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Review Article

Review: Targeting the Biosynthesis and Incorporation of Amino Acids into Peptidoglycan as an Antibiotic Approach Against Gram Negative Bacteria

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Abstract

The rise of multi-drug resistant bacteria causing life threatening infections is instigating researchers to shift their attention towards previously unexplored antimicrobial targets. Targeting the synthesis of peptidoglycan that contributes to the structure of the bacterial cell wall has been and is still an attractive pathway due to its essentiality in bacteria and absence in humans. Here, we examine the pathways involved in the synthesis of three key amino acids and their incorporation into the peptide stem in Gram negative bacteria. This review also summarises the current research investigating these pathways as novel antibiotic targets.

Keywords: *Biosynthesis; Amino Acids; Peptidoglycan; Antibiotic; Gram Negative Bacteria*

Introduction

In the last few decades, there has been a significant increase in antibiotic resistance, resulting in multi drug resistant (MDR) and extremely drug resistant (XDR) bacteria [1]. Due to these increasing levels of resistance, the need for new antibiotic targets is more urgent than ever before. Ever since the development of the first antibiotic over 70 years ago, peptidoglycan synthesis has been a major target of antibiotics. It is regarded as an ideal target due to its essentiality to bacterial survival and its absence in humans and other eukaryotes. However, with the emergence of resistance against many antibiotics that target peptidoglycan and its synthesis, researchers started to examine other antibiotic targets upstream to the synthesis of the peptidoglycan precursors and their components.

Peptidoglycan itself consists of long strands of alternating N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc) residues. Interestingly, the length of these glycan strands is not indicative of the thickness of the peptidoglycan layer, with both Gram positive and Gram negative bacteria having long and short strands depending on the species [2]. The length of these strands can also vary depending on the strain and growth conditions [3]. The strands of MurNAc and GlcNAc are cross-linked by a pentapeptide stem, connecting two opposing strands. This peptide stem varies between species, but is most commonly found to be L-alanine–D-glutamate–meso-DAP–D-alanine–D-alanine in Gram negative bacteria, with the terminal D-alanine cleaved during the formation of the mature peptidoglycan [4]. In the majority of Gram negative bacteria, the crosslinking between the peptide stems is a direct link between the side chain of the third amino acid, meso-DAP, and the carboxyl group of the fourth residue, D-Ala, on the opposing strand [2]. This is in contrast to the interbridge between residues found in Gram positive species [2]. Penicillin binding proteins are responsible for catalysing these crosslinking reactions, and as the target for many established antibiotics, have been comprehensively studied [5]. This review will focus upstream of the crosslinking, specifically on the biosynthesis of the three key amino acids found in the peptide stem and their incorporation into the peptidoglycan layer in Gram negative bacteria. We will also explore the targeting of the biosynthesis of these amino acids and their incorporation into the peptide stem as novel antimicrobial strategies. Although this review will focus primarily on Gram negative bacteria, it will also include *Mycobacterium* as they contain a similar pentapeptide to that of other Gram negative bacteria [6].

Alanine

Synthesis

Alanine plays many key roles in eukaryotes, including humans, and bacteria. In most Gram positive and Gram negative bacteria, the L- and the D- isomers of alanine are found in the first and last positions of the peptide stem, respectively [7]. The first L-alanine is responsible for binding to the peptidoglycan precursor; UDP-MurNAc, and in a subsequent reaction, binds to D-Glutamate, the next amino acid in the peptide chain [2]. L-Alanine is typically synthesised by transaminases (also referred to as aminotransferases) from pyruvate via the transamination of L-glutamate or L-valine to L-alanine (Figure 1) [8]. *E. coli* contains both AlaA and AlaC glutamate-pyruvate aminotransferases, which can reversibly convert L-glutamate and pyruvate into L-alanine and α-ketoglutarate (Figure 1) [8]. Transaminase C (an alanine-valine transaminase) is believed to catalyse the last step in valine synthesis, producing pyruvate and valine from 3-methyl-2-oxobutanoate and alanine [9]. However, it also plays a significant role in synthesising alanine due to the reversibility of this reaction (Figure 1) [9]. Up to eight other possible alanine synthesis pathways have been described in *E. coli*, however this is believed to be a secondary function for these enzymes, with the physiological contribution of these reactions remaining unclear [8].

