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Targeting the Bacterial Transglycosylase: Antibiotic Development from a Structural Perspective

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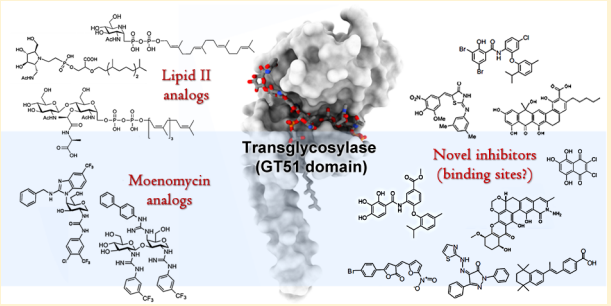
ABSTRACT: One of the major threats to human life nowadays is widespread antibiotic resistance. Antibiotics are used to treat bacterial infections by targeting their essential pathways, such as the biosynthesis of bacterial cell walls. Bacterial transglycosylase, particularly glycosyltransferase family 51 (GT51), is one critical player in the cell wall biosynthesis and has long been known as a promising yet challenging target for antibiotic development. Here, we review the structural studies of this protein and summarize recent progress in developing its specific inhibitors, including synthetic substrate analogs and novel compounds identified from high-throughput screens. A detailed analysis of the protein−ligand interface has also provided us with valuable insights into the future antibiotic development against the bacterial transglycosylase.



KEYWORDS: *bacterial transglycosylase, peptidoglycan glycosyltransferase, antibiotic development, moenomycin, lipid II analogs, structural analysis*

Bacterial transglycosylase, also called the peptidoglycan glycosyltransferase, participates in the biosynthesis of the peptidoglycan cell wall that exists in almost all bacterial species.[1] This peptidoglycan layer, with a unique mesh-like architecture composed of polymerized linear glycan chains interlinked with short peptides, maintains the structural integrity of the bacterial cell and provides mechanical strength against osmotic challenges.[2] Proteins involved in the process of peptidoglycan synthesis and regulation are hence attractive targets of antibiotics, because they are indispensable for bacterial survival, and they are accessible to drug treatments as they locate on or outside of the cell membrane.[3] Indeed, the first true antibiotic used in humans, penicillin, was discovered in 1928 as a microbial natural product and later revealed to inhibit peptidoglycan cross-linking by competitively binding to the active site of the transpeptidase domain of penicillin binding proteins (PBPs).[4] This discovery changed the paradigm of antibiotic development and led to the continuous discovery and development of numerous penicillin derivatives and β-lactam antibiotics, bringing forth great medical benefits.[5] Although both transpeptidase and transglycosylase are domains of the same bifunctional PBPs, no transglycosylase-specific antibiotic has ever been found, with the exception of moenomycin, which was discovered in 1965 from a microbial source.[6] Unlike penicillin, moenomycin has not been developed or improved for use in humans, because it contains a long 25-carbon fatty acid tail, which results in poor pharmacokinetic properties and low bioavailability, making it used solely as a growth promoter in feed additives for farmed animals.[7] Yet surprisingly, compared to the globally increasing resistance against β-lactam antibiotics, almost no evolved resistance against moenomycin has been observed after decades of animal feeding.[8,9] Its potential antiresistance advantage has drawn attention back to the bacterial transglycosylase, which is predicted to be a more promising target than transpeptidase for antibiotic development.[7,10,11] If a clinically useful antibiotic against transglycolysae could become available one day, it may save millions of lives per year, which could have been lost due to antibiotic resistance.[12]

Toward this goal, decades of research efforts have been devoted into this field, aiming to provide a clearer view of peptidoglycan biosynthesis and the mechanism by its potential inhibitors. It was recently found that, in addition to the canonical transglycosylase (glycosyltransferase family 51, GT51, domain of PBPs), there are other bacterial membrane proteins functioning as the peptidoglycan polymerase in alternative pathways.[13,14] These newly discovered transglycosylases, including RodA and FtsW of the SEDS (shape, elongation, division, and sporulation) family, may also be appealing therapeutic targets; however, no antibiotic development has yet been reported. Therefore, if not otherwise specified, the transglycosylase discussed in this Perspective only denotes the glycosyltransferase family GT51 (EC 2.4.1.129). Other interesting topics in this field such as the development of substrate binders as potential antibiotics[15−17] and the dynamic regulation of peptidoglycan biosynthesis[18−20] are not included in this article. Here, we focus on the canonical bacterial transglycosylase and its specific inhibitors, revisit structural details of its active site, and summarize recent

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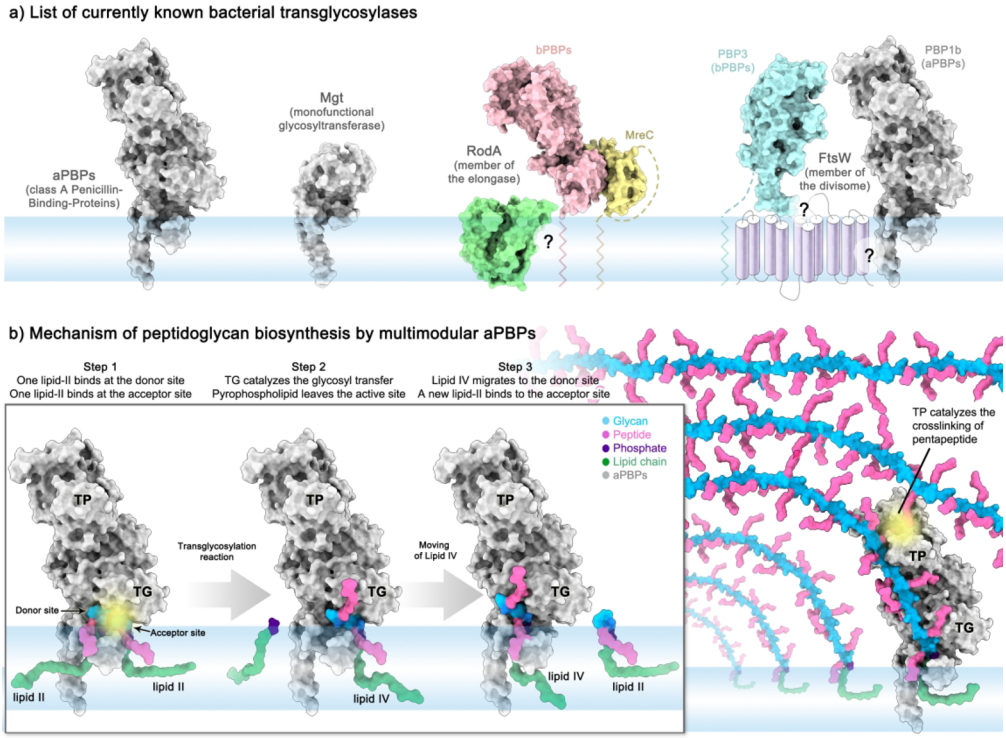

Figure 1. Bacterial transglycosylase and peptidoglycan biosynthesis. (a) List of currently known bacterial transglycosylases and their protein complexes, including aPBPs (PDB 5hlb), Mgt (PDB 3vmt), RodA (PDB 6bar) with its modeled interactors bPBPs and MreC (PDB 5lp5), and FtsW (structure unknown) with interactors *E. coli* PBP3 (PDB 4bjp) and PBP1b (PDB 5hlb). The unknown interfaces were marked by "?". (b) Mechanism of peptidoglycan synthesis by aPBPs. Lipid II molecules were modeled onto *E. coli* PBP1b (PDB 5hlb), with glycans in blue, pentapeptide in pink, pyrophosphate in purple, and the C55 chain in green. The catalytic reaction is proposed to take place in three steps as described. All molecular graphics and analyses in this article were performed with UCSF ChimeraX.[101]

advances in its inhibitor development from a structural perspective.

