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Novel antimicrobial agents inhibiting lipid II incorporation into peptidoglycan

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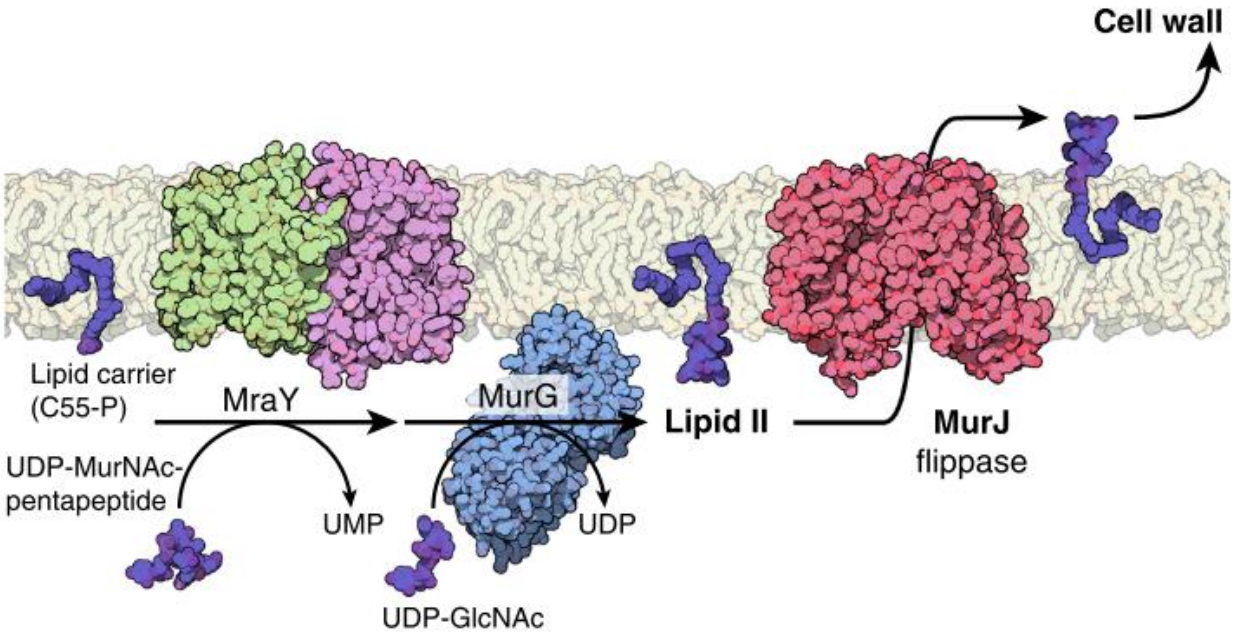

Mark Nijland
S3265978

Supervisor:
Prof. Dr. Dirk-Jan Scheffers
Molecular Microbiology
University of Groningen

Content

Abstract

Antibiotic resistance development in bacteria towards the currently available antibiotics forms a global health threat. Moreover, the antibiotic pipeline is running dry due to the limited success of the conventional culture-based method to introduce novel antibiotics. Innovative platforms have been established that greatly expand the natural product source from which novel antimicrobial compounds can be discovered. Here we focus on five recently identified antimicrobials that were mainly revealed by the application of such platforms. The mode of action of these antimicrobials involve the inhibition of peptidoglycan synthesis by either targeting lipid II (teixobactin, tridecaptin and malacidin) or the recently identified lipid II flippase MurJ (humimycin and Lys^{M}). In both cases, lipid II is sequestered from the penicillin binding proteins, thereby preventing synthesis of the peptidoglycan layer, which is fatal for the cell. These compounds are mainly effective against Gram-positive bacteria, with the exception of tridecaptin, and reveal robustness towards resistance development *in vitro*. The latter is an important feature that make them interesting potential candidates for therapeutic applications.

Keywords: Lipid II, teixobactin, tridecaptin, malacidins, humimycins, Lys^{M}

1.0 Peptidoglycan biosynthesis of bacteria

Antibiotics have drastically decreased the morbidity and mortality of bacterial infections ever since their introduction. However, antimicrobial resistance (AMR) has been reported for all the currently available clinical antibiotics and, more importantly, resistant strains have already emerged to which the conventional treatments are not effective[1]. To date, the most prominent antibiotic resistant infections are caused by the so-called 'ESKAPE' organisms, which include *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* species[1,2]. The ongoing AMR development is a global health concern that urgently requires the discovery and development of novel antibiotics that are robust to the development of resistance mechanisms. Strikingly, the biosynthetic pathway of the bacterial cell wall represents the most successful and predominant target of the currently available antibiotics (>50%)[3]. The cell wall is essential for bacteria, whereas humans lack analogous pathways and homologues structures, making it an attractive antimicrobial target[4]. Peptidoglycan (PG) is the major cell wall component of virtually all bacteria, its biosynthesis (**Fig 1a**) is therefore commonly referred to as the Achilles' heel of bacteria[3,5,6]. PG is comprised of alternating N-acetylmuramic acid (MurNAc) and N-acetylglucosamine (GlcNAc) sugars that are cross-linked via the stem peptide anchored to the MurNAc molecules[7,8]. This network is responsible for maintaining the shape and rigidity of the cell and allows cells to resist turgor pressure[9,10].

