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Mur Ligases as Potential Drug Targets in *Mycobacterium tuberculosis*. A review

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ABSTRACT

An inhibitor targeting a single-enzyme has high probability of being rendered ineffective due to gain of resistance caused by bacterial mutations. However, inhibitors that target more than one enzyme are likely to remain effective for longer duration, since bacteria may not be able to acquire resistance against such inhibitors by altering its multiple proteins that serve as the targets for the inhibitors. Hence multi-target inhibition offers to be a promising approach in the drug-discovery efforts. The peptidoglycan synthesis in *Mycobacterium tuberculosis* (Mtb) involves Mur ligases (MurC-MurF); key enzymes that catalyze the cytoplasmic steps of the Muramic acid synthesis pathway. Mur ligases are the enzymes that can be the potential targets for multi-target therapy since their mechanism of action is comparable and the residues in their active sites are highly conserved. Also, Mur ligases are promising target proteins for efficient structure-based optimization of inhibitors.

Keywords: Drug targets; Peptidoglycan; Mur ligases; Multi-target therapy

INTRODUCTION

Tuberculosis (TB) is since one of the lethal infectious disease that humanity continues to suffer. As per WHO estimates (1), approximately 10.4 million people are affected with TB. In spite of the availability of vaccine (BCG) and effective drug therapy, the pathogen continues to claim more lives than any other single infectious agent on the world's population. The key feature that contributes *M. tuberculosis* as successful pathogen is its unique characteristic lipid rich cell wall (2). The thick, rigid and waxy mycobacterial cell wall envelope consists of peptidoglycan-arabinoglactan polymers with outer lipid membrane enriched in mycolic acids, linked covalently to the arabinogalactan layer (2, 3). This complex structure of the cell wall makes conventional antimicrobial drugs nearly impermeable which limits the discovery of effective agents against tuberculosis (3). This mandates longer half-life of anti-*M.tuberculosis* drugs and/or a preventive therapy for complete clearance of bacteria from the host.

The genomic/post-genomic eras are witnessing a continuous expansion of database with respect to the genome sequences and protein structures. With the advancements in high-throughput experimental methods and screening techniques to analyze big data (whole genomes and proteomes), a wealth of information is now available on the gene-expression, regulation and protein–protein interactions in any organism (4). The availability of complete genome sequence of *M. tuberculosis* and various mycobacterium species was a major step towards expanding our knowledge about the bacterium, which further led to identification and detailed biological characterization of various genes and their proteins as antigens, diagnostic markers, vaccines, drugs targets for tuberculosis (5).

PEPTIDOGLYCAN BIOSYNTHESIS

Peptidoglycan (murein) is an essential component of the cell wall found on the outside of the cytoplasmic membrane of both Gram-positive and Gram-negative bacteria (6). Its main function is to preserve the integrity of the cell by withstanding the turgor pressure and to contribute towards the maintenance of cell shape (7, 8). Peptidoglycan also acts as a support to anchor several components (proteins and teichoic acids) of the cell envelop (9). Hence, an interference in its biosynthesis or its specific degradation can cause cell lysis.

The main structural features of peptidoglycan consists of linear glycan strands made up of alternating N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAM) residues linked via β (1 →4) linkages with side chains of amino acids cross-linked by trans peptide bridges (3).

The biosynthesis of peptidoglycan in *M.tuberculosis* begins in the cytoplasm with the formation of uridine diphosphate-N-acetylglucosamine (UDP-GlcNAc), catalyzed by the acetyltransferase and uridyltransferase activities of a bifunctional enzyme, GlmU (Rv1018c) (10). The UDP-GlcNAc is formed in two steps: (i) acetyl-CoA transfers its acetyl group to glucosamine-1-phosphate (GlcN-1-P) to produce N-acetylglucosamine-1phosphate (GlcNAc-1-P) and (ii) then UDP from UTP is transferred to from UDP-GlcNAc. The next step involves the generation of the UDP-N-acetylmuramic acid (UDP-MurNAc) catalyzed by MurA (Rv1315) and MurB (Rv0482) enzymes. Further addition of amino acids to UDP-MurNAc to form UDP-MurNAc-pentapeptide catalyzed by Mur ligases MurC (Rv2151c), MurD (Rv2155c), MurE (Rv2158c) and MurF (Rv2157c) in a sequential manner takes place (8). UDP-MurNAc-pentapeptide is then transferred to decaprenyl phosphate in the cytoplasmic membrane to form Lipid I by MurX (Rv2156c), also known as MraY (11). MurG (Rv2153c) then attaches GlcNAc from UDP-GlcNAc to Lipid I via a β (1→4) linkage between GlcNAc and MurNAc/Glyc to form Lipid II, the final monomeric unit of PG synthesis (12). Lipid II is translocated across the plasma membrane (by Lipid II flippase), where trans glycosylation and transpeptidation catalyzed by bifunctional PonA1/PBP1 and PonA2/PBP2 occurs to form the classical (3→4) cross-linkages between m-DAP and D-ala (13).