BACTERIAL TRANSGLYCOSYLASE IN PEPTIDOGLYCAN BIOSYNTHESIS

The bacterial transglycosylase (abbreviated as TG, PGT, TGase, or GTase) acts either monofunctionally or as an N-terminal domain of the class A multimodular penicillin binding proteins (aPBPs) (Figure 1a).[1] Compared with the aPBPs, which contain the N-terminal transglycosylase (TG) domain and the C-terminal penicillin-binding transpeptidase (TP) domain for coupled activities of glycosyl transfer and peptide cross-linking, the class B PBPs (bPBPs) only have the C-terminal TP domain but lack the TG domain.[21] By forming multicomponent peptidoglycan synthases with other transglycosylases from either the divisome (FtsW) or the elongasome (RodA) of the SEDS family, bPBPs are indispensable in bacterial cell division and cell wall elongation[22−25] (Figure 1a). The existence of multiple machineries for cell wall synthesis (aPBPs and SEDS complexes) is significant since each of them has distinct subcellular dynamics and roles, and the reduction in peptidoglycan synthesis by inactivating one group cannot be fully rescued by the other.[26,27] Moreover, even within the class of the aPBPs, there are multiple homologous proteins, such as *E. coli* PBP1a and PBP1b, which were thought to be redundant but are now revealed to be specialized for different environmental conditions to promote overall fitness while maintaining cell wall integrity.[28] The monofunctional glycosyltransferase (Mgt), on the other hand, contains only the TG domain but lacks the TP (Figure 1a) and is a dispensable protein for bacterial growth as tested in *Staphylococcus aureus*.[29] Because of its high sequence similarity with the TG of multimodular aPBPs, the smaller-size *S. aureus* MgtA protein has been used for structural and functional studies.[30,31]

Similar to the mammalian glycosyltransferase (E.C.2.4.1.17), which catalyzes the transfer of the saccharide moiety from an activated nucleotide sugar to a nucleophilic glycosyl acceptor molecule, the bacterial transglycosylase catalyzes the connection between saccharide moieties of its substrates, lipid II precursors, resulting in the polymerization of a nascent peptidoglycan chain[32] (Figure 1b). The lipid II precursor, serving as the fundamental building block of the bacterial cell wall, is composed of a undecaprenol−pyrophosphate tail anchored in the cell membrane and a disaccharide−pentapeptide head being invariably formed by an *N*-acetylglucosamine (GlcNAc), an *N*-acetylmuramic acid (MurNAc), and a pentapeptide (Ala-Glu-Lys-(D-Ala)-(D-Ala)) attached to the 3-acetylamide of MurNAc.[2] A general mechanistic model of transglycosylation for *de novo* peptidoglycan biosynthesis by the aPBPs can be divided into three steps. First, it was proposed that the two active sites, the donor and the acceptor sites of TG, are each occupied by one lipid II molecule.[30] This triggers the chemical reaction to connect MurNAc of the donor lipid II with GlcNAc of the acceptor lipid II (step 2), resulting in a "lipid IV" product with two pentapeptide branches at an ∼120° angle and an extra undecaprenol−pyrophosphate.[33,34] The phospholipid molecule then leaves the enzyme and is recycled, while lipid IV is proposed to translocate from the acceptor to the donor site in

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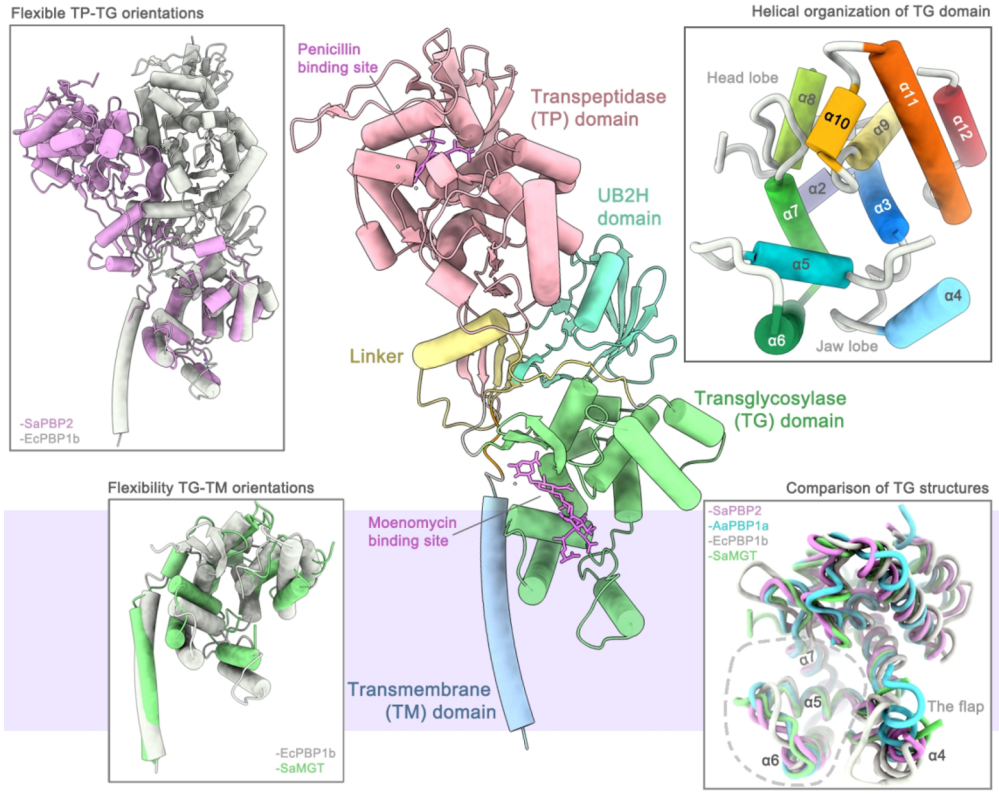