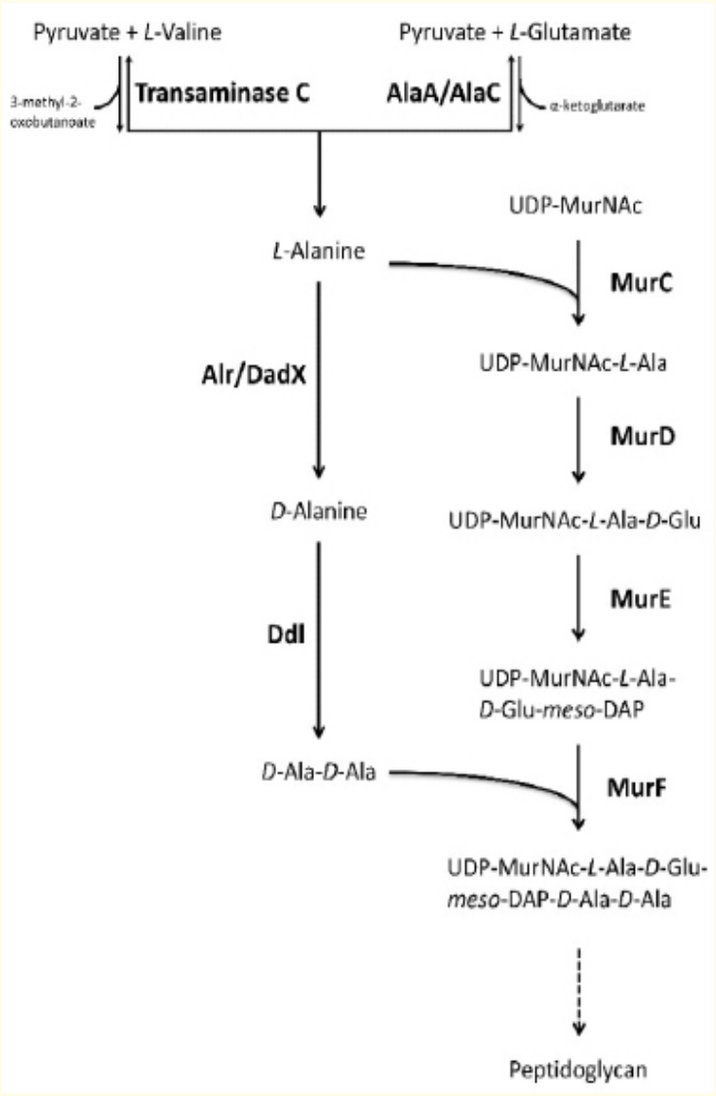

Figure 1: *L-alanine synthesis in Gram negative bacteria. L-alanine biosynthesis in E. coli by transaminase C and AlaA/AlaC enzymes followed by the conversion to D-alanine and ligation of the D-Ala–D-Ala dipeptide before incorporation of both to the peptide stem of the UDP-MurNAc peptidoglycan precursor.*