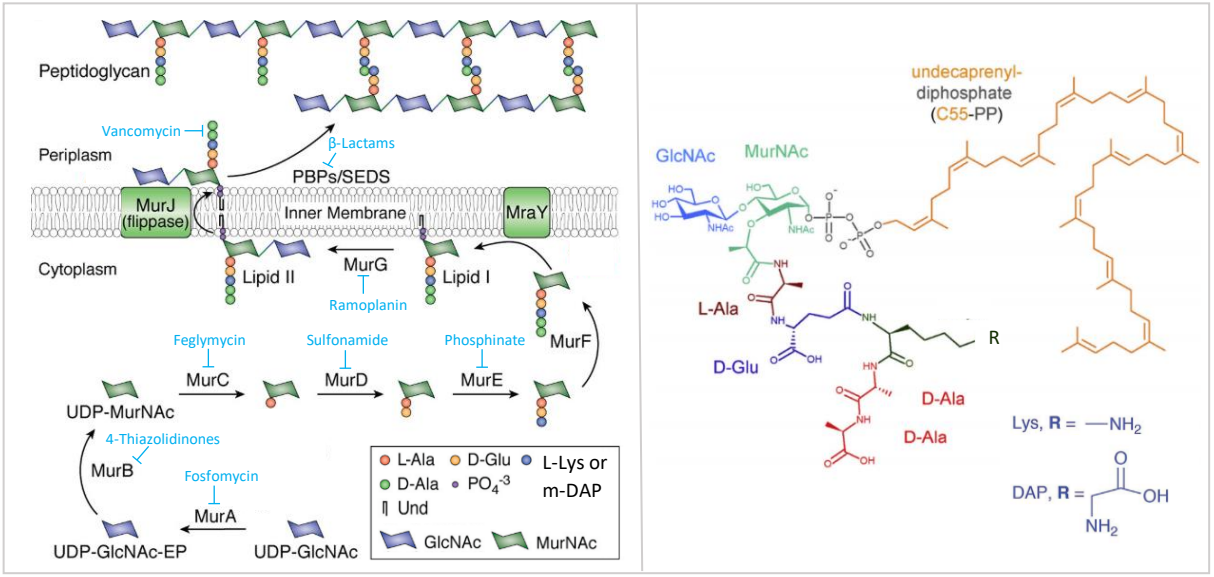

Figure 1. a) PG biosynthesis starting from the cytoplasmic substrate UDP-GlcNac to the incorporation of lipid II into the growing PG layer. Examples of compounds known to inhibit a particular enzymatic reaction are depicted in blue (adapted from K. Chamakura *et al.*[12]). **b)** Chemical structure of lipid II. The third amino acid residue of the stem peptide is depicted as R and differs for Gram-positive (L-Lys) and Gram-negative bacteria (m-DAP). Picture modified from F. Grein *et al*[35].

PG biosynthesis is initiated in the cytoplasm, where MurA catalyses the transfer of an enolpyruvate to uridine diphosphate (UDP)-GlcNAc[11] followed by the reduction of this moiety by MurB, yielding UDP-MurNAc (**Fig. 1a**)[12]. Subsequently, the Mur ligases MurC, D, E, F successively add the amino acids L-Ala, D-Glu, L-Lys (Gram-positive) or *meso*-diaminopimelic acid (Gram-negative), D-Ala and D-Ala to the fourth carboxyl group of the intermediate to generate the stem pentapeptide[13]. Although the amino acid sequence is highly conserved among most bacterial species, some strains possess a modified variant[14]. The UDP-MurNAc-pentapeptide is then linked to the lipid carrier undecaprenyl phosphate (C_{55}-P) by the membrane embedded protein MraY, leading to the formation of the membrane-associated intermediate lipid I[12]. MurG eventually mediates the last intracellular step in which a sugar GlcNAc molecule is attached to the MurNAc moiety of lipid I, to produce lipid II (**Fig 1b**)[12]. This lipid-

linked disaccharide pentapeptide is the central building block of peptidoglycan and is translocated across the hydrophobic membrane towards the extracellular side[10,12]. Initial research towards the identity of the protein responsible for lipid II translocation was controversial, where MurJ, FtsW and its homologue RodA displayed features to serve as flippase. These findings are extensively discussed elsewhere[3,15,16]. Over the past few years, compelling evidence has accumulated revealing that MurJ is the main protein mediating the flipping process[17–24]. Besides, the flippase Amj (alternate to MurJ) has recently been identified in *Bacillus subtilis*, which has shown to rescue the lethal *E. coli ΔmurJ* strain *in vivo*[19]. The membrane bound penicillin-binding proteins (PBSs)[3,25,26] eventually mediate the incorporation of lipid II into a growing PG network. These proteins catalyse a combination of glycosyltransferase reactions, in which the disaccharide of lipid II is linked to the glycan strand, and transpeptidation reactions to accomplish cross-linking between the neighbouring stem peptides[3,25,26]. Recently, members of the shape, elongation, division and sporulation (SEDS) family were also reported to catalyse glycosyltransferase reactions[27–29].

In principle all the enzymes and substrates involved in PG production serve as potential target for antimicrobial compounds (**Fig. 1a**)[30]. A combination of multiple factors make lipid II the most prominent antimicrobial target among the PG biosynthetic pathway, particularly for Gram-positive pathogens[3,4]. First, the structure of the membrane-anchored precursor is highly conserved throughout eubacteria, especially the pyrophosphate and sugar moieties[31]. Recognition of lipid II by the PG biosynthesis machinery is required to allow enzymatic processing, which greatly complicates structural modification without lethal consequences[3–5,32]. Thus, it is difficult for bacteria to acquire AMR towards lipid II-targeting antibiotics[3–5,32]. This is illustrated by the fact that it took 30 years to observe AMR against vancomycin, a clinical relevant antibiotic that targets the stem peptide of lipid II[33]. Second, the molecule is exposed at the periplasmic side of the inner membrane after its translocation, which is therefore readily accessible even for larger antibiotics[4,5]. Nonetheless, it should be noted that the outer membrane of Gram-negative bacteria acts as a permeable barrier that complicates its accessibility[34]. Another important aspect is that lipid II is only present in minimal numbers (few thousand molecules per cell), where the carrier lipid has to be constantly recycled for efficient PG synthesis[4,6]. Hence, any compound that sequesters the precursor molecule is a potential antimicrobial[6]. Lastly, multiple potential interaction sites are present (sugar molecules, stem peptide, pyrophosphate), despite its relative small size[10].

