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Bacterial transglycosylase inhibitors

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The spread of bacterial resistance to known antibiotics has inspired interest in previously underexploited drug targets. The transglycosylation reaction remains a 'black box' in the generally well-studied process of bacterial peptidoglycan biosynthesis, which is a very attractive target for chemotherapeutic intervention. Here, we summarize recent progress in the study of bacterial transglycosylases and the compounds that inhibit them. The transglycosylation reaction is readily targeted by several different classes of natural products, implying that it should be possible to develop drugs that inhibit this process once efficient high-throughput screens and appropriate compound libraries have been developed.

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Introduction

Antibiotic-resistant bacterial infections pose a serious threat to human health. Resistance has emerged to every class of antibiotics in clinical use, and many common pathogens are resistant to several different antibiotics [1]. The need for new drugs to treat antibiotic-resistant infections has led to a resurgence of interest in bacterial metabolism and pathogenesis, and it is hoped that a detailed understanding of important bacterial enzymes and processes will lead eventually to the discovery of new antibiotics.

Most broad-spectrum antibiotics function by inhibiting one of a small number of highly conserved metabolic processes in bacteria [2]. The biosynthesis of peptidoglycan, the major component of the bacterial cell wall, is one of those processes. Peptidoglycan is a polymeric mesh that surrounds bacterial cells and functions as a protective exoskeleton. It comprises linear strands of repeating GlcNAc-β-(1,4)-MurNAc (NAG-NAM) disaccharide units that are linked together via crossbridges between peptide moieties attached to the MurNAc sugars (Figure 1). One of its major functions is to stabilize bacterial membranes against high internal osmotic pressures, and anything that disrupts the integrity of the peptidoglycan layers therefore threatens the viability of bacterial cells [3]. A large number of natural-product antibiotics, including penicillin, cephalosporin, fosfomycin, cycloserine and vancomycin interfere with peptidoglycan biosynthesis, attesting to the importance of this pathway as a target for therapeutic intervention [2].

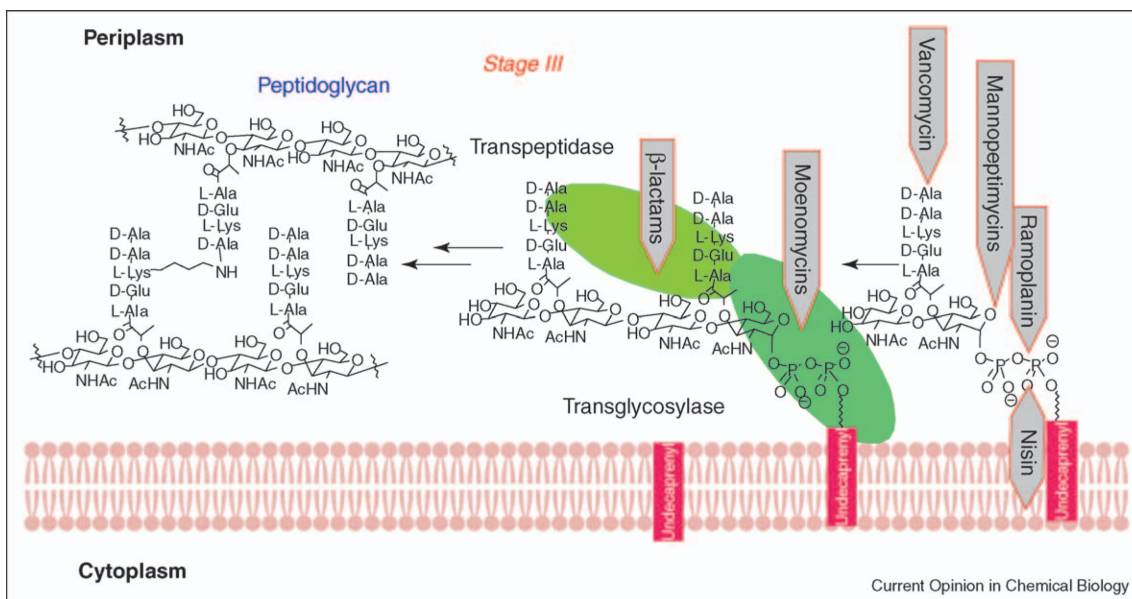
The emergence of antibiotic resistance has prompted efforts to obtain detailed structural and mechanistic information on every enzyme in the biosynthetic pathway to peptidoglycan and to discover new inhibitors for each enzyme. Significant progress has been made on both fronts for most of the intracellular enzymes and for several types of extracellular penicillin binding proteins ([4–6,7•,8]; [9] and references therein). However, for one group of enzymes, the bacterial transglycosylases (TGs) that catalyze the polymerization of the carbohydrate chains of peptidoglycan, progress has been exceptionally slow. Here, we review recent work on these enzymes and discuss the compounds that are known to inhibit them. We consider the validity of these enzymes as antibiotic targets; and address what might be done to accelerate the discovery of useful inhibitors.

