Identification of novel natural MurD ligase inhibitors as potential antimicrobial agents targeting Acinetobacter baumannii: In silico screening and biological evaluation

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**ABSTRACT**

The increased multidrug resistance in Acinetobacter baumannii (A. baumannii) to the present-day known antibiotics has stimulated academic and industrial efforts globally for the development of novel antibacterial agents. Natural compounds as potential drug leads are gaining significant attention due to their less toxic and more tolerant nature. In the current study, the natural product-based compounds were explored as probable inhibitors of UDP-N-acetylmuramoyl-L-alanine-D-glutamate (MurD) ligase from A. baumannii (AbMurD) to provide a new class of drug leads. The prepared natural library of 3,16,714 compounds from ZINC database was screened into the active site of AbMurD using in silico high-throughput virtual screening which resulted in 100 compounds having high binding affinities. Further screening through flexible molecular docking yielded four potential compounds selected on the basis of estimated binding affinity (\(\Delta G\)) and favorable protein-ligand interactions. MD simulation of these four compounds under physiological conditions and free binding energy calculations using MM/PBSA (molecular mechanics with Poisson-Boltzmann and surface area solvation) approach revealed three compounds ZINC08879777, ZINC30726863, and ZINC95486217 as potential binders of AbMurD. The calculated physicochemical and ADME properties of these compounds revealed that they can be exploited and modified to improve their binding affinity with the enzyme. Two compounds were purchased and tested against bacterial cell cultures of A. baumannii, Salmonella Typhi, and Staphylococcus aureus to determine their broad-spectrum antibacterial activity. The results suggest that the identified compounds can be exploited as potential herbal leads to target both Gram-positive and Gram-negative pathogens.

**Abbreviations:** A. baumannii: Acinetobacter baumannii; AbMurD: MurD from A. baumannii; ATP: Adenosine triphosphate; DS: Discovery Studio; MD: Molecular dynamics; MIC: Minimum inhibitory concentration; MurD: UDP-N-acetylmuramoyl-L-alanine-D-glutamate ligase; PDB: Protein Data Bank; RMSD: root-mean-square deviation; RMSF: root-mean-square fluctuation

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MurD; Molecular dynamics simulation; Molecular docking; Virtual screening; MMPBSA energy; MIC assay

1. Introduction

Multi-drug resistance in clinically relevant pathogens has become a serious concern all over the world. Acinetobacter baumannii (A. baumannii), an aerobic, non-motile, gram-negative bacterium is designated as a “red alert” human pathogen and classified under priority I (critical) pathogen by World Health Organisation (Cerqueira & Peleg, 2011). A. baumannii possesses an enhanced ability to acquire and upregulate antibiotic resistance and has thus gained clinical significance (Montefour et al., 2008). A recent study reports the resistance acquired by clinical isolates of A. baumannii against the last resort drugs, carbapenem, and polymixin (Skariyachan et al., 2019). The bacterium targets the most vulnerable hospitalized patients and often has a predilection for Intensive Care Unit patients, which, not only lengthens hospital stay but also increases health care costs of the patients (Alsan & Klompas, 2010). A recent perspective on the virulent factors associated with A. baumannii and the need for the identification of virulent targets and newer therapeutics for the drug resistant A. baumannii has been highlighted (Skariyachan et al., 2019). Targeting enzymes responsible for the biosynthesis of the bacterial peptidoglycan layer, which are involved in the structural integrity of the cell by combating the inner osmotic pressure and preserving its shape, results in cell degradation or lysis leading to the death of the pathogen (Bugg et al., 2011).

Mur ligases (Mur C, D, E, and F) are enzymes responsible for consecutive addition of five amino acid residues i.e. L-
alanine, D-glutamate, a diamino acid (lysine or diaminopimelate), and D-alanyl-D-alanine to UDP-N-acetylmuramic acid (UDP-MurNAc) of a peptidoglycan layer and, hence, assist in the biosynthesis of the bacterial peptidoglycan layer (Sangshetti et al., 2017). All the Mur ligases belong to the family of ADP-forming ligases and form an attractive target for the design and production of novel antimicrobial agents. MurD Ligase (UDP-N-acetylmuramoyl-L-alanine:D-glutamate ligase) is the second enzyme in the series of Mur ligases responsible for the ATP-dependent addition of D-Glutamate (D-Glu) to UDP-N-acetylmuramoyl-L-alanine (UMA) of the growing peptidoglycan layer to form a final product UDP-N-acetylmuramoyl-L-alanyl-D-glutamate (UMAG) according to the reaction (Bertrand et al., 1997).

\[
\text{UMA} + \text{D-Glu} + \text{ATP} = \text{UMAG} + \text{ADP} + \text{Pi}
\]

The L-Ala-D-Glu linkage is conserved in all eubacteria making it an essential drug target (Sink et al., 2013). The three-dimensional crystal structure for MurD ligase from \textit{A. baumannii} (AbMurD) is not available, though the high resolution structure of MurD from \textit{E.coli} has been determined at 1.46 Å in the presence of thiazolidine-2,4-dione based inhibitors (VSV) containing glutamic acid (PDB ID – 2XSO; Zidar et al., 2010). The \textit{E.coli} MurD is expressed as a monomer of 437 amino acid residues with a molecular weight of 47 kDa, and manifests a three-domain topology structure comprising N-terminal, Central, and C-terminal domains. The N-terminal domain binds to the nucleotide precursor (UMA) while other substrates like ATP and D-glutamate bind to the central and C-terminal domain respectively (Bertrand et al., 1999).

MurD from \textit{E.coli} has been well studied for a variety of inhibitors with distinct structural classes, none of which have proved very promising in their therapeutic action (Barreteau et al., 2012). Several categories of inhibitors such as peptidic, non-peptidic, glutamic acid-based inhibitors, and second-generation sulphonamide inhibitors have been designed against \textit{E.coli} MurD (Kotnik et al., 2007; Sosić et al., 2011; Tomasić et al., 2011). Moreover, another class of inhibitors, thiazolidine-2,4-dione- and rhodanine-based MurD \textit{E.coli} inhibitors, containing glutamic acid were identified as MurD product analogs (Zidar et al., 2010). All these inhibitors, however, lack antibacterial activity for MurD orthologs from other pathogenic bacteria mainly due to differences in the active site sequences and topologies which play a crucial role in inhibitor recognition (Barreteau et al., 2012). The catalytically important residues (Lys 115, Asn 138, and His 183 in \textit{E.coli} MurD) are conserved in MurD orthologs which play a significant role in catalytic function and mechanism. In many previous studies, MurD from \textit{A. baumannii} (AbMurD) is considered as a potential and efficient drug target (Ahmad et al., 2019; Amera et al., 2019; Kaur et al., 2013). Hence, AbMurD forms a novel and attractive drug target as it is vital for the survival of the pathogen and is absent in humans (Moraes et al., 2015).

