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Recent developments on UDP-N-acetylmuramoyl-L-alanine-D-gutamate ligase (Mur D) enzyme for antimicrobial drug development: An emphasis on in-silico approaches

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ABSTRACT

Introduction: The rapid emergence of antibiotic resistance among various bacterial pathogens has been one of the major concerns of health organizations across the world. In this context, for the development of novel inhibitors against antibiotic-resistant bacterial pathogens, UDP-N-Acetylmuramoyl-L-Alanine-D-Glutamate Ligase (MurD) enzyme represents one of the most apposite targets.

Body: The present review focuses on updated advancements on MurD-targeted inhibitors in recent years along with genetic regulation, structural and functional characteristics of the MurD enzyme from various bacterial pathogens. A concise account of various crystal structures of MurD enzyme, submitted into Protein Data Bank is also discussed.

Discussion: MurD, an ATP dependent cytoplasmic enzyme is an important target for drug discovery. The genetic organization of MurD enzyme is well elucidated and many crystal structures of MurD enzyme are submitted into Protein Data bank. Various inhibitors against MurD enzyme have been developed so far with an increase in the use of *in-silico* methods in the recent past. But cell permeability barriers and conformational changes of MurD enzyme during catalytic reaction need to be addressed for effective drug development. So, a combination of *in-silico* methods along with experimental work is proposed to counter the catalytic machinery of MurD enzyme.

1. Introduction

The expeditious emergence of antibiotic resistance strains within microbes because of the progressive and extensive utilization of antibiotics has been one of the major concerns of the healthcare system across the globe (Klugman and Black, 2018). It has jeopardized the traditional means of successful prohibition and cure of a range of infections caused by various pathogens (Li and Webster, 2018). World Health Organization (WHO) has stated antimicrobial resistance - as one of the most high-priority threats of the current time and has asked to submit the antimicrobial surveillance reports from different countries in 2020 for input to its Global Antimicrobial Resistance and Use Surveillance System (GLASS) (www.who.int). In the contemporary scenario, there is an intense demand for the development of advanced inhibitors with an innovative mode of action Liu et al. (2019). The existence of a trivial number of efficacious therapeutic targets has resulted in an inadequate number of antibacterial agents scare to deal with escalating challenges of multi-drug resistance among bacterial pathogens (Liu et al., 2017). In this context, for the development of novel antibacterial agents, screening of a large number of compounds against newer targets will surely play a pivotal role.

The enzymes catalyzing the various steps of bacterial cell wall biosynthesis have been one of the most substantiated targets for the discovery of novel drugs. (El Zoeiby, Sanschagrin and Levesque, 2003). The bacterial cell wall is made up of peptidoglycan polymer which acts as mechanical endurance in addition to supporting the cell exterior efficiently (Barreteau et al., 2008). Apart from being indispensable, peptidoglycan is exclusively found in the bacterial world, contriving it an appropriate target for the design and development of novel antibacterial agents to target critical bacterial biosynthetic pathways (Vollmer et al.,

Abbreviations: PG, Peptidoglycan; UDP-GlcNAc, UDP-N-acetylglucosamine; UDP-MurNAc, UDP-N-acetylmuramicacid; UDP-Mpp, UDP-N-acetylmuramylpentapeptide; PEP, Phosphoenolpyruvate; UNAG, UDP- N-acetylglucosamine; UMA, UDP N-acetylmuramoyl-l-alanine; HTS, High Throughput Screening; PDB, Protein Data Bank; MD, Molecular Dynamics; SAR, Structural Activity Relationship; MIC, Minimum Inhibitory Concentration.

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2008). The structure of peptidoglycan consists of linear chains of alternate units of *N*-acetylglucosamine and *N*-acetylmuramic acid (Sibinelli-Sousa et al., 2021). These are cross-linked by small pentapeptides that are attached to muramyl residues directly or through other short peptides - forming a mesh-work. (El Zoeiby, Sanschagrin and Levesque, 2003). The biosynthesis of peptidoglycan has been well characterized in recent literatures (Ogasawara and Dairi, 2021). The biosynthesis of peptidoglycan takes place in three sections of the cell i.e., cytoplasm, membrane, and periplasm, catalyzed mostly by enzymes acting in sequential order (Miyachiro et al., 2019).. Lipid II is the major constituent of peptidoglycan that is synthesized through the activity of Mur enzymes in the cytoplasm of the cell (Miyachiro et al., 2019). After synthesis, the enzyme called flippases catalyze the movement of Lipid II to the flip side of the bacterial membrane where transpeptidation and glycosyltransferase reaction are carried out through the activity of penicillin binding proteins (pbp) (Miyachiro et al., 2019).

UDP-N-acetylglucosamine (UDP-GlcNAc) is the starting precursor for the cytoplasmic stages of peptidoglycan synthesis resulting in the formulation of UDP-MurNAc, the reaction being catalyzed by enzymes, UDP-N- acetylglucosamine enolpyruvyl transferase (MurA) and UDP-N-acetylenolpyruvylglucosamine reductase (MurB) (Miyachiro et al., 2019). MurA brings about the transference of the enolpyruvyl group from its substrate phosphoenolpyruvate (PEP) to UDP-N-acetylglucosamine (UNAG) resulting in the formation of UDP-N-acetylglucosamine enolpyruvate (UNAGEP) (Miyachiro et al., 2019). The succeeding step involves the reduction of UNAGEP through the activity of MurB by utilizing NADPH, resulting in the formation of UDP-MurNAc(Barreteau et al., 2008). The successive addition of peptides takes place by the activity of four ATP-dependent ligases, MurC, MurD, MurE, and MurF (Jha et al., 2020). These enzymes catalyze the addition of L-Ala, D-glutamic acid (D-Glu), meso-diaminopimelic acid (m-DMPA) (or L-Lys), and D-Ala--D-Ala respectively to UDP-N acetylmuramic acid (UDP-MurNAc) (M. A. Azam and Jupudi, 2019).

Mur ligases (C, D E & F) present as one of the appealing groups of targets that can be employed for the discovery of newer drugs (Hrast et al., 2014). Since the last decade, a wide range of studies has been done on Mur ligases, resulting in the finding of a novel and varied group of inhibitors. All the Mur ligases have a common mechanism of action and an organized kinetic mechanism (Hrast et al., 2019). Also, all the Mur ligases have a highly conserved ATP binding site having similar sequences which varies in the range of 22–26%. Other common features among Mur ligases include the presence of a P-loop having an abundance of glycine residues and varied residues like glutamic acid and histidine involved in the regulation of Mg^{2+} ions (El Zoeiby et al., 2003). Despite these similarities, there are differences in the configurational topology and arrangement of residues present in the catalytic site of MurD from diverse groups of bacterial species like *Staphylococcus aureus*, *Borellia burgdoferi*, *Escherichia coli (E. coli)*, *Mycobacterium tuberculosis* and, many more (M. A. Azam and Jupudi, 2020).

MurD enzyme along with other Mur ligases plays an important role in the synthesis of peptide stem of N-acetylmuramic acid during cell wall synthesis. The structure and mechanism of action of the MurD enzyme have been characterized thoroughly (Zdouc et al., 2018). It is the second enzyme in the series of Mur ligases that catalyzes the joining of D-glutamic acid residue to its substrate UDP-MurNAc-l-Ala (UMA) which includes acyl-phosphate and tetrahedral intermediates (El-Sherbeini et al., 1998). MurD enzyme is highly specific for its substrate D-glutamic acid and its homologue is not present in mammalian cells making it an excellent target for the design of novel inhibitors (Perdih et al., 2014).

High throughput virtual screening (HTS) of compounds for drug discovery has entrenched with the exploration of bacterial genome sequence information in the earlier stages of the 1990s along with the accessibility of substrates and enzymes taking part in earlier steps of cell wall biosynthesis (Bugg et al., 2011). Computer-assisted drug discovery has become a leading technology in the modern drug development process. This procedure helps in making effective and efficient use of chemical modifications. Structure-based molecular docking particularly employs molecular docking procedures (Talevi, 2018). For the identification and development of lead compounds against the potential targets for drug discovery, there has been a gradual increase in the use of computer-aided drug design methods. (Turk et al., 2009). Based on binding sites, molecular docking includes virtual screening of large chemical libraries to provide potential drug candidates. Molecular simulations give details about the structural and thermodynamic characteristics of target proteins on various levels that help in recognizing drug binding sites and illustrating the mechanism of action of the drug (Lin et al., 2020).

In the past several comprehensive reviews demonstrating crucial features of the MurD enzyme along with advances made in the development of inhibitors have been published (M. A. Azam and Jupudi, 2020; Šink et al., 2013). The present review gives a comprehensive insight into the structural and functional characteristics of the MurD enzyme and gives an information about the various structures of the MurD enzyme from different bacteria developed and inducted into Protein Data Bank so far along with the recent development of inhibitors against MurD enzyme with an emphasis on *in-silico* methods.