Lipid II is targeted by various classes of antimicrobials including lantibiotics, lipo(depsi)peptides, defensins and (lipo)glycopeptides[3,4]. These peptides generally sequester the precursor from the PBPs upon lipid II binding and thereby prevent extension of the existing PG layer, which is fatal to the cells[10]. In some cases lipid II sequestering is associated with an additional mechanism of action, like the formation of pores by the lantibiotic nisin[34]. Besides, inhibition of PG synthesis by targeting the involved enzymes that are localized outside the cell has also shown its potency[32]. For example, β-lactams are currently one of the most clinical relevant antibiotics that inactivate the PBPs[31,35]. The recently identified flippase MurJ is a currently underexploited target that may provide effective novel antimicrobials[36]. Although inactivation of enzymes is a valuable approach for therapy, it is in general easier for pathogens to develop AMR against such antibiotics due to the proteinaceous nature of their target[34]. Here we will provide more details about five exciting new recently discovered antimicrobials that target lipid II (teixobactin[37], tridecaptin[38] and malacidin[39]) or the lipid II flippase MurJ (humimycin[40], Lys^{M}[41]).

2.0 Novel antimicrobial agents

2.1 Teixobactin

The approach to discover novel antibacterial agents by culturing microbes under laboratory conditions has shown its success, but it encounters one major limitation. We are only able to grow a small proportion (~1%) of the microbes from the external environment in the laboratory[40], while the remaining uncultured organisms provide a potential source to discover novel antibiotics[42]. Recently, T.

Schneider *et-al.* circumvented this problem by applying a novel cultivation technique using an isolation chip (iChip)[37]. This method allows to isolate multiple bacteria from in this case soil samples, that are diluted in such way that only a single bacterium is trapped into separate channels[37]. These channels are covered with semi-permeable membranes, which allows diffusion of nutrient and growth factors[37]. The isolated bacteria can thereby grow under natural conditions when the iChip is placed into soil, which stimulates growth of unculturable bacteria[37]. Interestingly, most microbes that were previously uncultivable are subsequently able grow to *in vitro* once a colony is formed[43]. The iChip approach in particular is estimated to enhance the cultivability of microbes to approximately 50%[37]. Extracts from 10,000 isolates were analysed and has led to the discovery of the cell wall targeting macrocyclic depsipeptide teixobactin (**Fig. 2a**), which is non-ribosomally produced by the β-proteobacterium *Eleftheria terrae*[37]. The peptide consists of 11 amino acid chains among which four have a D-conformation and position 10 is occupied by the rare residue L-*allo*-enduracididine[37]. It shows potent antimicrobial activity against Gram-positive bacteria including *S. aureus*, *Mycobacterium tuberculosis*, *Clostridium difficile*, *Bacillus anthracis*, *Streptococcus* and *Enterococcus* species[37]. A tremendous amount of research towards the characterisation of teixobactin has been conducted after its discovery in 2015, due to its high potential as therapeutic.