MUR ENZYMES of *Mycobacterium tuberculosis* as the POTENTIAL DRUG TARGETS

Mur pathway involved in the peptidoglycan synthesis is one such metabolic pathway that can be exploited for developing new drugs against *M.tuberculosis*. It involves the synthesis of UDP-MurNAc-pentapeptide and the enzymes that catalyze various step in Mur pathway are called Mur enzymes and are expressed during infection of the host (14, 15). The cytoplasmic phase of peptidoglycan synthesis can be divided into two sets of reactions: (i) formation of UDP-MurNAc from UDP-GlcNAc and PEP & (ii) assembly of the peptide stem leading to UDP-MurNAc-pentapeptide (7). In the subsequent stage, a five residue

polypeptide chain is formed by four ATP-dependent amide bond ligases by stepwise assembly of L-alanine (catalyzed by MurC), D-glutamic acid (catalyzed by MurD), a diamino acid, generally meso-diaminopimelic acid (catalyzed by MurE) and dipeptide D-Ala-D-Ala (catalyzed by MurF) onto the D-lactoyl group of UDP-MurNAc (14, 16) (Figure I).

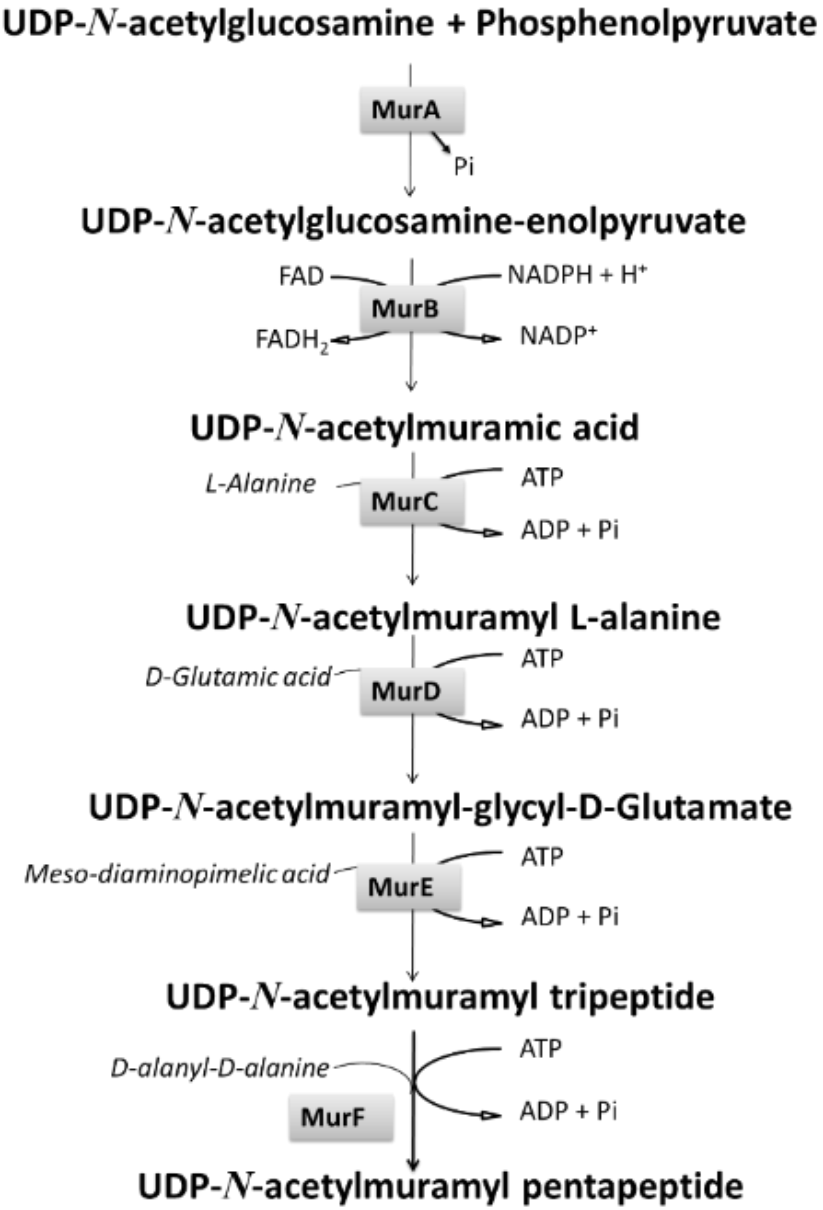

Figure I. Cytoplasmic steps of Mur pathway in *Mycobacterium tuberculosis*. (17)

Mur LIGASES

Mur ligases are good targets for the development of antibacterial because they are considered essential for bacterial survival (14, 16), are highly conserved and don't have counterparts in eukaryotic cells (18, 19). Their elucidated enzymatic mechanisms have been suggested to be suitable for high-throughput screening of potential inhibitors (14, 15, 18, 20). Position of genes coding for Mur ligases are close to each other in the cell division/cell wall (dcw) cluster and it is believed that their co-transcription may be important for mycobacterial growth. It is thought so since the cell elongation and formation of septum during the cell division requires proteins involved in peptidoglycan biosynthetic as well as in cell division (21).

Mur ligases catalyse the formation of an amide or a peptide bond with simultaneous ATPase activity. Mur ligases share three indistinguishable characteristics: (i) they generate an acyl phosphate intermediate and ADP; (22), (ii) contain six invariant residues and an ATP-binding consensus sequence (18, 19) and (iii) have similar native structures (N-terminal domain binds to UDP-precursor, central domain binds to ATP, and the C-terminal binds to the amino acid or dipeptide (14).