Natural products are bioactive compounds emerging as preferred therapeutics in the market due to comparatively lesser side effects when compared to synthetic compounds (Harvey, 2008). The natural products are highly distinctive due to the presence of a diversity of scaffolds with a large number of chiral centers (Feher & Schmidt, 2003). This property has led to a large number of drugs in the market that are derived from natural products directly or indirectly. The alkaloids, flavones, tannins, and phenolic compounds are reported to be active against various drug targets of Acinetobacter species (Liu et al., 2018). A natural compound (epiestriol-16) showed profound binding potential to four other prioritized targets of multi-drug resistant \textit{A. baumannii} (MDRab) compared to conventionally used antibiotic faropenem (Skariyachan et al., 2020). However, studies have yet not been undertaken to screen herbal products against AbMurD drug target to the best of our knowledge. Therefore, in this study, the quest was to identify a potent natural product inhibitor of AbMurD ligase through \textit{in silico} screening of natural compounds library from the ZINC database. The rationale behind selecting a natural product library was to explore and identify more natural leads with diverse scaffolds against the potential drug target AbMurD. The structural model for the AbMurD protein was generated through homology modeling. The identified hits from virtual screening were further filtered and refined by MD simulations to identify the possible hits. Selected potential compounds were purchased and tested by MIC assay for their broad spectrum inhibitory potency against \textit{A. baumannii}, \textit{Salmonella Typhi} and gram-negative \textit{Staphylococcus aureus}.

### 2. Material and methods

#### 2.1. 3D-Structure modeling and refinement of AbMurD

The MurD ligase sequence for \textit{A. baumannii} strain ATCC 19606 (accession number ENW73371) was acquired from GenBank (NCBI). The protein modeling was achieved using program Modeller (version 9.2) (Fiser et al., 2000) available in Discovery Studio v4.0 (AccelrysInc, San Diego, CA, USA). This program performs BLAST (Basic Local Alignment Search Tool) (http://www.ncbi.nim.nih.gov/blast) to search for the best template for query sequence (Altschul et al., 1990). The \textit{E. coli} MurD ligase [PDB ID: 2XSO] was selected as the template based on query coverage and sequence identity. The lowest DOPE score model was chosen and minimized to remove any short contacts. OPLS-AA/L all-atom force field was applied to protein atoms to correct the bond order and also add charges. Energy minimization was done by applying 50,000 steps of the steepest descent minimization algorithm with a convergence threshold of 1000KJ/mol/nm. Energy minimized model coordinates were refined by the Molecular Dynamics simulation (MD) procedure. This refined structure was evaluated using PROCHECK server (Laskowski et al., 1996), Verify3D (Eisenberg et al., 1997), and ProSA-web (Wiederstein & Sippl, 2007).

The active site residues for AbMurD protein were predicted using the known active site information of the template. For this, the amino acid sequences of both proteins (AbMurD and template) were aligned and corresponding residues of the target protein were identified. These corresponding residues assumed to be the active site residues of...
2.2. In silico screening of molecular docking of the natural product database

The evaluated and verified protein model structure was used as a receptor for the molecular docking-based screening of the natural product database. The 3D coordinates of natural compounds downloaded from the ZINC 15 database contain over 230 million (Sterling & Irwin, 2015) purchasable compounds in ready-to-dock format with almost 85,000 natural product molecules which are regularly updated (Sorokina & Steinbeck, 2020). Precise docking, requires a reasonable ligand configuration, conformation (3D), ionization states, and stereochemistry which was achieved through the ligand preparation wizard in Maestro (Schrödinger, LLC, New York). This program calculated the protonation state of each compound in the pH range of 6 to 8. Tautomers and isomers of each possible compound were also generated. This yielded a total of 3,16,714 molecules wherein each was energy minimized using force field parameter OPLS-2005 (Harder et al., 2016).

Initially, virtual screening was accomplished with default parameters in Vina wizard of AutoDock-based virtual screening program called PyRx 0.8.x (Dallakyan & Olson, 2015) to eliminate unfavourable compounds with steric clashes. The active site of the receptor (AbMurD) was defined as the binding site for the screening of compounds. This pocket corresponds to the site where the inhibitor N-(1,2-Dioxo-1,3-Thiazolidin-5-Ylidene)Methyl[Phenyl]Amino)Methyl[Phenyl]Carbonyl]-D-Glutamic Acid (VSV) is complexed with E. coli MurD in the template (PDB ID: 2X5O) and hence the grid box was built accordingly. The top 100 compounds having lower binding energy were selected for flexible docking procedure in AutoDock Tools v4.2 (Morris et al., 2009). More negative the binding energy, the better is the probability of binding of the ligand in the active site.

To validate the docking methodology before proceeding with the molecular docking of screened compounds, control docking studies were undertaken using the reference/control ligand VSV. The reference ligand was docked into the active site of energy minimized AbMurD model using Lamarckian Genetic Algorithm (Morris et al., 2009) (a type of stochastic conformational sampling approach) with default parameters (GA run = 10, population size = 150, rate of gene mutation — 0.02, rate of crossover = 0.8 and maximum number of evaluation — 25 million). The generated docked conformations (poses) were scored using a derived semi-empirical scoring function which gives an estimated ΔG in kcal/mol and hence dissociation constant (Kd) or binding affinity. The grid box was defined on the active site at −10.08, 28.55, 19.35 (grid centered xyz coordinates) and 60, 60, 60 (grid xyz dimensions). The top best low energy conformation was selected and evaluated for protein-ligand interactions and calculation of structural and RMSD deviation between the crystal and docked conformation of VSV.

Top 100 hits having the lowest energy conformation obtained after virtual screening were docked using the flexible mode of AutoDock v4.2 with the same grid box and docking parameters as that used in control docking. For this, the initial conformation of the top hits was taken from the prepared natural product library. The best hits were selected based on protein-ligand interactions and estimated binding free energy (ΔG) of the docked pose of the ligand in the binding pocket of the receptor.