Bacterial transglycosylases

The carbohydrate chains of peptidoglycan consist of repeating NAG-NAM disaccharide units. The NAG-NAM subunit is synthesized inside the bacterial cell as an activated undecaprenyl diphospho-sugar, which is commonly known as Lipid II because it is the second membrane-anchored intermediate in the biosynthetic pathway starting from UDP-GlcNAc (Figure 1). Once made, Lipid II is transported to the external surface of the bacterial membrane where it is proposed to react with the reducing end of the growing peptidoglycan chain in a reaction catalyzed by TGs. The peptide chains attached to the MurNAc sugars of the growing glycan chains are then crosslinked by transpeptidases (TPs). All organisms contain multiple TGs, which come in two known forms: as N-terminal domains of bifunctional proteins that also contain TP domains; and as separate proteins. The former are called Class A PBPs and the latter are known as monofunctional glycosyltransferases (MGTs) [10]. Recent work suggests that there are other types of TGs as well, but their genes have not yet been identified [11••,12••].

The first biochemical work on bacterial TGs was carried out in the 1960s [13], yet more than 40 years later there is

Figure 1



Peptidoglycan biosynthesis. Antibiotics that inhibit the transpeptidation and/or transglycosylation steps are shown as arrows pointing at the presumed stage of their intervention.

still no detailed structural or mechanistic information on these enzymes. Efforts to study bacterial TGs have been hampered by difficulties in obtaining appropriate substrates and in handling the enzymes. The Lipid II substrate is present in very small quantities in bacterial cells and cannot be isolated in large quantities from natural sources. Several preparative routes to Lipid II have been reported in the past few years [14–17], but several other challenges remain. One major challenge is to obtain 'well-behaved' TGs that are amenable to analysis. All TGs contain N-terminal transmembrane anchors that make them hard to purify. Although there have been several attempts to express soluble TG variants in the cytoplasm by truncating the transmembrane domains, the enzymes produced have extremely low activity [18]. It may be possible to obtain soluble, active enzyme by removing the transmembrane domain following periplasmic expression, but this strategy has apparently not yet been explored. Instead, efforts have focused on developing conditions to monitor enzymatic activity of intact proteins. Since synthetic Lipid II became available, three studies of *Escherichia coli* PBP1b and variants thereof have been carried out [19–21]. Although these studies have provided some insight into the behavior of bacterial transglycosylases, it has become clear that PBP1b is a cumbersome model system. It contains a longer N-terminal cytoplasmic tail than many other Class A PBPs and it also contains a long insertion of unknown function between the end of the TM helix and the beginning of the TG domain. These two features increase the size and hydrophobicity of PBP1b relative to many other TGs, and more tractable TG model systems are desirable. There is a particular interest in TGs from Gram-positive organisms, in part because several known inhibitors of transglycosylation are active only against Gram-positive strains. Low activity has been reported for two different Gram-positive TGs lacking TM domains [22,23]. The first report of a purified Gram-positive TG with reasonable kinetic activity appeared in 2005 [24••], when *Staphylococcus aureus* PBP2 was expressed in *E. coli* with its TM domain intact. *S. aureus* PBP2 is the primary TG in a significant pathogen [25]. This example demonstrates that active Gram-positive TGs can be expressed in reasonable quantities for study, and lays the groundwork for mechanistic and structural analysis of TGs.