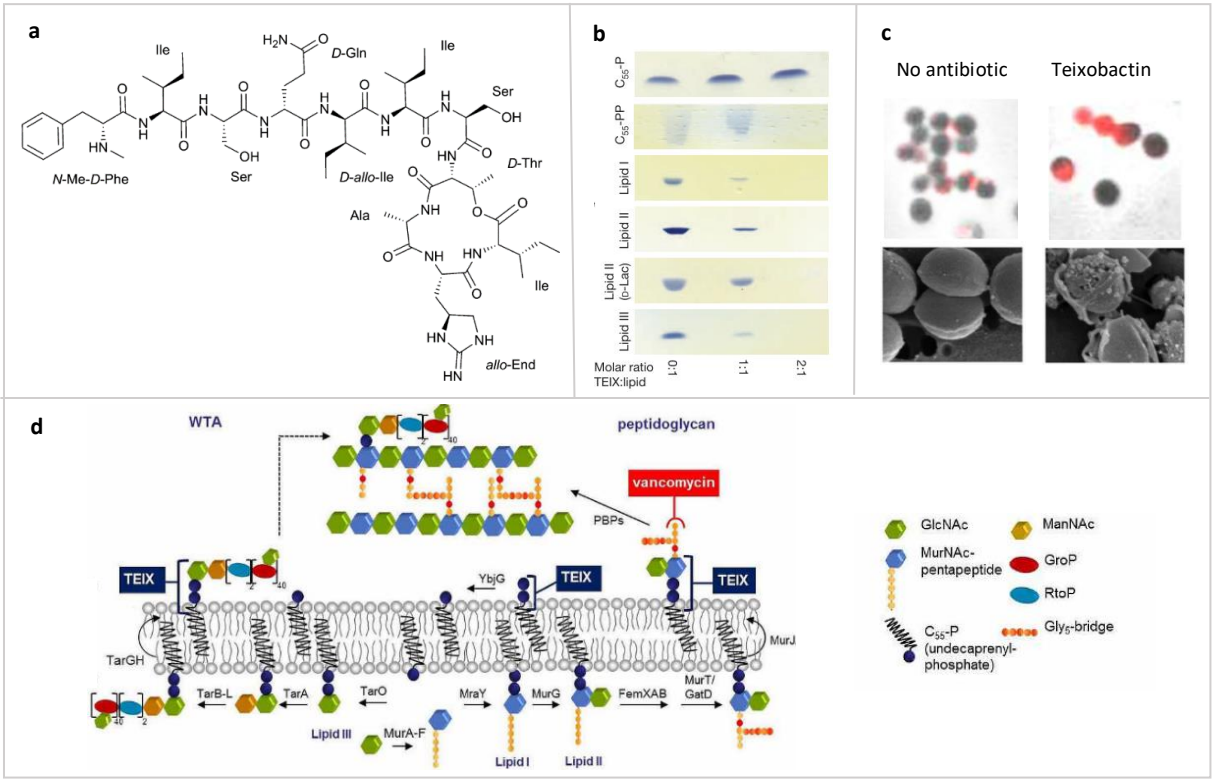

Figure 2. a) Chemical structure of teixobactin[37]. **b)** Binding assay of teixobactin with various precursor substrates. Teixobactin is visualized by thin-layer chromatography, diminished intensities indicate binding of teixobactin to the substrate[37]. **c)** Treatment of *S. aureus* with teixobactin results in delocalization of the autolysin Atl (upper) and cell wall damage (lower)[44]. **d)** Mechanism of action of teixobactin. The peptide binds lipid II (precursor PG) and lipid III (precursor TA) once transported towards the periplasmic site of the membrane. This results in a simultaneous inhibition of the PG and TA biosynthesis[37].

Initial research towards the identification that teixobactin targets the cell wall was acquired by growth of *S. aureus* in presence of various radioactive labelled precursor molecules, where it was shown that the peptide strongly represses PG synthesis[37]. Moreover, treatment of *S. aureus* with teixobactin is associated with intracellular accumulation of the last soluble PG substrate UDP-MurNAc-pentapeptide[37]. This phenomenon is also observed for vancomycin and is highly indicative for inhibition of PG synthesis by blocking one of the membrane-associated steps[37]. Accordingly, it was

confirmed by thin-layer chromatography that teixobactin binds bactoprenol derivates lipid I, lipid II and lipid III (**Fig 2b**)[37]. Lipid III is a precursor molecule involved in teichoic acid biosynthesis in Gram-positive bacteria and represents, like lipid II, a valuable target for antibiotics[44]. Teixobactin exhibits its bactericidal activity by simultaneously inhibiting the biosynthesis of peptidoglycan and teichoic acid (TA, **Fig. 2d**)[44,45]. This generates a synergistic mode of action that leads to increased cell wall damage, compared to lipid II targeting alone, and delocalization of autolysins **(Fig. 2c)**, which eventually leads to cell lysis[37,44]. Importantly, teixobactin binds to the pyrophosphate moiety and therefore allows binding of lipid II and III regardless of the identity of their sugars and alterations in the pentapeptide[46]. In contrast, teixobactin does not bind mature PG, which was shown by the lack of ability by purified PG to reduce antimicrobial activity of the peptide[37]. Indeed, it is effective against bacteria with increased cell wall density such as the vancomycin-intermediate *S. aureus* (VISA)[37]. The peptide also binds lipid I *in vitro*, but this interaction is believed not to be physiologically relevant as the precursor is not exposed outside the cell[37].

More insights into the mode of action of teixobactin were recently obtained by identifying structural changes of the peptide upon binding to lipid II residing in dodecylphosphocholine (DPC) micelles, using magic angle spinning (MAS) solid-state NMR[46]. No apparent differences are observed for the C-terminal pyrophosphate binding cage (residues 8-11) and this part of the molecule presumably contributes to the interaction with lipid II[46]. Structural changes are largely confined within the residues 2-6 (**Fig. 3**)[46], which were initially believed to be primarily associated with cell wall anchoring[42]. These residues are disordered in unbound state, while it adopts an extended β-sheet formation when bound to lipid II, leading to aggregation of the peptide-lipid II complex[46]. This phenomenon is also observed *in vivo*[46], is supported by X-ray studies using aqueous solution[47] and the binding mode in agreement with molecular dynamic studies[48], signifying the potential importance of this process under physiological conditions. Interestingly, no aggregation is observed when teixobactin binds a lipid II mimic lacking the sugar molecules, implying that binding of the peptide to this moiety is essential for aggregation[46].

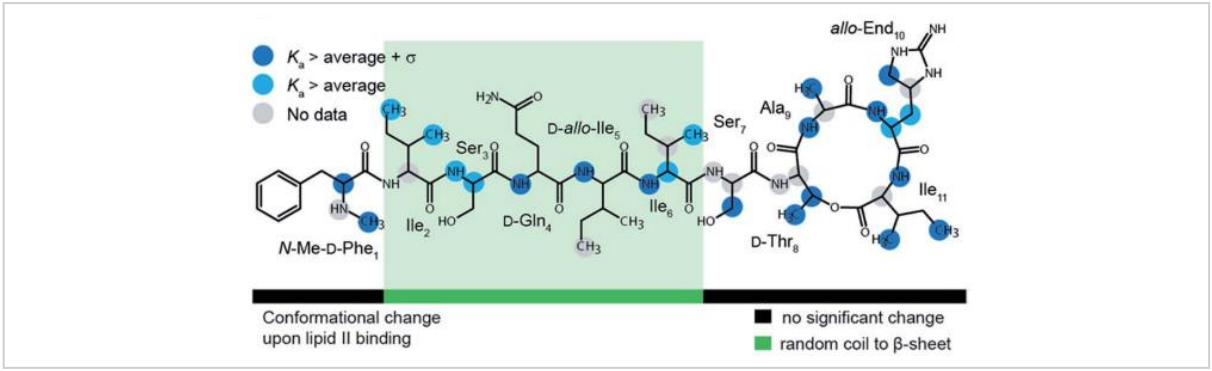

Figure 3. Solid-state NMR analysis upon titration of teixobactin with DPC micelles containing lipid II of Gram-negative bacteria. The region of teixobactin that undergoes structural changes, compared to the unbound state, is highlighted in green. The blue color represents the binding affinity towards lipid II[46].

