Review

Glutamate racemase as a target for drug discovery

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Summary
The bacterial cell wall is a highly cross-linked polymeric structure consisting of repeating peptidoglycan units, each of which contains a novel pentapeptide substitution which is cross-linked through transpeptidation. The incorporation of D-glutamate as the second residue is strictly conserved across the bacterial kingdom. Glutamate racemase, a member of the cofactor-independent, two-thiol-based family of amino acid racemases, has been implicated in the production and maintenance of sufficient D-glutamate pool levels required for growth. The subject of over four decades of research, it is now evident that the enzyme is conserved and essential for growth across the bacterial kingdom and has a conserved overall topology and active site architecture; however, several different mechanisms of regulation have been observed. These traits have recently been targeted in the discovery of both narrow and broad spectrum inhibitors. This review outlines the biological history of this enzyme, the recent biochemical and structural characterization of isozymes from a wide range of species and developments in the identification of inhibitors that target the enzyme as possible therapeutic agents.

Introduction
The use of antibiotics to treat microbial infectious diseases represents one of the most important advances in modern medicine. Remarkably, the current major classes of antimicrobial agents target only four cellular processes: cell wall biosynthesis, protein synthesis, DNA replication and repair and folate coenzyme-dependent thymidine biosynthesis (Walsh, 2003). Within this small set of targets, it can be argued that cell wall biosynthesis has achieved the most extensive clinical utility as inhibitors to this pathway comprise more than 60% of the total antibacterial market, now estimated to be worth more than 25 billion dollars. Recently, the progress of developing agents against the early phases of peptidoglycan biosynthesis has been the subject of a number of reviews (van Heijenoort, 2001; Katz and Caufield, 2003; Silver, 2006; Kotnik et al., 2007). The aim of this report is to review the biology and drug discovery potential of glutamate racemase (MurI), an enzyme involved in the early phases of peptidoglycan biosynthesis.

The bacterial cell wall is a highly cross-linked polymeric structure consisting of repeating peptidoglycan units of disaccharides (joined in β1-4 linkage) which contain a novel pentapeptide substitution (for review see van Heijenoort, 2001). Cross-linking occurs through transpeptidation of the peptide linkages between adjacent glycan strands resulting a structural mesh that acts as a cellular skeleton that protects the cell from rupture due to the osmotic pressure gradient. Inhibition of cell wall production renders the bacteria susceptible to lysis by osmotic pressure and inhibitors that target this pathway are generally cidal.

Peptidoglycan biosynthesis is classified into three distinct phases based on the cellular location of the synthetic machinery. The majority of the clinical success has been achieved through inhibition of the Phase III segment, which involves the extracellular cross-linking and final maturation of the cellular envelope. While discovery of improved agents that target this phase remains a dominant area of research and development, the burden of resistance continues to rise and limit the therapeutic utility of both the existing and future compounds within these classes. Further, the emergence of multi-drug-resistant bacteria, such as methicillin-resistant Staphylococcus aureus, MDR-Pseudomonas aeruginosa and MDR-Acinetobacter baumannii, has resulted in significantly increased mortality rates with limited or no options for therapeutic intervention (Klevens et al., 2007; Perez et al., 2007). This crisis has resulted in a call for the discovery of drugs that have a novel mode of action (Spelberg et al., 2004).
Glutamic acid racemase: Biology of glutamate racemase

The role of glutamate racemase in peptidoglycan biosynthesis was not always so clear. Pioneering work in Lactobacillus arabinosus suggested the presence of glutamate racemase activity in cellular extracts (Ayengar and Roberts, 1952; Narrod and Wood, 1952) and implicated its role in supporting growth when D-glutamate was substituted for L-glutamate in the growth medium. However, the primary route for D-glutamate production was hypothesized to involve D-amino acid transaminases (D-AAT). This was supported by the observation that extracts from Bacillus subtilis produced significant amounts of D-glutamate via D-AAT coupling to D-alanine pools produced by alanine racemase (Thorne et al., 1955). While the generic mechanism across the bacterial kingdom for D-glutamate production remained unresolved, the purification and biochemical characterization of glutamate racemase from L. arabinosus (Glaser, 1960) and Lactobacillus fermenti (Tanaka et al., 1961) provided direct evidence of glutamate racemase enzyme activity in lactic acid bacteria. The biochemical studies conflicted on the cofactor dependence of the enzyme, initially characterizing it as pyridoxal phosphate-dependent (Glaser, 1960) then as a flavoprotein (Tanaka et al., 1961). Both of these assertions were proven inaccurate upon further purification of the L. fermenti enzyme, which demonstrated that the enzyme required no cofactors for catalytic activity (Diven, 1969). Additional biochemical studies with the enzyme derived from Pediococcus pentosaceus confirmed that the enzyme was a member of the cofactor-independent family of racemases (Nakajima et al., 1986); however, cellular extracts across a range of species were found to be devoid of glutamate racemase activity, suggesting that the enzyme was exclusive to lactic acid bacteria (Nakajima et al., 1988) and that the general mechanism for D-glutamate production was through D-amino acid aminotransferase.

