In silico identification of albendazole as a quorum sensing inhibitor and its in vitro verification using CviR and LasB receptors based assay systems

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Abstract

Introduction: Quorum sensing inhibition (QSI) is one of the vital tools to overcome emerging virulence of pathogenic bacteria which aims at curbing bacterial resistance. Targeting QS (quorum sensing) as chemotherapy is less likely to generate resistance among pathogens as it targets only the adaptation and not the survival mechanism of the pathogen. Several QS inhibitors were developed in the recent past but none of them managed to have clinical application due to known toxic effects for human consumption. A rapid development of QS inhibitor drugs could be achieved by verification of the QSI activity of drugs which are already in clinical use with known pharmacology. Recently, a known FDA approved clinical drug niclosamide belonging to an anthelmintic class is found to exhibit QSI activity.

Methods: We have focused our study on Albendazole, another FDA approved clinical drug belonging to the same class for its potential to act as QSI. The structure-based molecular docking is used for finding putative interactions made by this drug with the CviR and LasB receptor protein of Chromobacterium violaceum and Pseudomonas aeruginosa, respectively. Further, the in vitro activity of this drug has been evaluated by employing CviR and LasB receptor-based bioassay. The efficacy of this drug alone and in combination with antibiotic Tobramycin to inhibit P. aeruginosa based biofilms was also analyzed by developing the biofilms on chambered glass slides and performing anti-biofilm assay.

Results: Further, this drug found to inhibit purple pigment violacein production in C. violaceum, which is under the control of C6-AHL-CviR mediated QS in this human pathogen. The in vivo bioassays results suggested that albendazole has great potential to act as a QS inhibitor as found inhibiting violacin production in C. violaceum and biofilm formation in P. aeruginosa, respectively.

Conclusion: It is that structure-based molecular docking guided bioassay evaluation is an efficient tool for finding the new therapeutic use of old drugs which could have more chances to come easily in clinical application for their newly identified therapeutic uses.

Introduction

Researchers all around the world are engaged in finding remedies for the problems that are associated with chemotherapy against Pseudomonas aeruginosa and Acinetobacter sp. in the immune-compromised persons. These organisms have emerged to pose deadly threat for hospitalized patients.1,2 Mostly, the patients, who have gone through the organ transplant or undergoing cystic fibrosis treatment often caught infections from either of these pathogens.3-5 Moreover, these organisms have developed multiple drug resistance along with their behavior to dwell in complex biofilms which further rescue them from the...
attack of antibiotics. The main driving force for bacterial biofilm formation and virulence gene expression comes from a well-known process of quorum sensing (QS), which is cell density-dependent mode of communication used by these bacteria. Both the Gram-positive and Gram-negative bacteria share the same basic principles of QS-regulated gene expression but the signaling and molecular mechanism operating in the QS circuit get deferred. The interruption of QS is the way to fight against these pathogenic bacteria. The discovery of halogenated furanones from the marine alga Delisea pulchra has generated interest among the scientific community to screen several compounds of natural and synthetic origin against QS signaling. Various antibiotics like azithromycin, ciprofloxacin and ceftazidime are known to inhibit bacterial QS at the concentrations below their respective minimum inhibitory concentrations (MICs). In several other reports, conjugates of antibiotics (e.g., ciprofloxacin, gentamicin, tobramycin, clarithromycin, Piperacillin, etc) and N-Acyl homoserine lactones (AHL) analogues (antibiotic-AHL) have been administered to the biological systems. It was found that these conjugates enhance the uptake of antibiotics and hence shown better outcomes in terms of bacterial clearance load. Previously, aminoglycoside antibiotic tobramycin in the sub-MICs, found to disrupt biofilm formation in P. aeruginosa as well as affecting the swarming motility and protease production. However, this organism has shown resistance against several marketed antibiotic formulations.

Several QS inhibitors have been developed with a great potential of QS inhibition activity, but unfortunately, none of them so far is available for the clinical use due to the pharmacokinetics and toxic implications. Here, we have tried to use the principle of evaluating "old drugs for new use" to curb the QS-mediated virulence in P. aeruginosa. The screening of known drugs for the other activities by employing the SOSA (selective optimization of side activities of drug molecules) approach had been already implied by Imperi et al and thereby niclosamide was found to possess a QS inhibition (QSI) activity. This approach is highly advantageous as the resultant hits can be directly tested under the clinical studies or can be used as a lead on drug discovery platform. This process can yield safe drug like compounds at extremely reduced cost than what generally associated with drug discovery pipeline.