As mentioned previously, in addition to L-alanine, D-alanine is present in the peptidoglycan crosslink. D amino acids are formed by either racemases or aminotransferase enzymes, with the racemase enzymes being the most prominent for D-alanine production [10]. Several Gram negative bacteria, including *Escherichia coli, Pseudomonas aeruginosa* and *Salmonella typhimurium*, have two alanine racemases, encoded for by the *alr* and the *dadX* genes [7]. The *dadX*-encoded racemase has been found to be inducible, and expressed only when L-Ala has been sequestered as a carbon source [7]. On the other hand, the *alr* racemase is expressed constitutively, and thus, thought to be of more physiological importance [7]. This has led to Alr being more thoroughly studied. These racemases are pyridoxal-5'-phosphate (PLP)-dependent, involving a two-step binding event with the co-factor and the substrate [10]. Once L-alanine has been converted to D-alanine, it is ligated together to form a D-Ala–D-Ala dipeptide by the enzyme D-Ala–D-Ala ligase (Ddl) for use in the peptidoglycan peptide stem [7]. Typically, the Ddls synthesise the D-Ala–D-Ala in bacteria, yet some vancomycin resistant Gram positive bacteria have D-Ala–D-Ser or D-Ala–D-Lac in these terminal positions [11]. Two Ddl genes (*ddlA* and *ddlB)* have been observed in multiple Gram negative bacteria, including *E. coli* [12] and *S. typhimurium* [13]. While the products are of different sizes, both have been purified and shown to have similar kinetic characteristics, including comparable substrate affinities [7]. Despite the fact that the terminal D-ala of the pentapeptide chain is cleaved in mature peptidoglycan, it is added to the precursor UDP-MurNAc-L-Ala–D-Glu–meso-DAP as a dipeptide [2].

Addition of L-Ala onto the peptidoglycan precursor UDP-MurNAc is commenced by the MurC enzyme, which exhibits high specificity for L-Ala, lower affinity for glycine and L-serine, but no affinity for D-Ala [7,14]. *Mycobacterium tuberculosis* and *Mycobacterium leprae* show a higher affinity for a glycine substrate than other MurC enzymes, however it has been noted that glycine is the first residue of the peptide stem for *M. tuberculosis*, which could explain this difference in affinities [15]. The MurF enzyme ligates the D-Ala–D-Ala dipeptide to the peptide stem, and is similar between Gram positive and Gram negative bacteria. These enzymes show equal affinity for both the meso-DAP and L-Lys containing substrates [16].

Inhibition

A vast amount of research has focused on the development of inhibitors of Alrs and Ddls, as these enzymes are not found in humans. One such inhibitor, which was further developed into a treatment, is cycloserine, marketed as the drug Seromycin. Cycloserine is a structural analogue of D-Ala, which inhibits both Alr and Ddl *in vitro*, while exhibiting a higher affinity for Ddl *in vivo* [17]. While mainly used as a treatment for MDR tuberculosis, questions have been raised about its safety [18], indicating that to target these enzymes, the development of new inhibitors is required. Accordingly, *de novo* drug design has been employed for the development of inhibitors against Ddl, but resulting compounds displayed weaker potency compared to the current Alr and Ddl inhibitors [19].

Further downstream of L- and D-alanine synthesis, the MurC and MurF enzymes also represent promising antimicrobial targets. Phosphinate inhibitors for MurC have been reported to have IC_{50} values as low as 49 nM, utilising phosphinate inhibitors from other Mur enzymes as a structural backbone [20]. Identification of an inhibitor of MurF was achieved by virtual screening of compound libraries. The identified compound has an IC_{50} of 63 μM against the *E. coli* MurF enzyme, but lacks antimicrobial activity [21]. Affinity-based high-throughput screening was also employed, with resulting inhibitors reported to have IC_{50} values between 20 - 70 nM, but again, little to no antibacterial activity [22]. Little progress was made for almost a decade after this until MurF inhibitors were developed that showed promising antimicrobial activity. These inhibitors were reported to have MIC values ranging between 4 - 64 μg/ml for the different *E. coli* strains tested [23]. While gaining antimicrobial activity was a success, these compounds are unlikely to undergo any further development due to off-target effects caused by eukaryotic cytoplasmic membrane damage [23].