These conclusions were challenged through a series of elegant genetic studies in Escherichia coli. Using the mutant strain WM355 (Hoffman et al., 1972), which requires D-glutamate for growth, glutamate racemase activity was unambiguously assigned to an open reading frame encoding a protein of 289 amino acids in the 90 min region of the chromosome, near the murA and murB genes, but quite distant from the remaining Phase I peptidoglycan biosynthetic genes (Doublet et al., 1992). Disruption of this gene, denoted murI using the nomenclature adopted for the peptidoglycan biosynthetic pathway, was found to alter the peptidoglycan precursor pool distribution and ultimately lead to cellular lysis (Doublet et al., 1993). Subsequent studies confirmed the essentiality of mutI for cellular growth through more detailed genetic dissection of the WM355 mutant strain (Dougherty et al., 1993) and plasmid-based complementation of mutI.
Phase I peptidoglycan biosynthesis corresponds to the intracellular steps of the overall pathway and therefore glutamate racemase resides in the cytoplasm of the cell. While the cellular localization of the enzyme is well established, relatively little is known about the transcriptional regulation of the glutamate racemase genes. The most detailed work in this area has been performed in *B. subtilis* in an effort to resolve the functions of the two homologues. Expression levels of were measured using direct measurements of RNA or indirectly using reporter genes fused to the chromosomal promoter regions of each gene. The racE/glr gene was expressed in both rich and minimal media whereas yprC expression was only observed in minimal media (Kimura et al., 2004). Further, a separate report demonstrated that the racE/glr gene was actively transcribed during exponential growth and continuously at a low level in stationary phase but the yprC gene was expressed at a very low level throughout growth (Kada et al., 2004). Along these lines, the regulation of the *E. coli* murl gene has been studied using the d-glutamate auxotroph strain WM355. In this case, the auxotrophy was complemented with plasmids carrying either *E. coli* murl or the *Staphylococcus haemolyticus* D-amino acid amidotransferase gene under the control of the native *E. coli* murl promoter; however, the levels of D-glutamate and enzyme activity (Murl and D-AAT) were below detection (Liu et al., 1998). Based on these results, the authors postulated that growth is dependent on a threshold glutamate concentration and that transcriptional regulation may be required to sustain growth at specific occasions in the growth cycle. Finally, while there are caveats with interpreting negative results from gene expression profiling studies, no differences in glutamate racemase gene transcription were noted in *Streptococcus pneumoniae* in response to exposure to penicillin (Rogers et al., 2007), sub-lethal concentrations of translation inhibitors (Ng et al., 2003) or changes in growth temperatures (Pandya et al., 2005). Similarly, no differences in *Helicobacter pylori* murl expression were observed upon growth-phase transition (Thompson et al., 2003). Finally, differential expression of the *E. coli* murl gene was not detected when cultures were exposed to either bacteriostatic or bactericidal antibiotics (Kohanski et al., 2007) or upon colonization in a murine infection model (Motley et al., 2004). Given the importance of maintaining adequate levels of both enantiomers of glutamate for cellular survival (Mengin-Lecreulx et al., 1982), additional, more definitive studies of glutamate racemase expression are warranted.

**Biochemical and structural characterization**

To date, glutamate racemase enzymes have been biochemically characterized from a wide range of species.
including *B. subtilis* (Ashiuchi et al., 1998; 1999; Taal et al., 2004), *B. anthracis* (Dodd et al., 2007; May et al., 2007), *Bacillus pumilus* (Liu et al., 1997), *Bacillus subtilis* (Fotheringham et al., 1998), *L. fermenti* (Gallo and Knowles, 1993), *Lactobacillus brevis* (Yagasaki et al., 1995), *P. pentosaceus* (Nakajima et al., 1986), *Aquilax pyrophilus* (Kim et al., 1999), *S. haemolyticus* (Pucci et al., 1995), *Brevibacterium lactofermentum* (Malathi et al., 1999), *E. coli* (Yoshimura et al., 1993), *M. tuberculosis* (Sengupta et al., 2006), *M. smegmatis* (Sengupta and Nagaraja, 2008), *Streptococcus pyogenes* (Kim et al., 2007), *S. pneumoniae* (de Dios et al., 2002), *S. aureus*, *Enterococcus faecium*, *Enterococcus faecalis* and *H. pylori* (Lundqvist et al., 2007). On a technical note, several reports have observed that coexpression of chaperone proteins, such as GroEL/GroES, can significantly enhance the overproduction of soluble, active enzyme (Ashiuchi et al., 1995; Kohda et al., 2002; Lundqvist et al., 2007). A wide range of assays for measuring enzyme activity have been reported, including continuous spectrophotometric assays using polarimetry (Schönfeld and Bornscheuer, 2004), circular dichroism (Tanner et al., 1993; May et al., 2007), detection of derivatized products following HPLC separation (de Dios et al., 2002) or direct spectrophotometric detection of product after chiral HPLC separation (Kim et al., 2007). However, the most common methods involve coupled enzyme systems measuring either L-glutamate (e.g. Nakajima et al., 1986; Gallo and Knowles, 1993) or D-glutamate production (Lundqvist et al., 2007). The most popular assay format – from both cost and convenience perspectives – is the use of L-glutamate dehydrogenase as a coupled enzyme to L-glutamate production where catalysis is measured spectrophotometrically as a function of NADH formation during the conversion of L-glutamate to α-ketoglutarate and ammonia. From a biochemical perspective, a number of general traits emerge upon review of the enzymes studied to date. First, pH titrations of enzymatic activity indicate that optimal enzymatic activity is achieved in pH ranging from 6.0 to 8.0 and that catalysis requires two basic centres. Second, the enzymatic activity requires reducing agents in the buffer to maintain the cysteine side-chains as free thiols. Thiol-modification agents, such as 2-nitrophenylthiobenzoic acid (Choi et al., 1992), result in rapid and complete inactivation of the enzyme. Third, in contrast to most other racemase and epimerase enzymes, no additional cofactors – such as pyridoxyl phosphate or pyruvate – are required for catalytic activity. All of these observations are consistent with the classification of glutamate racemase within the cofactor-independent, two thiol-based family of racemases. Finally, the enzyme has an extremely specific substrate preference for glutamate, as even structurally related amino acids, including aspartic acid, α-aminobutyric acid or α-methylglutamic acid, are neither substrates nor inhibitors of the enzyme.