Our study is focused on albendazole (Fig. 1), a benzimidazole derivative that is given orally for the treatment of parasitic worms. We are working in the area of QS and screening several secondary metabolites produced by microorganisms subsequent to their QSI activity. We have standardized various bioassays by testing several known inhibitors of QS against violacein pigment production in Chromobacterium violaceum and also green fluorescence protein production in P. aeruginosa lasB-gfp strain. As mentioned earlier, both of the assayed traits are under the control of QS. Interestingly, we found that albendazole has anti-QS activities in sub-MICs in a dose-dependent manner. Therefore, it can inhibit violacein production in C. violaceum. Albendazole also interferes with virulence production and biofilm formation in Pseudomonas aeruginosa lasB-gfp strain. Further, the key interactions and binding pose of albendazole inside the active site pocket of CviR and LasR receptor proteins were evaluated by performing molecular docking analysis. To the best of our knowledge, this is the first report on (i) QS inhibitory property of albendazole, and (ii) demonstrating a reduction in virulence gene expression and biofilm formation at sub-MIC concentration.

Materials and Methods

Molecular docking studies

Molecular docking is one of the most frequently used methods in the structure-based drug design strategy, where small ligands are docked into the active site of target receptor proteins. These ligands are then ranked on the basis of their binding affinity and orientation in the active site. This technique is highly useful for understanding the ligand-binding interactions into the active site, and the ligand-receptor intermolecular forces were analyzed based on the docking score of reference ligand. For performing molecular docking analysis, the following three steps have to be carried out: (1) ligand and protein preparation, (2) grid generation, and (3) ligand docking.

Protein and ligand preparation

The structure of CviR (PDB ID: 3QP5) and LasR (PDB ID: 3IX3) protein co-crystallized with chlorolactone (HLC) MF: (C$_n$H$_{14}$ClNO$_2$) and 3-Oxo-N-[(3S)-tetrahydro-2-oxo-3-furanyl]-dodecanamide (OHN) MF: (C$_{26}$H$_{32}$NO$_4$) ligands were taken from the depository PDB (protein data bank). The protein structures were prepared using the Protein Preparation Wizard incorporated in Maestro. This tool helps in assigning right bond orders to amino acids as well it adds hydrogen atoms to the protein. Finally, the minimization was done using Impref and up to the described state, where the average root mean square deviation (RMSD) of all the atoms comes down to 0.3 Å. Further, for preparation of ligands, Lig-prep module of Maestro was utilized, where low energy ionized, tautomeric and stereoisomeric states of the ligands within a pH range of 7.0±2.0 were generated using the OPLS 2005 force field.
**Receptor grid generation**

Receptor Grid generation module of GLIDE (grid-based ligand docking with energetics) software[^1] was used to generate the grid for molecular docking purpose. The co-crystallized ligand HLC and OHN was used as a reference for the grid generation. The inner grid box of 10 Å was defined around the centroid of bound ligands (HLC and OHN), whereas outer box was extended up to 20 Å.

**Ligand docking**

For the validation of docking protocol, bound ligand was extracted and then was re-docked to generate the same docking poses as found in their co-crystallized forms. Finally, a set of optimized ligands were docked using Ligand Docking module of GLIDE, and they were analyzed on the basis of their GLIDE docking score and intermolecular interactions.

**Visualization software**

For the purpose of molecular visualization and binding site analysis, the academic version of PyMOL (https://pymol.org/academic) was used.

**Bacterial strains, growth conditions and chemicals used**

In this study C. violaceum (CV12472) wild-type strain and P. aeruginosa plasB-gfp (ASV) strain was used. In the wild type strain CV12472, production of purple violacein pigment was under the control of QS.[^2] In strain plasB-gfp (ASV), gene expression of green fluorescent protein (GFP) that results in green fluorescence was under the control of QS.[^3] The assay plates for the C. violaceum were made using the Luria Bertani (LB) (tryptone 1%, yeast extract 0.5%, NaCl 1% and 1% agar). The plasB-gfp strain was grown using the AT media as described previously.[^4] The known QS inhibitor, 4-nitropyridine-N-oxide (4-NPO), was purchased from Sigma-Aldrich and was used at 1 mM concentration as a positive control. Propidium iodide (PI) dye was used for staining biofilms and in 2.5 mM stock) were added to the first well in each row and 100 µL was serially transferred to the next well up to the fifth well. The last well in each row was added with DMSO as a negative control. Finally, 100 µL of 1:40 diluted O/N culture was added to all the wells resulting in 1:80 dilutions of O/N culture. In test tubes, 10 mL of LB broth was inoculated with plasB-gfp (ASV) strain and was kept in incubation (overnight (O/N), 37 °C). An O/N growth culture was diluted 1:40 (about 125 µL of O/N culture was added to 4.875 mL of LB broth). In each row, 170 µL of the LB broth was added to the first well and 100 µL to the rest of the wells in 96-well microtiter plate. To this, 30 µL of the albendazole (1 mM stock) were added to the first well in each row and 100 µL was serially transferred to the next well up to the fifth well. The last well in each row was added with DMSO as a negative control. Finally, 100 µL of 1:40 diluted O/N culture was added to all the wells resulting in 1:80 dilutions of the culture with concentration doses ranging (7.5–4.6 mg/mL) of albendazole. The effect of albendazole on the growth and GFP expression was measured by taking OD at 450 nm and fluorescence under the excitation of 485 nm and emission at 535 nm.