Glutamate

Synthesis

Several Gram negative bacteria have two primary pathways for glutamate synthesis; via the glutamate dehydrogenase (GDH) or glutamine synthetase (GS)-glutamate synthase (GOGAT) enzymes. The GDH enzyme catalyses the formation of glutamate through the reductive amination of 2 oxoglutarate (Figure 2) [24]. On the other hand, the GOGAT pathway results in the amidation of L-glutamate to glutamine by GS, before GOGAT catalyses the transfer of the amide group to 2 oxoglutarate (Figure 2) [24]. Previous work indicated that mutations in both the GDH and GOGAT encoding genes in *E. coli* required supplementation with external glutamate for survival. However,

mutations in only one of either of the genes showed glutamate production equivalent to the wildtype [25]. This indicates that only one of the two pathways is necessary for sufficient glutamate production. This is further validated by studies done on the Gram negative bacteria *Erwina carotovora*, which lacks the GDH enzyme and, as such, only contains the GOGAT pathway for glutamate production [26]. Other pathways for glutamate synthesis exist, with the transamination reactions involving the transfer of the amino groups from other amino acids to 2-oxoglutarate to produce glutamate. An example of this is the aspartate aminotransferase reaction (Figure 2) [24]. This reaction is similar to that of the amino-transferases involved in alanine synthesis and is also dependant on the PLP cofactor for activity [26]. However, as glutamate synthesis is present in many organisms, including humans, the most common antibiotic target for glutamate synthesis is the pathway that synthesises the bacteria-specific D-glutamate.

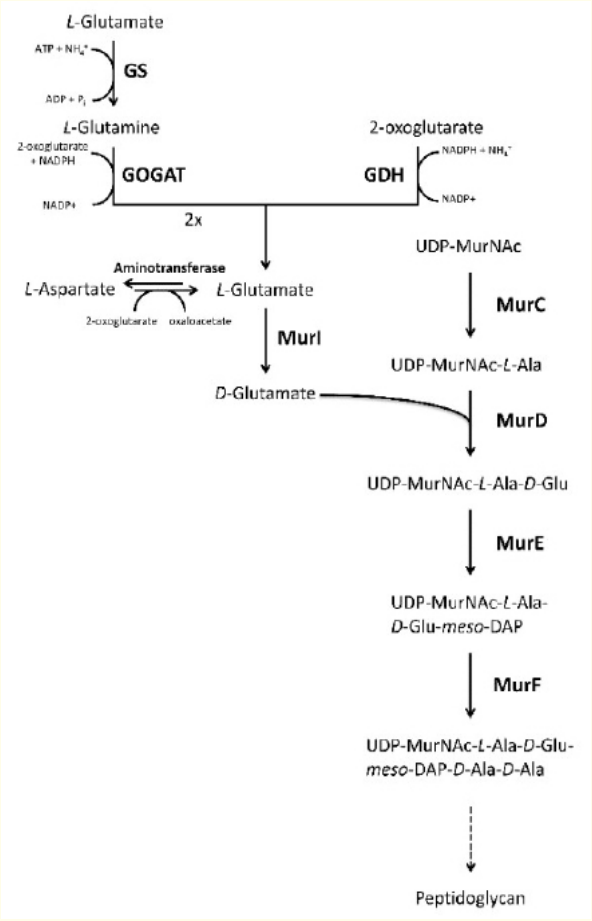

Figure 2: *D-glutamate synthesis in Gram negative bacteria. D-glutamate synthesis by glutamate dehydrogenase (GDH) and the glutamine synthetase-glutamate synthase (GOGAT) pathways, and the incorporation into the peptide stem of peptidoglycan precursor glycan strands.*

D-glutamate is synthesised by two enzymes; glutamate racemase (MurI) and D-aminotransferase (Dat). Unlike the alanine racemase discussed earlier, the MurI enzyme is PLP-independent and, as such, requires no cofactor to function [27]. Typically for Gram negative bacteria, the glutamate racemase is the only route for D-glutamate synthesis, however, several studies on Gram positive species report the synthesis of D¬-glutamate using D-aminotransferase (Dat) [7].