2. Genetic aspects and regulation of MurD enzyme

The D-glutamic acid adding enzyme (MurD enzyme) is encoded by the MurD gene. This gene is found to be positioned in the 2-min region of the *E. coli* genome which contains an assemblage of genes of pbpB (penicillin-binding protein B) to envelope A (envA). The order of genes in this region is pbpB-murEmurF-X-murD-Y-murG where X and Y chromosomal fragments code for unknown proteins and E and F genes are coupled together. Earlier MurD enzyme was contemplated to be a product of MurG gene but later it was found that MurD gene was positioned 2.5 kb upstream of murG gene (Mengin-Lecreulx et al., 1989). This 2-min region in *E. coli* is assigned as mra (from murein A)cluster. Seven peptidoglycan synthesizing (murein) genes including Mur ligases, mraY and ddl are located in this cluster along with genes associated with cell division (fts genes) and genes for synthesis of lipopolysaccharide (envA) (Hara et al., 1997). In *Mycobacterium tuberculosis*, the location of MurD gene is also reported to be present in a cluster of genes positioned near genes like ftsW that are involved in cell division. There is 64% homology within MurD gene of *E. coli* and *M. tuberculosis* (Thakur and Chakraborti, 2008). Gram-negative bacteria *E. coli* and *Haemophilus influenzae* have a conserved series of genes in the entire mra cluster. However, in Gram-positive bacteria, there are slighter variations in the position of genes. The pbp1 succeeds mraY in *Staphaureus*, *Enterococcus faecalis*, and *Streptococcus pyogenes*, while the series of genes murD, murG, divIB, ftsA, ftsZ remains unchanged in *E. faecalis*, *E. hirae*, and *St.. pyogenes* (Watanabe et al., 1997). Location of Mur D gene in *Staph aureus* was displayed by sequence analysis and was found to be positioned in an array of genes present in the order of pbp1 (encoding penicillin-binding protein 1), mraY, murD, divIB, ftsA, ftsZ, orf, divIVA, ileS (encoding isoleucyl tRNA synthetase). Compared *to E. coli*, these genes were supposed to be part of the same operon as were transcribed by the identical portion of DNA and carried short intergenic sections (El-Sherbeini et al., 1998). Three genes present in the 4.4 kb region of *Bacillus subtilis* chromosome-map between the distal portion of spoVD promoter sequence and start of spoVE gene were found to have sequence homology with the genes murE, mraY and murD present in *E. coli* (Daniel and Errington, 1993). Several genes present in the 133″ regions of *B. subtilis* are known to have functional similarity with the cluster of genes present in the *E. coli* 2min region. *B. subtilis* chromosome apart from having a similar series of genes to that of *E. coli*, differs in the absence of three genes and presence of more comprehensive intergenic regions in *B. subtilis* genome as compared to *E. coli* (Daniel and Errington, 1993).

A single mra promoter controls the transcription of genes contained in mra operon of *E. coli* including the murD gene. Studies on mutant *E. coli* strains have shown that reduced expression of murD gene in the mra

promoter results in lysis of the bacterial cell displaying the essential nature of MurD gene for bacterial survival (Hara et al., 1997). Serine/threonine protein kinases were found to have a potential role in the regulation concerning biosynthetic machinery of peptidoglycan in mycobacteria as were found to phosphorylate the MurD enzyme. The concentration of MurD enzyme and incubation time were found to play a major role in the phosphorylation reaction catalyzed by the labelled Serine/threonine protein kinase (PKnA) (Thakur and Chakraborti, 2008).

MurD gene of *Staph aureus* contains an open reading frame of 449 amino acids (El-Sherbeini et al., 1998). A significant level of homology exists between MurD protein from *Staph. aureus* and that of the other bacteria i. e*E.coli* (54%), *H. influenzae* (55%), *B. subtilis* (65%) and *Strep. pyogens (*66%). Numerous residues present in the homologous regions of MurD enzyme are conserved among various groups of bacteria, which also includes the region of the C-domain containing the ATP binding site (El-Sherbeini et al., 1998).

All the base sequences in the mra region of 12 kb chromosomal portion containing genes for Mur ligases E, F and D, mra Y, and MurG have been elucidated (Fig. 1.) (Ikeda et al., 1990). In *E. coli*, MurD enzyme encoding gene is about 2.6 kb and its location on chromosome map is in between the murF and ftsW genes at 2min region (Genebank ID 944818). Elucidation of sequences showed the occupancy of two open reading frames in this region, one encoding for Mur D enzyme and another is open reading frame Y located downstream to MurD. The Open reading frame of MurD consists of 1314 bp and encodes a protein of 438 amino acids with a molecular weight of 46938 (Ikeda et al., 1990).

In multi drug resistance strains of *Enterococcus faecalis*, antibiotic resistance genes are present in 3 clusters namely C1, C2 & C3. The 6 mur enzymes $murA_B$, murB, murC, murD, murE and murF are present in Clusture C1 and play a role in peptidoglycan biosynthesis (Naha, A et al., 2020).

3. Functional aspects of mur D enzyme

The crystal structures of MurD enzymes with various ligands have been elucidated from various bacteria e.g. *E. coli* (Bertrand et al., 1999), *Thermotoga maritime* (Favini-Stabile, Contreras-Martel, Thielens and Dessen, 2013), *Streptococcus agalactiae* (serogroup V) by Stein et al. in 2010. Apart from that, several structures have been generated by homology modeling of various pathogenic bacterial species *Staph aureus* (M. A. Azam and Jupudi, 2019), *Leptospira interrogans* (Amineni et al., 2010)) to develop potential inhibitors by utilization of *in-silico* tools. But the most earlier and extensive work has been done on *E. coli* MurD in last couple of decades (Bertrand et al., 1997; Humljan et al., 2008; Kotnik et al., 2007). MurD enzyme (UDP-*N*-acetylmuramyl-L-alanine:D-glutamate ligase), has a molecular weight of 46,973 Da and is the second enzyme in the series of Mur ligases (M. A. Azam and Jupudi, 2020). MurD brings about the addition of D -glutamic acid (d-Glu) residue to its substrate UDP-N-acetylmuramyl-l-alanine (UMA) which leads to the creation of a peptide bond joining the amino residue of D-glutamic acid and the carboxyl group of UDP-N-acetylmuramyl-L-alanine.The reaction results in the utilization of an ATP molecule with the production of an ADP and release of the orthophosphate group (Walsh et al., 1999).

Resolution of crystal structures of associations of MurD enzyme from *E. coli* with several ligands and their products like quaternary complex of MurD enzyme with UMA, ADP and Mg^{2+} ion and Mn^{2+}ion, product ADP and UMA and binary complex of the enzyme alongside the product UDPN-acetylmuramoyl-L-alanine-D-glutamate (UMAG) were studied in order to understand the mechanism of action (Bertrand et al., 1999). The mechanism of catalysis involves the initial phosphorylation of carboxylic acid at C-terminal position of substrate UMA by the gamma phosphate group of ATP with the generation of acyl phosphate intermediate. Nucleophilic attack by the amino group of the inbound D-Glutamic amino acid on acyl phosphate results in the formation of a high-energy tetrahedral intermediary that is finally converted to the amide product and subsequently inorganic phosphate is released (Fig. 1.) (Bertrand et al., 1999; Humljan et al., 2008). The primary phosphorylation of the substrate UMA takes place in the cleft present in between the central and C-terminal domains. Entrance of reactive part of UMA into the cleft occurs close to the N-terminal domain and the ATP molecule enters from the opposing end (Hrast et al., 2019).

Two bivalent cations (Mn^{2+}& Mg^{2+}) are needed by the MurD enzyme to transmit a phosphoryl group amongst a pair of anionic substrates (Bertrand et al., 1999). These divalent cations are not required for the binding of the substrates UMA and ADP, but play a crucial role in the ligase action of the MurD enzyme (Kotnik et al., 2007).

The principle residues of the MurD enzyme of *E. coli* which take part while interacting with the substrate UMA involves amino acid residues Leu15, Thr16, Asp35, Thr36, Arg37, Gly73, and Asn138, while His183 interacts with Mg^{2+}(Bertrand et al., 1997, 1999, 2000; Bertrand et al.,

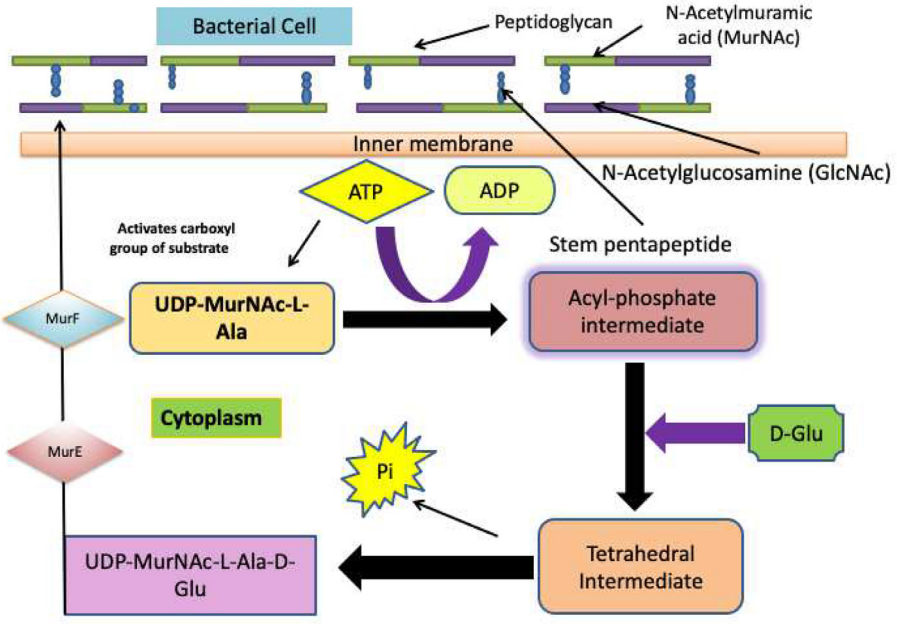

Fig. 1. Mechanism of action of MurD enzyme.