The substrate problem has been solved, and it is now clear that suitable enzymes are not as difficult to obtain and handle as was previously believed. Although some engineering of candidate TGs will probably be required to obtain diffracting crystals of a TG domain, a structure is anticipated within the next few years. In the meantime, there is a need for additional studies on the mechanisms of these enzymes. The enzymology is challenging because TGs are believed to be processive enzymes, and the reaction involves conversion of one substrate, Lipid II, into a large and heterogeneous polymer. Although both continuous and discontinuous assays to quantify total product exist, these assays do not permit dissection of the mechanism [19,20]. It is imperative that methods be developed to evaluate polymer length distributions, to verify the proposed direction of chain elongation, and to identify any intermediates that may be released in the course of the reaction. Without appro-

priate methods of analysis, we cannot understand either the mechanism of the enzyme or the mechanisms by which various inhibitors block transglycosylation.

Inhibitors of bacterial transglycosylases

Screening efforts

Because pure Lipid II has only recently become obtainable, high-throughput kinetic assays for TG activity have not yet been developed. However, moderate-throughput assays using crude membrane extracts supplemented with Lipid II precursors have been developed to monitor TG activity, and these assays have provided some information about known TG inhibitors and inhibitor analogues [26–28]. For example, a crude membrane assay was used to show that the antibiotic ramoplanin, which was previously proposed to target an earlier step of peptidoglycan biosynthesis, also targets the TG reaction [29]. Crude membrane assays have also been used to assess the activity of several moenomycin analogs (see below) [30]. In addition to these kinetic assays, a filtration assay based on displacement of PBPs from beads derivatized with moenomycin has been reported [31]. Finally, scientists at Millenium Pharmaceuticals have reported a cell-based screen for cell wall active inhibitors in which an outer membrane permeable *E. coli* strain containing the genes for β-lactamase (*ampC*) and its regulator (*ampR*) is treated with various compounds. Cell wall inhibitors, including moenomycin, vancomycin, ramoplanin, fosfomycin, cycloserine and cefoxitin [32], were shown to induce expression of β-lactamase and promote survival in the presence of β-lactams. A high-throughput screen conducted to look for other compounds that induced *ampRC* led to the identification of a partially purified natural product extract that inhibits transglycosylation with an IC_{50} of 10 μg/ml [33].

It is expected that the availability of both Lipid II substrate and purified active TGs will enable the development of other assays, including high-throughput kinetic assays, that can be used to screen for TG inhibitors [34,35]. More screening may identify lead compounds that will provide insight into how to design better TG-based drugs.

Natural product inhibitors

What we currently know about TG inhibitors rests almost entirely on what we have learned from studying the handful of natural products that inhibit these enzymes. There are two major categories of natural product inhibitors of bacterial TGs. The first includes substrate binders, compounds that recognize structural elements present in Lipid II and nascent peptidoglycan (Figure 2); and the second category includes compounds that bind to the TGs themselves.