MurC

UDP-N-acetylmuramate-L-alanine ligase (MurC) adds the first amino acid, L-alanine (L-Ala) to form the peptide stem. L-alanine is the amino acid of choice in most bacterial species, is but in some cases, glycine or L-serine is used in place of L-alanine (23). The MurC enzyme of *M. tuberculosis* has shown greater specificity towards L-Ala under *in vitro* condition, than with Gly and L-Ser (21). ATP binds first to the enzyme followed by UDP-MurNAc binding and then the amino acid L-Ala. The ATP-binding site in the MurC enzyme lies at the interface between the second and the third domains with key interactions with the adenine ring and α- and β- phosphates (24). It has been shown that *E.coli* MurC enzyme has activity in both mono- and dimeric forms *in vitro* and exists in a balance between each form (25). As shown in Figure IIA, the interaction of loops from domain II and from domain I from different molecules results in the formation of the dimer interface.

However, the corresponding residues involved in interactions are not conserved in *M. tuberculosis*.

Several inhibitors have been reported against MurC enzymes. Benzylidene rhodanines have shown *in vitro* activity against MurC from *E.coli* with an IC_{50} values between 12 μM to 27 μM, but failed to show antibacterial activity against *E.coli*. These compounds have revealed selective inhibition against the Methicillin Resistant *Staphylococcus aureus* (MRSA) but were found to be cytotoxic to mammalian cells indicating its nonspecific mechanism of action (26). Using high-throughput screening, AstraZeneca tested their chemical library against MurC enzyme of *E.coli* and found a number of benzofuran acyl-sulfonamides as inhibitors of MurC enzyme with one compound showing an IC_{50} value of 2.3 μM. However, the inhibitor showed significant affinity to BSA too, hence proving to be nonspecific, off-target protein binding (27).