2.3. Molecular dynamics simulation

MD simulation is a computational technique that considers both ligand and protein as flexible and measures the physical movements of atoms and molecules within a fixed time period. The individual AbMurD-ligand complexes and apo AbMurD were subjected to MD simulations using Gromacs v5.1 (Prönk et al., 2013) in the presence of GROMOS96 53a6 force field parameters. All complexes and apo AbMurD were first minimized and the topology and coordinates of each selected compound were obtained using an external program, PRODRG. All structures were solvated in a cubic box extended to 1 Å in all three directions by adding spc216 water models. The solvated systems were neutralized by adding counter ions (6 chlorides) followed by energy minimization for 1000 steps using the steepest descent with a convergence threshold of 1000KJ/mol/nm. All solvated complexes were equilibrated for 100 ps each in NVT ensemble condition followed by NPT at 300 K and 1 bar. Subsequently, 100 ns of the production run was performed for each system under NPT condition 300 K and 1 bar. Each simulation was performed in periodic boundary conditions in the presence of LINCS constraints (Hess et al., 1997). The particle-mesh-Ewald (PME) method was applied for long-range electrostatic calculation.

Various structural parameters were calculated for analyzing the stability, compactness, and interaction between protein and ligand such as root mean square deviation (RMSD), root mean square fluctuation (RMSF), radius of gyration (Rg), and intermolecular hydrogen bonds (H-bonds) by the gmx rms, gmx rmsf, gmx gyrate and gmx hbond utility modules respectively provided with GROMACS. All graphs were plotted with the help of Xmgrace, a 2D plotting tool.

2.4. Binding free energy calculation (MMPBSA)

Additionally, the protein-ligand binding free energy was determined using the Molecular mechanics Poisson–Boltzmann surface area (MM-PBSA) method with the gmx mmpbsa package included in Gromacs v5.1. It’s a widely used method to calculate final binding free energy, the free energy of solvation (polar+ non-polar solvation energies), and molecular mechanics potential energy (electrostatic + van der Waals interaction) (Kumari et al., 2014). This equation computes polar solvation energy, while the nonpolar solvation energy was assessed through the solvent-accessible surface area (SASA) method (Gilson & Honig, 1988).

AbMurD protein formed a cleft which was defined as the binding site for ligand docking and virtual screening.
The final 10 ns of the MD trajectory of each protein-ligand complex were employed for the determination of MM-PBSA.

2.5. In silico ADME prediction

ADME profile for the selected compounds was predicted using QikProp module v5.4 (Schrodinger, LLC, New York) (QikProp, 2017). This module is widely used for calculating the physicochemical and pharmaceutically relevant profiles of small molecules. QikProp compares the molecular properties of a compound to 95% of known drug profiles and accurately reports the absorption, distribution, metabolism, and excretion profile of the compound. The following descriptors were applied for the selected compounds: SASA, FOSA, molecular weight, donorHB, acceptHB, QPlogPoct, QPlogPw, QPlogPov/w, logS, QPlogBB. The toxicity predictions for identified natural compounds were calculated using the freely accessible Cambridge University small-molecule pharmacokinetics prediction (pkCSM) web server (University of Cambridge, UK) (http://biosig.unimelb.edu.au/pkcsmprediction) (Pires et al., 2015).

2.6. Determination of minimum inhibitory concentration

The final hits were purchased to measure their biological activities by determining the minimal inhibitory concentration for A. baumannii, S. aureus, and S. Typhi. The bacterial inoculum was further diluted to 10^6 CFU/ml. Plates were incubated at 37°C for 24 h. The results were analyzed by the addition of MTT dye (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) which changes color from yellow (tetrazolium salt) to purple (formazan) in the living cells due to their respiratory activity (Wang et al., 2010).

3. Results and discussion

3.1. 3D-Structure modelling and refinement of AbMurD

The experimental X-ray crystal structure for AbMurD was not available, hence, the homology model was developed using the 3D structure homology modeling approach. The BLAST search for the closest protein homolog resulted in template Ecoli MurD (PDB ID: 2X5O_A) with 96% query coverage and a maximum score of 317 with a sequence identity of 42% (Supplementary Material Figure S1). The superimposition of the backbone atoms gave an r.m.s. deviation (RMSD) of 0.19 Å reflecting high structural conservation between the template and query (Figure 1A). The modeled structure of AbMurD ligase shows a three-domain topology comprising N-terminal (residues 1-93), central (residues 94-298), and C-terminal (residues 299-448) domains (Figure 1B). Two insertions were observed in AbMurD which projected out as loops (residues 241-245 and 338-345) (Figure 1A). These loops exposed to the surface were situated away from the active site selected for the screening of compounds. The structure of AbMurD was further refined to determine its stability and flexibility through 100 ns MD simulations in the water system. The protein structure after simulation was found to be steady in terms of temperature, pressure, and energetics.

The protein backbone stability within the solvent system was analyzed through the root mean square deviation (RMSD). The RMSD plot for the 100 ns run indicates that the enzyme stabilizes considerably after 25 ns of MD timeframe with minor fluctuations (Figure S2A). The flexibility of AbMurD was evaluated by calculating the root mean square fluctuation (RMSF) of each residue with respect to time during the simulation. The RMSF plot for 100 ns MD timeframe exhibits a higher fluctuation of around 0.43 nm in the region of residues 176-190 (central domain) (Figure S2B). These residues form a loop close to the vicinity of the active site and project outward of the protein surface. The remaining residues displayed low fluctuations close to 0.2 nm. The stability of the overall system signifies the maintenance of proper simulation conditions throughout the MD run and indicates the suitability of AbMurD for inhibitor screening. The lowest energy conformation of the protein was extracted from the trajectory and evaluated for the model quality.

3.2. Evaluation of AbMurD model quality

The refined model AbMurD structure was evaluated for stereochemical errors and quality. ProCheck analysis revealed that 90% of residues fall in the favored region, 7.8% in the additionally allowed region, and 0.5% in the disallowed region of the Ramachandran map (Figure S3). These findings substantiate the good quality of the
stereochemical parameters and the reliability of the protein structure. Verify3D score was 0.2 for 92% of total residues suggesting the agreement between sequence-structure compatibility (Figure S4). ProSA-web is a reliable diagnostic tool based on the statistical analysis of known protein structures for evaluating the predicted structure from modeling for any potential errors. The calculated Z-score of $-9.92$ indicates the good overall quality of the protein (Figure S5). All these parameters obtained from various analyses validate the correctness of the modeled AbMurD structure. These values suggest the reliability of the developed model for screening potential natural inhibitors and to investigate the protein-ligand interactions.