Figure 2. Overall structural of the bacterial transglycosylase aPBPs. *E. coli* PBP1b (PDB 5hlb) was used as the representative shown as a cartoon model in the middle with each domain colored differently. Top left, superposition of *S. aureus* PBP2 (PDB 2olv, purple) onto *E. coli* PBP1b (PDB 5hlb, gray) showing flexible orientations between TP and TG domains. Bottom left, superposition of *S. aureus* MgtA (PDB 3vmr, green) onto *E. coli* PBP1b (PDB 5hlb, gray) showing flexible orientations between TG and TM domains. Top right, the conserved fold of the TG domain has 11 helical fragments, colored in the order from N to C terminus. Bottom right, superposition of four different TG structures (*S. aureus* PBP2, PDB 2olv in purple; *A. aeolicus* PBP1a, PDB 3d3h in cyan; *E. coli* PBP1b, PDB 5hlb in gray; *S. aureus* MgtA, PDB 3vmr in green) reveals a structurally conserved core consisting of α5, α6, and α7 (dashed line) and the most flexible region of α4.

a mechanism not yet understood, waiting for a new lipid II binding to the acceptor site to start the next round of reactions[30,35] (step 3). As this reaction continues, the growing glycan chain extends upward to reach the active site of the transpeptidase (TP), enabling the cross-linking of pentapeptides with existent peptidoglycan chains and ultimately weaving them into a multilayered peptidoglycan mesh with concerted functions of many other enzymes and regulatory proteins in the peptidoglycan synthase complexes[36−38] (Figure 1b).

OVERALL STRUCTURE OF BACTERIAL TRANSGLYCOSYLASE

Although functional characterizations of the bacterial transglycosylase (TG) have been documented in as early as 1960s, a structural understanding of this protein was not available until 2007 with the first crystal structure of *S. aureus* PBP2 being solved by the Strynadka group[39] (PDB 2olv) and a TG-only structure of *Aquifex aeolicus* PBP1a being reported by the Walker group[40] (PDB 2oqo) (Figure 2). Both structures contain a moenomycin molecule bound to the donor site, albeit without the transmembrane helix of PBPs, which was later found to be critical for enzymatic activities and moenomycin binding of TG.[41] When the first structure of a membrane-bound full-length *Escherichia coli* PBP1b was determined in 2009 (PDB 3vma), it was proposed that the TG domain is partially embedded in the membrane, pointing to the importance of a membrane environment for TG functions.[34,35] In the same year, the TG-only structure of *S. aureus* MgtA was reported, exhibiting a high structural similarity with other TG domains of PBPs.[31] It took another three years for the determination of the membrane-bound structure of *S. aureus* MgtA, which for the first time revealed a substrate lipid II analog bound at the acceptor site (PDB 3vmt) (Figure 3b).[30]

As exemplified by the *E. coli* PBP1b structure,[34,35] the multimodular aPBP is composed of a transmembrane (TM) domain, a transpeptidase (TP) domain, a transglycosylase (TG) domain, a linker region, and a UB2H (UvrB domain 2 homologue) domain that exists in *E. coli* PBP1b but not in *S. aureus* PBP2 and was found to interact with periplasmic cofactors such as LpoB[42,43] (Figure 2, middle). Structural superpositions revealed flexible orientations of the TP domain toward TG and of the TG domain toward the TM helix (Figure 2, left). The interdomain flexibility between TP and TG may be important for accommodating the need of the peptidoglycan chains to grow in flexible angles as well as a better controlled "self-regulation" of peptidoglycan chain length as previously proposed.[44] The TG−TM flexibility may reflect the flexible positions of TG embedded in the cell membrane,[34] which need be considered toward a more realistic understanding of the environment of potential TG inhibitors.

In terms of the protein fold, the TG domain adopts a unique fold that is different from all other glycosyltransferase structures solved before.[39] This fold is structurally conserved in all known TG structures of aPBPs and Mgt, consists of

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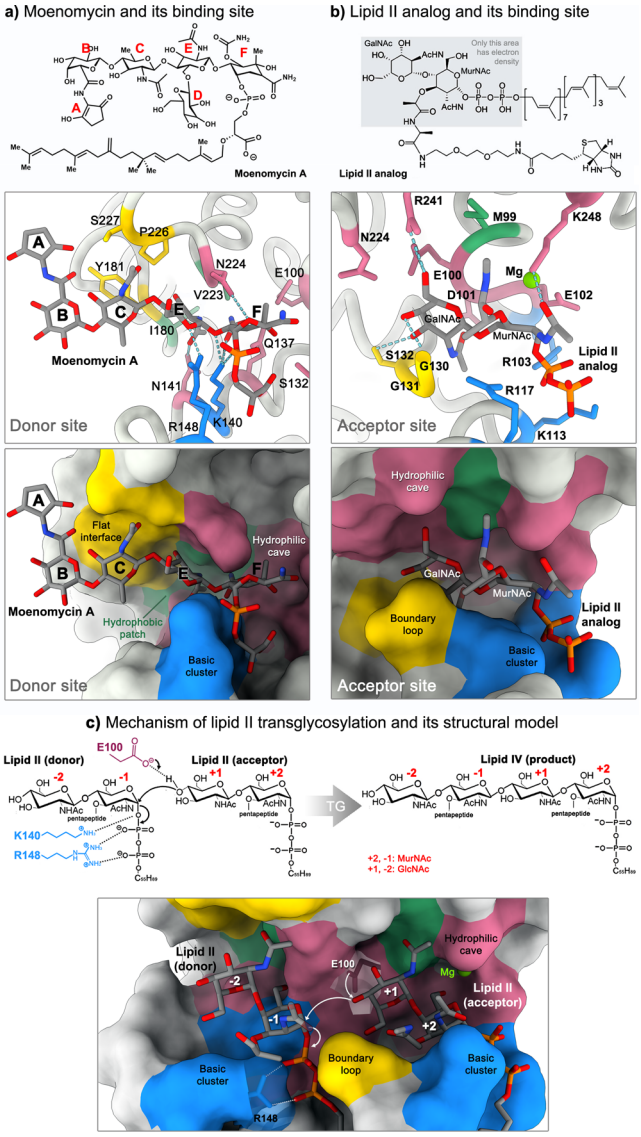

Figure 3. Graphic view of the TG active site and its catalytic mechanism. (a) The moenomycin A chemical structure (upper panel) with its binding site on TG (donor site) shown in either cartoon (middle panel) or surface (lower panel) mode with the ligand drawn in stick. Noninteracting ring D was omitted for clarity. (b) The chemical structure of a synthetic lipid II analog (upper panel) with its binding site on TG (acceptor site) shown in either cartoon (middle panel) or surface (lower panel) mode with the ligand in stick and Mg cation in sphere. (c) A schematic model of lipid II transglycosylation in either chemistry model (upper) or structural model (lower) with lipid II molecules (modeled) and key catalytic residues drawn in stick mode. Color code of active-site residues: magenta, polar residues in hydrophilic interactions with the ligands; blue, positively charged residues that mainly interact with the phosphate moiety; yellow, residues that interact with the ligand through their backbone or aromatic side chain; green, hydrophobic residues. PDB 3vms and 3vmt were used as representative structures. Residue numbering is based on the *S. aureus* MgtA sequence with equivalent residues in other structures listed in Table 1.

