

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 anti-bacterial 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 (Murl), 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 β 1-4 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 (Spellberg *et al.*, 2004).

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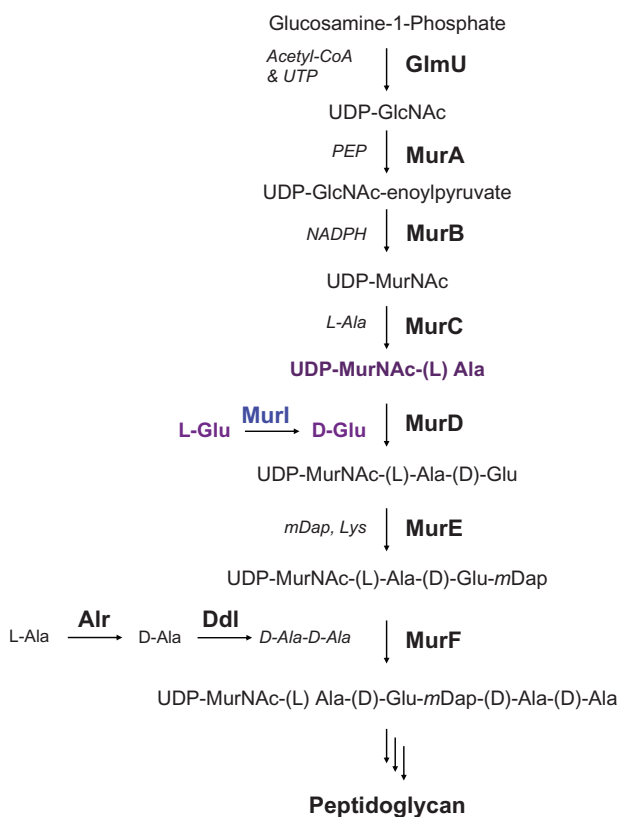


Fig. 1. Schematic of Phase I peptidoglycan biosynthesis. Adapted from van Heijenoort (2001) – reproduced by permission of The Royal Society of Chemistry.

The Phase I portion of cell wall biosynthesis has relatively few clinically validated targets but has been the subject of intense discovery efforts over the past decade. Phase I biosynthesis includes the first committed step to peptidoglycan synthesis – conversion of glucosamine-1-phosphate to UDP-*N*-acetylglucosamine (UDP-GlcNAc) – and culminates with the production of UDP-*N*-acetylmuramic acid-pentapeptide (UDP-MurNAc-pentapeptide) (see Fig. 1). A key feature of the peptidoglycan is the incorporation of *D*-amino acids, *D*-glutamate by the ligase MurD and *D*-alanine as a dipeptide formed by *D*-ala-*D*-ala ligase, which is a substrate of the ligase MurF. It is presumed that these unusual residues provide a defence against hydrolysis of the bacterial capsule, as host proteases are unable to recognize sequences containing *D*-amino acid residues. While there is some variation in the peptide composition of the pentapeptide intermediate, incorporation of *D*-glutamate as the second amino acid is strictly conserved (van Heijenoort, 2001). Cellular pool levels of *D*-glutamate are derived from *L*-glutamate, which are transformed by glutamate racemase, the enzyme implicated in the production and maintenance of *D*-glutamate in bacteria that produce a cell wall envelope.

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 *murl* 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 *murl* for cellular growth through more detailed genetic dissection of the WM355 mutant strain (Dougherty *et al.*, 1993) and plasmid-based complementation of *murl*

strains using either the *E. coli murl* gene (Baliko and Venetianer, 1993) or the *murl* gene from *P. pentosaceus* (Pucci *et al.*, 1994).