MurD

UDP-N-acetylmuramoyl-L-alanine-D-glutamate ligase (MurD) catalyses the addition of D-glutamate (D-glu) to UDP-N-acetylmuramoyl-L-alanine, the product of MurC enzyme (28). D-amino acids are one the most characteristic feature of the peptidoglycan, and might be providing resistance to degradation by external enzymes (29). MurD enzyme has been identified as one of the most promising target for the discovery of selective antibacterial agents (30, 31). It displays exceptional specificity for D-amino acids as its substrate (30, 20, 32) and catalyzes the regulatory step in the peptidoglycan synthesis required to maintain the relative thickness of the peptidoglycan layer. Furthermore, *M.tuberculosis* MurD is a substrate for regulation by PknA kinase, which is speculated to toggle between synthesis of peptidoglycan and the cell division process in mycobacteria (33). The catalytic mechanism of *E. coli* MurD is well established (15, 34). The mechanism of MurD reaction is through ATP dependent phosphorylation of the UDP-MurNAc-L-Alanine carboxylic acid, resulting in an acyl-phosphate intermediate (34). MurD from *M. tuberculosis* has been shown to be similar to *E. coli* MurD in both structure and function (35) with a protein sequence identity of 31% and similarity of 45%, respectively. Similar to MurC enzyme, MurD enzyme also has three domains, N-terminal, central and the C-terminal domains which binds UDP-MurNAc-L-Ala, ATP and D-glutamate, respectively (Figure IIB).

Using structure-based virtual screening Turk et al discovered 9H-xanthene derivatives and polycyclic compounds as the inhibitors of *E. coli* MurD enzyme with an IC_{50} of 10 μM (36). Another group designed N-benzylidene-sulfono-hydrazides against MurD enzyme from *E.coli* to imitate the diphosphate moiety of its substrate UDP-MurNAc-L-Ala which however showed weak inhibition but another class of inhibitors with sulfonohydrazone motif showed potent inhibitory activity with an IC_{50} of 30 μM (37, 38). Small-molecule inhibitors reported against MurD enzyme can be grouped as those with glutamic acid motif or without (39). Since glutamic acid is regarded as the major structural motif for inhibition of MurD enzymes, substitution in the other parts of the molecules have also been studied (40–42). Sulfonamides are also considered to be important class of MurD inhibitors and their X-ray co-crystal structure (MurD-sulfonamides) are also solved (43). The benzylidene-2,4-thiazolidin-dione and 2-thioxothiazolidin-4-one substituted glutamic acids are the other family of MurD inhibitors which was the first series of inhibitors developed containing both D-glu and L-glu residues (44, 45). Refinement in the chemical structures of these inhibitors by altering the aromatic rings and by insertion of a short linker between the glutamic acid part improved the activity of these inhibitors. The former modification showed IC_{50} of 3 μM and the latter showed IC_{50} of 10 μM against MurD enzyme from *E.coli* (45).

MurE

UDP-N-acetylmuramoyl-alanyl-D-glutamate-2, 6 diaminopimelate ligase (MurE) is the third ligase which adds mDAP (meso-diaminopimelic acid) in the growing peptide chain. MurE enzyme is the only Mur ligase that varies among bacteria with respect to its substrate-specificity. Classically, in Gram-negative bacteria and Mycobacteria, MurE incorporates m-DAP residue at the third position of the growing peptide while in Gram-positive bacteria, it adds L-Lys residue (16). This is considered critical because the third residue in the peptidoglycan is involved in cross-linkages between the glycan strands and therefore plays the crucial role in maintaining cell wall integrity. In fact, various studies showed morphological changes and bacterial lysis as a consequence of incorrect substitution (by another amino acid) (46, 47). Amongst the Mur ligases in *M.tuberculosis,* structure of only MurE is solved and the available X-Ray crystallographic structure is in complex with UDP-MurNAcL-Ala-D-Glu, product of MurD (48). *M.tuberculosis* MurE is

found to be structurally similar to other MurE ligases that contain the three domains. Domains I and II of the enzymes bind to UDP-MurNAc-L-Ala-D-Glu (UDP portion binds to domain I and the L-Ala-D-glu to domain II). Interestingly, topology of domain I of MurE from *M.tuberculosis* is closer to MurF structure from *E. coli* or *S. pneumoniae* than to MurE from *E. coli* (49). However, the degree of closure of the complex between the enzyme's active site with the substrate is similar to that of MurE-product structure in *E. coli* (Figure IIC). Multiple sequence alignment of MurE sequences from various bacterial species shows tetrapeptide sequences Asp-Asn-Pro-Arg (for m-DAP recognition) and Asp-Asp-Pro-Arg (for L-Lys recognition) to impart specificity of MurE enzyme to its amino acid substrate. (50, 51).