There are various desirable characteristics making teixobactin a promising therapeutic agent; (I) it shows favourable pharmacokinetics[37] (II) it efficiently treats mice infected with either MRSA or *Streptococcus pneumonia*[37] and (III) resistance to teixobactin was not detected in various *in vitro* studies[37]. Nonetheless, it is possible that resistance mechanisms have been developed in nature that could eventually lead to resistance within pathogenic bacteria as result of genetic transfer. There are currently also comprehensive efforts undertaken towards the production of teixobactin analogs in which the unusual residues are replaced to simplify its structure[49–55]. Details are left out of the scope of this review, but it has been shown that L-*allo*-enduracididine at position 10 can be replaced by other residues without severely affecting its antibacterial activity. This modification greatly facilitates the production process of the peptide, especially in large-scales.

2.2 tridecaptin A_1

The lipopeptide Tridecaptin A_1 ($TriA_1$, **Fig. 4a**) is produced by *Bacillus polymyxa* and its discovery by J. Shoji *et al.* dates back to 1978, where it was found by the traditional culture-based strategy[56]. Remarkably, research towards a further characterisation of the antimicrobial peptide was only conducted very recently[38,57]. $TriA_1$ inhibits PG synthesis by binding to lipid II[38] and has the rare ability to display potent antimicrobial activity against Gram-negative strains including *K. pneumoniae*, *A. baumannii* and *E. coli*[57]. Oct-$TriA_1$ is a structural analog, which lacks the hydroxyl and methyl moieties at the lipid tail (**Fig. 4a**), and shows similar antimicrobial activity. Even though many antimicrobials are currently reported that eliminate Gram-positive bacteria, novel therapies for Gram-negative bacteria are considerably more difficult to develop as a consequence of the additional outer membrane[58]. Notable is that other antimicrobials that target lipid II generally fail to traverse the outer membrane and are consequently only active against Gram-positive bacteria[3]. Binding assays of various tridecaptin analogues using isothermal titration calorimetry (ITC) indicate that $TriA_1$ realizes this by interacting with a chiral target of lipopolysaccharide (LPS)[38]. The N-terminal lipid tail is not required to facilitate this interaction[38], although it is essential for its antimicrobial activity[57]. The exact mode of action to penetrate through the membrane has not been determined, but it known to sensitize Gram-negative bacteria towards hydrophobic antibiotics such as rifampicin and vancomycin[59]. $TriA_1$ is subsequently able to reach its target lipid II in order to exert its bactericidal effect. Strikingly, it was shown with a combination of antimicrobial inhibition assays, *in vitro* binding assays and ITC that $TriA_1$ specifically binds to lipid II from Gram-negative bacteria[38]. The difference between the lipid II variants from Gram-negative and Gram-positive bacteria is the presence of a *meso*-diaminopimelic acid (mDAP) and L-Lysine residue, respectively, at the third position of the pentapeptide **(Fig 1b)**[38]. Accordingly, the NMR structure of $TriA_1$ bound to a Gram-negative lipid II analog determined in DPC micelles reveal that $TriA_1$ binds this particular amino acid[38]. This specific target also explains the reduced antimicrobial activity against Gram-positive bacteria.

Using an adapted variant of the prodye 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxy-fluorescein acetoxymethyl ester (BCECF-AM), it was shown that bactericidal effect is associated with the disruption of the proton motive force, presumably by the formation of small pores (**Fig. 4b**)[38]. The peptide antibiotic nigericin targets the same essential cellular process[60] and yields similar observations[38]. However, obvious pore formation could not be observed in the NMR structure where $TriA_1$ binds lipid II residing within micelles[38]. Although most structural data on lipid II-peptide interactions are determined in non-physiological conditions like micelles, the controversial nature of such assays are prone to artefacts[61]. Hence, to get a better understanding into the physiological mode of action, experiments should be conducted in more physiological relevant conditions such as liposomes. $TriA_1$ displays low cytotoxicity and no persistent antibiotic resistance could be developed *in vitro* even after 30 days exposure of sub-lethal concentrations[57,59]. $TriA_1$ has also shown to successfully treat mice infected with *K. pneumonia*[38], making it a promising potential therapeutic agent.

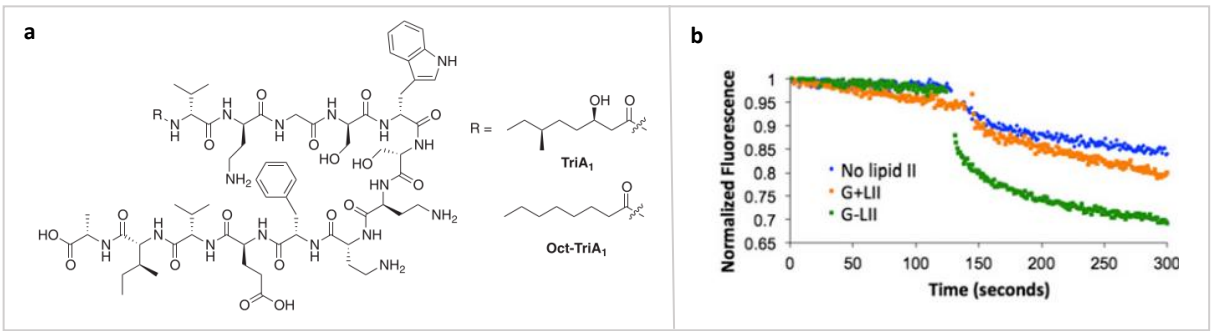

Figure 4. a) Chemical structure of $TriA_1$ and its structural analogue Oct-$TriA_1$[38]. **b)** Fluorescence analysis of BCEF-AM prodye encapsulated in large unilamellar vesicles (LUVs) containing various lipid II content. Treatment with $TriA_1$ causes a decrease in fluorescence only for LUVs containing lipid II from Gram-negative bacteria as a result of drop in pH. This is indicative for pore formation[38].