Given the history of glutamate racemase biology, it is not surprising that efforts to elucidate the catalytic mechanism were initiated on enzymes isolated from *L. fermenti* and *Pediococcus* spp. The enzymes from these species have similar kinetic profiles (*K_m, k_cat*) for D- and L-glutamate (symmetrical processing). Studies using isotopically labelled substrates, or reactions performed in D_2O, confirmed that the enzyme utilizes a two-base mechanism for catalysis (Choi et al., 1992; Tanner et al., 1993), wherein one base acts to abstract a proton from the substrate to form an enolate-stabilized transition state while the second base donates a proton on the opposite face to form the product (for review see Tanner, 2002, see Fig 2). These studies suggested that two forms of the enzyme exist during catalysis, perhaps differing in the protonation state of the active site bases; however, the absence of substrate inhibition for either substrate indicated that the two enzyme forms rapidly equilibrate under physiological conditions (Fisher et al., 1986). Two conserved cysteines (Cys73, Cys184 L. *fermenti* numbering) were identified through analyses of primary sequence conservation across a number of species (Gallo and Knowles, 1993), and recent comprehensive phylogenetic analyses of glutamate racemase genes from over wide range of species confirm that these residues are strictly conserved across the bacterial kingdom (Hwang et al., 1999; Kim et al., 1999; Lundqvist et al., 2007; May et al., 2007). Mutation of these residues to alanine abolished activity and replacement with serine resulted in a ~1000-fold drop in activity, thereby supporting the hypothesis that residues act as the catalytic bases (Gallo et al., 1993; Glavas and Tanner, 1999).

The similarities in biochemical properties and primary sequence conservation suggest that the glutamate racemase enzymes share an active site architecture and common biochemical catalytic mechanism. This hypothesis has been supported by a plethora of recent high-resolution structural data from a wide range of species including *A. pyrophilus* (Hwang et al., 1999), *B. subtilis* (Ruzheinikov et al., 2005), *B. anthracis* (both isozymes, May et al., 2007), *E. coli*, *S. aureus*, *E. faecium* and *E. faecalis*, *H. pylori* (Lundqvist et al., 2007) and *S. pyogenes* (Kim et al., 2007). It is evident from these reports that the glutamate racemase enzymes share a conserved topology and fold. The enzyme is comprised of two domains that are nearly symmetric in structure and which are joined by a two-stranded hinge region (see Fig 3). A number of these structures contain substrate bound at the active site, thereby providing a basis for the unambiguous assignment of the active site region and hypotheses regarding the functional roles of participating residues.
The active site is formed at the interface of these two domains, with each domain contributing the catalytic machinery required for enantiomeric proton abstraction/donation. The N-terminal domain contains the catalytic base and conserved residues involved in deprotonation of D-glutamate (D7, S8, C70, T72 H. pylori numbering, see Fig. 2) while the C-terminal domain encodes the residues involved in L-glutamate deprotonation (E150, C181, T182, H183, see Fig. 2). These residues, along with other conserved amino acids in the active site, have been shown to be essential for activity by site-directed mutagenesis (Glavas and Tanner, 1999; 2001).

Analyses of the active site indicate that the cavity is relatively hydrophobic, buried from solvent with well-defined hydrogen bond networks. For example, the substrate carboxylate group is positioned in a ‘threonine pocket’ – a highly ordered hydrogen-bonding network formed by the conserved residue side-chains of T72, T116 and T182 (see Fig. 2), water, and main chain interactions (May et al., 2007). These features, along with similar strong hydrogen-bonding networks at the substrate amino group, have been rationalized in the context of the difficult and remarkable chemical transformations performed by the enzyme given the solution state pKa values of cysteine (pKa ~ 10) and the α-carbon of glutamate (fully protonated form, pKa ~ 21). Computational and physical chemistry measurements suggest the hydrophobic environment, the hydrogen-bonding networks to form and stabilize the amino-protonated, main chain cationic form of substrate, and the soft ionization energy of the thiolate base work in concert to minimize both the penalty of desolvation of the substrate upon binding and the large pKa differential between the substrate and enzyme at the transition state (Rios et al., 2000; 2001; 2002; Puig et al., 2005; 2006; 2007). Additional studies have implicated dynamics as a key element in overcoming the activation barrier for catalysis (Möbitz and Bruice, 2004).