1999). At the same time, ADP interacts with Gly114, Lys115, Ser116, Thr117, Asn271, Arg302, and Asp317 residues. Interaction of D-glutamic acid includes residuesThr321, Lys348, Ser415, Leu416, and Phe 422 (Bertrand et al., 1999) (Table 1).

Closed configuration of MurD crystal structure obtained by interaction with enantiomeric Glutamic acid derivatives showed an 180^0 turnover of Leu13-Gly14 and Pro41-Gly42 bonds resulting in a small movement of N-terminal domain from Leu13 to Val150. Minute contortion of the central domain also takes place particularly at Ile139 and Gly140 amino acids as a result of the binding of inhibitors in the enzyme active site (Kotnik et al., 2007). After hydrolysis of ATP molecule, the resulting ADP molecule binds to P-loop of the enzyme molecule, consisting of residues from 108 to 116 forms a part of mononucleotide -binding fold. The ADP molecule binds within an area located in the middle of the central and C-terminal domains. The studies on D-glutamic acid-based inhibitors have, shown that most of the hydrogen bonds with the amino acids present in the active site of the MurD enzyme are formed by the D-Glu portion of the inhibitor that inhabits the binding site of the D-Glutamic- acid region and the heterocyclic ring occupying the uracil-binding region (Tomašić et al., 2011). The MurD enzyme is specific for its substrate D-Glutamic acid which is displayed by its interaction with its product UMAG (PDB 4UAG), in which the carboxylic group at alpha position of D-Glu forms hydrogen bonds with Thr321 residue and interacts in a charge based manner with nitrogen of Lys348 residue while the γ-carboxylate is hydrogen-bonded to amino acid residues Ser415 and Phe422 (Humljan et al., 2008). MurD enzymes from gram-negative bacteria, *E. coli*, and *H. influenzae* exhibit feedback inhibition by the substrate UMA when the concentration exceeds the 15 and 30 μM level while MurD enzymes from gram-positive bacteria *E. faecalis* and *S. aureus* have little effect on UMA concentration (Table 2.) (Walsh et al., 1999).

4. Structural features & physiochemical properties of MurD

So far, 25 crystal structures of the MurD enzyme have been elucidated and submitted to the PDB (Table 3). The crystal structures from many bacterial species like*E.coli* (Zidar et al., 2011) (Šink et al., 2016)*Thermotoga maritima* (Favini-Stabile, Contreras-Martel, Thielens and Dessen, 2013), and *Streptococcus agalactiae* serogroup V by Stein et al., 2009) in both open and closed forms have been resolved so far (Fig. 2.). Considerable resemblances were observed in the domain organization of these crystal structures. Three globular domains i.e. N-terminal domain, central domain and C-terminal domain constitute the binding pocket of MurD enzyme (Bertrand et al., 1997). N-terminal domain is attributed for binding of UDP component of UMA. The central domain acts as ATP binding region and is conserved in the entire Mur enzyme family. The C-terminal domain takes part in the binding of D- Glutamic acid. The cleft between the central and C-terminal domains forms the active site of the MurD enzyme (M. A. Azam and Jupudi, 2017). N-terminal domain involves amino acid residues from 01 to 93. It is composed of five lateral strands of B-sheet encircled by a quartet of α-helices. The C-terminal domain comprehend 94 to 298 amino acids and is composed of a β-sheet of parallelly organized six strands enclosed by seven α-helices and an antiparallel β-sheet consisting of three strands (Bertrand et al., 1999).

Table 1
Amino acid residues present at various domains of MurD enzyme.

Domain	Binding substrate	Amino acid Residues	References
N-terminal domain	UMA	Val18, Thr19, Asp39, Asp40, Gly75, Asn147, Gln171 &His192	Kotnik et al., 2007
Central domain	ATP	Gly123, Lys124, Thr125, Thr126, Glu166, Asp283, and Arg314	Mustafa Alhaji Isa.,2019
C-terminal domain	D-Glutamic acid	Arg382, Ser463, and Tyr470	Mustafa Alhaji Isa.,2019

Table 2
Relative efficiencies of MurD enzymes from various bacterial species.

Organism	KmATP (μM)	Km UMA (μM)	Km D-Glu (μM)	References
Strep. pneumoniae (MurD)	2000 (Approx)	96 ± 39	190 ± 26	H.Barreteau et al., 2012
Borellia burgdoferi (MurD)	53 ± 12	63 ± 30	110 ± 29	H.Barreteau et al., 2012
Myco. tuberculosis (MurD)	710 ± 290	340 ± 10	700 ± 180	H.Barreteau et al., 2012
E.coli (MurD)	97 ± 9	7 ± 0.6	42 ± 5	M.Simčič et al., 2014
Staph.aureus (MurD)	5400	41	100	H.Barreteau et al., 2012
E.faecalis (MurD)	47 ± 4	36 ± 7	118 ± 14	Walsh et al. (1999)
Haemophilus influenzae (MurD)	102 ± 6	8 ± 4	169 ± 20	Walsh et al. (1999)

During the catalytic reaction of MurD enzyme, there are successive changes in the morphology of these domains from open to closed states through an intermediate semi-closed position. Obliteration of domain motion of each ligand takes place following each stage of ligand binding. During the catalytic reaction, the apo form of the MurD enzyme exhibits twisting as well as opening, and closing of its domains. Twisting is repressed as a result of the binding of ATP while binding of inhibitor results in suppression of open-closed method (Nakagawa et al., 2021). *E. coli* MurD enzyme shows two open configurations, one in the free state and the other bound to its substrate UMA (Šink et al., 2016). Based on NMR spectroscopy and X-ray crystallography studies on the manners of binding of inhibitors to MurD catalytic site a new crystal structure of MurD (2XPC) was resolved with the inhibitor 4-aminocyclohexane-1, 3-dicarboxyl.A charge-based interaction was observed by the contribution of the carboxyl group placed at position 3 in the cyclohexyl ring with the nitrogen atom of Lys348 (Sosič et al., 2011).

During the catalysis reaction, the MurD enzyme exhibits a semi-closed form. The MurD enzyme undergoes several changes in its shape because of the attachment of its ligand that in turn controls ligand binding sequence and also regulates its binding affinity to the succeeding ligand (Saio et al., 2015). C-domain of MurD enzyme assumes most distinct conformations compared to other domains. The C-terminus of the MurD enzyme exhibits the most flexibleness and does not collude with the central domain and in the same asymmetric section displays two non-identical configurations (Favini-Stabile et al., 2013).

So far, work conducted on crystallographic structures of MurD enzyme suggested a possible configurational drift of the C- terminal domain to the center of the structure. This change is due to binding of the ligand molecule, resulting in the closing of the enzyme (Bertrand et al., 1999, 2000). In open structures of MurD enzyme (pdb:1EEH) greater energy is required for closure of the C-terminal domain when its location is outside the plane of the central and N-terminal domain as compared to the open crystal structure when the C-terminal domain is restricted within the plane of these domains (PDB:1EOD) (Perdih et al., 2014).

The NMR spectrum has shown that domain 3 of the MurD enzyme undergoes drastic conformational changes during its catalytic process. During its Apo state, the MurD enzyme shows a shifting from open to close form (Saio et al., 2015). In contrast, the closed configuration of the enzyme is sustained by coupling of the ligand molecule (PDB IDs 2X5O and 2JFF). The progression in which the various ligands bind the MurD enzyme includes the initial binding of ATP in the ubiquity of Mg^{2+} that is followed by the binding of the UMA, succeeded by the hydrolysis of the ATP molecule. At the end, binding of D-Glutamic acid takes place (Saio et al., 2015).

E.coli MurD enzyme showed sequence identities of 31% with Bacillus subtilis and 62% with Haemophilus influenza respectively (Bertrand et al., 1997). Conserved amino acid sequences are also reported between E. coli and *Staphylococcus aureus* (Bouhss et al., 1999). Amino acid

Table 3
PDB entries of MurD enzyme.

S. No	PDB Entry	Ligand/s	Resolution (A°)	Organisms	References
1.	1UAG	UMA[a], SO_4	1.95	*E.coli*	Bertrand et al. (1997)
2.	2UAG	UMA,ADP[b], Mg^{+}ion	1.7	*E.coli*	Bertrand et al. (1999)
3.	3UAG	UMA,ADP, EPE[c], Mg^{+}ion	1.77	*E.coli*	Bertrand et al. (1999)
4.	4UAG	UMA, sulphate ion	1.66	*E.coli*	Bertrand et al. (1999)
5.	1EEH	UMA	1.9	*E.coli*	Bertrand et al. (2000)
6.	1E0D	Sulphate ion	2.4	*E.coli*	Bertrand et al. (2000)
7.	2JFF	LK2[d],SO_4	1.89	*E.coli*	Kotnik et al., 2007
8.	2JFG	UMA,ADP,SO_4	1.52	*E.coli*	Kotnik et al., 2007
9.	2JFH	LK1[e],SO_4	1.97	*E.coli*	Kotnik et al., 2007
10.	2UUP	LK4[f],SO_4	1.88	*E.coli*	Humljan et al., 2008
11.	2JFH	LK1,SO_4	1.97	*E.coli*	Kotnik et al., 2007
12.	2VTE	LK4,SO_4	2.2	*E.coli*	Humljan et al., 2008
13.	2VTD	LKM[g],SO_4 ion	1.94	*E.coli*	Humljan et al., 2008
14.	2UUO	LK3[h],SO_4	2.5	*E.coli*	Humljan et al., 2008
15.	2WJP	D17[i], azide ion, Cl^{-}ion, DMS[j]	1.6	*E.coli*	Tomašić et al., 2010
16.	2X5O	VSV[k],SO_4,SO_3,Azide, Cl ion	1.46	*E.coli*	Zidar et al. (2010)
17.	3LK7	Cl & SO_4 ion	1.5	*Strep.agalactiae*	Stein et al., 2009
18.	2Y68	DMS, Azide ion & Cl ion	1.49	*E.coli*	Tomašić et al., 2011
19.	2Y66	NO_4, sulphate ion, sulphite ion, DMS,Cl	1.49	*E.coli*	Zidar et al. (2011)
20.	2Y67	N21[l], sulphate ion	1.85	*E.coli*	Zidar et al. (2011)
21.	2Y10	DMS,SO_4	1.49	*E.coli*	Tomašić et al., 2012
22.	4BUC	Glycerol,PO_4,PO_3, Cl ion,NH_4^{+}ion	2.17	*T. maritima*	Favini-stabile et al. (2013)
23.	2XPC	051[m],DMS,SO_4,Cl^{-} ion	1.49	*E.coli*	Sosic et al.,2013
24.	5A5E	UMA,ADP, Malonate ion	1.9	*E.coli*	Sink et al.,2016
25.	5A5F	UMA,ADP	1.9	*E.coli*	Sink et al.,2016