mainly alpha helices, and can be divided into 11 helical regions, namely, α2 to α12, while α1 is the N-terminal transmembrane helix[34,35] (Figure 2, top right). The 11 helices of TG are further grouped into two subdomain lobes, with helices α4, α5, and α6 forming the lower jaw lobe and all the other helices forming the upper head lobe, which resembles the fold of the phage λ lysozyme[39,40] (Figure 2, top right). When superimposing different TG structures, a subset of helices (α5, α6, and α7) turn out to be structurally more conserved than the others (Figure 2, bottom right), and this conserved core overlaps with the TG donor site and corresponds to three of the five sequentially conserved motifs, motifs I, II, and III, while motifs IV and V mainly contribute to the structural stability of TG as previously described.[44,45] However, located adjacent to the conserved core is the most flexible and the least conserved region of TG, including the α4 helix and part of the α4−α5 linker (Figure 2, bottom right). This region is usually referred to as the "flap", which may undergo a conformational change during the transglycosylation reaction,[35,39,46] because it lacks a consistent pattern of conformations in different TG structures, and in some cases, it carries large missing areas due to weak electron densities.[34,39]

STRUCTURAL DETAILS OF THE TRANSGLYCOSYLASE ACTIVE SITE

The active site of TG is located at the junction of its head and jaw lobes, forming a long and wide groove across the entire protein[35] (Figures 1b and 4). This groove can be further divided in two halves, a donor site on the left and an acceptor site on the right, with their central boundary region defined by a set of critically conserved residues. Since the structural information at the interface between its acceptor site and the substrate lipid II analog (PDB 3vmt) is available, here, we use residue numbering of *S. aureus* MgtA throughout the article and equivalent residues in other TG structures can be found in Table 1. The conserved residues in the central region include: the proton abstractor E100 that initiates the electrophilic migration between two substrates,[39] the polar residue Q137 that makes an electrostatic interaction with E100,[35] and three small-size residues G130, G131, and S132 on the α4−α5 linker.[30] The α4−α5 linker, or the boundary loop (Figure 3b), serves as a steric divider between the donor and the acceptor site, and its importance was verified by mutagenesis studies that showed single mutations like G130A or S132A could lead to greatly reduced transglycosylation activities.[30] While much emphasis has been placed on either the donor or the acceptor site as the target for inhibitors, it will be interesting to know if a catalytic-intermediate analog can be found as an inhibitor that binds to the conserved central area, spanning over the boundary loop.

On the left side of the boundary loop is the TG donor site to which moenomycin binds; it is sandwiched between the α5−α6 fragment at the bottom and the α9−α10 linker on the top. While specific interactions may differ slightly between TG and moenomycin in different structures, the architecture of the donor site can still be graphically generalized (Figure 3a). On its inner side is a hydrophilic cave (magenta) including several polar residues that interact with the hydroxyl groups of moenomycin sugar rings E and F, partially overlapping with the central area including residues E100, Q137, and S132. On the lower side is a basic cluster (blue) including conserved positively charged residues that attract the phosphate moiety of moenomycin. On the far-left edge is a flat interface (yellow) consisting of the backbone of the α9−α10 linker and a tyrosine side chain (Y181), which makes a π−π interaction with sugar ring C. Finally, in the middle is a small hydrophobic patch (green) composed of two conserved nonpolar residues, I180

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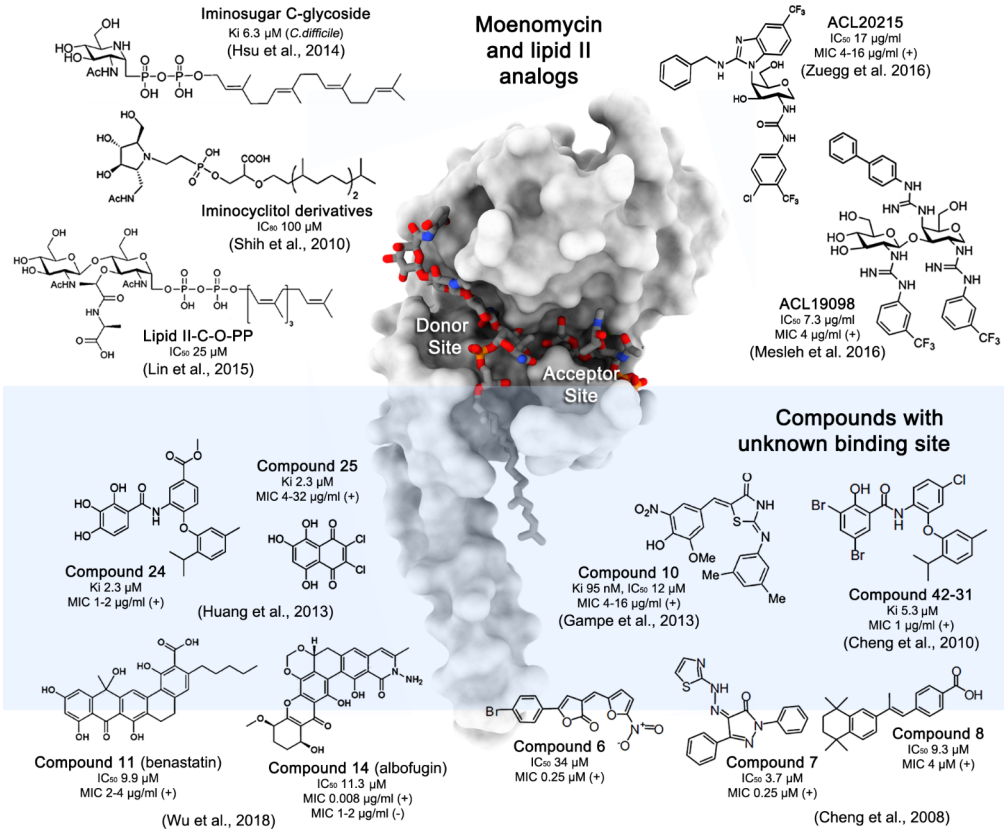

Figure 4. Summary of potential inhibitors against the bacterial transglycosylase (GT51 domain). The lipid II-analog-bound *S. aureus* MgtA structure (PDB 3vmt) with an additional moenomycin A modeled in the donor site (PDB 6ftb and 3hzs) was shown in the middle. Shown in the surroundings are selected moenomycin and lipid II analogs[66,67,76,79,81] on the top and novel non-saccharide[41,87−91] inhibitors on the bottom, with compound name, brief information on the activities, and reference labeled for each. MICs tested in Gram-positive or -negative bacteria were labeled (+) or (−). MICs (−) were not shown for inhibitors inactive against Gram-negative strains. More detailed information (such as the assay target) can be found in the inhibitor section of the article.

and V223, which interact with the *N*-acetyl methyl group of sugar ring E.