Substrate binders

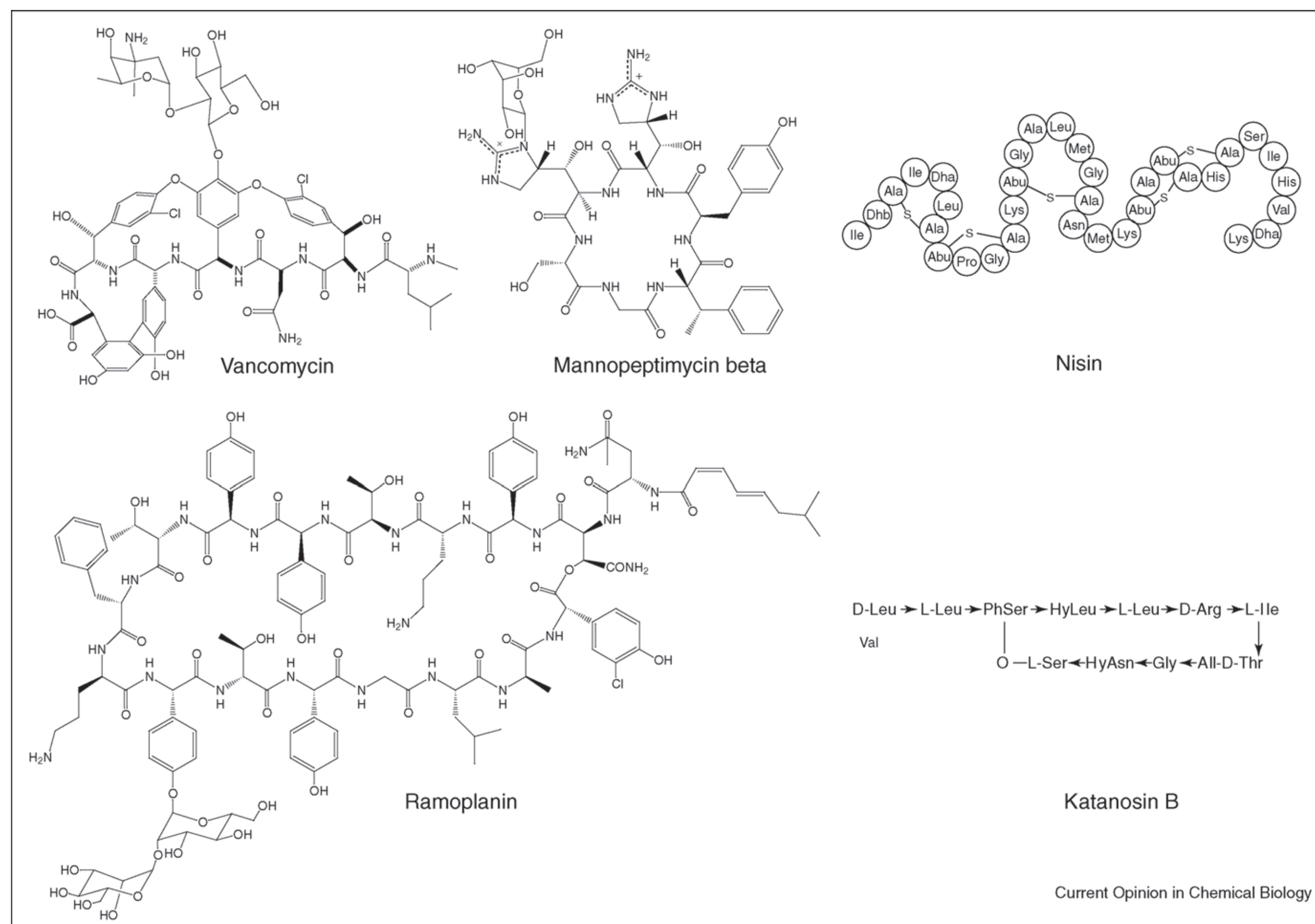
Natural products that bind to TG substrates include the glycopeptides vancomycin and teicoplanin, which bind to D-Ala-D-Ala [36], and several members of the lantibiotic family of antibiotics such as nisin and mersacidin [17,37•,38], which are believed to recognize the diphospho-sugar portion of Lipid II. The lipoglycodepsipeptide antibiotic ramoplanin is also thought to recognize the diphospho-sugar portion of Lipid II [39]. Finally, there are several other putative substrate binders that block transglycosylation, including the cyclic depsipeptides katanosin and plubascin A_3 [40], as well as the novel mannopeptimycin glycopeptides [41•]. These compounds are thought to bind to Lipid II at sites that are distinct from where vancomycin binds, but what they recognize is not yet clear.