[a] UMA: UDP –N-acetylmuramoyl-L-alanine.
[b] UDP:Uridine -5′-Diphosphate.
[c] EPE: 4-(2-Hydroxyethyl)-1-Pipeazine ethanesulphonic acid.
[d] LK2: N-[(6-Butoxynapthalene-2-YL)sulfonyl]-D-Glutamic acid.
[e] LK1:N-[(6-Butoxynapthalen-2-yl)sulfonyl]-L-Glutamic acid.
[f] LK4: N-({6-[4-cyanobenzyl)oxy]Naphthalen-2-yl}phonyl)-D-Glutamic acid.
[g] LKM:N-({6-[4-cyano-2-fluorobenzyl)oxy]Naphthalen-2-yl}sulfonyl)-D-Glutamic acid.
[h] LK3: N-{[6-(Pentyloxy)Naphthalen-2-yl]sulfonyl}-D-Glutamic acid.
[i] D17: N-({3-[({4-[(Z)-(4-oxo-2-thioxo-1,3-thiazolidin-5-ylidene]methyl]phenyl}amino)methyl]phenyl}carbonyl)-L-Glutamic acid.
[j] DMS:Dimethyl sulphoxide.
[k] VSV: N-({3-[({4-[(Z)-(2,4-dioxo-1,3-thiazolidin-5-ylidine)methyl]phenyl}amino)methyl]phenyl}carbonyl)-D-Glutamic acid.
[l] N21: (2R)-2-[[4-[[4-(Z)-(2,4-dioxo-1,3-thiazolidin-5-ylidine)methyl]phenoxy}methyl]phenyl}sulfonylamino]pentanedioic acid.
[m] 051: (1R,3R,4S)-4-[({6-[(4-cyano-2-fluorobenzyl)oxy]naphthalen-2-yl}sulfonyl)amino]cyclohexane-1,3-dicarboxylic acid.

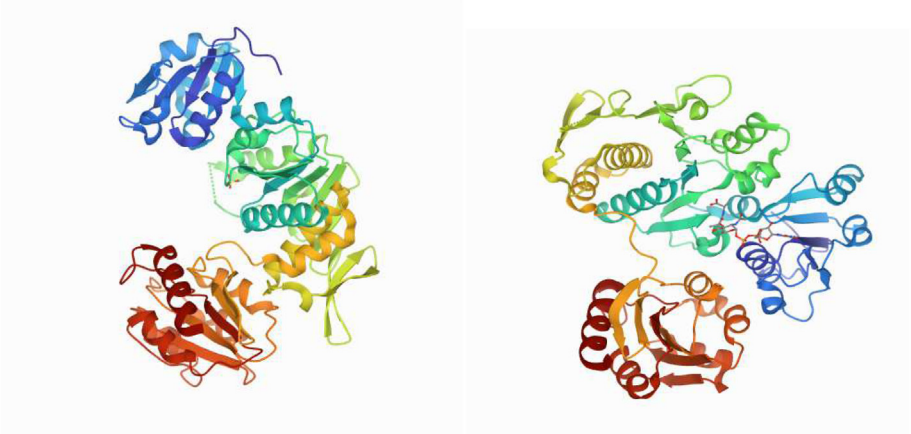

Fig. 2. Open structures of MurD enzyme from *E. coli* (PDB:1E0D,1EEH).

sequence identity was said to be 31% between MurD from E. coli (PDB ID 1EEH, 1UAG & 2JFF) and that from *Mycobacterium tuberculosis* while similarity was at 45%. (Barreteau et al., 2012). The crystal structure of the MurD enzyme from E. coli (PDB ID 2JFH) was considered most suitable for homology modeling of M. tuberculosis MurD enzyme. The crystal structure displayed 45% sequence similarity and 31% sequence identity with the query sequence. (Shinde et al., 2021). In Xanthomonas oryzae, MurD2 enzyme (X00_1320) was identified as the first MurD enzyme that was able to ligate L-Glutamic acid (Fig. 3). This MurD2 displayed 26% identity with canonical MurD enzyme from E. coli. (Feng Ruoyin, 2019).

MurD from *E. coli* is closely related to MurD from Haemophilus influenza and belongs to a single clade. Both are closely related to the Mycobacterial MurD enzyme in terms of phylogeny and all are part of a single ingroup. MurD from E. coli is distantly related to all the other organisms tested (Fig. 4).

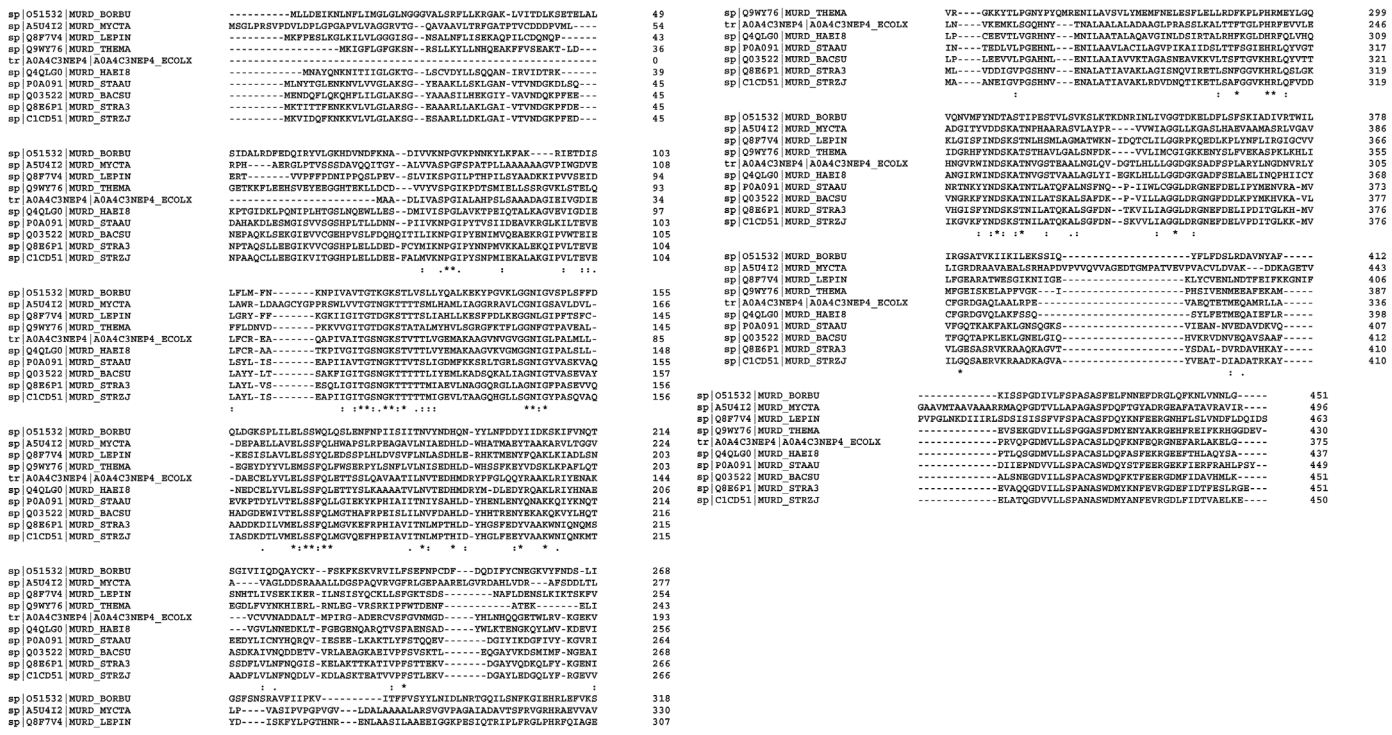

Fig. 3. Amino acid sequence comparison: Multiple sequence alignment of MurD from *E. coli* (Accession no. A0A4C3NEP4), *M. tuberculosis* (Accession no. A5U4I2), Bacil*lus subtilis* (Accession no. Q03522), *Thermotoga maritima* (Accession no. Q9WY76), *Streptococcus agalactiae* (Accession no. Q8E6P1), *Borrelia burgdorferi* (Accession no. O51532), *Staph. aureus* (Accession no. P0A091), *Leptospira interrogans* (Accession no. Q8F7V4) *Streptococcus pneumoniae* (Accession no. C1CD51), *Haemophilus influenzae* (Accession no. Q4QLG0). (output of Clustal Omega) (MurD Accession no. from www.unipro.org).