It is noteworthy that, while the binding of moenomycin covers a relatively large area of TG involving more than ten conserved residues, its bottom side is rather wide and exposed (Figure 3a). This may be understood as a way to accommodate for the flexible pentapeptide as the glycan chain grows,[34] and it may cause difficulties in structure-based drug design by simulated molecular docking. Another often-ignored feature of the moenomycin binding site is that it is composed of a mixture of hydrophobic and hydrophilic residues, with the latter being the majority.[35,39,47] Hence, compounds with large hydrophobic groups may have a much lower chance of binding as tightly as moenomycin. However, the small conserved hydrophobic patch of the donor site was largely unexplored (Figure 3a), which is only partially occupied by a methyl group of moenomycin sugar ring E. Structure-based drug modifications are encouraged to optimize the occupancy of an inhibitor on the hydrophobic patch, as nonpolar interactions are usually preferred due to an energy drop upon binding.[48]

In contrast to the well-established donor site, much less is understood about the TG acceptor site since only one structure is available[30] (PDB 3vmt). Yet similarly, the acceptor site is wide and hydrophilic (Figure 3b). Aside from the boundary loop (yellow), which makes extensive interactions with the first sugar residue of the lipid II analog, several polar residues from helices α3, α9, and α11 come together to form a hydrophilic cave (magenta), surrounding a nonconserved hydrophobic residue M99 (green), with three positively charged residues on the α4 helix forming another basic cluster at the lower interface (blue). This α4 helix, being the least conserved region of TG, is partially buried in the membrane between the lipid tails of two substrates[34,35] (Figure 1b). The nonconserved flexible α4 helix at the acceptor site is a major difference with the donor site, as the donor-site residues are generally conserved[44] (Table 1). Given that the α4 helix is a major part of the flap region of TG, which must undergo either localized unfolding or conformational change to facilitate the translocation of lipid IV,[35,44] its importance in the transglycosylation mechanism is undeniable, but its actual role in this process remains elusive.

MECHANISTIC UNDERSTANDING OF LIPID II TRANSGLYCOSYLATION

The detailed atomic model of the TG active site mentioned above has significantly improved our understanding of the structural basis of the lipid II transglycosylation mechanism. When two lipid II precursors occupy the donor and acceptor sites, as modeled on the moenomycin- and lipid II-analog-bound structures,[30,31] GlcNAc 4-OH of the acceptor lipid II is proposed to be located close enough to MurNAc C1 of the donor lipid II (Figure 3c). The carboxyl group of E100 serves as a Brønsted-Lowry general base to accept a proton from the donor GlcNAc 4-OH, resulting in the simultaneous

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Table 1. Equivalent Active-Site Residues of the Bacterial Transglycosylase Structures from Different Species[a]

	S. aureus PBP2	*A. aeolicus* PBP1a	*E. coli* PBP1b	*S. aureus* MgtA
Moenomycin binding site				
Hydrophobic patch	I195	I164	V314	I180
	P231	P200	V354	V223
Basic cluster	K155	K124	K274	K140
	R167	R132	R286	R148
Hydrophilic cave	E114	E83	E233	E100
	Q150	Q119	Q271	Q137
	D156	N125	N275	N141
	Q232	K201	K355	N224
	S147	S116	S266	S132
Flat interface	Y196	Y165	Y315	Y181[b]
	P234	P203	A357	P226
	N235	A204	S358	S227
Lipid-II binding site				
Boundary loop	G145	G114	G264	G130
	A146	G115	A265	G131
	S147	S116	S266	S132
Basic cluster	R117	N86	H236	R103
	K127	V96	Y246	K113
	R128	R100	R250	R117
Hydrophilic cave	R249	R218	R372	R241
	Y255	R225	K378	K248
	E114	E83	E233	E100
	D115	D84	D234	D101
	N116	R85	R235	E102
	Q232	K201	K355	N224
Hydrophobic residue	T113	T82	T232	M99

[a]Conserved residues are highlighted in gray. [b]Although the same amino acid is found at this position in all these structures, it is not conserved in other species.[30,44]

nucleophilic attack toward the acceptor MurNAc C1, which then dissociates the negatively charged undecaprenol−pyrophosphate (stabilized by K140 and R148) from the donor lipid II.[30,35,39] The catalytic product lipid IV thus has a new β1−4-linkage between sugar residues −1 and +1 and only one lipid tail (Figure 1b), and subsequently, it needs to migrate from the acceptor site to the higher-affinity donor site,[49] possibly with the assistance of the flap region,[35,39] to start the next round of catalysis for peptidoglycan polymerization.

The idea of the flap region that facilitates catalysis-related movement is not uncommon among enzymes.[50] However, unlike an expected flap, which usually has a pattern of distinct conformational stages[51,52] (e.g., open and closed), the flap of TG exhibits a variety of conformations (Figure 2, bottom right). It is noteworthy that this flap is often found to be involved in the crystal packing of TG structures[30,31,40] (PDB 2oqo, 3hzs, 3vms, etc.), probably due to its semihydrophobic nature that tends to form intermolecular interactions. Although the chemical reaction mechanism by TG is generally understood, the enigma about the steric passage for lipid IV translocation has not been resolved. In a recent report, on the basis of isothermal titration calorimetry data and the re-refined *S. aureus* MgtA structure combined with molecular dynamics simulations, Punekar et al. found two moenomycin-binding sites on MgtA with different affinities and positive cooperativity and suggested a hypothetical model for processive lipid II polymerization in which a swaying movement of the flap from the open (similar to PDB 3vms) to the closed (PDB 3hzs) state would continue while the lipid IV product moved from the acceptor site to the donor site, thus allowing another lipid II molecule to bind to the acceptor site.[46] Further verification of this hypothesis will lead to a more thorough understanding of the transglycosylation process and may even provide unexpected insights into the antibiotic development targeting TG.

ANTIBIOTIC DEVELOPMENT FOR BACTERIAL TRANSGLYCOSYLASE

Antibiotic development targeting the bacterial transglycosylase started long before any TG structures were available, with the initial focus on the improvement of its natural inhibitor moenomycin and lipid II substrate analogs,[53] which inactivate TG by competitively binding to either the donor or the acceptor site. The main goal is to find an improved analog of moenomycin or lipid II with desirable inhibitory efficiency but higher bioavailability, because both compounds contain a long lipid tail that prevents them from being easily absorbed.[15] An alternative approach aims to find novel inhibitors that are structurally distinct from the phospholipid−saccharide scaffold using high-throughput screening methods.[10,54] Summarized below are previous reports on these three classes of potential antibiotics against TG: moenomycin analogs, lipid II analogs, and novel inhibitors.