Subsequently, D-glutamate is added to the L-Ala component of the peptide chain by the enzyme MurD [7]. When compared to the Gram positive MurD enzymes, those from the Gram negative bacteria *E. coli* and *Haemophilus influenzae* have been found to be more highly regulated [28]. This could indicate that Gram negative bacteria have a tighter overall regulation on peptidoglycan synthesis than Gram positive bacteria.

Inhibition

Disruption of the *murI* gene ultimately leads to cell lysis as a result of alteration of peptidoglycan synthesis, making it a promising antibiotic target [29]. It also has a high protein sequence similarity at the active site between many species, including *E. coli, Helicobacter pylori* and *Aquifex pyrophilus*, giving rise to the potential development of a broad spectrum inhibitor [30]. The first attempts to inhibit the MurI enzyme involved the development of substrate analogues that were able to covalently modify the enzyme, but these were shown to be ineffective [27,31]. Other inhibitors were designed based on the product, D-glutamate, to avoid any off-target effects resulting from L-glutamate [32]. These compounds were effective against *S. pneumoniae* strains, however inhibition did not translate to any other tested bacterial strains [32]. Allosteric inhibitors were then developed against *H. pylori*, though again these had selectivity for *H. pylori* only [33]. Recently, a whole-cell vaccine strategy against *Pseudomonas aeruginosa, Acinetobacter baumannii,* and *Staphylococcus aureus* has been developed. The proposed vaccine would involve inactivating genes encoding both MurI and the Dat enzymes (if applicable), resulting in an attenuated strain dependent on supplemented D-Glu [34]. This promising strategy has shown that the attenuated strains yield significant immune responses combined with a reduced virulence and no observed phenotypic reversion to the wildtype [34].

In addition to MurI, the MurD enzyme has also been targeted for antibiotic development. The first inhibitors were developed based on the intermediate product and bound to the transition state of the enzyme [35]. This included the phosphinate based inhibitors, with the best compound reporting an IC_{50} of 0.68 μM [35]. This was optimised through the incorporation of muramic acid, resulting in an IC_{50} of 20 nM [36]. This spurred further development of phosphinate inhibitors, however these alterations were often ineffective and resulted in no improvement in the inhibition of MurD. To design a new class of inhibitor against MurD from *P. aeruginosa*, techniques such as phage display, coupled with extensive biopanning, were used [37]. This resulted in peptides with IC_{50} values of ~4 μM, however much optimisation is still required for these to be able to cross the bacterial membrane [37]. Virtual screening has also been employed to target the MurD enzyme, with design aimed towards the ATP-binding site as opposed to the active site [38]. While these inhibitors showed weak activity, they provide a novel pharmacophore for drug development.

Lysine

Synthesis

The third position of the pentapeptide chain is typically the point of difference between most Gram positive and Gram negative cell walls. Lysine or meso-DAP generally occupy this position, respectively, although there are exceptions [7]. Peptide stems from the Gram positive bacteria *Thermotoga maritima* have revealed L-Lys or D-Lys in the third position, as opposed to meso-DAP [39]. In bacteria, both L-Lys and D-Lys residues are synthesised via the diaminopimelate (DAP) pathway (Figure 3). The first committed step of the DAP pathway involves the condensation reaction between pyruvate and L-aspartate semialdehyde (ASA) catalysed by the enzyme dihydrodipicolinate synthase (DHDPS) [40,41]. The product of this reaction, 4-hydroxy-2,3,4,5-tetrahydro-L-L-dipcolinic acid (HTPA), is then reduced to L-2,3,4,5-tetrahydrodipicolinate (THDP) by dihydrodipicolinate reductase (DHDPR) [42,43]. Depending on the organism, L,L-2,6-diaminopimelate (LL-DAP) is produced by one of four different sub-pathways. Since all documented Gram negative bacteria use the succinyl pathway, this will be the focus in this review. The succinyl sub-pathway commences with the enzyme 2,3,4,5-tetrahydropyridine-2-carboxylate N-succinyltransferase (THPC-NST) catalysing the production of N-succinyl-L-2-amino-6-ketopimelate (NSKAP) from THDP [44]. NSKAP is then converted to N-succinyl-L,L-2,6-dimainopimelate (NSDAP), before desuccinylation to LL-DAP by N-succinyldiaminopimelate aminotransferase (NSDAP-AT) and succinyldiaminopimelate desuccinylase (SDAP-DS), respectively [44]. DAP epimerase then catalyses the conversion of LL-DAP to meso-DAP, which can then be incorporated into the peptide chain covalently linked to UDP-MurNAc in Gram negative bacteria [44]. It is important to note that in Gram negative bacteria, meso-DAP can be converted to lysine by the enzyme DAP decarboxylase (DAP-DC) for use in protein synthesis as opposed to incorporation into the peptidoglycan layer [45].