The number and structural diversity of known or putative substrate binders that block transglycosylation is remarkable and implies that substrate binding is an evolutionarily successful strategy for blocking this particular step of peptidoglycan biosynthesis. Substrate binders may have several advantages over other types of inhibitors that account for their success. Typically, they block multiple steps in a pathway and/or combine two different killing mechanisms in the same molecule. For example, vancomycin can block both transglycosylation and transpeptidation by binding to D-Ala-D-Ala. Furthermore, certain analogs of vancomycin are proposed to block transglycosylation by binding to *both* the substrate and the enzyme [20,35]. Similarly, nisin is proposed to bind to Lipid II, thereby blocking transglycosylation, *and* to permeabilize bacterial membranes by forming Lipid II-containing 'pores' [38]. It has also been observed that high-level resistance does not readily develop to substrate binders *except* by horizontal transfer of genes originating in the producing organism. As therapeutic agents, however, substrate binders have some notable disadvantages. They are large and relatively polar, which means they are not orally bioavailable, and they cannot penetrate the outer membrane of Gram-negative bacteria. Therefore, they must be administered parenterally and their spectrum of activity is restricted to Gram-positive microorganisms. Nevertheless, vancomycin and teicoplanin are in clinical use and have been very important drugs for decades. Ramoplanin is also in clinical trials, and some lantibiotics are being investigated for possible clinical development [38,39]. With resistance to vancomycin widespread after 50 years of clinical use, we need alternatives to treat life-threatening infections, and substrate binders deserve serious consideration despite the limitations posed by their physical properties.

Enzyme binders

The only known natural products that bind directly to TGs belong to a small family of antibiotics called the moenomycins [42,43]. The most prominent member is moenomycin A (Moe), a pentasaccharide decorated with a C25 isoprene chain on one end and a chromophore on the other (Figure 3). Moe is extremely potent, with minimum