Moenomycin Analogs. The minimal pharmacophore of moenomycin, consisting of sugar rings C, E, and F, a phosphoglycerate, and a C25 lipid chain, was determined several decades ago together with detailed structure−activity relationship studies[55−57] (Figure 3a). The removal of the C ring maintains its TG inhibition but results in the loss of antibacterial activity,[58] while shortening the lipid chain gradually reduces its potency.[59] On the basis of this knowledge, a combinatorial library was established containing 1300 analogs of the moenomycin disaccharide core (ring E and F) with a variety of modifications,[60] which was followed by functional verifications of mono-, di-, and trisaccharide moenomycin analogs[61,62] as well as hybrid glycopeptides of moenomycin fused with vancomycin or ampicillin.[63] Most of these analogs synthesized during that time exhibited modest inhibition of TG with MIC (minimum inhibitory concentration) values of 1−4 μg/mL against a broad panel of Gram-positive bacteria,[11,54] but none of them were potent enough to replace moenomycin as a new antibiotic with comparable antibacterial activities and improved pharmacokinetic properties.[60]

The crystal structure of the moenomycin-bound TG structure started a new page in the field of antibiotic development and shifted the research focus onto structural investigations of TG in complex with different moenomycin analogs.[39,64] A neryl-moenomycin-bound *Aquifex aeolicus* PBP1a structure (PDB 3d3h) showed that a novel moenomycin intermediate, which does not contain the putative minimal pharmacophore, can still serve as an effective TG inhibitor, as long as the interactions with the six critically conserved residues are maintained.[47] On the other hand, the less explored carboxylate group of the phosphoglycerate moiety in moenomycin was found to be important as it interacts with an invariant K140 at the donor site (Figure 3a). A decarboxylated or methylphosphoryl neryl-moenomycin failed to produce clear electron density for the ligands (PDB

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3nb6, 3nb7), but replacing the carboxylate with a more negatively charged acylsulfonamide group could further enhance its inhibitory efficiency.[65] These results have encouraged further studies of synthetic analogs with the hope that the loss of inhibitory activity due to shortening of the lipid tail can be compensated by optimizing the polar contacts at the active site.[54]

A novel strategy in developing moenomycin-analog inhibitors was later reported by two groups several years ago. By removing the entire lipid tail of moenomycin, drug-like building blocks were used to attach the saccharide core to achieve moenomycin-like activities.[66,67] Zuegg et al. established a monosaccharide compound library and identified the two most potent pyranose-scaffold inhibitors with similar chemistry, ACL20215 and ACL20965 (Figure 4), with an IC_{50} of 17 and 11 μg/mL against *S. aureus* Mgt.[67] Like moenomycin, these compounds are inactive against Gram-negative *E. coli* but display activity against a broad range of Gram-positive strains, including methicillin-resistant *S. aureus* (MRSA), with an average MIC of 4−16 μg/mL. Mesleh at al.[66] synthesized a disaccharide-core compound library and obtained their best hit, ACL19098 (Figure 4), with an IC_{50} value of 7.3 μM against *S. aureus* Mgt and an MIC of 4 μg/mL against *S. aureus*. The aromatic branches of the saccharide core in these studies are interestingly unique, but it becomes difficult to predict their actual binding modes since their chemical structures have been noticeably changed from the original moenomycin blueprint. Structural information on the TG−inhibitor complex will then be necessary for further improvement of these compounds in terms of enhanced inhibition activities and cell wall permeability in Gram-negative bacteria.

Not only can the chemical structure of moenomycin be optimized toward the goal of its clinical application, but also the way moenomycin is applied can be innovatively reconsidered. Although the biggest obstacle of moenomycin lies in its poor bioavailability, this should not become a limitation if moenomycin is expected to be effective only in the human gastrointestinal tract for the treatment of related diseases such as *Helicobacter pylori* infections.[68] Indeed, antibiotic activities were observed when treating clinical isolates of multidrug resistant *H. pylori* with moenomycin, pointing to an interesting alternative for future applications of this well-known but clinically yet-unavailable antibiotic.[69]

Lipid II Analogs. While moenomycin is thought to mimic the transglycosylation product lipid IV,[49] lipid II analogs are designed to mimic the substrate lipid II, and they differ in several aspects other than the number of saccharide units (Figure 3b). First, they have different sugar types and modifications that may interact with TG differently. Second, lipid II has a pentapeptide moiety attached to its MurNAc, which is usually absent in moenomycin analogs. Third, the phosphate moiety of moenomycin is a phosphoglycerate, but in lipid II, it is a pyrophosphate.

In the early years, lipid II analogs received much less attention than moenomycin, partly because they have much weaker potency compared to moenomycin analogs and, most importantly, they are much more difficult to obtain either enzymatically or synthetically.[70−72] In the 2010s, with advanced methods for lipid II synthesis being developed[49,73,74] and the first structure of a lipid II-analog-bound TG being solved,[30] there was a revived interest in the field of lipid II analogs. This structure not only revealed a detailed acceptor−site interface but also provided a structural explanation of how the epimerization of the C4−OH group from GlcNAc to GalNAc in lipid II converted it from a substrate into an inhibitor.[75] During that time, chemical modifications on different moieties of lipid II were extensively explored to obtain a better understanding of this molecule as a potential TG inhibitor.

For the saccharide moiety of lipid II, Shih et al. found that lipid II analogs with a longer sugar chain led to an increased affinity with TG;[49] since lipid II can bind to either the acceptor or the donor site, an elongated sugar chain should have stronger binding at the donor site. It was also found that the *N*-deacetylation of the GlcNAc of lipid II analogs led to a notable decrease in inhibition activities,[75] in agreement with the structural observation that the *N*-acetyl methyl group of the moenomycin sugar ring E interacts with the conserved hydrophobic patch at the donor site (Figure 3a).

For the peptide moiety of lipid II, the minimal requirement of the pentapeptide to maintain its antibacterial activity has been determined to be the first two residues (Ala-Glu), while the terminal D-Ala-D-Ala residues are dispensable.[76] It was further found that the two methyl groups on the minimal dipeptide of lipid II are particularly important for substrate activity, the removal of which not only affects the hydrophobic interaction but also may lead to a conformational change of the peptide moiety.[76] Indeed, this finding is supported by structural data[30] in which the first methyl group is located in the proximity to the backbone of the α4−α5 linker at a distance of 4.0 Å to Cα of G130 and the second methyl group is close (4.8 Å) to the Cε of M99 (Figure 3b).