In addition to the effects on peptidoglycan biosynthesis, effects on nucleoid separation and supercoiling of plasmids were observed in strains overexpressing the *E. coli murl* gene (Baliko and Venetianer, 1993). These observations led the authors to suggest that elevated levels of *E. coli* Murl lead to inhibitory effects on the topoisomerases responsible for DNA replication, gyrase and topoisomerase IV. Subsequent studies demonstrated that *E. coli* Murl is a potent inhibitor of gyrase-supercoiling activity (Ashiuchi *et al.*, 2002) and similar findings have been observed with the glutamate racemase proteins from *Mycobacterium tuberculosis* (Sengupta *et al.*, 2006), *Mycobacterium smegmatis* (Sengupta and Nagaraja, 2008) and *B. subtilis* (Ashiuchi *et al.*, 2003). Mechanistic studies utilizing the recombinant enzymes from *M. smegmatis* and *M. tuberculosis* indicate that Murl inhibits DNA binding to gyrase and that Murl overexpression *in vivo* provides protection against the action of the gyrase inhibitor ciprofloxacin. The physiological role of this inhibition remains an open question.

The dawn of the genomic era rapidly established the presence of glutamate racemase in all species of bacteria encoding a cell wall and its essential role in peptidoglycan biosynthesis has been confirmed in species spanning the bacterial kingdom, including Gram-positive organisms that encode the D-AAT pathway for D-glutamate production (Bae *et al.*, 2004; Kada *et al.*, 2004; Kimura *et al.*, 2004; Song *et al.*, 2005). Furthermore, the D-AAT gene in *S. aureus* has been shown to be non-essential based on a high frequency of transposon insertions mapped throughout the gene encoding this activity (Bae *et al.*, 2004). Interestingly, several *Bacillus* species, including *Bacillus anthracis* and *B. subtilis*, have been identified that encode two homologues of glutamate racemase. A hallmark of these organisms is the ability to produce γ -polyglutamic acid (γ -PGA), a repeating polymer that contains significant D-glutamate content, which has been implicated in virulence (Drysdale *et al.*, 2005) and is valued as a foodstuff in Japan (Kada *et al.*, 2004). The physiological role of the two homologues remains somewhat controversial, as there are conflicting reports on the essentiality of the individual gene products (Ashiuchi *et al.*, 2003; Kada *et al.*, 2004; Kimura *et al.*, 2004; Shatalin and Neyfakh, 2005). Subsequent biochemical studies have confirmed that both genes encode functional glutamate racemase enzymes with similar pH and substrate preferences; however, differences have been noted between the isozymes in their thermostability (Ashiuchi *et al.*, 1999), solution oligomeric state (Dodd *et al.*, 2007); May *et al.*, 2007) and, for the *B. subtilis* enzymes, their overall catalytic efficiency.

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 aminotransferase 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-Lecreux *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 sphaericus* (Fotheringham *et al.*, 1998), *L. fermenti* (Gallo and Knowles, 1993), *Lactobacillus brevis* (Yagasaki *et al.*, 1995), *P. pentosaceus* (Nakajima *et al.*, 1986), *Aquifex 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-nitrocyanothiobenzoic 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 D₂O, 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.