2.3 Malacidins

It is possible to sequence the genetic information of uncultured microbes[40], which in combination with bioinformatic tools provides a new concept to reveal novel potential therapeutic agents[62]. By the use of degenerated primers, a pool of amplicons that contain coding sequences of natural products can be amplified from a total DNA extract of an environmental sample[63]. To identify malacidins, primers targeting the adenylation domains of non-ribosomal peptide synthetases were used and provided the amplicons with a 'barcode' to allow individual sequence read-outs[39]. The generated sequence data allows to identify potential antimicrobial candidates using bioinformatic tools. To reveal novel calcium-dependent antibiotics, the presence of genes encoding the conserved calcium binding motif (Asp-X-Asp-Gly) was analyzed throughout different soil samples[39]. One of the soil samples containing interesting candidates was used for further analysis by generating a cosmid library, using phage transfection[39]. The bacteriophages are provided with various plasmids containing random blunt-end fragments generated from the soil total DNA extract[39]. Transfection using these bacteriophages results in the formation a large library that can be screened for the expression of compounds exhibiting antimicrobial features[64].

This led to the discovery of the two lipopeptides malacidins A and B, which contain a 10-amino acid cyclic ring (**Fig. 5a**)[39]. The peptides display antimicrobial activity in a calcium-dependent manner against Gram-positive bacteria including various multi-drug resistant *S. aureus* strains, *E. faecium* and *S. pneumoniae*[39]. Characterized members within the calcium-dependent antibiotic family display distinct modes of action and exhibit their antibacterial activity by affecting the cell wall integrity or inhibiting cell wall biosynthesis[39]. Accordingly, it was demonstrated with ultra-performance liquid chromatography–mass spectrometry (UPLC-MS) that *S. aureus* accumulates the last soluble PG precursor UDP-MurNAc-pentapeptide in the cytoplasm upon exposure to malacidin A (**Fig. 5b**)[39]. As described for teixobactin (see above), this is indicative for the inhibition of PG biosynthesis[39]. Binding assays further reveal that malacidin A binds lipid II (**Fig. 5c**) [39], although it remains inconclusive which part of the precursor molecule represents the binding site. The peptide does not bind the lipid carrier C_{55}-P, indicating that it most probably interacts with the sugars or the stem peptide. Similar as other lipid II targeting antimicrobials, *S. aureus* failed to develop resistance against malacidin A, even after 20 days exposure to sub-MIC levels[39]. Malacidin A has also shown to successfully sterilize MRSA-infected wounds of mice and did not show toxicity and haemolytic activity[39]. Malacidins were only reported in 2018, meaning that its characterization is still in its initial phase, but the results so far are promising for potential use as therapeutic agent.

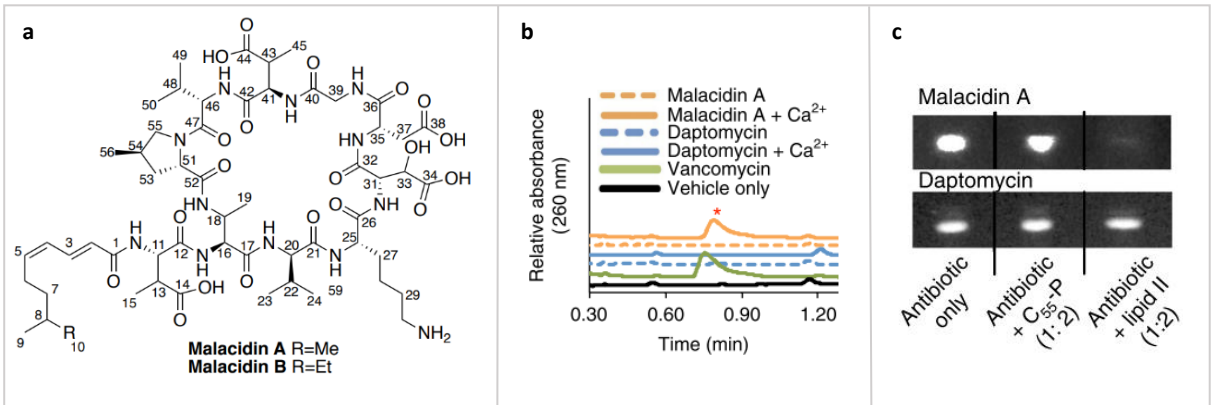

Figure 5. **a)** Chemical structure of malacidin A and B[39]. **b)** UPLC-MS trace showing *S. aureus* treated with malacidin A accumulate the precursor molecule UDP-MurNAc-pentapeptide inside the cell in a calcium dependent manner (similar to vancomycin). UDP-MurNAc-pentapeptide is indicated with a star[39]. **c)** Binding assay of malacidin A with C_{55}-P and lipid II. Malacidin A is visualized with thin-layer chromatography, where diminished signal intensities indicate substrate binding[39].

2.4 Humimycins

In addition to the fact that we are only able to culture a small proportion of all existing microbes, many potential interesting microbial metabolites are not produced under laboratory conditions[65]. Nonetheless, the currently available technology provides the ability to predict structures of (supressed) metabolites from solely the sequence of (uncultured) microbes. In a proof of principle study, the readily available sequence data of the human microbiome[66,67] was used to predict structures of peptides that are encoded by non-ribosomal peptide synthase gene clusters[68]. The predicted structures were subsequently chemically synthesized to produce the so-called synthetic-bioinformatic natural products (syn-BNPs)[68]. J. Chu *et al.* synthesized a total of 25 syn-BNPs which were used for an initial antimicrobial activity screening against pathogenic bacteria[68]. Two 'natural product mimics', humimycin A and B (**Fig. 6a**), were used for further analysis and are predicted from gene clusters found in the genomes of *Rhodococcus equi* and *Rhodococcus erythropolis*, respectively [40].