For the phosphate moiety of lipid II, Dumbre et al. replaced the pyrophosphate with a phosphoglycerate of moenomycin and synthesized a set of disaccharide/dipeptide lipid II analogs with a shorter C16 lipid tail (Figure 4).[77] All phosphoglycerate compounds they tested showed a 3- to 10-fold increase in inhibitory activity compared to their cognate monophosphate analogs; significant morphological defects of the bacteria were observed under the microscope, but their antibacterial activities were modest with an MIC larger than 128 μg/mL against *S. aureus*.[77] Another way to improve lipid II analogs is to try to change the saccharide−phosophate-linkage to an uncleavable 1-C-glycoside bond, which is believed to increase the chemical stability of lipid II analogs.[71,78,79] Lin et al. synthesized an 1-C-glycoside-linked lipid II analog, namely, lipid II−C−PP, which had no inhibition activity even at 1 mM.[79] However, when replacing the 1-C-linkage with an elongated sugar−phosphonate bond, they obtained a lipid II−C−O−PP compound, which exhibited a much higher inhibition activity with an IC_{50} of 25 μM against *Clostridium difficile* PBP[79] (Figure 4). From the structural analysis, this can be understood as the loss of the oxygen atom in 1-C-linked analogs becomes unfavorable in the basic-cluster environment of both active sites (Figure 3a,b), while an elongated C−O−P-linkage may provide certain flexibility for the phosphate moiety to fit in the binding site properly.

In addition, a completely different class of the sugar type, the iminocyclitol or iminosugar derivatives, was also explored as their protonated forms are known to mimic the oxonium ion transition state of the transglyosylation reaction.[80] An iminocyclitol-based library was developed to screen for potential TG inhibitors, and the best hit obtained is composed of an iminocyclitol core, a C15 short lipophilic tail, and a phosphoglycerate-like moiety with >80% inhibition at 100 μM

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against *E. coli* PBP1b[73] (Figure 4). This is so far the smallest lipid II analog inhibitor being reported. Besides, Hsu et al. explored a variety of imino-C-glycosides of α-D-GlcNAc-1-phosphates, and the best inhibitor obtained (compound 2a) contains a C20 lipid tail with a K_i value of 6.3 μM against *C. difficile*, compared to the K_i of moenomycin at 0.0015 μM[81] (Figure 4). In a following study, Wang et al. improved compound **2a** with an added GlcNAc and a truncated peptide moiety, obtaining compound **2b** which showed 70% TG inhibition at 50 μM against *A. baumannii*.[82] It is noteworthy that, from the structural point of view, the nitrogen atom of iminosugar may have a stronger interaction with E102 than the oxygen of MurNAc (Figure 3b). Although E102 is not a conserved residue (Table 1), this information could be useful for the future optimization of TG inhibitors, especially when a species-specific preference is needed.

Non-Saccharide Transglycosylase Inhibitors. In order to overcome the bioavailability problem resulting from the long lipophilic tail of both moenomycin and lipid II analogs, attempts have been made to identify structurally novel inhibitors using high-throughput screening (HTS) of small molecule libraries. In the early 2000s, several HTS experiments that target TG were summarized as follows: an affinity-competition assay was designed to screen for compounds that competitively displace PBPs from moenomycin-bound beads;[83] an *in situ* screen assay was developed using Triton X-100 to turn on and off the peptidoglycan synthesis in cell membrane isolates tracked with radiolabeled UDP-GlcNAc;[84] a cell-based screen monitored β-lactamase induction as a result of cell wall synthesis inhibition in an outer-membrane-permeable *E. coli* strain.[85,86] However, few positive results were reported following these screens, except for one compound with unpublished chemical structure (AC98) that was identified from the lactamase-induction assay from a partially purified natural product and found to inhibit transglycosylation with an IC_{50} of 10 μg/mL.[86]

The first novel TG inhibitors with reported structures were discovered from a fluorescence-anisotropy-based HTS assay in 2008[41] (Figure 4). Instead of using cell membrane isolates or whole cells, Cheng et al.[41] used purified full-length *E. coli* PBP1b protein and measured the change in fluorescence anisotropy of fluorophore-labeled moenomycin in a competitive inhibition assay, obtaining three hits (compounds **6**, **7**, and **8**) with IC_{50} of 34, 3.7, and 9.3 μM against *E. coli* PBP1b and MIC values of 0.25−4 μM against several Gram-positive strains. The discovery of these non-saccharide inhibitors has fulfilled a long-term pursuit that TG inhibitors with completely different structures from moenomycin or lipid II analogs can be found. At the same time, more questions were raised about their overall hydrophobic nature, which made them less likely to bind in the same way as moenomycin even though they were supposed to be moenomycin-competitive compounds. It then became important to know their actual binding mode so as to facilitate further structure-driven optimizations, which unfortunately, has not yet been realized.

A different group of novel TG inhibitors containing a salicylanilide core was repeatedly reported from two high-throughput screening studies: Cheng et al. screened 2 million compounds using the combined transglycosylase binding and activity assays to identify inhibitors with antimethicillin-resistant *S. aureus* (anti-MRSA) potency;[87] Huang et al. designed a quantitative FRET (fluorescence resonance energy transfer)-based HTS assay that measures the quenching of FRET signals between fluorescently labeled phosphate and pentapeptide of lipid II during the transglycosylation reaction.[88] Representative hits (compounds **42**-**31** and compound **24**) from these two independent reports exhibited a strikingly similar scaffold composed of a salicylanilide core with halogens or hydroxyl branches (Figure 4), and their activities were evaluated by K_i (competitive binding constant) values of 5.3 and 2.3 μM against *C. difficile* and MIC values of ∼1 μg/mL against Gram-positive strains including MRSA. Another hit (compound **25**) from the FRET screen has a much simpler chemical structure and a higher MIC value (32 μg/mL) against MRSA (Figure 4). Molecular docking of the salicylanilide-based inhibitor revealed a predicted binding site with ∼32% occupancy near the flat interface of the donor site, possibly forming a π−π stack with Y181 and extending to the hydrophobic patch by contacting the side chain of I180[87] (Figure 3a). Since no structural data are available to support this binding mode, other possibilities exist due to the concern that the flat interface is exposed and it may not serve as an optimal binding pocket (Figure 3a).

Moreover, an interesting modification of the high-throughput screen to discover the novel TG inhibitor was reported by Gampe et al. Instead of using the original moenomycin for their fluorescence polarization displacement assay, they used the fluorescently labeled moenomycin pharmacophore from which the inhibitors with lower affinities could also be identified.[89] The hit molecule, compound **10** (Figure 4), showed a good inhibition efficiency with K_i of 95 nM against *E. faecalis* PBP2a, an IC_{50} of 12 μM against *S. aureus* MgtA, and MIC values of 4−16 μg/mL against Gram-positive strains.[89] Again, being structurally distinct from moenomycin, compound **10** may not bind to TG in the same way as moenomycin, but it is more likely to adopt a similar binding mode than that of the salicylanilide derivatives.[87,88] The structural similarity among the above-mentioned novel inhibitors (compound **7**,[41] compounds **42**-**31**,[87] compound **24**,[88] and compound **10**[89]) that all have three aromatic rings arranged in a "V" shape (Figure 4) points to an intriguing possibility that these compounds may share a novel binding site with an unexpected inhibition mechanism. There may be a long way to go before a consensus chemical scaffold is found from these novel compounds; nonetheless, it may turn out to be a new class of TG-specific inhibitors.