Several unique contributions to the active site composition are observed in the A. pyrophilus glutamate racemase structure (Hwang et al., 1999). While it contains a
similar overall topology and fold, this enzyme forms a homodimer where the active sites are placed in close proximity (head-to-head dimer). As a result, the active site is formed from both inter-domain and inter-monomer interactions. Most notably, the side-chain of residue E147 (Apyrophilus numbering) makes no contribution to the intra-monomer active site, but is fully inserted in the trans-dimer active site. The physiological relevance of this structural form of the enzyme has been debated and remains controversial because this interaction, along with other active site trans-dimer interactions, are not observed in any of the other structures solved to date (Ruzheinikov et al., 2005; Lundqvist et al., 2007; May et al., 2007; Puig et al., 2007). Putting aside the unique Apyrophilus interactions, the glutamate racemase enzymes share highly conserved active site architectures and overall fold across the bacterial spectrum that provides both exquisite substrate specificity and remarkable catalytic potential.

Despite these general structural similarities, a number of important biochemical differences have been identified in the enzymes isolated across the bacterial kingdom. For example, the glutamate racemases from Gram-positive organisms vary in their oligomeric state in solution. While all of the structures solved for this group of enzymes were homodimeric, which is consistent with the solution studies performed on the Apyrophilus, S. pyogenes, S. aureus, E. faecalis, E. faecium and the B. anthracis RacE2 enzymes, the enzymes from S. pneumoniae (de Dios et al., 2002), L. fermenti (Gallo et al., 1993), B. subtilis (RacE, Taal et al., 2004) and B. anthracis (RacE1, Dodd et al., 2007; May et al., 2007) are monomeric in the absence of substrate and, at least in the case of the enzymes from Bacillus spp., form dimers at saturating substrate levels. The dimerization interface of the Gram-positive enzymes structurally solved to date occurs across a C2 symmetry axis using interactions from both domains of the monomer in the hinge region (see Fig. 3). This mode of dimerization places the active sites at opposite poles of the dimer, fully exposed to solvent (tail-to-tail). Elegant work on the B. anthracis enzymes RacE1...
and RacE2, which show differing propensity to form dimers in solution but exhibit nearly identical substrate-bound crystallographic structures, suggests that the dimerization interactions are finely tuned (May et al., 2007). In particular, it was noted that residue R214 in the RacE2 protein (homodimeric) forms a hydrogen bond network with P99, T103 and E215. In contrast, the RacE1 enzyme, which is a monomer in the absence of substrate, encodes an isoleucine at this position (I217), thereby eliminating a key hydrogen bond between the two monomers. Mutation of the R214 residue to alanine was found to be sufficient to convert the RacE2 protein to a monomer in solution in the absence of substrate. These studies, along with analyses of the range of hinge motion exhibited in the crystal structures (see Fig. 3), suggest that dimerization in these species requires a balance between stabilization of the enzyme while retaining sufficient flexibility required for catalysis.

Another example of biochemical diversity among the glutamate racemase family is regulation of the E. coli enzyme activity by UDP-MurNac-Ala, the peptidoglycan intermediate produced by MurC and co-substrate with D-glutamate for MurD (see Fig. 1, Doublet et al., 1994; Ho et al., 1995). In the presence of this metabolic intermediate, the E. coli enzyme was found to be activated more than ~500-fold. The activation can be attributed to effects on both $K_M$ (approximately fivefold) and $k_{cat}$ (~100-fold) and early mutagenesis work indicated that the N-terminal region of the protein was involved in the activation mechanism (Doublet et al., 1996). The protein exists as a monomer in the unit cell of the crystal structure and this is consistent with biophysical measurements in the solution state (Doublet et al., 1994; Lundqvist et al., 2007). It is possible to rationalize the regulation mechanism from the crystal structure of the enzyme, which was solved as a complex with substrate and the activator. The activator binds at the hinge region in a pocket that is not confined to the N-terminus of the enzyme, but contacts in this region are critical for activation (see Fig. 3). As a result, the activator is presumed to both stabilize and focus the dynamics of the protein, and therefore increase the population of functionally productive enzyme states. This control mechanism is consistent with physiological measurements of L-glutamate levels which are substantially higher than D-glutamate in growing cells; activation of the enzyme as a function of peptidoglycan biosynthesis (UDP-MurNac-Ala production) allows for 'just-in-time' delivery of D-glutamate, while preserving high levels of L-glutamate pools for central metabolic processes (Mengin-Leclercq et al., 1982).

Finally, the H. pylori enzyme exhibits unique kinetic and structural features that are distinct from the other species studied to date. In this case, the enzyme exhibits a high degree of asymmetry in substrate processing, with a $K_M$ and $k_{cat}$ ~10-fold lower for D-glutamate than for L-glutamate (Lundqvist et al., 2007). In addition, severe substrate inhibition is observed with D-glutamate only. These observations suggest that, unlike the other species studied to date, the enzyme does not rapidly equilibrate between forms at steady state, but exists in an oversaturated state (Fisher et al., 1986). As a result of these features, the D-glutamate-bound form of the enzyme is the dominant enzymatic species at steady state and, from a physiological perspective, these factors are predicted to constitute another mechanism for regulation of L-glutamate levels as the D-glutamate production is expected to be tightly kinetically controlled. The crystal structure of the enzyme reveals additional areas of distinction. The enzyme forms a homodimer, consistent with studies in the solution state, but in this case the active sites are in close proximity and shielded from solvent by the dimerization interface (head-to-head dimer, see Fig. 3) thereby severely restricting substrate access to the active site. As expected from the kinetic profile, the structure contains exclusively D-glutamate and may represent a fully closed form of the enzyme. The dimerization interface contains contacts from both domains; however, contributions of the C-terminal domain are more extensive and are presumed to remain intact during the catalytic cycle.