3.3. Identification of active site residues

The active site of an enzyme constitutes the site where the substrate binds to undergo a catalysis reaction. Targeting the active site residues can hinder the catalysis reaction by the enzyme thereby inhibiting its function. The AbMurD ligase active site and catalytic residues were determined through sequence-structure alignment with the closest homolog from E. coli. The inhibitor, VSV, in the template E. coli is bound at the active site between the three globular domains of the protein (Zidar et al., 2010). The sequence alignment of the template and target proteins aided the determination of active site residues for AbMurD ligase by locating the corresponding residues from E. coli (Figure S1, Table S1) (Kotnik et al., 2007). The identified corresponding active site residues located in AbMurD ligase in the N-terminal domain were Asp36, Ser37, Arg38, Gly74, and Leu75, in the Central domain were Asn139, Ser160, Asp183, and His184 and in the C-terminal domain were Thr325, Lys357, Ser426, Phe427 and Tyr433 (Figure 2A) (Table S1). These residues were conserved in both structures except at four positions in AbMurD ligase, Ser 37 (Thr 36 in template), Leu 75 (Ile 74 in template), Tyr 433 (Phe 422 in template), and Phe 427 (Leu 416 in template) (Figure S6).
3.4. Control docking

The predictive ability of the docking program used to screen the library of natural inhibitors was validated by docking the ligand with known binding mode and binding affinity in the active site of AbMurD ligase through control docking using AutoDock 4.2. The bound ligand (VSV) in the template (PDB ID: 2X5O) was extracted, prepared, energy minimized, and docked into the active site of AbMurD ligase with grid-centered coordinates as \( x = 10.08, y = 28.55, z = 19.35 \) and dimensions 60, 60, 60. The reference ligand docked with an RMS deviation of 1.8 Å with respect to the crystal pose (Figure S7). An RMSD value \( \leq 2.00 \) Å is considered as successful molecular docking for reproducing the experimental conformation of the ligand. The predicted pose as evaluated from the calculated RMSD reveals that AutoDock was successful in predicting and scoring a pose close to the crystal pose. This demonstrates the reliability of the docking program selected for screening, prediction, and scoring of the best conformation of the ligand as well as for the evaluation of protein-ligand interaction. The estimated binding energy of this docked pose was \(-6.42 \) kcal/mol. The reference ligand, VSV, binds at the center of the active site to interact with residues from the three domains, through two hydrogen bonds with active site residues, Thr325 and Lys357 (Figure 2B). The residues involved in the hydrophobic interactions with VSV are Pro73 and Thr325.

3.5. Structure-based virtual screening and molecular docking of natural compounds

Virtual screening is a computational approach to identify probable and promising drug hits based on binding energy and ligand interaction with the receptor (Cheng et al., 2012). The initial structure-based virtual screening of the prepared...
library of natural compounds revealed top 100 compounds having estimated binding energy of interaction equivalent to or better than \(-10.0 \text{ kcal/mol}\). This correlates with a previous study where the estimated binding energy from the screening of chemical library against MurD ligase was also found to be equivalent to \(-10.0 \text{ kcal/mol}\) (Jha et al., 2020). Further, these 100 compounds were subjected to a more exhaustive search using the flexible docking mode of AutoDock Tools v4.2 to remove false positive hits. A ligand-based virtual screening approach has also been used to identify chemical compounds against four drug targets of \(A.\text{baumannii}\) including MurD ligase (Amera et al., 2020).

Molecular docking is a computational tool to predict the binding mode and orientation of the ligands in the receptor active site to explore potential inhibitors based on protein-ligand interaction and binding energy. The identified 100 compounds were docked into the active site of the protein. The side chains of residues Arg38, Phe162, Arg187, Lys357 were kept as flexible as these were blocking the passage to the active site and might have hindered the binding of the upcoming inhibitors. Manual analysis and refinement of compounds screened down false positives based on two criteria. The first criterion was to select compounds with higher negative binding energy than \(-6.42 \text{ kcal/mol}\) as this was the determined binding energy of the reference ligand, VSV during control docking. This screening resulted in 16 compounds with estimated binding energy lower than \(-6.42 \text{ kcal/mol}\). The second criterion was based on the agreement between predicted binding energy with respect to the feasible docked conformation of the ligand and the interactions formed between the ligand and receptor atoms. Hence, compounds that did not interact with at least one protein active site residue or did not have a feasible docked conformation were excluded from further analysis. Thus, only four compounds ZINC08879777, ZINC30726863, ZINC85594516, and ZINC95486217 qualified to the list of probable potential hits due to reasonable bound docked conformation of the ligand at the active site and predicted low binding energy. The compounds that were screened out from the list of potential hits were either having high docking interaction energy than the reference ligand or not having a feasible docked conformation that interacted with active site residues. As the screening of compounds was targeted in the active site of the protein, the interaction of compounds with active site residues was a mandatory criterion. The strength of binding of a compound at the protein active site is exhibited through interactions such as hydrogen bonds, hydrophobic and ionic interactions. The docking binding energy along with protein-ligand interactions of all four

![Figure 4](image-url). The four hit compounds (ball and stick representation) superimposed in the active site of \(Ab\text{MurD}\) ligase (center figure, cartoon representation). Interactions observed between the protein amino acid residues and the compound (A) ZINC08879777 (red), (B) ZINC30726863 (green), (C) ZINC85594516 (yellow) and (D) ZINC95486217 (cyan). The hydrogen-bonded interaction is indicated as black dotted lines and salt-bridge in red dotted lines.
compounds and that of the reference ligand are summarized in Table 1 and the 2D structural formula of compounds is given in Supplementary Material (Table S2).