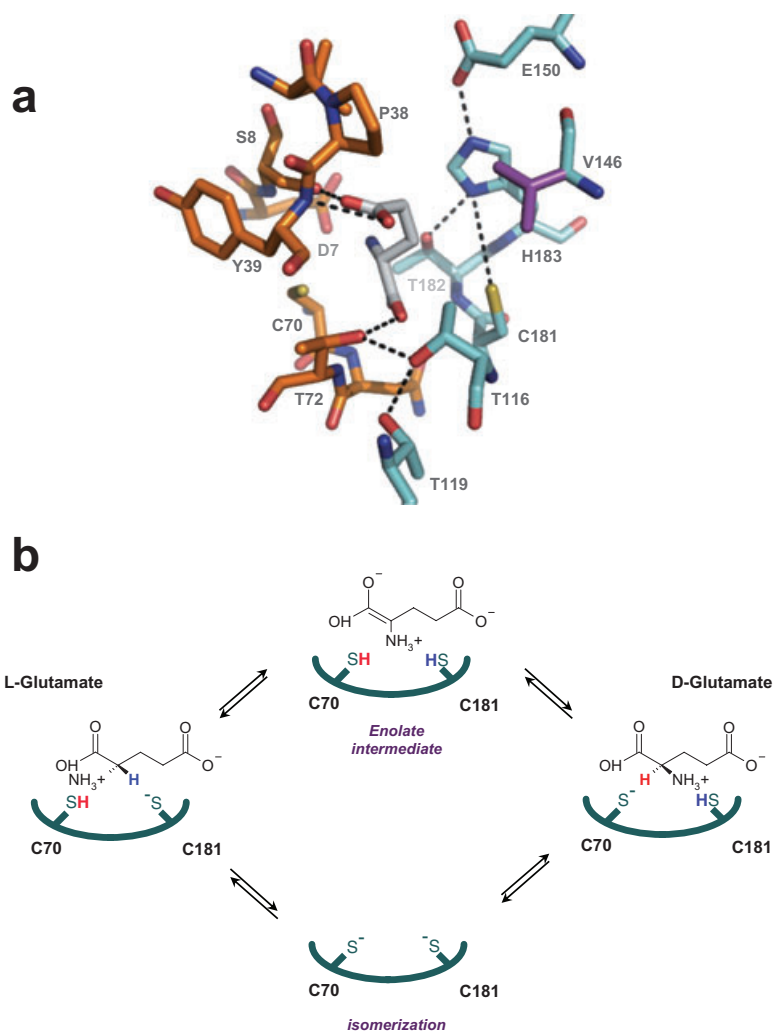


Fig. 2. Enzyme active site architecture and proposed catalytic mechanism.

A. Detailed view of the conserved active site residues from the *H. pylori* glutamate racemase structure complexed with D-glutamate (Lundqvist *et al.*, 2007). Putative hydrogen bonding interactions are shown (dashed lines). Residues are coloured by atom (nitrogen, dark blue; oxygen, red; sulfur, yellow; carbon: orange if derived from the N-terminal domain, blue if derived from the C-terminal domain; grey, D-glutamate substrate). The side-chain of V146 (purple) is shown for context with *S. pneumoniae* A148 (see Mechanism and Substrate-Based Inhibitors section of text).

B. Proposed catalytic mechanism of glutamate racemase. Conserved active site cysteines shown schematically (*H. pylori* numbering). Isomerization of the enzyme is represented proceeding through the fully deprotonated state; additional or other forms may be involved. (Copyright permission for Figure from the Royal Society of Chemistry)