In summary, the biochemical and structural features exhibited by these enzymes offer potential for discovery of both broad spectrum agents, through the judicious use of conserved features of the enzymes, and narrow spectrum agents through the exploitation – either by design or serendipitously – of the structural and biochemical differences observed across the bacterial kingdom.

### Mechanism and substrate-based inhibitors

The earliest reports of inhibitors targeted to glutamate racemase were based on mechanism-based designs to afford covalent modification of the enzyme. Several of these, L-serine-O-sulfate (LSOS, see Fig. 4, analogue 1, Ashiuchi et al., 1993), and D-N-hydroxyglutamate 2 (Glavas and Tanner, 1997), act as alternate substrates for the enzyme. In each case, the enzyme abstracts the $\alpha$-carbon proton of the inhibitor, resulting in an elimination process to form a reactive intermediate during the catalytic cycle. In the case of LSOS, sulfate is released to form aminoacrylate whereas in the case of N-hydroxyglutamate, elimination of water results in a glutamate-imine intermediate. Both of these reactive intermediates are subject to attack by the enzyme thiolate bases to form covalent enzyme : inhibitor complexes, but as the latter analogue resembles the proposed transition state of the enzyme it is possible that the inhibition can occur through non-covalent binding. In either case, the
intermediates are susceptible to hydrolysis to yield pyruvate in the case of LSOS or α-ketoglutarate. This approach has a number of merits in that the inhibitors are very selective for the enzyme and the inhibition is covalent and potentially irreversible; however, success is dependent on efficient partitioning of the intermediate to inhibition rather than turnover (hydrolysis). Based on this latter criterion, these inhibitors are relatively inefficient as the inhibitors are effective substrates of the enzyme when measured as a function of ketone formation.

The preparation of aziridino-glutamate (see Fig. 4, Tanner and Miao, 1994) represents another reactive substrate-based inhibitor strategy, although in this case the inhibitor is inherently reactive and does not require conversion by the enzyme. In this case, the active site thiols attack the aziridine ring to form a covalent adduct. While the inhibition is rapid, complete and irreversible, this approach suffers from the inherent chemical instability of the inhibitor.

The design and optimization of a series of substituted glutamate analogues represented a major breakthrough in the development of non-covalent inhibitors (de Dios et al., 2002). This series of compounds, as exemplified by analogues 4–7 (see Fig. 4), were based on D-glutamate, in part to avoid potential off-target biological effects associated with L-glutamate. Despite the strict substrate selectivity of the enzyme, potent inhibitors were identified that featured large, hydrophobic substitutions – particularly aryl-, heteroaryl-, cinnamyl- or biaryl-methyl – at the 4-position. It was noted that aryl groups featuring an anti-C4 substitution (2R, 4S) were preferred over alkyl or syn substitution (2R, 4R) and that extended or branched substitutions were not generally tolerated. This structure–activity relationship (SAR) led the investigators to infer that the substrate binding site encoded a large, linear hydrophobic pocket in the S. pneumoniae enzyme. Importantly, many of the analogues were found to inhibit S. pneumoniae growth and a good correlation between enzyme inhibition and whole-cell growth inhibition was generally observed. Finally, several of the top analogues (Fig. 4, analogues 4–6) demonstrated efficacy in a murine thigh S. pneumoniae infection model; all three compounds suppressed bacterial growth when administered intraperitoneally (40 mg kg⁻¹). A key drawback with these compounds was the lack of broad spectrum activity as the whole-cell activity was limited to S. pneumoniae strains. The rationale for the narrow spectrum of whole-cell activity remained unclear until recently when a structural basis for the selectivity was proposed using the crystal structures of the B. subtilis (Ruzheinikov et al., 2005), B. anthracis (May et al., 2007) and a co-crystal structure of the S. pyogenes (Kim et al., 2007) enzyme with analogue 7. In all of these structures, the inhibitor is predicted to bind in the active site region and utilizing a well-defined hydrophobic pocket that is formed by the side-chains of residues P41, P44, T118, G117, E119, P146, V149, P150 and Y188 (B. subtilis RacE numbering). Analysis of the primary sequence alignments indicates that a key

![Fig. 4. Substrate-based inhibitors. Selected biological data are shown; refer to the citations for experimental details and additional data. IC₅₀ values correspond to the concentration of inhibitor required for 50% inhibition of the enzyme activity; MIC values correspond to the minimum concentration of inhibitor required for complete inhibition of growth. Denoted species reflect the source of enzyme (IC₅₀) or organism (MIC).](image-url)
residue, V149, is an alanine in the *S. pneumoniae* enzyme (*S. pneumoniae*, A148, see V146 in Fig. 2A for context). This residue resides at the entrance of the hydrophobic pocket and the close approach of the branched side-chain of valine partially blocks access to the site. Most species encode valine at this position, including *S. aureus* and *B. subtilis*, and therefore would be expected to be resistant to these inhibitors. In further support of this hypothesis, V149A mutants of the *B. anthracis* RacE2 enzyme were prepared and tested for inhibition by members of this series (May *et al.*, 2007). While the wild-type enzyme exhibited weak inhibition by these analogues, potent inhibition was observed with the mutant enzyme. Considering all of this evidence together, the limited spectrum activity of the series is most likely due to subtle differences in the active site; however, differences in permeability across species or alternative pathways for D-glutamate production (e.g. D-amino acid aminotransferase pathway) may play a role as well.