Sulfonamide molecules have been reported to inhibit MurE enzyme from *E.coli* with an IC_{50} of 181 μM (52). MurE enzyme from *M. tuberculosis* has been reported to be inhibited by a plant derived 3-methoxynordomesticine and another inhibitor with an IC_{50} of 75 μM has recently been discovered from a plant source (53, 54). Tetrahydroisoquinolines, a reported inhibitor of MurE from *M.tuberculosis,* has been found to affect the whole-cell phenotype of the bacterium (55). In a promising study, structure based virtual screening and molecular dynamics (MD) simulation targeting the active site of MurE followed by *in vitro* experiments, a lead molecule ((2R)-2-[[1-[(2R)- 2-(benzyloxycarbonylamino) propanoyl] piperidine-4-carbonyl]amino]-5-guanidino-pentan) was identified which showed antibacterial activity close to the antibiotics used in anti-bacterial therapy (56).

MurF

The MurF enzyme, UDP-N-acetylmuramoy-L-alanyl-D-glutamyl-2, 6-diaminopimelate-D-alanyl-D-alanyl ligase catalyses the addition of dipeptide D-Alanine-D-Alanine (D-Ala-D-Ala) to the UDPMurNAc-tripeptide. This step is critical for the peptidoglycan assembly as the peptide bond formed during MurF enzyme reaction energizes the process for cross-linking of glycan strands in the periplasmic space where ATP is limiting (57). Commonly, D-Ala-D-Ala is dipeptide but MurF from vancomycin-resistant strains of enterococci have been shown to utilize D-Ala-D-Ser and D-Ala-D-Lac as the common acquired resistance mechanism against glycopeptide antibiotics (58). Reports have also shown that in response to environmental stresses such as high CO_2 level, change in temperature and when bacteria enters into the stationary phase, MurF can also use D-methionine as its substrate (59, 60).

The crystal structure of MurF enzyme from *E.coli* shows it to have the three domain open structure with a unique N-terminal domain which consists of a small αβ fold, the central domain being conserved and the C-terminal domain that adopts Rossmann fold, respectively (Figure IID). Lack of information about the active site of *E. coli* MurF enzyme makes it difficult to understand the *M. tuberculosis* MurF enzyme in detail.

Abbott Laboratories published a set of MurF inhibitors with IC_{50} values of 1 μM and 8 μM, by using a technology based on affinity selection. Further, structure based optimization of the above inhibitors have yielded a compound with an IC_{50} of 22 nM but none of the these compounds showed significant antibacterial activity (61, 62). In an earlier study, Johnson & Johnson identified a series of thiazolyl aminopyrimidine inhibitors against MurF from *E. coli* and a compound in this series showed inhibition with an IC_{50} of 2.5 μM (63).

