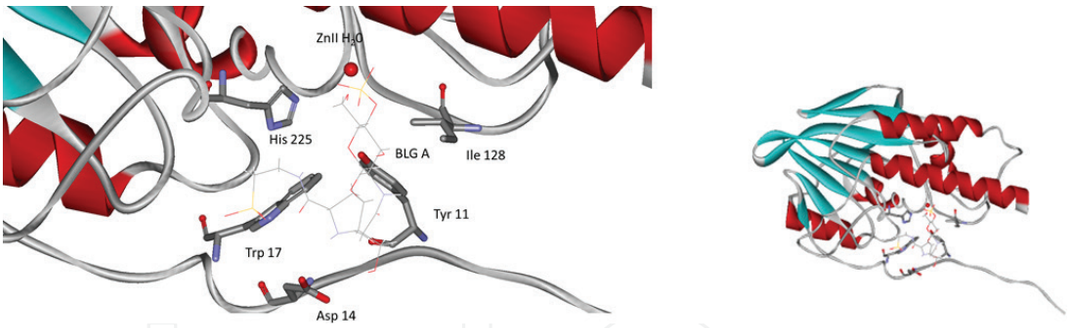

Figure 4.
L1 MBL (left) of Stenotrophomonas maltophilia with Bulgecin A; Bulgecin A sulphonates (yellow moieties, right) interacting with the ZnII site and with Asp 14 of the L1 protein.

appear to have specific toxicity in mice [51]. These investigators also studied Bulgecin A with *Neisseria gonorrhea* and *N. meningitides* strains that were resistant to penicillin and amoxicillin [48]. For strains with higher penicillin MICs not due to the presence of TEM-1 β-lactamase, Bulgecin A at concentrations of 19 mg/L, reduced the MICs from 0.5 to 0.09 mg/L for penicillin G, and 0.75 to 0.4 mg/L for amoxicillin.

Other investigators examined the effect of Bulgecin A as a metallo-β-lactamase inhibitor using L1 MBL of *Stenotrophomonas maltophila* as a model B1 (di Zn^{2+}) MBL enzyme (**Figure 4**). Simm et al. determined that the K_I for Bulgecin A was 150uM [52]. Later, our group investigated inhibition of VIM-1 using a Bulgecin A preparation from *B. mesoacidophila* and found that it also acted as an inhibitor of a second B1 MBL enzyme that is commonly found in *P. aeruginosa* in Europe, Asia and Canada, and rarely in the US [53].

Our group tested the Bulgecin A extracts from *B. mesoacidophila* against a variety of carbapenem resistant *P. aeruginosa* and *Acinetobacter baumannii* isolates with differing resistance mechanisms [53]. Although these were impure preparations, we found that small amounts were able to inhibit growth of these clinical isolates when combined with typical amounts of carbapenems to which the bacteria were otherwise resistant. The Bulgecin A-meropenem combinations proved effective whether carbapenem resistance was due to the presence of MBLs (VIM-1), hyperproduction of PDC (Amp C enzyme of *P. aeruginosa* in combination with OMP loss) or efflux. Tomoshige et al. using synthetic Bulgecin A were able to demonstrate bulge formation in *P. aeruginosa* PA01 as well as lysis in the presence of ceftazidime [36].

6. Slt, MltD and MltG are the main targets of Bulgecin A inhibition and potentiation of β-lactams that inhibit PBP2 and 3 in *P. aeruginosa*

Previously it was demonstrated that bulgecin A potentiated the bulge formation and lysis of *P. aeruginosa* in the presence of ceftazidime and meropenem [36] in a swarm assay [54]. Recently, Dik et al. [27] used individual transposon knockouts of. Lts in a susceptible *P. aeruginosa* strain, PA01 and further engineered a green fluorescent protein (GFP) gene into the bacteria. The various Lt knockout strains were exposed to ceftazidime, an inhibitor of PBP3 in *P. aeruginosa* and meropenem, an inhibitor of PBP2,3 and 4 [55] on agar medium containing propidium iodide. Bulge formation and bacterial cell lysis were monitored as a function of time by monitoring green fluorescence from viable cells, and red fluorescence during cell lysis, the red fluorescence arising from bacterial DNA interacting with the propidium iodide in the medium. In the presence of ceftazidime, the Slt and MltD knockouts formed bulges and showed lysis. The Slt knockout demonstrated significant bulge formation within 6 hours of exposure to ceftazidime, and lysis within

9 hours. Some of the other knockouts demonstrated minor bulge formation (MltA, MltG, MltF, SltB1, SltB3) at 9 hours but none showed cell lysis. The effect was even more dramatic in terms of rapidity of bulge formation and cell lysis when meropenem was used. In fact, this semi-qualitative assay that involves spotting the bacteria and β-lactam at a given distance onto agar had to be modified for meropenem, as lysis occurred too quickly compared to conditions for ceftazidime. In the case of meropenem, an inhibitor of PBP2, Slt showed the greatest bulge formation and lysis, followed by MltG.

The soluble forms of five of the Lt enzymes were purified and bulgecin A binding constants measured: Slt K_d = 8.5 ± 1.1 μM; MltD K_d = 1.4 ± 0.3 μM; MltG K_d = 24 ± 5 μM, SltB1 K_d = 160 ± 20 μM; RlpA K_d = 1200 ± 280 μM.

Dik et al. [27] also demonstrated via scanning electron microscopy that cell wall failure within the bulge is responsible for cell lysis, in the presence of β-lactams and Bulgecin A. Withdrawal of the β-lactam antibiotic leads to delayed recovery of cell morphology in the presence of Bulgecin A alone, suggesting further, the cooperative nature of the Lt and PBP functions in cell wall maintenance.

7. Future prospects for antibiotic enhancers to treat *P. aeruginosa* infections

Now that the syntheses of the bulgecins A, B and C have been accomplished and purified Lt enzymes of many bacteria are available with simple commercial high throughput assays such as ENZCHEK lysozyme™ (substrate is a fluorescein labelled sacculus from *Micrococcus lutei*), it should not be long before potent derivatives of bulgecins are developed through medicinal chemistry approaches. Combinations of enhancers with novel β-lactamase inhibitor/potent anti-pseudomonal β-lactams are possibilities in the future antibiotic arsenal.

8. Conclusions

Antibiotic resistance in *P. aeruginosa* is on the rise in both inpatient and outpatient settings. β-lactams remain among the most successful antibiotics due to their potency, efficacy and safety. Traditionally, β-lactamase inhibitors have proved able to extend the life of these valuable antibiotics. However, through a variety of resistance mechanisms, *P. aeruginosa* has eluded these approaches. Lts are novel cell wall enzymes that work in concert with PBPs to facilitate numerous cellular functions (insertion of secretion systems, cell division, etc.). When both Lts and PBPs are inhibited, bacteria exhibit abnormal bulging of cell wall and osmotic lysis. Bulgecins are naturally occurring compounds that inhibit Lts. Together with β-lactams, Bulgecin A can lead to effective bacterial killing, even when *P. aeruginosa* are resistant to the partner β-lactam antibiotics. Bulgecins are a novel β-lactam enhancer that may prove beneficial in the treatment of infections with resistant *P. aeruginosa*.

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Conflict of interest

The author is an employee of the U.S. Department of Veterans Affairs. The opinions expressed in this review are her own and do not reflect those of her employer.

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