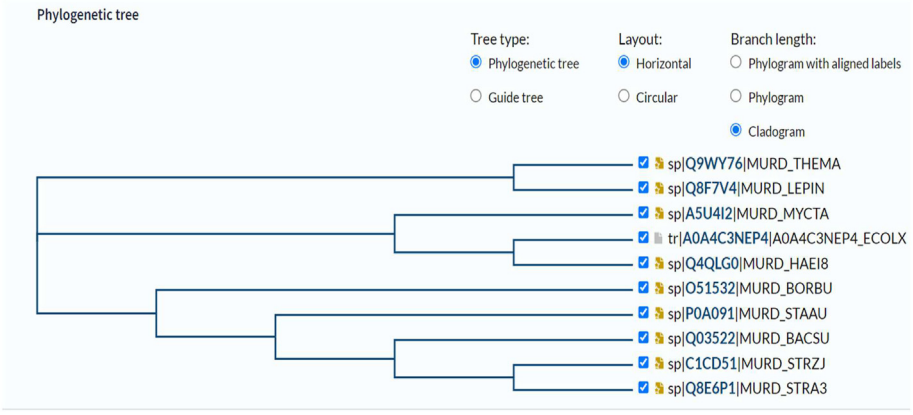

Fig. 4. Phylogentic tree of MurD enzyme from different bacterial species: MurD E. coli (Accession no. A0A4C3NEP4), MurD M. tuberculosis (Accession no. A5U4I2) MurD Bacillus subtilis (Accession no. Q03522), MurD Thermotoga maritima (Accession no. Q9WY76), MurD Streptococcus agalactiae (Accession no. Q8E6P1), MurD Borrelia burgdorferi (Accession no. O51532), MurD Staph. aureus (Accession no. P0A091), MurD Leptospira interrogans (Accession no. Q8F7V4)M, urD Streptococcus pneumoniae (Accession no. C1CD51), MurD Haemophilus influenzae (Accession no. Q4QLG0). (output of Clustal Omega) (Accession no. from www.unipro.org).

4.1. Physiochemical properties of MurD

Specific activities of the MurD enzyme from gram-positive bacteria E. faecalis and Staph aureus were found to be 2 to 6 times higher than their gram-negative counterparts from E. coli and H. influenza.In terms of efficiency MurD from Staph aureus was less efficient when compared with MurD from E. faecalis, E. coli, and H. influenza (Walsh et al., 1999). Studies on the effect of monovalent ions like NH4+ and K+ displayed these ions to increase the activity of MurD enzyme in gram-negative organisms which may be attributed to their role in conformational changes in proteins and stabilization of various reaction intermediates. But these monovalent cations displayed little or no effect on gram-positive organisms which was attributed to the thicker cell wall and subsequent higher demand for peptidoglycan precursors (Takahashi et al., 1984; Wedler and Ley, 1993). The transition state analog [1 (6-uridine diphospho)hexanamido](2,4-dicarboxybutyl) phosphinate was found to have an inhibitory effect on Mur D enzyme from all groups of bacteria probably due to similar transition state conformation. Most of the MurD enzymes from various groups of bacteria operate at optimum pH of 8–9.2. But studies on MurD from *Mycobacterium tuberculosis* displayed it to function best at alkaline pH. Further studies displayed that MurD orthologs from Borrelia burgdorferi and M. tuberculosis are less active (in terms of Km & Vmax) when compared with that from Staph aureus, Streptococcus pneumonia, and E. coli owing to slow growing nature of these bacteria (Barreteau et al., 2012).

In all the bacterial species, the Mur D enzyme is located in the cytoplasm of the cell (Table 4.) where it plays an important role in the biosynthesis of the peptidoglycan layer and a few bacteria undergo post-translational modification.

PknA, a eukaryotic type serine/threonine kinase that is encoded in the genome of *Mycobacterium tuberculosis* was found to trans-phosphorylate, mycobacterial MurD. The finding gave the possibility of

Table 4
Properties of MurD enzyme from different bacterial species.

MurD	Accession no.	Length (amino acids)	Mass (Dalton)	Subcellular Location	References
MurD *E.coli*	A0A4C3NEP4	375	40,036	Cytoplasm	https://www.uniprot.org/
MurD [a] *M.tuberculosis*	A5U4I2	496	50,354	Cytoplasm	https://www.uniprot.org/
MurD *Bacillus subtilis*	Q03522	451	49651	Cytoplasm	https://www.uniprot.org/
MurD *Thermotoga maritima*	Q9WY76	430	49154	Cytoplasm	https://www.uniprot.org/
MurD *Streptococcus agalactiae*	Q8E6P1	451	48972	Cytoplasm	https://www.uniprot.org/
MurD *Borrelia burgdorferi*	O51532	451	51054	Cytoplasm	https://www.uniprot.org/
MurD *Staph.aureus*	P0A091	449	49844	Cytoplasm	https://www.uniprot.org/
MurD *leptospira interrogans*	Q8F7V4	463	51298	Cytoplasm	https://www.uniprot.org/
MurD *Streptococcus pneumoniae*	C1CD51	450	48356	Cytoplasm	https://www.uniprot.org/
MurD *Haemophilus influenza*	Q4QLG0	437	47931	Cytoplasm	https://www.uniprot.org/

[a] Post translational modification/processing: Mycobacteria tuberculosis MurD is phosphorylated by PknA.

MurD being a substrate for the enzyme PknA and indicated that the kinase might be playing a role in the regulation of cell division and peptidoglycan biosynthesis (Thakur and Chakraborti, 2008). Overproduction of MurD ligase was observed in E. coli strains containing recombinant plasmids having the MurD gene under the control of lac or PR promoter (Pratviel et al., 1991).

5. Inhibitors of MurD

Among the ATP-dependent ligases involved in the synthesis of bacterial peptidoglycan, the MurD enzyme is considered one of the potential targets for the development of novel antibacterial agents. Several structures of MurD enzyme with co-crystallized ligands have been elucidated (Bertrand et al., 1997, 1999, 2000; Bertrand et al., 1999; Kotnik et al., 2007) providing the basis for the design and development of novel inhibitors. In the last two centuries, significant advancements have been made in understanding the structural properties and mechanism of action of MurD from several bacteria (*E.coli, Staph aureus, Strep. pneumoniae, Leptospira interrogans, Borellia burgdoferi*) and studies on orthologs of MurD from various pathogenic species have been done (Barreteau et al., 2012; Walsh et al., 1999). Several compounds acting as potential inhibitors of MurD, from the category of natural and synthetic components have been identified by structural activity relationship (SAR) and HTS methods (Table 6). But till date, none of the compounds have been successful for clinical use against the MurD enzyme.

In the current scenario, with the advent of computer-aided drug design methods (CADD) in combination HTS, several inhibitors forthe MurD enzyme have also been identified and designed using *in-silico* methods while screening large compounds libraries (M. A. Azam and Jupudi, 2017, 2019; Simčič et al., 2014).

5.1. Second-generation sulfonamide inhibitors

Binding means of a group of sulphonamide derivatives based on naphthalene-N-sulfonyl-D-glutamic acid were studied against the *E. coli* MurD enzyme. In these compounds, the D-glutamic acid fraction was replaced by rigid mimics. Molecular dynamics (MD) simulation studies on these inhibitors demonstrated the role of stretching forces while the interaction of sulphonamide inhibitors with C-terminal and the N-terminal domain and these must be acknowledged while designing novel inhibitors. Also suggested flexibility of inhibitor molecule to adjust to the conformational variations of MurD enzyme. A group of sulphonamide derivatives, which were based on the N-sulfonyl-D-Gluamic acid fraction, were studied against *E. coli's* MurD enzyme. The interaction between these derivatives and the N-terminal and C-terminal domains was studied. Molecular dynamics (MD) simulation studies on these inhibitors demonstrated the role of stretching forces while the interaction of sulphonamide inhibitors with C-terminal and the N-terminal domain (Simčič et al., 2012).

Table 5
Substituent groups attached to parent Phenoxyacetohydrazide structure in compounds from 4a-4k.

Cpd. marked	R1 Gp.	R2 Gp.	X Gp.	Ar Gp.
4a	-Cl	-H	>carbonyl	-2-furyl
4b	-H	$-OCH_3$	>carbonyl	-2-furyl
4c	-Cl	-Cl	>carbonyl	$-2\text{-Cl-}C_6H_4$
4d	-Cl	-Cl	>carbonyl	$-3,5\text{-diNO2-}C_6H_3$
4e	-Cl	-Cl	>carbonyl	$-3\text{-Br-}C_6H_4$
4f	-Cl	-Cl	>carbonyl	$-4\text{-Cl-}C_6H_4$
4g	-H	$-NO_2$	>carbonyl	$-2,4\text{-di–}CH_3O\text{–}C_6H_3$
4h	-H	$-NO_2$	>carbonyl	$-2,4\text{-di-Cl-}C_6H_3$
4i	-H	$-OCH_3$	>carbonyl	$-2\text{-}CH_3\text{-}C_6H_4$
4j	-Cl	-H	$>SO_2$	$-C_6H_4$
4k	-H	$-OCH_3$	$>SO_2$	$-4\text{-Cl-3-COOH-}C_6H_3$

5.2. Inhibitors obtained by structural modification of thiazolidine-4-one-based compounds

Dual inhibitors for MurD and MurE *from both E. coli and Staph aureus*were designed by structural modification of thiazolidine-4-one-based compounds. The most active compound displayed an IC_{50} value of 8.2 and 6.4 μM against MurD of *E. coli* and *Staph aureus* respectively and a MIC of 8 μg/mL against *Staph. aureus* and MRSA (Tomašić et al., 2012).