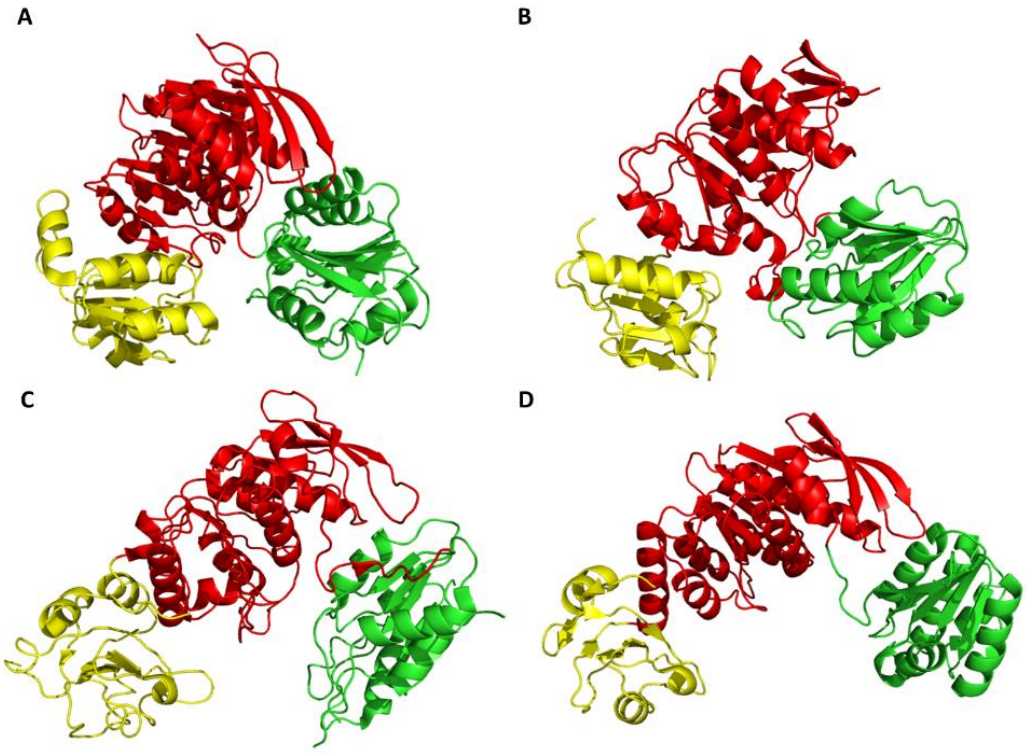

Figure II. Representation of the three-dimensional structures of Mur ligases. (A) MurC enzyme from *E.coli* (64)**. (B) MurD enzyme from *E.coli*** (28)**. MurE enzyme from *M.tuberculosis*** (48)**. MurF enzyme from *E.coli*** (49)**.**

Inhibitors with more than one target, their probability to remain effectual for longer duration is high since to acquire resistance against such inhibitors, bacterial population would have to evolve to alter multiple proteins (drug-targets) at the same time, which is less likely. Hence multi-target inhibitors offer great worth in the drug-discovery efforts. From our study, Mur ligases are the enzymes that can be the potential targets for multi-target therapy. Also, since these enzymes exhibit the same catalytic mechanism, development of molecules that efficiently target multiple Mur enzymes gets possible.

Phosphinate inhibitors which imitate the tetrahedral transition state of phosphoryl transfer in Mur ligases are the first class of inhibitors designed against Mur ligases (39). Biochemical characterization of these inhibitors showed that they acts as a mixed-type inhibitor with respect to all three of its enzyme substrates (65–69). MurC enzyme from *E.coli* has been shown to be inhibited by phosphinate inhibitors with an IC_{50} of 49 μM (65, 66). Inhibitors designed against MurD enzyme contain the glutamic acid motif and showed inhibitory activity with an IC_{50} of 680 nM (67). An IC_{50} of 1.1 μM was obtained when MurE enzyme from *E.coli* were tested against these inhibitors (69) and amino alkyl phosphinates designed against MurF enzyme from *E.coli* operated as reversible inhibitors (K_i between 200 μM to700 μM) (68). However, antibacterial activity was not reported for the phosphinate compounds, which could be attributed to their inefficiency in penetrating the membrane or their failure to accumulate up to the required inhibitory concentrations inside the cell. N-benzylidene sulfonohydrazide-based compounds designed against *E.coli* showed inhibition as a dual inhibitor of MurC and MurD enzyme (26). Similarly, feglymycin, has been identified as a reversible inhibitor of MurA and MurC enzymes in *E.coli* (70). In a significant study, Tomasic et al, 2010 (71) synthesized a series of hydroxy-substituted 5-benzylidenethiazolidin-4-ones and examined their potential to inhibit Mur ligases. One compound from this series was found to be active against MurD, MurE and MurF within IC_{50} values of 2-6 μM, hence appeared as an encouraging multitarget inhibitor. In another report, the naphthyl tetronic acids were identified as multiple inhibitors of MurA-E enzymes with IC_{50} values in the μM (72) by using Wyeth bacterial cell-wall program. Phosphorylated hydroxy ethyl amines, another class of compounds which are developed as the inhibitors of Mur ligases in *E.coli*, represented promising hit compounds at micromolar concentrations (73). Recently a set of benzene-1,3-dicarboxylic acid 2,5-dimethylpyrrole derivatives and furan-based benzene mono- and

dicarboxylic acid derivatives, showing inhibitory effects on Mur ligases from *E.coli* have been reported (74, 75). Two compounds from furan based mono- and dicarboxylic acid derivatives showed inhibition against MurE and MurF ligases of *M.tuberculosis* (17). In another study, docking analysis of four plant compounds Isojuripidine, Atroviolacegenin, Porrigenin B, and Nummularogenin showed their potential multi-target inhibitors on Mur enzyme from *Enterobacter aerogenes* (76).

CONCLUSIONS

The Mur ligases represent attractive targets for anti-tuberculosis drug development since (i) they belong to the same enzyme superfamily (ATPases), (ii) have similar structures with three domains where the N-terminal binds to the nucleotide precursor, central domain to ATP, and the C-terminal to amino acid or dipeptide and (iii) have common mechanism of action. So far, drugs targeting Mur ligases have not been reported for clinical use. To conclude, this review gives an update on structural characteristics of Mur ligases and on the inhibitors reported against them.

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