The compound ZINC08879777 (N-(2-(2-oxo-2,6,7,8-tetrahydrocyclopenta[g]-chromen-4-yl)benzofuran-3-yl)benzo[d][1,3]dioxole-5-carboxamide), with binding energy of $-7.77 \text{ kcal/mol}$, was stabilized through hydrogen-bonded interactions with the active site residue Asn 139 (Figure 3a). This residue is significant as it is initially involved in hydrogen bonding with enzyme-substrate UMA, however, this contact is lost after ligation of D-Glu to form UMAG (Bertrand et al., 1999). In addition, the active site residue, Phe 427 along with several other residues involved in hydrophobic interactions contribute to maintain the bound configuration of the compound in the active site pocket of the protein (Table 1). The functionally important residues such as His 184 and Phe 427 formed $\pi-\pi$ stacking interaction and residues Arg 187 and Arg 38 formed $\pi$-cation interaction with the compound. Several polar interactions involving active site residues Lys 357, and Thr 325 further contributed to the stabilization of the compound ZINC08879777.

The compound, ZINC30726863, (14S,27R)-22,33-dimethoxy-13,28-dimethyl-2,5,7,20-tetraoxa-13,28-diazaoctaacyclo[25.6.2.1\textsuperscript{6}.2\textsuperscript{16}.0\textsuperscript{13}.1\textsuperscript{9}.1\textsuperscript{19}.21.25.0\textsuperscript{14}.8.0\textsuperscript{31}.35.0\textsuperscript{14}.39] nonatriaconta-1\textsuperscript{33},3\textsuperscript{39},4\textsuperscript{39},9\textsuperscript{16},18,21\textsuperscript{36},22,24,31,34,37-dodecaene, most commonly known as cepharanthine, extracted from plant Stephania cephalantha Hayata, is used as a drug in Japan for the treatment of acute and chronic diseases such as leukopenia, snake bites, xerostomia and alopecia (Bailly, 2019). The drug is a naturally occurring alkaloid, known to have unique anti-oxidative, anti-inflammatory, anticancer, antiviral, and anti-parasitic pharmacological properties. It possesses a macrocyclic chemical structure with two benzylisoquinoline moieties and interacts with non-polar residues lining the binding
pocket of the target protein. This compound displays the highest binding affinity with an estimated $\Delta G$ of $-8.10$ kcal/mol compared to other compounds. The residues involved in hydrophobic contact with the compound are Pro 73, Leu 140, Phe 162, Tyr 194, Ala 425, Phe 427, and Tyr 433. Additionally, the active site residues, Tyr 433 and Phe 427 form non-covalent $\pi-\pi$ stacking interaction.

Figure 6. Number of hydrogen bond vs time plot for (A) AbMurD-VSV, (B) AbMurD-ZINC08879777, (C) AbMurD-ZINC30726863, (D) AbMurD-ZINC85594516 and (E) AbMurD-ZINC95486217.
also observed in compound ZINC08879777. Two hydrogen-bonded interaction was observed between the compound and active site residue Thr325 and Ser 426 (Figure 3b). Additionally, a salt bridge interaction involving active site residue Asp 183 explains the high binding affinity of this compound for the protein. These interactions along with several polar interactions utilizing both active site (Ser426, Thr325, and Ser160) and catalytic residues (Lys 116 Asn 139 and His 184) support the binding and strength of the compound in the binding pocket of the enzyme.

The compound, ZINC85594516 (1,4,5-trihydroxy-2-methyl-6-(4,5,9-trihydroxy-2-methyl-10-oxoanthracen-9-y)anthracene-9,10-dione), binds with an estimated interaction binding energy, $\Delta G$, of $-7.56 \text{kcal/mol}$. The compound commonly known as chryslandin, isolated from Kniphofia species is an anthraquinone (Dai et al., 2014). The polar atoms present in this compound engage in three H-bonds with residue Tyr 194 and a catalytic residue Lys 116. The three catalytic residues, Lys 116 Asn 139 and His 184, along with active site residues, Arg 38, Ser 160, and Thr 325 participated in polar interactions. The AbMurD- ZINC85594516 complex is further stabilized through hydrophobic interactions contributed by residues Leu 14, Pro 73, Phe 162, and two active site residues, Phe 427 and Tyr 433 (Figure 3c).

The compound, ZINC95486217, (7H,7H,8,8'-bi[1,3]dioxolo[4',5',4,5]benzo[1,2,3-de]benzo[g]quinoline), containing several aromatic rings, docks into the protein with an interaction energy of $-7.77 \text{kcal/mol}$, similar to compound ZINC85594516. This compound makes one hydrogen bond with the catalytic residue Lys 116. A salt bridge interaction engaging residue Arg 187 was also observed with the compound. The catalytic residues, His 184 and Asn 139, were observed to engage in polar interaction along with active site residues Asp 36, Arg 38, and Ser 160 (Figure 3d). Further, hydrophobic interactions are contributed by residues Leu 14, Pro 73, Leu 75, and Phe 162 as well as by active site residues Phe 427 and Tyr 433.

All the four natural compounds are bound to AbMurD with binding interaction energy between $-7.56$ to $-8.30 \text{kcal/mol}$ as estimated through flexible docking. The binding energy calculated from molecular docking of compound 16-epiestriol (a natural compound) in the binding pocket of four other identified drug targets of multi-drug resistant A. baumannii was also in the range of $-6.0$ to $-8.0 \text{kcal/mol}$ (Skariyachan et al., 2020). The compound ZINC30726863 binds with the most favorable interaction binding energy (lower energy) in comparison to other compounds and reference ligand. This may be supported by the formation of two H-bonded and a salt bridge interactions observed in this compound. The aromatic $\pi$-cation and $\pi$-$\pi$ stacking interaction, observed in compounds ZINC08879777 and ZINC30726863 is an attractive non-covalent interaction that plays a significant role in ligand recognition. These compounds were engaged in the formation of H-bonds with functionally important active site residues Asn 139 and Thr 325. The other two compounds, ZINC85594516 and ZINC95486217, formed H-bonds with a catalytic residue, Lys 116. All observed hydrogen bonds were in the bond length range of 2.6-3.2Å categorized as “moderate, electrostatic” hydrogen bonds which are most commonly found in proteins (Dannenberg, 1998). Interestingly, all the compounds bound at the active site located in the central region of the three globular domains of AbMurD protein and interacted with the functionally conserved residues. Also, these compounds are surrounded by common residues indicating they share a similar interaction mode in the protein active site (Figure 4).