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

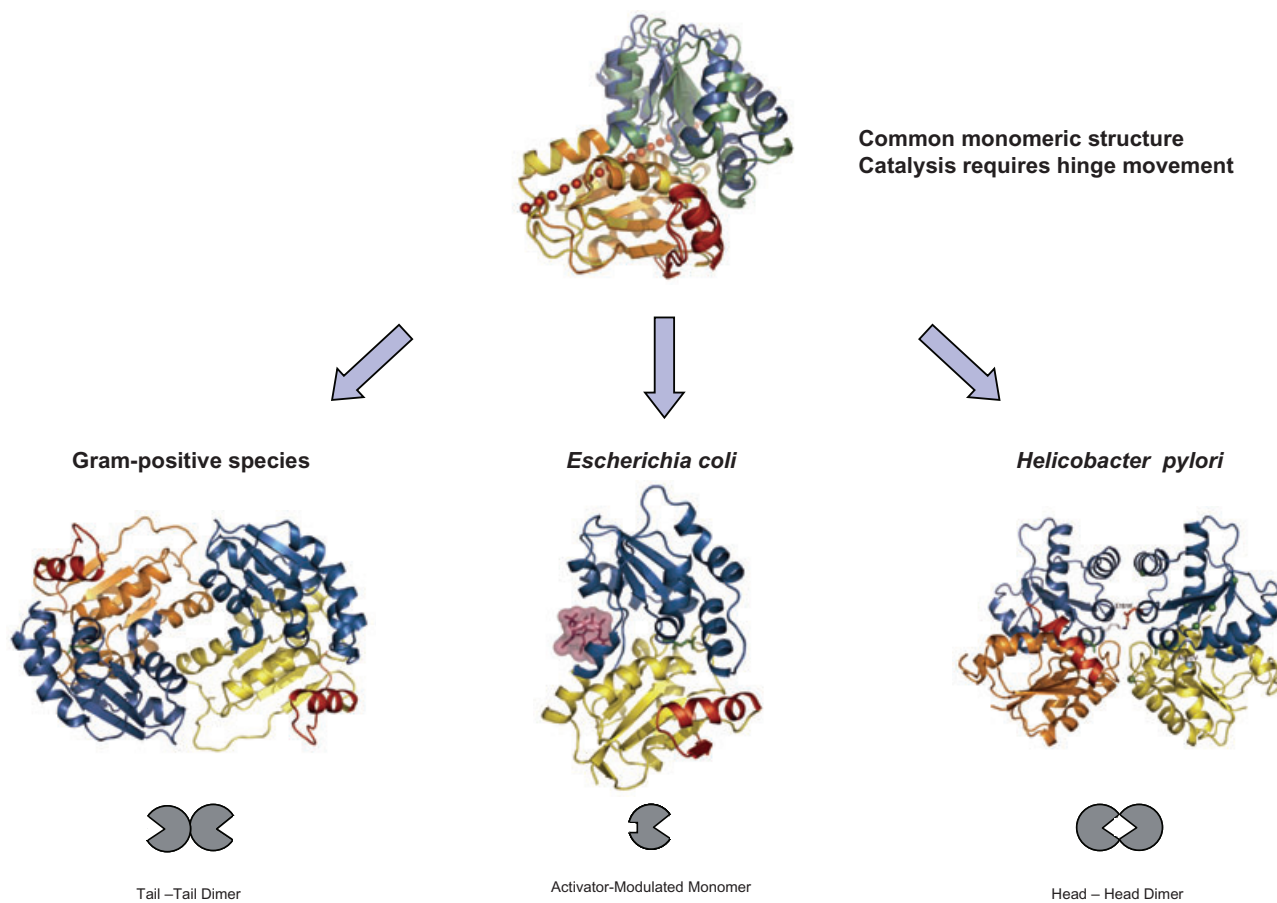


Fig. 3. Quaternary structural diversity across bacterial species. The common monomeric structure and the hinge movement and axis (red spheres) are illustrated through an overlay of monomers from an *Enterococcus faecalis* Murl structure. The *E. faecalis* glutamate racemase structure is shown representing the Gram-positive species. Purple mesh in the *E. coli* structure corresponds to the electron density of UDP-MurNAc-Ala. For all structures, the N-terminal domain (yellow, orange), C-terminal domain (blue), C-terminal helix (red) and glutamate substrate (green) are highlighted. For details see Lundqvist and colleagues (2007).

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 (*A. pyrophilus* numbering) makes no contribution to the intramonomer 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 *A. pyrophilus* 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 *A. pyrophilus*, *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-Lecreux *et al.*, 1982).

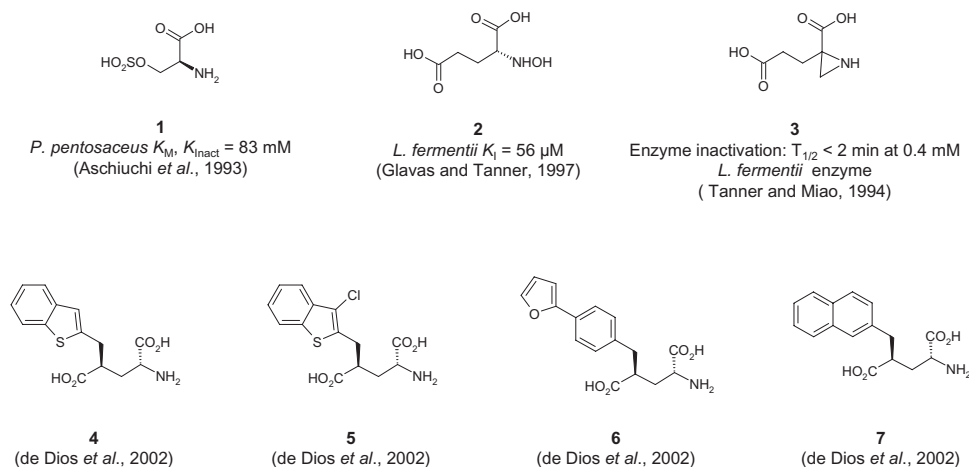
Finally, the *H. pylori* enzyme exhibits unique kinetic and structural features that are distinct from the other species studied to date. In this case, the enzyme exhibits a high degree of asymmetry in substrate processing, with a

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 α -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



Biological Assay	Analog			
	4	5	6	7
<i>S. pneumoniae</i> IC ₅₀ (μ g ml ⁻¹)	0.036	0.01	0.5	0.1
<i>S. pneumoniae</i> MIC (μ g ml ⁻¹)	0.024	0.5	0.24	0.25

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).