Humimycin A and B particularly show antimicrobial activity against *Streptococcus* and *Staphylococcus* species, including methicillin-resistant *S. aureus* (MRSA)[40]. Especially MRSA forms a major threat among the β-lactam resistant pathogens and urgently requires alternative approaches[69,70]. Spontaneous *S. aureus* mutants reveal mutations within the essential gene *sav1754*, a homolog of the flippase MurJ from *E. coli*, and overexpression of the gene inhibits the antibacterial activity of the humimycins[40]. Similar phenomena were observed for the MurJ targeting viral protein Lys^M (see below)[41]. This suggest that humimycins target the lipid II flippase (**Fig. 6c**), thereby inhibiting the transport of lipid II towards the periplasmic side of the membrane[41]. The interaction site of humimycins has been predicted based on the point mutations of the spontaneous resistant *S. aureus* strains and the available flippase structure of *Thermosipho africanus* that has ~20% identity (**Fig. 6e**)[36]. Nonetheless, it should be noted that direct evidence of the interaction partner, such as mentioned for teixobactin, $TriA_1$ and malacidins, is missing for humimycins[41]. Interestingly, humimycin A additionally displays synergistic activity in combination with β-lactams (**Fig. 6d**) and is able to drastically decrease the minimal inhibition concentration of carbenicillin and dicloxacillin against *S. aureus*[40].

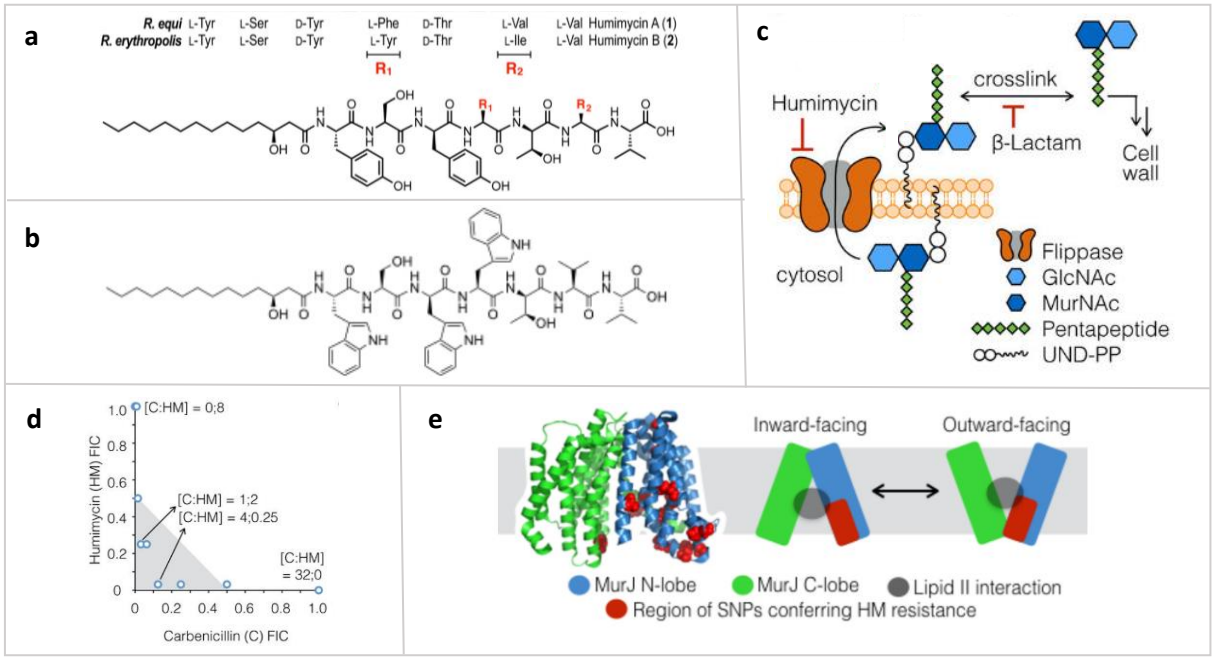

Figure 6. a) Chemical structure of humimycin A and B[40] and **b)** humimycin 17S[36]. **c)** Predicted mode of action of humimycin. Humimycins are predicted to bind the lipid II flippase and thereby impede its transport process[40]. **d)** Synergistical effect of humimycin A (HM) and carbenicillin (C) against *S. aureus*. A fraction inhibitory concentration (FIC) below 0.5 indicates synergy[40]. **e)** Predicted binding site of humimycin A based on point mutations of spontaneous humimycin A resistant *S. aureus* strains. The crystal structure of the flippase from *T. africanus* (~20%) is used as structure model[36].

Moreover, humimycin A restores susceptibility of the MRSA COL strain towards dicloxacillin, whereas both antimicrobials are not effective when supplied separately[40]. This phenomenon is also observed for other inhibitors that target early steps of cell wall biosynthesis[71,72]. The underlying principle is not exactly understood, but it is believed to arise due to the fact that the same pathway is inhibited at different stages (**Fig. 6a**)[36].

Follow-up research on humimycin A show that the antibacterial properties could be further improved upon replacement of all its aromatic residues (position 1,3 and 4) by a tryptophan (**Fig. 6b**)[36]. This compound, humimycin 17S, displays a broader spectrum of activity, including vancomycin resistant *E. faecalis* (VRE)[36]. Like MRSA, VRE forms a serious treat as result of antibiotic resistance development for which alternative treatments are requested[36]. Moreover, resistance development in *S. aureus* is 50-fold reduced for this analog, and these mutants remain susceptible to the synergistic activity in combination with carbenicillin[36]. Whether this is also true for humimycin A is not explicitly stated. *In vitro* resistance development could not be observed for *S. aureus* in the presence of both, humimycin 17S and carbenicillin, even after 14 days in presence of sub-lethal mixture of the two[36]. A mixture of humimycin A and carbenicillin on the other hand does result in resistance[40]. Important to address is that the target of humimycin 17S has not been tested, although the synergy with carbenicillin suggests that the analogue most probably utilizes a similar mode of action. Interestingly, treatment of MRSA infected mice with humimycin A in combination dicloxacillin has shown to drastically increase survival rate when compared to treatment with either compounds alone[68]. Hence, these humimycins might be able to prolong the lifetime of β-lactams as useful antibiotic.