The four screened compounds are highly hydrophobic due to the presence of aromatic rings that interact through non-polar interactions with hydrophobic residues at the binding pocket. The hydrophobic residues Leu 14, Pro 73, Phe 162, Phe 427, and Tyr 433 were involved in non-polar interactions with minimum of three compounds indicating that these residues may be important for molecular recognition (Figure 4). Additionally, functionally important residues such as Ser 160 and Thr 325 were observed to engage in crucial polar interactions with all four compounds. The catalytically conserved residue, His 184 also formed polar interaction with all ligands. The residue His 184 (His 183 in E.coli) is a significant residue that acts as a catalytic base during the catalysis process by reorienting the substrate UMA towards the substrate D-glutamyl acid for the formation of the peptide bond. Another conserved catalytic residue Lys 116 (Lys 115 in template) is known to stabilize the acyl phosphate intermediate formed during the catalytic mechanism by the transfer of gamma phosphate of ATP molecule to the UMA (Bertrand et al., 1999). The residue Lys 116 formed polar interaction with all ligands except ZINC08879777. Thus, lead molecules interact with the catalytic residues and successfully block these residues from performing their catalytic function. All the identified natural compounds were found to form at least one polar interaction with the catalytically conserved residues (His 184 and Lys 116) thereby indicating their ability to inhibit the vital catalytic activity (Figure 3).

Table 2. MMPBSA calculation for the last 10 ns of MD trajectory for each complex.

| Compounds      | van der Waal energy (kJ/mol) | Electrostatic energy (kJ/mol) | Polar solvation energy (kJ/mol) | SASA energy (kJ/mol) | Binding energy (kJ/mol) |
|----------------|-----------------------------|-----------------------------|--------------------------------|----------------------|------------------------|
| AbMurD-VSV     | $-162.19 +/- 20.67$         | $-91.83 +/- 111.86$         | $414.26 +/- 123.30$            | $-19.97 +/- 2.33$    | $-77.26 +/- 21.83$     |
| Zinc08879777   | $-252.96 +/- 10.64$         | $-95.50 +/- 9.76$           | $242.72 +/- 17.27$            | $-22.59 +/- 1.09$    | $-128.33 +/- 15.54$    |
| Zinc30726863   | $-272.16 +/- 12.22$         | $-588.53 +/- 33.69$         | $376.28 +/- 29.56$            | $-24.97 +/- 1.36$    | $-509.39 +/- 27.91$    |
| Zinc85594516   | $-251.93$                   | $-123.99 +/- 16.87$         | $342.89 +/- 32.02$            | $-24.19 +/- 1.25$    | $-57.82 +/- 21.98$     |
| Zinc95486217   | $-231.08 +/- 13.65$         | $-5.13 +/- 2.91$            | $95.77 +/- 20.33$             | $-21.55 +/- 1.40$    | $-161.99 +/- 16.05$    |
role in the identification and screening of favorable natural compounds. Therefore, computational biology, molecular modeling, screening, and docking play a significant role in identifying successful and efficient binders against carbapenem and polymixin-resistant *A. baumannii*.

All these four AbMurD - compound complexes were subjected to MD simulation for 100 ns and studied extensively by emulating the physiological conditions of the biological system and determining their structural deviation and stability.

3.6. MD Simulation

MD simulation study was undertaken to evaluate the stability of the docked pose of the screened natural compounds. This assisted in the refinement of the ligand position in the protein active site to obtain the optimum binding and maximum possible interactions between the protein and ligand. Ligand binding can lead to local conformational perturbations in proteins that can be evaluated using MD simulation studies. The dynamical stability of docked posture of 4 natural lead compounds along with reference ligand and apo AbMurD was examined as a function of time for 100 ns based on root mean square deviation (RMSD), root mean square fluctuation (RMSF), and radius of gyration (Rg).

3.6.1. Root mean square deviation (RMSD)

The RMSD of protein backbone atoms (C, C$_3$, O, and N) with reference to their respective initial structure was calculated for all complexes and apo AbMurD to understand their respective structural stability and flexibility. The RMSD plot illustrates that all complexes were stable during the 100 ns MD simulation timeframe (Figure 5A). The average RMSD value calculated for apo-AbMurD, AbMurD-VSV, AbMurD-ZINC08879777, AbMurD-ZINC85594516 and AbMurD-ZINC85594516 and AbMurD-ZINC95486217 were 0.40, 0.71, 0.51, 0.38, 0.48 and 0.40 nm respectively. The reference ligand, VSV, portrayed a high RMSD value and significant fluctuations after 50 ns in the RMSD plot as compared to other screened natural compounds. This indicated the comparatively lower stability of VSV in the protein active site. All the natural compound complexes attained stability soon after 10 ns except for ZINC0726863 which stabilized only around 20 ns. This may be due to the presence of the bulky and cyclic nature of the molecule which hinders the flexibility of the ligand inside the active site by providing a comparatively lesser degree of freedom. The average RMSD value for the two complexes, AbMurD-ZINC0726863 and AbMurD-ZINC95486217, was observed close to the apo structure indicating a conformation similar to that of the apo structure. Moreover, the small fluctuations observed in the RMSD plot upon binding of compounds may be a consequence of a change in ligand orientation in the active site during the simulation procedure.

3.6.2. Root mean square fluctuation (RMSF)

The Root Mean Square Fluctuation (RMSF) plot assesses the per residue flexibility revealing local flexible regions of the complexes. The regions of high flexibility in proteins correspond to the regions encompassing loops, turns, and coils. These regions are generally reflected by high RMSF value while a low RMSF value indicates a well-structured region. The RMSF trend indicated that all the complexes portrayed more pronounced residue fluctuations in the C-terminal (residue 299-448) domain accounting for its flexibility (Figure 5B). The C-terminal domain has been reported to be highly flexible in *E.coli* MurD ligase upon substrate binding (Kotnik et al., 2007). The average RMS fluctuation for apo-AbMurD, AbMurD-VSV, AbMurD-ZINC08879777, AbMurD-ZINC0726863, AbMurD-ZINC85594516 and AbMurD-ZINC95486217 were 0.16, 3.00, 0.13, 0.14, 0.16 and 0.14 nm respectively. The higher RMSF value reflects the instability of the AbMurD-VSV complex as compared to the screened compound complexes. The RMSF plot also indicates the high flexibility of reference ligand in the N-terminal and C-terminal domains. The lower RMS fluctuation for AbMurD-ZINC08879777, AbMurD-ZINC0726863, and AbMurD-ZINC95486217 complexes suggest that upon ligand binding the protein achieves a comparatively more stable and compact conformation to maximize the interactions with the ligand. The plot for the two complexes, AbMurD-ZINC08879777 and AbMurD-ZINC0726863, revealed high peaks of around 0.6 and 0.7 nm respectively for central domain residues, 238-250. These residues project out as a loop exposed to the surface which are generally unstructured flexible regions known to display higher fluctuations. Similarly, the loop formed by residues 176-190 situated close to the vicinity of the active site demonstrated a fluctuation of 0.5 nm in the AbMurD-ZINC08879777 complex and fluctuation of ~0.45 nm in other complexes.