In a more recent HTS report, Wu et al. developed an affinity-capture method using resin-immobilized *A. baumannii* PBP1b to screen small molecule libraries from natural products.[90] Among the four hits they identified, three are benastatin derivatives (compounds **11**−**13**) and one is an albofungin (compound **14**) with IC_{50} values of 9.9 μM against *A. baumannii* PBP1b (Figure 4). Surprisingly, the albofungin (compound **14**) was found to be significantly active against Gram-negative bacteria with an MIC of 1 μg/mL tested in *E. coli*, in comparison with the MIC of moenomycin A which is 128 μg/mL.[91] This finding is a breakthrough in the field, because all previously published inhibitors were only active against Gram-positive strains. The chemical basis of this feature may be explained by albofungin containing a primary amine (Figure 4), which was proposed to increase compound accumulation in Gram-negative cells.[92] Furthermore, it is noteworthy that K_i values of albofungin (e.g., 4.6 μM against *S. aureus*) are not correlated with its antimicrobial activities (MIC of 0.0008 μg/mL against *S. aureus*), implying there is an involvement of additional targets that may contribute to its low

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MICs in cell-based assays.[90] This is particularly interesting in the context of recent achievements in finding new proteins that interact with the aPBPs to form a supramolecular complex for cell wall synthesis, such as the transglycosylase FtsW,[13,24] the pyrophosphatase PgpB,[93] some bPBPs,[22,24] LpoB,[42] LpoP,[94] and other cofactors. Finally, their unique structure of a bulky planar molecule (Figure 4) may encourage further structural investigations to reveal their binding mode and inhibition mechanisms.

Current Challenges and Future Perspective. More than half a century has passed since the first discovery of the peptidoglycan glycosyltransferase in the 1960s;[95] there are still no clinically useful antibiotics specifically targeting this essential bacterial enzyme. Despite the difficulties in handling this essential protein and its special requirement of a membrane environment for activities, scientists have never given up the antibiotic development of bacterial transglycosylase (TG), hoping that TG inhibitors would become better antibiotics than the commonly used β-lactam-based TP inhibitors; the following reasons contribute to this promise: (a) the TG domain is relatively more conserved than TP, and the disaccharide moiety of its substrate has never changed among different bacteria; (b) TG-bound inhibitors may be less accessible to antibiotic destruction since TG is half-embedded in the membrane; (c) there is no report of naturally raised resistance against moenomycin.[7] Enormous efforts have been devoted to improve moenomycin and lipid II analogs as well as to identify new and more drug-like TG-specific inhibitors,[15,54] and now, we are at a crossroads with abundant information in each direction. While the low bioavailability of the moenomycin family of antibiotics remains a major obstacle, now, it could be bypassed by the newly identified nonlipophilic small-sized inhibitors.[41,66,67,87,89,90] However, the bottleneck in the current situation lies primarily in the structural studies in which the structures have yet to be determined with any of these novel inhibitors.

The lack of a novel-inhibitor-bound TG structure can be understood by several aspects. First, since the transmembrane helix of TG must be included to mimic the natural environment of an active transglycosylase,[41] the handling of a single-pass membrane protein has been shown to be difficult in crystallographic studies.[96] Besides, in order to obtain clear electron density of the ligand, the binding affinities with inhibitors cannot be too low.[97] Compounds with moderate affinities may fail to show up after structural determination, resulting from their low occupancies.[65] While increasing compound concentrations is usually useful to increase ligand occupancy in crystal structures, the presence of detergents and lipids in membrane protein crystallization may not be compatible with high concentrations of the ligand or DMSO used for dissolving the compounds.[98] However, these difficulties can always be overcome with a careful choice of inhibitors and accumulated experience in handling membrane proteins, and toward this goal, interdisciplinary joint efforts are especially important among structural biologists, biochemists, and synthetic chemists.

Another obstacle that hampers further improvements of TG inhibitors still lies in a limited understanding of its molecular mechanism. Even though the structural basis of the glycosyl transfer reaction seems clear, several aspects of the process have not been clarified. First, the oligomeric state of aPBPs has remained uncertain, and it may affect inhibitor binding. The multimodular aPBPs structures always exist as monomers,[34,35,39] which is biochemically conflicting with the finding that they readily form dimers in solution and maximal activities are achieved only in the dimeric state.[99,100] A dimeric TG domain of *A. aeolicus* PBP1a (PDB 2oqo) and even a trimeric *S. aureus* MgtA (PDB 3hzs) were reported;[31,40] however, clear steric clashes would be present if the TP domain was modeled onto these TG only structures,[40] leading to an argument whether this dimerization/trimerization is native or due to crystal packing. Therefore, a satisfactory solution to this problem is being awaited to discover some unknown factors that may give us new insights into designing TG inhibitors.

Second, a missing part of the mechanistic understanding of transglycosylastion is the role of the flap region, which is the least conserved and the most flexible region of the entire aPBP protein. It is, however, functionally indispensable because of its special location between the donor and the acceptor sites and half-embedded in the cell membrane[46] (Figure 1b). The poor understanding of the TG "flap" mainly results from its generally weak densities in crystal structures as it is loosely packed among the lipids or detergents.[34,35,39] If its intrinsic flexibility is actually the key to its catalysis-related functions, by either localized unfolding or a swaying movement to facilitate the translocation of lipid IV,[35,46] then a possible strategy for designing inhibitors would be to stabilize the "flap" and lock the enzyme in its reaction-intermediate stage.

Finally, as it is well-known that PBPs never work alone to achieve the high efficiency and accuracy of peptidoglycan biosynthesis during bacterial growth, it is now imperative to deepen our understanding about how all these different proteins collaborate with each other in a supramolecular machinery.[26,38] With new transglycosylases and division-regulatory proteins being continuously discovered in recent years,[13,14,94] many new questions are raised, such as how protein−protein interactions may generate a different molecular environment of the TG active site and maybe key information is neglected for the inhibitor design when we only study the isolated TG proteins. For structural studies of supramolecular complexes, the advanced cryo-EM (electron microscopy) technology may be suitable, although difficulties exist as most of the involved proteins in the peptidoglycan synthase complex are integral membrane proteins, which are challenging to obtain and handle. However, a breakthrough to get a more complete understanding of the complex machinery is indeed being anticipated, and it will likely provide new insights into targeting complexes of bacterial transglycosylase and possible other vulnerable sites for antibiotic development.

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Notes

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