**Allosteric inhibitors**

The recent report of selective, allosteric inhibitors of *H. pylori* glutamate racemase highlighted the structural and biochemical differences observed among bacterial species (Lundqvist *et al.*, 2007). This series of inhibitors, which are based on derivatization of a pyrazolopyrimidinedione core scaffold as exemplified by analogues 8–12 (see Fig. 5), were identified through a high-throughput screening campaign of *H. pylori* enzyme activity. The series exhibits time-independent, reversible, stoichiometric inhibition of the enzyme, but remarkably, substrate binding to the enzyme is required for inhibition. The requirement for substrate binding for inhibition (uncompetitive inhibition) is exceedingly rare for a single substrate enzyme and suggests that the inhibitor binds to a site that is distinct from the enzyme active site. Structural studies, using both NMR and X-ray crystallography, on the enzyme with the inhibitor confirmed the inhibitor binds to a cryptic site on the enzyme ~9 Å away from the catalytic centre that is primarily formed by a dislocation of the C-terminal helix (see Fig. 6). Overlays with the native enzyme structure indicate that the inhibitor-bound structure is highly similar and, importantly, no changes were observed in the active site. Upon dislocation of the C-terminal helix, the inhibitor binding site is formed by a displacement and rotation of the W252 side-chain; the pyrazolopyrimidinedione core forms a pi-stacking interaction with the W252 indole ring and the biaryl ring system (naphthyl ring in analogue 8) fills the pocket vacated by the W252 ring movement (see fig. 3 in Lundqvist *et al.*, 2007).

Optimization of the series was performed using both iterative structure-based design and medicinal chemistry.

![Image of inhibitors](https://example.com/inhibitors.png)

**Fig. 5.** Allosteric inhibitors of *Helicobacter pylori* glutamate racemase. Selected data are shown; refer to the citations for experimental details and additional data. See Fig. 4 for definition of terms.

| Biological Assay | Analog | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 |
|------------------|--------|---|---|---|---|---|---|---|---|
| *H. pylori* IC50 (µM) | 1.4 | 0.025 | 0.028 | 0.049 | 0.22 | 1.7 | 2.0 | 1.2 |
| *H. pylori* MIC (µg/mL) | 4.0 | 0.5 | 2.0 | 1.0 | 32 | 0.5 | 0.25 | 1.0 |

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strategies and very potent analogues were identified with excellent whole-cell activity against H. pylori (Gowravaram et al., 2005). Physiological studies on representative compounds confirmed that the primary mode of action was through inhibition of glutamate racemase (de Jonge et al., 2005). The series demonstrated exquisite selectivity for H. pylori as no activity was observed in panels of in vitro glutamate racemase enzyme or whole-cell growth inhibition assays covering a broad range of species. The narrow spectrum of these agents may be an advantage in this case, as selective, monotherapy treatments for diseases attributed to H. pylori infection could address the problems of existing therapies which suffer from poor patient compliance due to side-effects resulting from broad spectrum treatments and an increasing burden of resistance (Egan et al., 2007). The selectivity profile was rationalized from a structural perspective as the crystal structures of the E. coli enzyme and those derived from Gram-positive organisms (B. subtilis, S. aureus, E. faecalis, E. faecium) all contain a C-terminal extension that forms additional contacts that are predicted to limit the C-terminal helix movement and in some cases, critical residues for binding (including W252) are not conserved.

The potential for resistance was probed using optimized analogues (e.g. 9) and the rates for generating single-step mutants were found to be low (resistance rate \(< 10^{-9}\), Lundqvist et al., 2007). A set of mutants was characterized and all were found to map to the glutamate racemase gene, but the mutations were dispersed throughout the primary sequence of the protein. Two mutants were characterized biochemically and when compared with the wild-type enzyme, both were found to have profound changes in their substrate-processing kinetic profile despite the fact that the mutations were not located in the active site. In both cases the enzymes had elevated \(K_M\) values and the substrate inhibition by D-glutamate was either significantly reduced (A75T) or absent (E151K). Based on these results, it was hypothesized that the net effect of these mutations is to lower the population of the substrate-bound form of the enzyme – the target of these uncompetitive inhibitors – under physiological conditions while maintaining activity required for growth.

The physical properties of the inhibitor scaffold represented the primary drawback to these inhibitors. Despite extensive efforts to optimize the in vivo clearance, oral bioavailability and protein binding while maintaining or improving the microbiological potency, it proved difficult to identify analogues with all of these features combined (Basarab et al., 2005). Potent analogues were identified with good in vivo clearance, oral bioavailability and low susceptibility to bacterial efflux pump transport (analogues 10, 11), but improved microbiological potency was correlated with increased lipophilicity, an attribute associated with higher protein binding and lower solubility. As a result, the lack of efficacy in a murine mouse model of H. pylori colonization was attributed to insufficient free drug plasma concentrations observed with optimized analogues.