3.6.3. Radius of gyration (Rg)

The radius of gyration (Rg) is the measure of compactness and stability of a folded protein throughout the simulation. The protein tends to form a compact structure upon ligand binding to maximize the interactions with the bound ligand which may result in conformational changes. These changes can cause variations in Rg which may lead to additional time for a protein to attain stability during simulation. The Rg plot for all screened compound complexes reflects a decrease in Rg indicating that a more compact structure with improved protein folding is attained upon ligand binding in comparison to reference AbMurD-VSV structure (Figure 5C). The average Rg values calculated for apo-AbMurD, AbMurD-VSV, AbMurD-ZINC08879777, AbMurD-ZINC0726863, AbMurD-ZINC85594516 and AbMurD-ZINC95486217 were 2.44, 2.38, 2.30, 2.29, 2.40 and 2.31 nm respectively. The higher Rg value for the apo-AbMurD structure implies that in the absence of the ligand, the structure acquires a less compact and open conformation. The apo structure in *E.coli* MurD ligase has been reported in the open conformation which is known to form an active closed conformation upon binding of substrates (Bertrand et al., 1997). AbMurD-ZINC85594516
complex revealed an average Rg value close to that of apo structure indicating a less compact and open conformation after binding of the ligand. Moreover, the average RMSF value between the two structures was also similar. The Rg plot for the reference ligand depicted fluctuations after 50 ns exhibiting its lesser compact nature in comparison to AbMurD-ZINC08879777, AbMurD-ZINC30726863, and AbMurD-ZINC95486217 complexes. Hence, these three compounds are to a larger extent more tightly bound to the protein to attain a closed compact structure.

3.6.4. Intermolecular Hydrogen bond analysis
The time-dependent hydrogen-bonding pattern analysis was carried out by evaluating the dynamics of hydrogen bonds between atoms of protein and ligand for each docked complex (Figure 6). A strong hydrogen-bonding network was established between each protein-ligand complex. All the complexes exhibited a minimum of two stable hydrogen bonds which persisted throughout the trajectory. The reference ligand and compound ZINC85594516 formed three and four stable hydrogen bonds with the protein respectively (Figure 6A and D). The other three compounds ZINC08879777 (Figure 6B), ZINC30726863 (Figure 6C), and ZINC95486217 (Figure 6E) were able to maintain two stable and consistent hydrogen bonds throughout the MD trajectory. Though a few breaks were observed in the middle of the trajectory, the bonds were maintained till the end. The intermolecular hydrogen bonding pattern after MD simulation reveals an increase in the number of hydrogen bonds for each complex when compared to flexible docking results. The number of H-bonds formed between the ligand and protein is a significant factor during MD simulations. The higher number of H-bonds generally indicates a greater binding affinity of a ligand towards the protein molecule. Thus, all screened compounds ZINC08879777, ZINC30726863, ZINC85594516, and ZINC95486217 displayed substantial binding affinity towards the AbMurD ligase. The compound ZINC85594516 showed the maximum number of hydrogen bonds due to the greater number of polar atoms on its surface, but it failed to form a tight and closed, conformation upon interaction with the protein as observed from the Rg plot.

3.6.5. Binding free energy calculation
The Molecular Mechanics Poisson-Boltzmann Surface Area (MM-PBSA) is an efficient and widely used method to calculate biomolecular interactions and to evaluate the best binding partner based on binding energy. All four complexes were subjected to binding affinity calculation during the last 10 ns of MD simulation trajectory yielding 5000 snapshots or conformations for each complex. Protein-ligand interaction energy along with other energies (electrostatic, van der Waals, polar solvation, and solvent surface accessible area (SASA) energy) were calculated from MM-PBSA estimation (Table 2). The binding free energy for reference ligand was $-77.26$ and for the protein-ligand complexes were $-128.33$ kJ/mol (ZINC08879777), $-509.39$ kJ/mol (ZINC30726863), $-57.28$ kJ/mol (ZINC85594516), and $-161.99$ kJ/mol (ZINC95486217). The compound ZINC30726863 displayed strong interaction and high binding affinity for AbMurD with binding interaction energy of $-509.39$ kJ/mol. The strong binding of this compound with the protein was also supported by flexible docking results exhibiting binding energy of $-8.35$ kcal/mol with two hydrogen bonds and a salt bridge interaction (Table 1). Other compounds demonstrating better binding energy than the reference ligand are ZINC08879777 ($-128.33$ kJ/mol), and ZINC95486217 ($-161.99$ kJ/mol). The estimated negative value for binding free energy indicates favorable interaction between receptor and ligand. The van der Waals energy and polar solvation energy were the prime contributors of the binding free energy in all cases except for compound ZINC30726863. Compound ZINC85594516 having a binding energy of $-57.28$ kJ/mol revealed a low binding affinity for the protein. This compound was also observed to have minimum negative binding energy with the protein obtained after flexible docking.

The MD simulation and docking analysis suggest that compounds ZINC08879777, ZINC30726863, and ZINC95486217 performed consistently well in almost all aspects (RMSD, RMSF, Rg, H-bonds). These compounds were highly stabilized through hydrogen bonds, salt bridges, and several hydrophobic interactions in the active site of the enzyme. They formed polar contacts with the active site and catalytic residues leading to successful blocking of these functionally important residues.