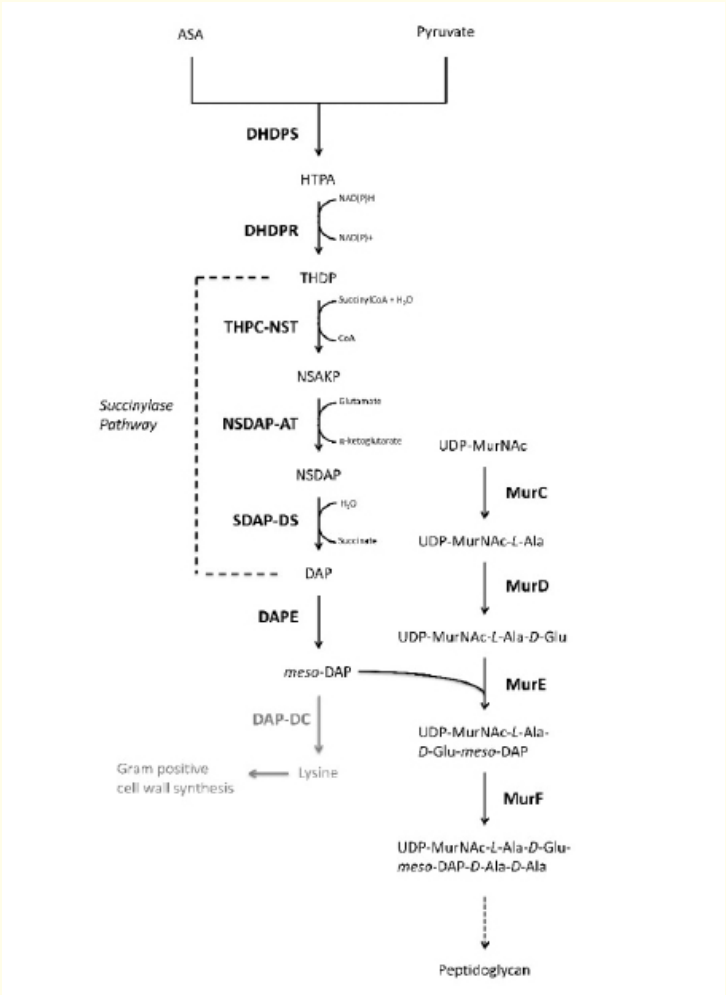

Figure 3: *meso-DAP and L-lysine biosynthesis via the diaminopimelate (DAP) pathway in bacteria. Synthesis in Gram negative bacteria occurs through the succinyl sub pathway before the incorporation of meso-DAP into the peptide stem of precursor peptidoglycan.*

The MurE enzyme catalyses the covalent linkage of meso-DAP to D-Glu on the peptide stem, with this enzyme displaying a species-dependent preference for either meso-DAP or lysine. Previous work suggested that incorrect incorporation of L-lys into the *E. coli* peptide chain resulted in lethality [46]. There are exceptions for this in Gram negative bacteria, with the MurE enzyme from *T. maritima* showing similar *in vitro* efficiency for adding L-Lys and meso-DAP, despite no meso-DAP being found in the peptidoglycan layer [39]. A preference for the addition of meso-DAP or lysine to the peptidoglycan layer has been hypothesised to be due to a difference in intracellular concentrations of these metabolites [39].