2.5 Lys^{M}

Lytic bacteriophages induce cell lysis in the final stage of the infection cycle to release their progeny into the extracellular environment[73]. The proteins involved in this process present a natural source of potential novel antibacterial agents. Small lytic bacteriophages, either containing single-stranded DNA (microviruses) or single-stranded RNA (leviviruses), encode only a single protein that realizes cell lysis without enzymatic degradation of PG[74]. K. R Chamakura *et al.* recently showed that Lys^{M}, the lysis protein encoded by Levivirus M, achieves this by inhibiting the translocation process of lipid II by targeting the membrane-embedded flippase MurJ[41]. The viral protein consists of 37 amino acids and has a single transmembrane segment[75] with the N-terminus oriented towards the cytoplasm, as demonstrated by the functional Lys^{M}-GFP fusion protein[41]. Overexpression of Lys^{M} in *E. coli* is associated with morphological defects, typical for PG biosynthesis inhibitors such β-lactams and the viral proteins A_2[76] and E[77], followed by cell lysis[41]. *E. coli* expressing elevated levels of MurJ or the heterologous flippase homolog Amj from *B. subtilis* lose their susceptibility towards the intracellular expressed peptide[41]. Moreover, spontaneous resistant mutants that could be isolated showed mutations within *murJ*[41]. The mutations were mapped on the available flippase structure of *T. africanus* similar as described previously for humimycins (**Fig. 7**)[41]. The positions of mutations are not comparable to those caused by humimycins (see above)[36]. The crystal structure of MurJ from *E. coli* is now available[20] and more recently conformational transition states of the transport cycle were also determined[24]. These structures should facilitate the interpretation of the interaction site. Radio-active based *in* vivo experiments further revealed that Lys^{M} perturbs lipid II availability on the periplasmic side of the membrane[41], supporting the hypothesis that the protein inhibits lipid II transport.

Although it is stated by the authors that Lys^{M} is a potential novel antibiotic, research towards this protein until now is not yet sufficient. All conducted experiments to show that the viral protein exhibits antibacterial activity were conducted with *E. coli* strains that express Lys^{M} intracellularly. It is not clear whether the viral protein is also effective when applied extracellularly and more importantly against which bacterial species it is active. Moreover, it is not known whether Lys^{M} could potentiate β-lactams or other antibiotics like humimycins and if this occurs in an AMR robust manner. This is of great importance as resistance to the protein alone was shown to develop relative easily[41]. Thus, further research is required to evaluate its potential use in clinical settings.

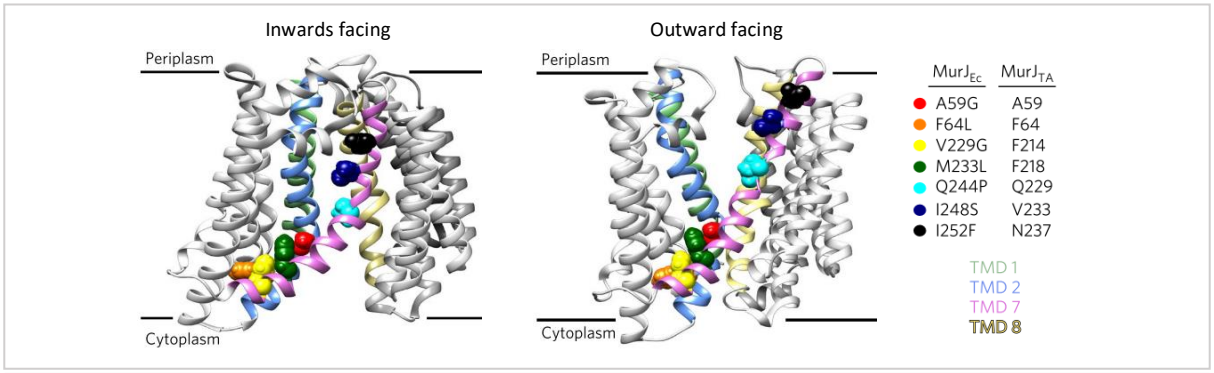

Figure 7. Mutation map of MurJ based on the point mutations of spontaneous resistant Lys^{M} *E. coli* strains. The flippase crystal structure of *T. africanus* serves as protein structure model for MurJ[41].

3.0 Concluding remarks

The above mentioned innovative drug discovery platforms have led to the identification of novel interesting antimicrobial compounds. These studies show that various approaches enable the ability to reveal novel therapeutic candidates in sources which were previously inaccessible. The continuing progress in technology should further facilitate this process in the future. The lipid II-targeting teixobactin, TriA$_1$ and malacidin A show features that are beneficial for clinical use, especially the robustness towards the formation of resistance. Teixobactin is for now seen as the most promising candidate, which is illustrated by the tremendous amount of research that has already been conducted on this peptide. Moreover, TriA$_1$ is very interesting due to its capacity to eliminate Gram-negative bacteria, against which even less antibiotics are being introduced nowadays. Malacidin A requires additional research to validate its potential, but the research so far is encouraging. Furthermore, inhibition of MurJ by humimycin 17S shows the capacity to prolong the life-time of the currently important β-lactams. This is interesting as they present an important fraction of clinical relevant antibiotics. Whether Lys^{M} could be used to potentiate β-lactam similar to humimycin 17S remains to be discovered.

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