Figure 2

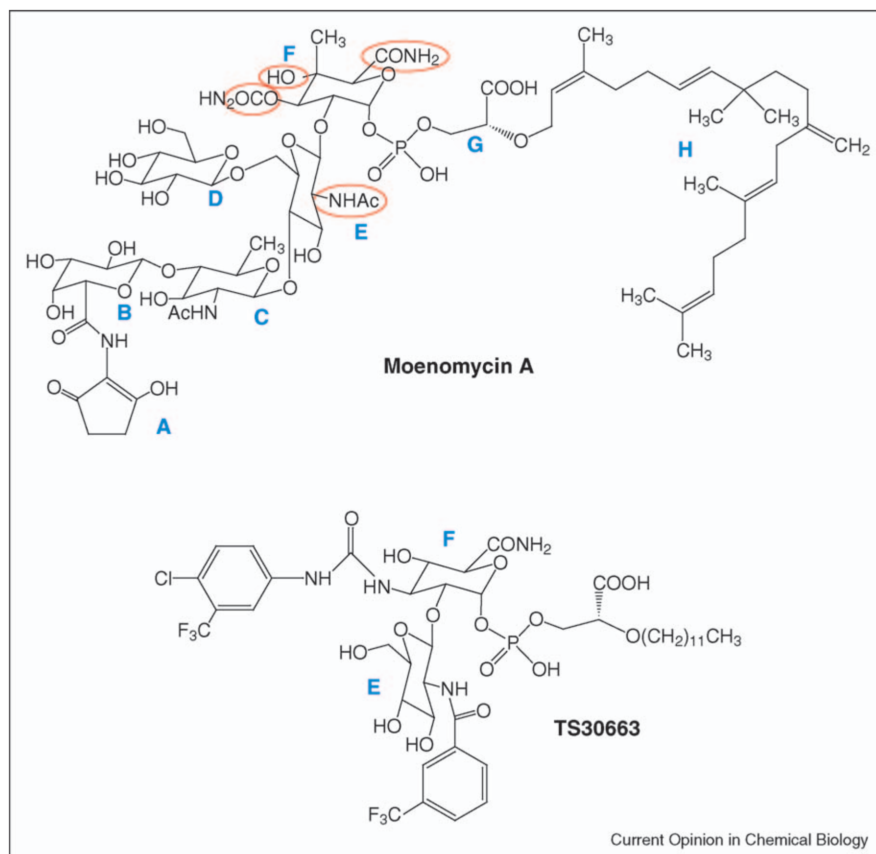


Structures of TG substrate binders. The following abbreviations are used to mark unusual amino acids in the molecules of nisin and katanosin: Abu, *S*-Ala-(2*S*, 3*S*, 6*R*)-3-methyllanthionine; Dha, 2,3-didehydroalanine; Dhb, (*Z*)-2,3-didehydrobutyrine; HyAsn, L-threo-β-hyudroxyasparagine; HyLeu, L-threo-β-hydroxyleucine; PhSer, L-threo-β-phenylserine.

inhibitory concentrations (MICs) in the range of 0.01 to 0.1 μg/ml [20]. It is used as a growth promoter in animal feed under the trademark Flavomycin, but is not used in humans. It is reported to have a long half-life and to show some toxicity upon parenteral administration, and it is not orally bioavailable. Nevertheless, it is believed that a better understanding of how Moe inhibits bacterial TGs could lead to the development of derivatives with more favorable properties. The structure–activity relationships of Moe derivatives have been studied extensively by Welzel and co-workers, who have identified features critical for bioactivity and for target interactions [43]. It was shown that the E-F disaccharide portion of Moe (Figure 3) retains inhibitory activity *in vitro*; however, the lipid chain is essential for activity *in vivo*. The lipid is evidently responsible either for anchoring Moe to the cell membrane or for interacting with hydrophobic regions of TGs. The carbamoyl group at C3′, the hydroxyl group at C4′ and the carboxamide entity at C5′ of unit F, as well as the acetyl group at C2′ of unit E, were shown to be critical for activity of the Moe pharmacophore [43,44].

As yet, there is no direct information on where Moe binds. Welzel and co-workers have suggested, on the basis of conformational analysis, that the inhibitor mimics the substrates as they react, and have concluded that Moe thus binds in the active site [45]. Kinetic analysis has recently shown that Moe inhibition cannot be overcome by increasing the concentration of substrate, raising questions about whether the molecule does, in fact, bind in the active site [20]. Because TGs are believed to be processive enzymes, and thus are presumed to have an extended binding pocket to accommodate the growing polymer chain, Moe could bind distally to the active site and block elongation. The development of better methods to dissect the mechanism of bacterial TGs should shed more light on the mechanism of inhibition.

Figure 3



Structure of Moe and a bioactive disaccharide analog developed by Sofia *et al*. [55]. Functional groups important for Moe disaccharide pharmacophore activity [18,44] are circled in red.

Synthetic transglycosylase inhibitors

There have been several attempts to make synthetic or semi-synthetic transglycosylase inhibitors. As with the natural-product inhibitors, these fall into two categories: those based on substrate binders; and those based on molecules that bind directly to the enzymes.

Substrate binders

Hydrophobic substituents appended to glycopeptides and mannopeptimycins have been found to increase activity against resistant microorganisms [20,36,46]. There is some evidence that 'hydrophobic' glycopeptides, in addition to binding peptidoglycan precursors, can also interact with bacterial TGs [36], and it has been proposed that biological activity against resistant microorganisms reflects these interactions. Four glycopeptide derivatives with hydrophobic substituents are currently in clinical trials: dalbavancin, oritavancin, TD-6424 and telavancin [36,47•]. Furthermore, there is ongoing research into methods to produce a wider variety of derivatives. For example, Kahne, Thorson, Walsh, Wohlleben and others have been investigating the utility of various glycopeptide glycosyltransferases, acyltransferases and halogenases for the production of novel glycopeptides that can be further functionalized and evaluated for biological activity [48,49,50•,51].

The substrate-binder ramoplanin has also been the subject of studies directed towards making synthetic derivatives. Boger and co-workers have developed a synthetic route to the ramoplanin aglycone [52,53], and have made a set of simplified analogues that maintain the biological activity of the parent compound [54]. Synthetic analogues could provide more insight into the mechanism of action of this molecule, which may in turn enable the design of analogues with improved properties.