The inhibitor was found to hinder the binding position of product UDP-MurNAc-L-Ala-D-Glu (UMAG). The inhibitor shows hydrophobic interactions with Leu416 residue and π−π interactions with Phe161 residue. Thr321, Lys348, Ser415, and Phe422 residues are involved in hydrogen bonding to the D-glutamic acid fraction of the inhibitor (Tomašić et al., 2012).

5.3. Naphthalene-N-sulfonyl-D-glutamic acid derivatives

A series of Naphthalene-N-sulfonyl-D-glutamic acid derivatives were identified as potential inhibitors for *E. coli* MurD enzyme through structural activity relationship (SAR). These compounds exhibited IC_{50} values in the range of 80–600 μM. These sulphonamides containing compounds were designed to mimic the tetrahedral intermediate state formed during the catalysis reaction of the MurD enzyme (Humljan et al., 2008) (Fig. 5.). γ-carboxylate group in D-glutamic acid derivatives were found to play a major role during the interaction with the enzyme. The carboxamide and sulphonamide series of substituent compounds were found to be inactive as compared to series containing bulky substituents of biphenyl and naphthalene, displaying IC50 values of 1720 and 810 μM. The series of compounds in which there was a direct attachment of naphthalene moiety to the D-Glu amino acid residue via the sulphonamide moiety, displayed inhibitory activity in contrast to those which do not have a direct contact. A three-fold increase in inhibitory activity was detected when length of side chain was increased by replacing methyl to pentyl group (590–170 μM). Arylalkyloxy substituents were found to be more effective as a substituted inhibitor (Humljan et al., 2008).

Table 6
Significant inhibitors of MurD enzyme identified so far.

Compounds	Mode of Inhibition/MOA[a]	IC50[b] (uM)	MIC (ug/ml)	References
N-sulfonyl-D- & L-Glu derivatives	Competitive inhibition towards D-Glutamic acid	280 & 710	Nd	Kotnik et al. (2007)
Napthalene-N-sulphonyl -D-glutamic acid derivatives	by interaction of alpha & gamma carboxylate gps of glutamate residue with a.a residues of enzyme	80–600	Nd	Humljan et al., 2008
Peptide inhibitors	Binds to active site of enzyme	140	Nd	Bratkovič et al., 2008
5-benzylidenerhodanine moeity (Glutamic acid based inhibitors)	D-Glu analogues	174 & 206	Nd	Tomašić et al., 2010
5-benzylidenerhodanine- and 5-benzylidenethiazolidine2,4-dione-based compounds	Competitive inhibition towards D-Glutamic acid	45–206	>128[c]	Zidar et al. (2010)
5-benzylidenethiazolidin-4-one moiety	Binds to active site of enzyme	3–7	Low activity[d]	Tomašić et al., 2011
5-benzylidenethiazolidin-4-one inhibitors	by H-bonding with active site residues, analogue of UMAG	28	128[e]	Zidar et al. (2011)
thiazolidine-4-one-based compounds	transition state analogues	6.4–180	8[f]	Tomašić et al., 2012
2-oxoindolinylidene based inhibitor	competitive inhibition towards product UMA	ne	128[g]	Simčič et al., 2014
Inhibitors from zinc database	interacts with central domain of enzyme	28	50uM[h]	Samal et al.,2015
benzothiazol-2-ylcarbamodithioate	vander wall interactions with active site residues	13.37	2–64[i]	Jupundi et al.,2019
H5 (EnamineT1827917)	interaction with active site residues	7	128,128,256[j]	Azam et al. (2019)
ATP-dependent Kinase inhibitors	competitive inhibition towards D-Glutamic acid	104	Inactive[k]	Hrast et al. (2019)
phenoxyacetohydrazide derivatives	vander wall interactions with active site residues	35.80 μM	64,128[l]	Jupundi et al.,2020

n.e not explained, n.d. not determined, UMAG:UDP N-acetylmuramoyl-l-alanine,D-Glutamic acid, UMA:UDP N-acetylmuramoyl-l-alanine.
[a] MurD activity was assayed by the detection of the orthophosphate generated during the reaction based on the colorimetric Malachite green method.
[b] The detrmination of MIC values were done on the basis of National Committee for Clinical Laboratory Standards (NCCLS).
[c] MIC values have been determined against*E. coli* ATCC 25922, *P. aeruginosa* 27853, *S. aureus* ATCC 29213, and *E. faecalis* ATCC 2921.
[d] MIC values have been determined against Staphylococcusaureus and *Enterococcus faecalis*.
[e] MIC values have been determined against *S. aureus* ATCC 29213, *E. faecalis* ATCC 29212.
[f] MIC values have been determined against *Staph aureus*& MRSA.
[g] MIC values have been determined against *Haemophilus influenzae* (ATCC49247) and *Enterococcus faecalis* (ATCC 29212).
[h] MIC values have been determined against *Salmonella typhimurium*.
[i] MIC values have been determined against *S. aureus* NCIM 5021 and S. *aureus* NCIM 5022.
[j] MIC values have been determined against *S. aureus* NCIM 5021, *S. aureus* NCIM 5022, and methicillin resistant *S. aureus* (MRSA strain 43,300).
[k] MIC values have been determined *E. coli*&*Staph aureus*.
[l] MIC values have been determined *S. aureus* NCIM 5022, methicillin resistant *S. aureus* ATCC 43300.

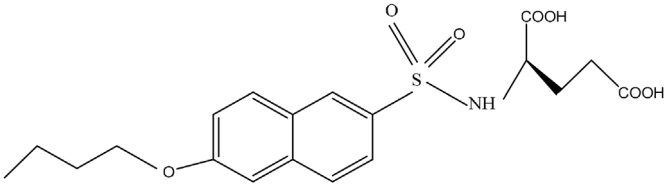

Fig. 5. Basic chemical structure of a sulphonamide inhibitor.

5.4. 2-Oxoindolinylidene based inhibitor

2-oxoindolinylidene based inhibitor (Fig. 6A) with a novel framework was evaluated using a steady-state kinetic mechanism against *E. coli* MurD enzyme. The derivative showed a competitive mode of inhibition for the substrate UMA. A MIC value of 128 μg/ml was obtained against *Haemophilus influenzae* (ATCC 49247) and *Enterococcus faecalis* (ATCC 29212) strains (Simčič et al., 2014).

NMR studies showed that unlike transition state analogues, the inhibitor interacted with the N-terminal and central domain, and there was no interaction with the C-terminal domain of the enzyme. As a result, its binding was unaffected by domain movements. 2-oxoindolinylidene ring was found to be located in the uracil-binding site. Hydrophobic interactions were mainly involved in the stability of the enzyme-inhibitor complex as demonstrated by MD simulation suggesting studies for the de novo examination of new structures with more powerful binding affinities (Simčič et al., 2014).

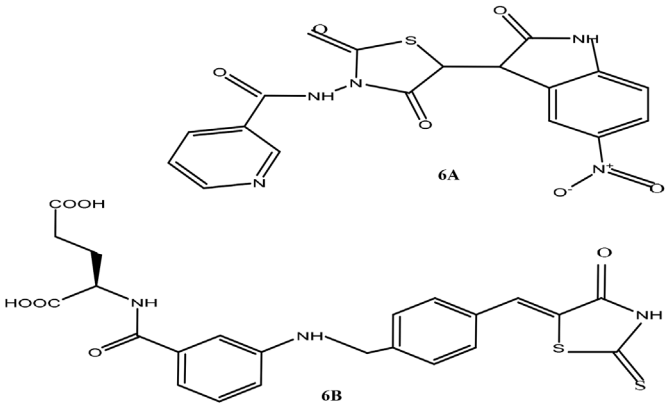

Fig. 6. 6A: Chemical structure of 2-oxoindolinylidene based inhibitor; 6B. Chemical structure of 2- Thioxothiazolidin-4-one based inhibitor.

5.5. Marine natural products as inhibitors

A marine natural product library was screened for potential inhibitors against modeled *Staph aureus* MurD enzyme with molecular docking and

simulation studies. Two compounds from seaweed database (SWMD) (http://www.swmd.co.in) showed a strong interaction within the binding pocket of the enzyme and displayed potential therapeutic effects and stable configuration with the target protein enzyme (Zheng et al., 2021). Compound 2 (46604) displayed hydrophobic interactions with amino acids Lys19 and Asn145 and hydrogen interactions with Glu166, Ser168, Lys328, and Thr330 residues. Compound 3 (46608) interacted by displaying hydrophobic interactions with Thr330 and Phe431 amino acids while Asn145, Ser168, and Lys328 residues were involved in H-bond formation (Zheng et al., 2021).

5.6. 2-Thioxothiazolidin-4-one based inhibitors

2-Thioxothiazolidin-4-one based marked as compound $1 (Fig. 6B) was studied as a potential inhibitor of homology modeled Staph aureus MurD enzyme through molecular docking and molecular dynamics studies. Major interactions between the inhibitor and MurD enzyme observed were salt bridge interactions, π-π stacking, and hydrogen bonding. Stabilization of inhibitor-enzyme complex was mainly contributed by residues Lys19, Gly147, Tyr148, Lys328, Thr330, and Phe431. Vander wall and electrostatic solvation energies were mainly involved in inhibitor binding. MD simulation showed the inhibitor and the modeled MurD enzyme to be in a stable configuration. During in-vitro validation, the compound was found to inhibit the enzyme with an IC_{50} value of 6.40 μM. Antibacterial activity assays showed the compound to be effective against commercial *Staph. aureus* and MRSA strains displaying MIC values of 8 μg/ml (M. Azam, Jupudi, Saha and Paul, 2019).