A second series of allosteric inhibitors targeted at the H. pylori enzyme has been reported. This series features a benzodiazepine core scaffold, as exemplified by analogues 13–15 (see Keating et al., 2006, Fig. 5). The series exhibited similar traits to the pyrazolopyrimidinedione series in that they were identified through high-throughput screening of H. pylori enzyme activity, they require substrate binding for inhibition and are selective inhibitors of the H. pylori enzyme. However, X-ray crystallography studies demonstrated that these inhibitors bind at a site that is clearly distinct and unaffected by the pyrazolopyrimidinedione binding site, as a crystal structure containing the enzyme:substrate:inhibitor complex showed both inhibitor classes binding at full occupancy (see Fig. 6). This novel binding site is formed at the dimer interface, along the dimer interface symmetry axis. Binding to the enzyme was determined to be chiral with an approximately sevenfold preference for the R-enantiomer, although most analogues were prepared and tested as the racemate. Unlike the pyrazolopyrimidinediones, this series was largely unaffected by efflux pump expression and as a result, excellent whole-cell growth inhibition was observed for relatively weak enzyme inhibitors. Physiological studies confirmed that growth inhibition was a function of glutamate racemase inhibition in the cell and that the series was cidal with kinetics similar to \(\beta\)-lactam antibiotics (e.g. amoxicillin; de Jonge and Kutschke, 2008).
These studies also identified moderate spontaneous rates of resistance as a potential risk for the series (resistance rates \(< 10^{-7}\)). In contrast to the pyrazolopyrimidinedione series, optimized benzodiazepine analogues generally exhibited improved physical properties. Excellent protein binding, solubility and overall lipophilicity and molecular weight were hallmarks of the series (e.g. analogues 14, 15; Geng et al., 2006). Despite this profile, attempts to demonstrate efficacy in a murine \(H. pylori\) colonization model were unsuccessful and this lack of activity was attributed to insufficient free plasma levels of the drug due to high intrinsic clearance in mice (Newman et al., 2006). Comparative studies were performed using amoxicillin to establish the pharmacodynamic requirements of cell wall biosynthesis inhibitors for efficacy in this model. The pharmacodynamic index for amoxicillin was determined to be time above minimum inhibitory concentration (MIC), with a long duration requirement (T \(> 23\) h) over 4 days of dosing. As a result, extended dosing schemes were attempted with the benzodiazepine analogues and increased exposure was achieved on the first day of the dosing when the compounds were co-administered with a general P450 inhibitor (e.g. aminobenzotriazine); however, progressively lower and insufficient drug levels were observed over the course of the 4-day dosing regime required by the animal model.

Recently, two series of allosteric inhibitors have been reported that have activity against Gram-positive organisms. The first series features a purine scaffold (Fig. 7, analogues 16–18; Geng et al., 2007) and was identified using a high-throughput screen of \(E. faecalis\) MurI enzyme activity. Early analogues in this series exhibited good potency against the enzymes from \(E. coli\) spp. and crystallographic studies with the \(E. faecalis\) enzyme revealed that the compounds bind to a site that partially overlaps with the region corresponding to the \(H. pylori\) pyrazolopyrimidinedione binding site. Importantly, both series of inhibitors utilize a displacement of the C-terminal tryptophan (W252 in \(H. pylori\); W254, \(E. faecalis\)) for binding; however, the movement of this residue, and the C-terminal helix as a whole, is less extensive in formation of the purine binding pocket relative to that observed for the pyrazolopyrimidinedione binding site. Analysis of the \textit{in vitro} inhibition structure activity relation-

\[
\begin{array}{c|cccc}
\text{Biological Assay} & \text{Analog} \\
\hline
\text{E. faecalis IC50 (µM)} & 16 & 17 & 18 & 19 & 20 \\
\text{E. faecium IC50 (µM)} & 18 & 2.7 & 5.5 \\
\text{S. aureus IC50 (µM)} & >400 & >400 & >400 & 8 & 1.1 \\
\end{array}
\]

Fig. 7. Allosteric inhibitors of Gram-positive glutamate racemase enzymes. Selected data are shown; refer to the citations for experimental details and additional data. See Fig. 4 for definition of terms.
ships suggests that hydrophobic substitutions at the 2- and 9-positions of the purine ring are required for binding, whereas the 6-position is positioned near the solvent front and hydrophilic substitutions are tolerated. Despite efforts to improve the analogues through iterative structure-based drug design strategies, only modest improvements in potency were observed for the Enterococcus spp and no activity was observed against the S. aureus enzyme. Further, analogues in the series exhibited relatively poor solubility and only moderate antibacterial activity (MIC = 16–64 μg ml\(^{-1}\)).

Subsequent focused screening efforts based on the purine series scaffold lead to the identification of a pteridine-based series (Fig. 7, analogues 19, 20, Breault et al., 2007). This series exhibits expanded spectrum activity with most analogues exhibiting equipotent inhibition against the E. faecalis and S. aureus enzymes. Lead optimization efforts in this series resulted in analogues with moderate whole-cell growth inhibition across a range of Gram-positive organisms including S. pneumoniae, S. aureus and E. faecalis (MIC = 4–8 μg ml\(^{-1}\)). While crystal structures of the enzyme : substrate : inhibitor complexes were not solved for this series, overlapping SAR with the purine series and molecular modelling studies indicate that the pteridine series binds in the purine binding site. The expanded spectrum exhibited in this series represents a breakthrough in the development of broad spectrum small molecule inhibitors, but this series generally exhibits poor equilibrium solubility. Attempts to improve the physical properties of these inhibitors through the incorporation of polar substitutions were moderately successful.