Inhibition

Similar to the other Mur class enzymes, MurE has been targeted in efforts to produce novel antimicrobials. The first MurE inhibitor, like the MurD inhibitors, was a phosphinate compound, which targeted the transition state of the enzyme. This inhibitor has an IC_{50} value ~ 1 μM, but lacks antibacterial activity [47]. Phage display studies resulted in the discovery of a peptide with MurE inhibitory properties, but the degree of inhibition was reduced compared to published inhibitors. Despite this, this work provided another avenue of investigation for novel inhibitor development. More recently, other compounds that exhibit MurE inhibitory activity, such as tetrahydroisoquinolones, have been investigated for treatment of *M. tuberculosis*. The inhibitors have been shown to display moderate antibacterial *activity in vivo*, but they lack broad spectrum activity against Gram negative bacteria [48].

Unlike alanine and glutamate synthesis, meso-DAP and lysine are not synthesised by humans, thus making the DAP pathway an ideal target for antibiotic development upstream of the MurE enzyme. The first enzyme in the DAP pathway, DHDPS, is considered a prime target for antibiotic development since it catalyses the rate limiting step of the pathway. Several groups have previously attempted to develop DHDPS inhibitors by targeting either the active or allosteric site [49-51]. The first inhibitors were generated by analogy to its substrates, pyruvate and ASA, or to dihydrodipicolinate, which was originally thought to be the product of the DHDPS-catalysed reaction [51]. Until recently, the most successful of these compounds were planar and heterocyclic, but these were not effective at sub-millimolar concentrations [49]. With the identification of HTPA as the DHDPS product, various heterocycles with oxygen functionality have been tested [49]. However, these compounds were only marginally more effective at inhibiting DHDPS than the first generation substrate analogues [49]. Recently, rational inhibitor design approaches have been employed to demonstrate that α-ketopimelic acid, a structural analogue of pyruvate, binds the active site of *Mycobacterium tuberculosis* DHDPS and partially inhibits the enzyme with micromolar potency [52].

Additionally, a bislysine analogue that binds to the allosteric site of *Campylobacter jejuni* DHDPS has recently been described with sub-μM potency [50].

While less explored than DHDPS, the subsequent enzyme DHDPR also presents as a promising antibiotic target due to its essentiality to bacteria and absence in humans [53]. Several inhibitors have been designed to target both the substrate and the cofactor binding sites simultaneously [54]. One such example is 2, 6-pyridinedicarboxylic acid (2,6-PDC), with a K_i of 0.9 mM against *E. coli* DHDPR [51]. A molecular modelling approach has also been employed to identify several sulphonamides that inhibit DHDPR, with the most potent of these having IC_{50} values ranging from 10 to 90 μM [53]. These inhibitors provide a platform for future inhibitor development against DHDPR.

As DHDPS and DHDPR are positioned above the junction of the multiple sub-pathways employed in all bacteria to produce meso-DAP, they remain the focus for new broad spectrum antimicrobial development. However, despite extensive research into the structural and functional characterisation of these enzymes, there have been no published DAP pathway inhibitors with antibacterial activity to date.

Conclusion

In this review, we have summarised how peptidoglycan synthesis can be targeted upstream of the crosslinking of the glycan strands by examining the precursors and their components. While over 3 decades of research has focussed on inhibiting the Mur class of enzymes and their role in the addition of amino acids onto the peptide stem, there are still promising under-explored avenues for antibiotic discovery targeting peptidoglycan synthesis. Exciting new research is emerging, including the development of the bacterial whole cell vaccine against the MurI enzyme as well as lysine biosynthesis inhibitors, which further validate the importance of targeting pathways not directly involved in peptidoglycan synthesis. Further exploration of these areas could yield new classes of antibiotics, which are urgently needed to negate the steady rise in antibiotic resistance worldwide.

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