3.7. Physiochemical and ADME property
The four hit molecules (ZINC08879777, ZINC30726863, ZINC85594516, and ZINC95486217) were further evaluated for their molecular physicochemical and pharmaceutical properties utilizing QikProp. The physicochemical descriptors of the four selected natural compounds are within the permissible reference range except for the reference ligand that violates the hydrogen bond donor descriptor reference range (Table 3). The compound ZINC85594516 violated the brain/blood (QPlogBB) descriptor while other natural compounds were within the recommended range for each descriptor (Table 4). The compound ZINC30726863 has the highest molecular weight and possesses the largest solvent surface accessible area (SASA) and hydrophobic component (FOSA) among all. The human oral absorption (HOA) descriptor is a fundamental property of a drug that determines the oral drug bioavailability. All identified compounds predicted higher human oral absorption compared to the reference...
The compound ZINC85594516 exhibited a weaker affinity for the target compared to the control ligand-bound protein (Figure S8). The MD simulation for 300 ns in terms of RMSD, RMSF, and Rg revealed a higher binding affinity (-8.35 kcal/mol) and high binding energy (-509.39 kJ/mol, MMPBSA calculation) compared to compound ZINC30726863 which gave a MIC value of 200 µg/ml. The computational drug likeliness and ADMET predictions of the three compounds, ZINC08879777, ZINC30726863, and ZINC95486217, also suggest that they are ideal lead molecules that can be further explored and optimized to obtain enhanced physicochemical and pharmaceutical properties. Out of these three compounds studied computationally and identified as probable candidates, only two compounds (ZINC08879777 and ZINC30726863) could be purchased and tested for MIC due to the unavailability of compound ZINC95486217 (Table S3). The MD simulation of the two purchased compounds (ZINC08879777 and ZINC30726863) was extended to 300 ns to further evaluate the stability of the protein-ligand complex. The analysis revealed robust binding of the ligand to the protein as observed from the stable trajectory for 300 ns in terms of RMSD, RMSF, and Rg as compared to the control ligand-bound protein (Figure S8).

### 3.8. Biological evaluation: In vitro antimicrobial activity

Experimental analysis is required to evaluate the effectiveness of the screened compounds against the target as computational studies do not fully mimic the physiological environment of the cell (Gimeno et al., 2019). The bacterium E.coli has been extensively exploited for the identification of MurD ligase inhibitors which failed in any therapeutic action probably due to the problems associated with the bacterial cell entry. Studies have shown that designed inhibitors reported for E. coli MurD turned out to be weaker inhibitors of other MurD orthologs (Barreteau et al., 2012). Thus, antimicrobial activity for the two compounds purchased (ZINC08879777 and ZINC30726863) was tested against gram-negative, A. baumannii (ATCC 19606), Salmonella Typhi (ATCC 19430), and gram-positive Staphylococcus aureus (ATCC 43300) bacteria using the broth microdilution method (Figure S9). Both compounds ZINC08879777 and ZINC30726863 exhibited potent antimicrobial activity with MIC values of 100 and 200 µg/ml respectively for A. baumannii (Table 5).

The lower MIC value for compound ZINC08879777 indicates better inhibition of the target protein than compound ZINC30726863 which gave a MIC value of 200 µg/ml. Although compound ZINC30726863 reported minimum binding energy (-8.35 kcal/mol) and high binding affinity (-509.39 kJ/mol, MMPBSA calculation) compared to compound ZINC08879777, but it yielded a higher MIC. This can be due to the cyclic nature of compound ZINC30726863 which restricts its penetration inside the cell. However, both compounds confirmed their substantial binding through various polar and non-polar interactions with functionally...
important residues and lower binding free energy of docking (Table 1). The reported inhibition against *A. baumannii* by these compounds accounts for the fact that structure-based in silico screening was successful in identifying good inhibitors against this valuable drug target. Overall, both the compounds evaluated made an average of two stable hydrogen bonds during MD simulation and were considered very stable in terms of RMSD, RMSF, and Rg in the protein active site. Hence, these compounds could be explored as potential lead inhibitors of *A. baumannii* and can be further modified and optimized to improve their potency.

*A. baumannii* and *Staphylococcus aureus* belong to the ESKEAPE pathogen family which is characterized by increased resistance to antibiotics such as penicillin, vancomycin, and carbapenems (Santajit & Indrawattana, 2016). Thus, to evaluate the broad-spectrum antimicrobial inhibition of these compounds, the MIC for Gram-negative *Salmonella* Typhi (ATCC 19430), and Gram-positive *Staphylococcus aureus* (ATCC 43300) was also determined. In the present study, the compound ZINC08879777 showed good inhibition against both pathogens *S. aureus* and *S. Typhi* with a MIC value of 200 μg/ml. This indicates that further optimization of the compound, ZINC08879777, with an improved binding affinity towards both Gram-positive and Gram-negative bacteria can lead to the development of broad-spectrum MurD inhibitors. The protein sequence alignment of all three pathogens illustrates that the active site residues in the two Gram-negative pathogens are more conserved than the Gram-positive pathogen (Figure S10). The presence of similar residues between *S. Typhi* and *A. baumannii* predicts that the compounds may bind to *S. Typhi* with a similar binding affinity as that in *A. baumannii*. The compound ZINC30726863 presents a MIC of >1600 μg/ml for *S. aureus* and *S. Typhi* which can be attributed to its bulky nature and less conserved active site residues between Gram-positive and Gram-negative pathogens.

### 4. Conclusion

The present work successfully screened two natural potent lead compounds (ZINC08879777 and ZINC30726863) against drug target AbMurD ligase through high-throughput in silico screening and biological evaluation. These compounds revealed a good binding affinity for the enzyme estimated from MM-PBSA and stable trajectories throughout the MD timeframe of 100 ns. The compounds were highly stabilized into the enzyme active site through a stable interaction network comprising hydrogen bonds, aromatic interactions, polar and non-polar interactions. These compounds were found to form polar interactions or H-bonds with the functionally important active site and catalytic residues. An analysis of the protein-ligand complexes reflects the importance of residue Asn 139 in the screening of favorable and potent natural compounds for the target. Thus, this residue should be considered in further studies for selective screening of more potent inhibitors in near future. The MIC assay displayed that the compound ZINC08879777 could inhibit both Gram-positive and Gram-negative pathogens. This study is the first report of a natural herbal-based lead molecule against AbMurD ligase which can also target other bacterial species as indicated by antibacterial assay of the screened identified natural compounds. These two compounds provide the basic scaffold for designing new modified and effective natural inhibitors. Hence, they can be utilized to further guide the design of drug leads targeting other bacterial pathogens and optimized by improving the interactions with the active site residues to enhance their binding affinity. Hence, the combined experimental and computational modeling approach can be used to design antimicrobial agents having elevated activity.

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### Disclosure statement

No potential conflict of interest was reported by the authors.

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