Enzyme binders

In addition to the aforementioned studies of substrate binding antibiotics, there have been efforts to design inhibitors that bind to the enzyme. These inhibitors have been patterned on either Moe or Lipid II, which are among the few compounds known to bind directly to TGs. For example, Sofia *et al.* developed a combinatorial library approach to synthesize disaccharide analogues of Moe [55]. 1300 analogs were prepared and tested, and

some compounds with activity approaching that of Moe were identified [30,56] (Figure 3). These compounds exhibited interesting differences in *in vivo* activity against different microorganisms, suggesting structural and functional differences in the TGs expressed within and among bacteria. It is possible that the various inhibitors targeted different subsets of TGs. This finding would not be unprecedented: β-lactam antibiotics are known to display differential activities against different PBPs [57]. If Moe analogues are similarly able to discriminate amongst TGs, they could be useful tools to dissect the functions of the different enzymes that participate in cell wall assembly. Two other recent papers describing modest TG inhibitors based on Moe and Lipid II analogs have been reported [45,58].

Are transglycosylases good antibiotic targets?

The integrity of the bacterial cell wall, which is essential for bacterial survival, depends on the proper functioning of bacterial TGs. Within the peptidoglycan biosynthetic pathway, the bacterial TGs would seem to be particularly attractive targets because there are no human homologues of these enzymes, and they are located outside the bacterial cell membrane. This extracellular location is advantageous for two reasons. First, a wider range of structural types can be explored as inhibitors because cell penetration is not required for efficacy. (This advantage is limited to antibiotics of last resort that are administered parenterally.) Second, there are not as many mechanisms by which resistance can develop to compounds that inhibit extracellular targets. Nevertheless, whether TGs are *in fact* good targets has recently been questioned for several reasons. First, it has been shown that in many organisms several TGs can be deleted without destroying viability under laboratory conditions [11••,12••]. Thus, individual TGs are typically not essential, although TG activity clearly is. In addition, some organisms in which TGs have been deleted become resistant to Moe, raising concerns that resistance to direct inhibitors of these enzymes may develop readily [12••,18]. Finally, efforts to screen small molecule libraries for TG inhibitors have not yet borne fruit, leading to speculation that effective TG inhibitors must have the structural complexity (and attendant disadvantages) of Moe. With regard to the argument about essentiality, it is worth noting that individual transpeptidases are typically not essential, although TP activity clearly is; and the β-lactam antibiotics have been a hugely successful class of drugs. It should also be pointed out the Moe kills bacteria that have been shown to contain some Moe-insensitive TGs. This implies that inhibition of a subset of TGs, *none of which are essential on their own*, can produce a lethal effect. In this regard also, the TGs resemble the TPs involved in peptide crosslinking and hydrolysis, and it is hoped that TG inhibitors that are as efficacious as the β-lactams can eventually be discovered.

Conclusions

Recent work has substantially increased our knowledge of bacterial TGs, and has laid the groundwork for future research. More information about these enzymes should accelerate the development of new drugs. The structure of a TG domain would be the single most important advance in the search for TG inhibitors. No structures of any TG domains have yet been reported, although Macheboeuf *et al.* recently reported the structure of the TP domain of a class A PBP from *S. pneumoniae* [7•]. The structure of a TG domain bound to substrate and to Moe would be particularly useful, and could facilitate the design of compound libraries tailored to the TG target. Along with traditional chemical synthesis, further advances in chemoenzymatic production of novel glycopeptides [59,60] should significantly contribute to the generation of new compound libraries that can be investigated as TG inhibitors. Biosynthetic studies should also be extended to other unique antibiotics (e.g. the moenomycins) to provide us with new tools for combinatorial biosynthesis. Advances in the generation of more diverse libraries of drug-like compounds must be paralleled by improvements in the screening for TG inhibitors. With better compound libraries and improved TG inhibitor screens in hand, we will be able to tackle the problem of bacterial multidrug resistance.

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