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

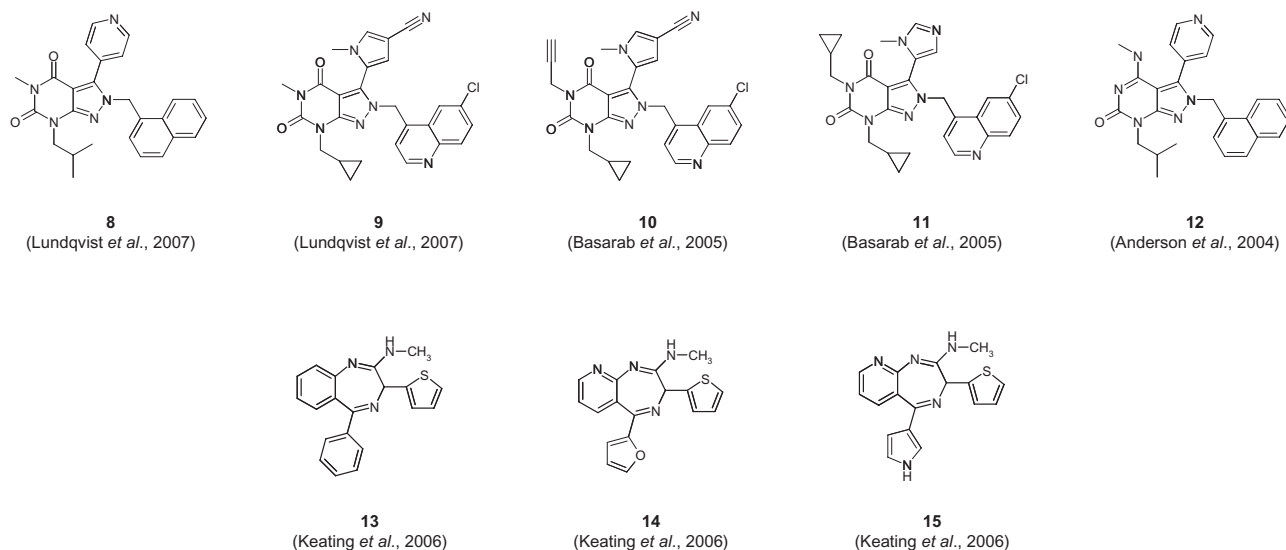
residue, V149, is an alanine in the *S. pneumoniae* enzyme (*S. pneumoniae*, A148, see V146 in Fig. 2A for context). This residue resides at the entrance of the hydrophobic pocket and the close approach of the branched side-chain of valine partially blocks access to the site. Most species encode valine 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 pyrazolopyrim-

idinedione 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



Biological Assay	Analogue								
	8	9	10	11	12	13	14	15	
<i>H. pylori</i> IC ₅₀ (μM)	1.4	0.025	0.028	0.049	0.22	1.7	2.0	1.2	
<i>H. pylori</i> MIC (μg/mL)	4.0	0.5	2.0	1.0	32	0.5	0.25	1.0	

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.