**Large molecule inhibitors**

Aside from small-molecule inhibitors, there are two reports of large-molecule (molecule weight > 650) inhibitors. The first report identified haemin as a moderately potent inhibitor of the P. pentosaceus enzyme (Choi et al., 1994). The authors noted that, based on the primary sequence, glutamate racemase shares a high level of similarity with mammalian myoglobins. Upon incubation with the enzyme, haemin formed a stoichiometric complex that completely inactivated the protein. The inhibition was found to be non-competitive (\(K_I = 3.7 \mu M\)) and the complex was stable to gel filtration chromatography. Importantly, complex formation was found to be specific to glutamate racemase as similar experiments with the mechanistically related enzyme aspartate racemase did not yield appreciable complex formation or inhibition of the enzyme. Spectroscopic studies indicated that the haem environment in the haemin : glutamate racemase complex resembled that of haemoglobin under similar conditions. The authors concluded that the haemin inhibits glutamate racemase either by binding at the active site, or by binding at a distal site and inducing a conformational change.

A series of peptide ligands identified through phage display library panning comprise the second report of large molecule inhibitors of glutamate racemase (Kim et al., 2000). In this study, the authors targeted the E. coli enzyme with a library of random dodecapeptides displayed on the surface of bacteriophage. Selection of peptides with specific binding to the enzyme was accomplished using an enzyme-linked immunosorbent assay (ELISA). A total of 27 phage clones were analysed after three rounds of selection and these clones were found to encode seven unique peptide sequences, the most frequent of which was His–Pro–Trp–His–Lys–Lys–His–Pro–Asp–Arg–Lys–Thr. Biochemical studies using the selected phage and the synthetic peptide confirmed that these agents inhibited the E. coli enzyme with relatively weak potency (IC\(_{50} = 160 \mu M\)).

**Future prospects**

The conservation and confirmed essentiality of the enzyme in species that span the bacterial kingdom, coupled with the precedent of clinically successful agents targeted to peptidoglycan biosynthesis, provides a solid foundation for identification of glutamate racemase as a target for drug discovery. The recent reports of inhibitor optimization demonstrate not only that it is possible to identify leads that have intrinsic potency against the target, but also that potent in vitro inhibition can translate into whole-cell growth inhibition through the desired mode of action. The demonstration of in vivo efficacy with the D-glutamate analogues and the low resistance rates observed for the pyrazolopyrimdinedione series provide two key precedents for features required for full target validation and progression to the clinic.

However, even with these attributes, significant questions remain about the scope and suitability of glutamate racemase as a target for discovery of agents that meet the demands of the clinical setting. For example, it is clear that the enzyme is susceptible to a wide range of lead matter utilizing both allosteric or substrate competitive modes of inhibition, but, as a general observation, it has proven difficult to extend the spectrum of the leads to meet the bacterial spectrum required for treating diseases in the clinic. Clearly, for those settings where exquisite selectivity is desired, such as in the treatment of H. pylori infections, the narrow spectrum provides a significant advantage in terms of minimizing the development of cross-resistance and potential side-effects due to disturbances of the gastric flora. However, the majority of antibacterial infections in the clinic are attributed to a variety of opportunistic pathogens and effective treatment requires activity across a range of pathogenic species.
The extension of spectrum observed in the pteridine class of inhibitors, along with the recent modelling studies on the D-glutamate analogues, suggests that the development of broader spectrum agents is possible with suitable scaffolds and a sufficient understanding of the target biology and binding site architecture. Rapid development of resistance to a new clinical agent remains one of the key concerns with any antibacterial drug discovery effort. To mitigate this risk, potential mechanisms of resistance are evaluated well before agents enter the clinic, most typically through frequent profiling of lead compounds to assess the spontaneous rates of resistance. In the latter case, it is clear from the data on the pyrazolopyrimidinedione and benzodiazepine classes of inhibitors that the spontaneous rates of resistance can vary between series within a bacterial species. In these cases, all of the mutants mapped to the glutamate racemase gene, suggesting that the differences depend on the scaffold and the specific binding site. While this is likely to be a general phenomenon across all species, it is also possible that some species may encode additional mechanisms for resistance. For example, compensation of inhibition by an orthologue has been shown to provide an intrinsic resistance mechanism resulting in a limited spectrum of some advanced antibacterial programmes targeted to the FabI and MetRS enzymes (for review see Payne et al., 2007). It is unclear at this stage whether similar effects would be observed for inhibitors targeted to Bacillus spp. which encode two orthologues of glutamate racemase (e.g. B. anthracis), but clearly analogues which demonstrate equipotent activity against both isozymes would be preferred in these cases. The D-AAT pathway encoded in some Gram-positive species represents a second potential source of resistance as overexpression of this gene has been shown to be sufficient to complement a deletion of the glutamate racemase gene in E. coli (Pucci et al., 1995; Fotheringham et al., 1998; Liu et al., 1998). While it is clear that glutamate racemase is essential for growth, it remains an open question whether significant selection pressure will result in the evolution of resistance mechanisms utilizing this pathway.

Even with these potential concerns in hand it is evident that, on balance, the case for glutamate racemase as a target for antibacterial agent discovery is strong and continues to grow. The combination of detailed understanding of the target biology, derived from biochemical, structural and physiological studies spanning more than 40 years, the wide range of compelling inhibitor series identified through both rational and screening strategies to date, and the confirmation that optimized analogues can lead to efficacy in defined animal models of infection provides a foundation for drug discovery that is rare for a novel target without a clinical precedent.

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