Molecular simulation studies on the inhibitor $1 showed that its configurational flexibilities were mainly dependent on rotations of a single bond around the C_6H_4–CO–NH-,C_6H_4–CH_2–NH–C_6H_4- group along with the structural features of D-glutamic acid. As 2-thioxothiazolidin-4-one ring has a non-significant contribution towards the binding process, a series of new inhibitors (D1, D2, D3 & D4) (Fig. 7) were designed. These were prepared by replacing the C6H4–CO–NH- a group with nitrogen-containing heterocyclic rings and replacement of D-Glu fraction with an aromatic ring containing –COOH,-OH, and other polar groups to enhance the binding affinity. These alterations resulted in improvement of glide score from −6.75 to −8.88 kcal/mol and increase in binding free energy from −61.36 to 83.71 kcal/mol (M. Azam et al., 2019).

5.7. Screening of a group of commercial compound libraries

In 2019, Azam et al. screened a group of commercial compound libraries containing about 1.6 million small molecules against modeled MurD enzyme from *Staphaureus* through HTS and in-vitro validation. Based on binding free energy calculations and interaction of various ligands with the residues present in the catalytic pocket, the top ten compounds marked from H1 to H10 and were shortlisted for further studies. Crucial forces supporting ligand binding were found to be van der Waals and coulomb energy while electrostatic solvation energies opposed binding of the ligand molecules (M. A. Azam and Jupudi, 2019).

The highest inhibitory activity was displayed by compound H5 EnamineT6806127 (https://enamine.net/) (Fig. 8) with an $IC_{50}$and MIC value of 7 μM and 128 μg/ml respectively showing the repressive effect on several commercial strains e.g., *Staph. aureus* NCIM 5021, *Staph. aureus* MRSA 43,300. No inhibitory effect was displayed by compound H10 (T0500-2187) against any of the tested strains which might be attributed to its poor permeability inside the bacterial cell. Ligand H5 (EnamineT6806127) interacted through forming a series of hydrogen bonds with the residues Lys19, Glu23, Gly80, and Gly147 and π-cation interaction with Lys19 residue to provide stability to the complex (M. A. Azam and Jupudi, 2019).

Some of the established MurD inhibitors were docked against *E. coli* MurD binding site and molecular simulation studies were carried out. Docking results revealed a common mode of interaction which exhibited by most of the inhibitors during recent studies. Major residues involved in the interaction between enzyme and inhibitor were Thr36, Arg37, His183, Lys319, Lys348, Thr321, Ser415, and Phe422. Non-polar interactions like Vander walls force was found to play a significant role in binding-stability of the inhibitor-protein complex while electrostatic interaction presented a minor role (M. A. Azam and Jupudi, 2017).

A total of 10,344 compounds from the Zinc (https://zinc.docking.org/) and PubChem database were screened against modeled MurD enzyme of *Mycobacterium tuberculosis* H37Rv based on molecular docking, Lipinski rule, and pharmacokinetic studies. The top four compounds ZINC11881196, ZINC12247644, ZINC14995379, and PubChem6185 were subjected to molecular simulation and MM-GBSA (molecular mechanics energies combined with generalized Born and surface area continuum solvation) and showed better values of binding free energies than the ATP. This study presented the above mentioned compounds as potential inhibitors of MurD which can be further endorsed for use as therapeutic drug (Isa, 2019).

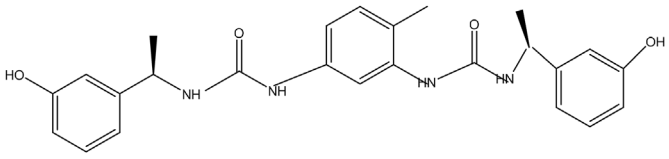

Fig. 8. Chemical structure of compound H5 (EnamineT6806127).

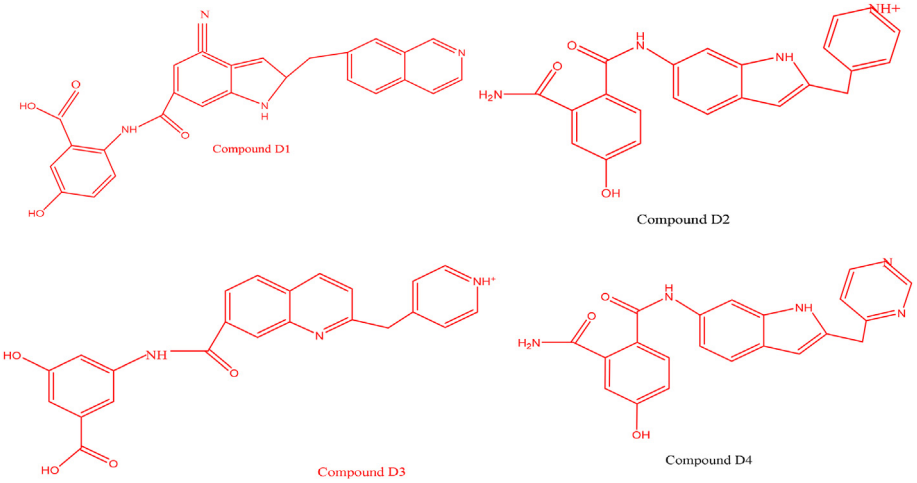

Fig. 7. Chemical structures of compounds D1,D2, D3 & D4 synthesized by modification of 2-thioxothiazolidin-4-one ring.

5.8. Aza-stilbene derivatives

A library of synthesized ATP-competitive kinase inhibitors were screened against Mur ligases (Mur C, D, E & F) from E. coli. Four new scaffolds were identified that displayed a probability for the development of new drugs. Aza-stilbene derivative marked as 1 (Fig. 9A) was found as a potential inhibitor of the MurD enzyme (Hrast et al., 2019). NMR studies indicated that the binding of this inhibitor to the D-glutamic acid binding site is present in the C-terminal domain of the MurD. The compound acted in a competitive inhibition manner for D-Glutamic acid and disturbed the signals of the Leu416 methyl groups and was independent of the closing of the enzyme aided by ATP.

IC_{50}value of compound 1 against MurD enzyme was found to be 104 μM. No inhibitory effect was displayed by compound 1 against any of the tested gram-negative or gram-positive organisms (*E.coli & Staph aureus*). The reason for this might be attributed to their low penetration effect which suggested further structural modifications need to be done to the parent compound to increase the inhibitory potency against various groups of Mur ligases (Hrast et al., 2019).

5.9. Molecular docking and simulation studies on compounds contained in zinc database

Molecular docking and simulation studies were done on about 6,42,759 compounds from the Zinc database (https://zinc.docking.org/) against the MurD enzyme from *Acinetobacter baumannii* prepared from *E. coli* (PDB ID: 4UAG) by homology modeling (Jha et al., 2020). Two compounds - ZINC19221101 and ZINC12454357 (Fig. 9 B)with binding free energy values of -62.6 ± 5.6 kcal/mol and -46.1 ± 2.6 kcal/mol respectively were the most promising candidates discovered for further validation. MD simulation studies showed compound ZINC19221101 to interact via hydrogen bonds with Ala122, Lys123, Ser124, and Asn146 residues. At the same time, it showed π-interactions with Lys123, Glu165, Lys330, Phe434, and Tyr440 residues and Vander wall interactions with residues Arg313, Thr332, and Lys364 (Jha et al., 2020).

Compound ZINC12454357 interacted with the residue Lys123, Asn146 (conserved residue), Lys364, and Tyr440 of MurD enzyme through hydrogen bonds. π-interactions were formed with the residues Glu165, Phe169, His191 (conserved residue), and Lys330 while Vander Walls interactions were formed with residue Pro80, Gly81 (catalytically conserved residue), Ser120, Asn121, Ala122, Leu147, Gly148, Ser167, Phe434, Ser439, and Asn441(Jha et al., 2020).

5.10. Phenoxyacetohydrazide derivatives

A set of phenoxyacetohydrazide derivatives (marked as 4a to 4k, Fig. 10, Table 5)were manufactured and characterized against *Staph aureus* MurD. Antibacterial activity analysis showed that compounds 4a, 4j, and 4k displayed an inhibitory effect on *Staph aureus*NCIM 5022 having MIC of 64 μg/ml. These compounds also revealed an inhibitory effect on MRSA strain ATCC 43300 with MIC of 128 μg/ml. Amongst all the examined compounds 4c and 4j showed the most powerful activity, sequentially against *B. subtilis* strain NCIM 2545 and *Klebsiella pneumoniae* strain NCIM 2706. Compound 4d expressed activity on *Pseudomonas aeruginosa* strain NCIM 2036 with MIC value of 64 μg/ml. Enzyme assay revealed that compound 4k exhibited the greatest inhibitory effect on *Staph aureus* MurD with an IC50 value of 35.80 μM. Sulfonylhydrazides derivatives were found to be more effective than hydrazides which might be due to the presence of SO_2 group (Jupudi et al., 2021).