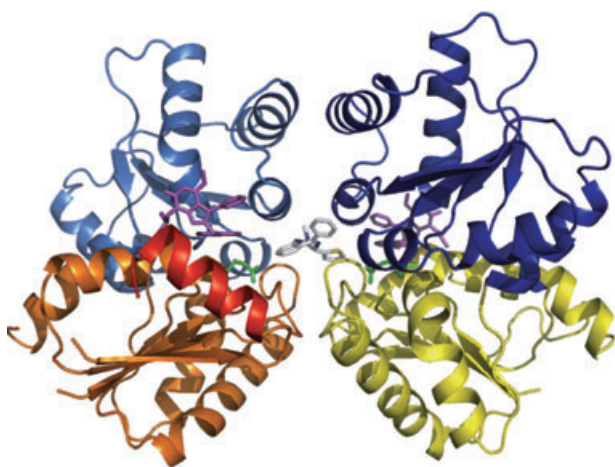


Fig. 6. Structure of *H. pylori* glutamate racemase in complex with D-glutamate and pyrazolopyrimidinedione and benzodiazepine series inhibitors. Overall protein orientation and colour scheme as described for Fig. 3. D-Glutamate (green), pyrazolopyrimidinedione analogue **12** (purple) and benzodiazepine analogue **13** (coloured by element; carbon, grey; nitrogen, blue; sulfur, yellow) are highlighted.

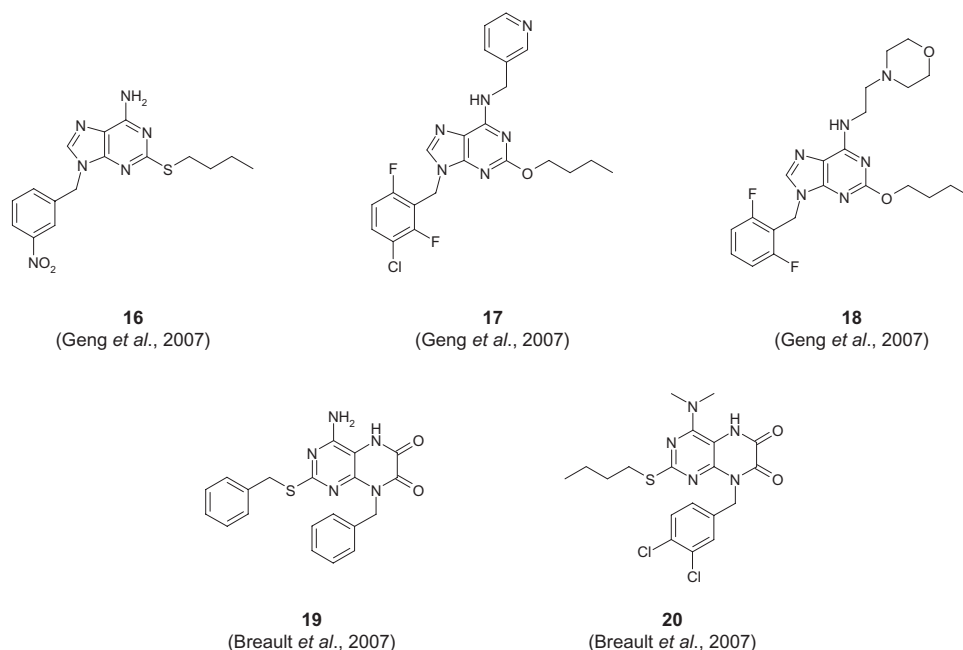
strategies and very potent analogues were identified with excellent whole-cell activity against *H. pylori* (Gowravaram *et al.*, 2005). Physiological studies on representative compounds confirmed that the primary mode of action was through inhibition of glutamate racemase (de Jonge *et al.*, 2005). 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 β -lactam antibiotics (e.g. amoxicillin; de Jonge and Kutschke,



Biological Assay	Analog				
	16	17	18	19	20
<i>E. faecalis</i> IC50 (μM)	9.4	2.0	5.8	21	1.0
<i>E. faecium</i> IC50 (μM)	18	2.7	5.5		
<i>S. aureus</i> IC50 (μM)	>400	>400	>400	8	1.1

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.

2006). 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* Murl enzyme activity. Early analogues in this series exhibited good potency against the enzymes from *Enterococcus* 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 *in vitro* inhibition structure activity relation-

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 was 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 $\mu\text{g ml}^{-1}$).

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 $\mu\text{g mL}^{-1}$). 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 ($K_i = 3.7 \mu\text{M}$) 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 ($\text{IC}_{50} = 160 \mu\text{M}$).

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 pyrazolopyrimidinedione 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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