Molecular docking and binding free energy calculations on MurD from *Staph aureus* via homology modeling showed that the vander Walls interaction is to be the major contributor for inhibitor-protein binding stability rather than electrostatic interactions (Jupudi et al., 2021).

5.11. Aza-Stilebin analogues

A set of 20 synthetic aza-stilbene derivatives were evaluated against Mur ligases C, D, E & F from *Staph aureus*. Weak antibacterial activities towards *E. coli* and *Staph aureus*were seen by most of these derivatives. Two compounds marked as 30 and 31 (Fig. 11) showed moderate activities towards *Staph aureus* exhibiting MIC values of 0.125 and 0.031 mM, respectively.

Molecular docking studies showed that the tetrazole ring interacts with the enzyme with a higher number of hydrogen bonds while pyridine, phenyl, and furan fractions forms weak interaction with the enzyme. These study suggested that possible structural modifications are needed to increase the binding affinity and inhibitory effect (Hrast et al., 2021).

5.12. Screening of CHEMBEL database against mycobacterial mur enzymes

CHEMBL database (https://www.ebi.ac.uk/chembl/) was screened by molecular docking and simulation studies against homology modeled Mur enzymes (Mur A, B, C, D, E & F) of *Mycobacterium tuberculosis* (Mtb). CHEMBL446262 (Fig. 12) was found out to be the best-docked compound against all the modeled Mur enzymes of Mtb. The compound was found to form active Hydrogen -bond interactions with Ser129, Arg141,Leu144, Ile148, Ser150, Glu166, Asp194, Arg446, and Met448 residues of the enzyme. Suggested further experimental validation for the development of new inhibitors (Kumari and Subbarao, 2021).

6. Conclusion and future prospect

The MurD is an ATP- dependent cytoplasmic cell wall biosynthesis enzyme that is gaining interest due to its important role in bacterial growth and its absence in humans. Structural studies on MurD has revealed details about its substrate binding pattern and catalysis mechanism (Bertrand et al., 1997, 1999; Bertrand et al., 1999; Humljan et al., 2006; Tomašić et al., 2010). The MurD along with other Mur ligases (Mur C, E & F) have a common mechanism of action and a conserved ATP binding site having similar sequences (El Zoeiby et al., 2003). Despite the many promising effects of MurD inhibitors identified so far, they have not been exploited significantly for antibacterial activity studies. This is presumably due to the failure of inhibitors to traverse the cytoplasmic barrier of the bacteria. Therefore, attempts need to be made to devise novel inhibitors or make structural and chemical modifications in the existing inhibitors to subdue cell membrane barrier obstacles. Further, as the MurD enzyme undergoes several conformational changes in its domain structure during its catalytic reaction (Perdih et al., 2014), some challenges need to be addressed while devising new inhibitors against such flexible moving target.

In silico or structure-based drug designing may be used to develop new inhibitors that can restrain the action of MurD enzyme. Currently, *in-silico* techniques have been used successfully in the development of drugs for different diseases like HIV/AIDS (amprenavir) and influenza (zanamivir) (jSimmons et al., 2010). In the recent past, there has been a gradual increase in the use of various computer-aided drug design methods (Turk et al., 2009)like molecular docking to design novel inhibitors against various biological targets like Type 1 TGF beta - receptor kinase inhibitor&aurora kinase A inhibitor (Sethi et al., 2019) including the MurD enzyme. Earlier, most of the drug discovery processes included conventional methods that comprised of tedious and repetitive experimental techniques and identification of ligands for the targets (protein/enzyme)by utilizing various chemical and proteomics strategies. These are usually extensive, expensive, and time-consuming. Nevertheless, the advancements in bioinformatics synchronously with progress in

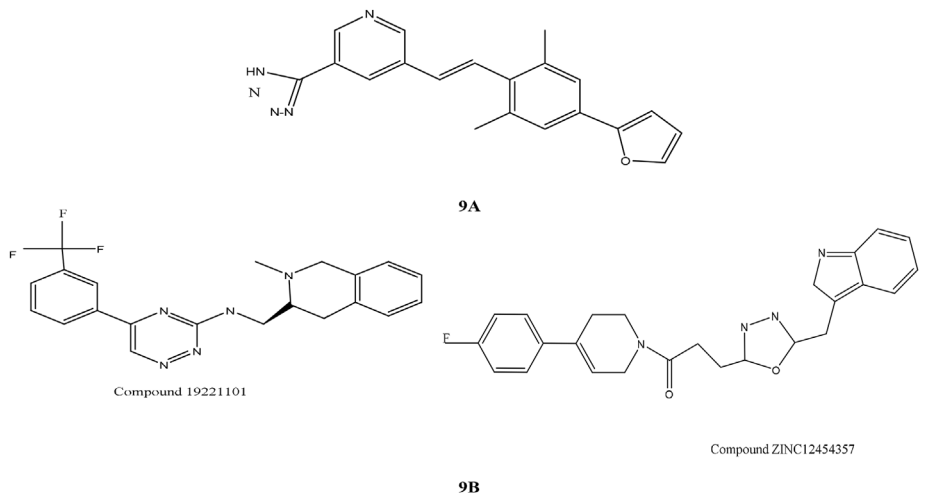

Fig. 9. 9A. Chemical structure of ATP-dependent kinase inhibitors (aza-stilbene derivative); 9B. Chemical structures of compounds ZINC19221101 & ZINC12454357.

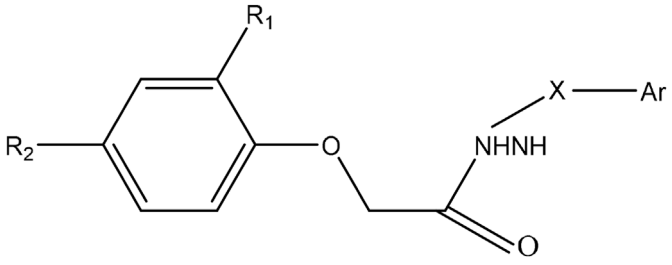

Fig. 10. Chemical structure of Parent phenoxyacetohydrazide structure.

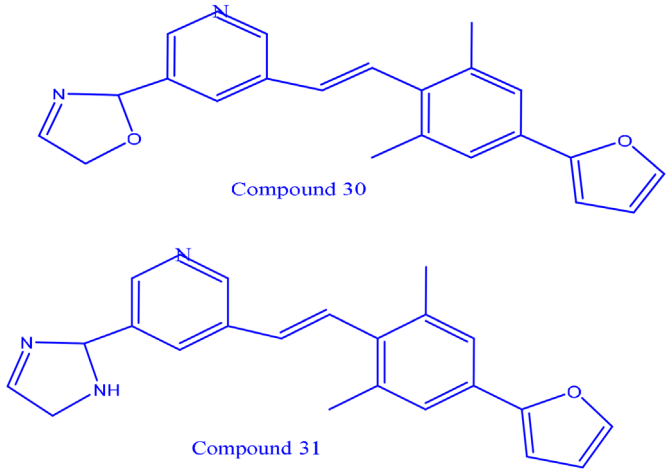

Fig. 11. Chemical structures of Aza-stilbene derivatives marked as compound 30 and 31.

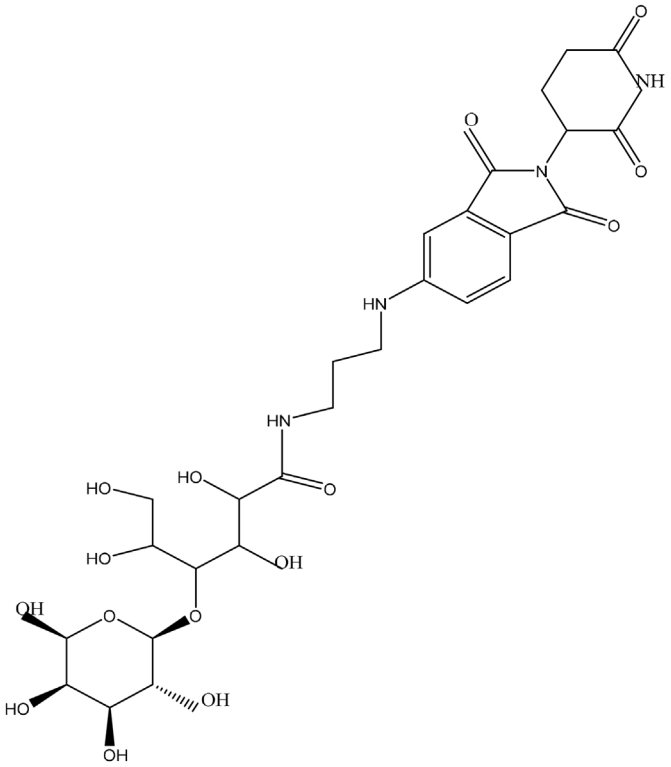

Fig. 12. Chemical structure of compound CHEMBL426262.

computer application tools and means have transformed the drug designing process against novel targets. Hence, there is a need for the effective use of various *in-silico* methods in combination with experimental work to design effective inhibitors against MurD enzyme. At the same time, various challenges like cell membrane permeability barrier and conformational changes need to be effectively managed which needs more experimental research data in the current field. So, in order to counter increasing resistance among bacterial pathogens toward the existing antibiotics, there is a growing need for the persistent development of clinically important novel antibiotics with entirely novel modes of action (Bratkovič et al., 2008; Zidar et al., 2010).

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CRediT authorship contribution statement

Vinita Gaur: Conceptualization, Writing – original draft, Data curation. **Surojit Bera:** Conceptualization, Writing – original draft, Data curation, Writing – review & editing, Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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