**Violacein inhibition assay**

The production of characteristic purple pigment violacein is under the control of the QS system.[^5] In this wild-type strain, violacein is inducible by the auto-inducer C6-AHL.[^6] Hence, the presence of anti-QS compounds may compete for the binding in the active site of the receptor and thereby results in inhibition of violacein production. The QSI activity of albendazole was checked on C. violaceum using the agar diffusion method. To determine the MIC of albendazole for QS inhibition activity, agar well diffusion method was performed in which a stock solution of albendazole (1 mg/mL) and subsequent dilutions (0.1, 0.2, 0.4, 0.6 & 0.8 mg/mL) were made and added to the wells of seeded CV12472 plates and incubated overnight at 30°C.

**lasB-gfp assay**

Followed by preliminary assay on C. violaceum, further analysis of QSI potential of albendazole was carried out using plasB-gfp (ASV) bioassay. We have performed this assay as previously described in one of our work.[^7] In this study, violacein was inducible by the auto-inducer C6-AHL. Hence, the presence of anti-QS compounds may compete for the binding in the active site of the receptor and thereby results in inhibition of violacein production. The QSI activity of albendazole was checked on C. violaceum using the agar diffusion method. To determine the MIC of albendazole for QS inhibition activity, agar well diffusion method was performed in which a stock solution of albendazole (1 mg/mL) and subsequent dilutions (0.1, 0.2, 0.4, 0.6 & 0.8 mg/mL) were made and added to the wells of seeded CV12472 plates and incubated overnight at 30°C.

**Biofilm inhibition assay**

The biofilms of plasB-gfp (ASV) were established on microscopic coverslips. 10 µL of an overnight culture was added to the chambers having 200 µL of the LB broth and such 5 chambers assembly was kept for incubation at 37°C. After every six hours, the media was replaced with fresh LB broth. About after 24 hours of incubation, the chambers were added with 10 µL of albendazole (1 mM) and then kept for incubation (for 24 hours).[^8] The 3-day old mature biofilms stained with PI, washed twice with phosphate buffer saline (PBS) to remove the unattached cells and subsequently the stained biofilms were fixed using fixative. The biofilms on coverslips were visualized on Nikon Confocal microscope and analyzed using NIS-elements software.

**Effect of albendazole on biofilms**

The biofilms of P. aeruginosa were developed on microscopic cover-slips. The plasB-gfp (ASV) strain was inoculated in 10 mL AT media and incubated overnight at 37°C with 180 rpm shaking. The O/N culture then was diluted to OD 0.2 and 150 µL of this added to the microfuge-coverslip assembly and kept for incubation for 48 hours at 37°C. A water reservoir has been kept in the incubator to prevent drying of the biofilms. The two days old biofilms then treated with sub-MIC concentration (0.16 µg/mL) of albendazole alone and along with tobramycin (100 µg/mL). Since the GFP expression of this strain is under the control of QS, hence green fluorescence served as an indicator of live cells and propidium iodide

[^1]: GLIDE
[^2]: C. violaceum
[^3]: plasB-gfp
[^4]: AT media
[^5]: C6-AHL
[^6]: C6-AHL
[^7]: C. violaceum
[^8]: plasB-gfp
[^9]: BioImpacts
[^10]: 2018, 8(3), 201-209
Results

Molecular Modeling studies

CviR and LasR protein

The x-ray crystal structure of full-length CviR (PDB ID: 3QP5) bound with antagonist chlorolactone (HLC) in a resolution of 3.25 Å was reported by Chen et al. It is only known structure for CviR so far, which is co-crystallized with its antagonist. CviR is a homodimer with two binding domains (Ligand binding domain [LBD] and DNA-binding domain [DBD]) that placed in a “crossed-domain” conformation. In this closed conformation of the protein, 2 DBD units are apart which have reduced affinity to bind with DNA as shown in Fig. 2. The antagonist HLC stabilizes this closed conformation of CviR as compare to the agonist and thus prevents the QS activity.

Similarly, the structure of LasR protein, which directs the synthesis of autoinducer OHN [N-(3-oxododecanoyl)-L-homoserine lactone], is provided in Fig. 2. The bound autoinducer forms the hydrogen bonding interaction with key amino acid residues viz. Ser129, Asp73, Trp60. In addition, the hydrophobic interactions played the significant role in stabilizing the protein-ligand complex. This finding is also noticed by us in the previous study where \( N,N \)-disubstituted biguanides with aryl groups have shown a significant correlation between \( \pi \) stacking ability and observed biological activity.

Molecular docking studies were performed to investigate the binding potential of albendazole towards CviR and LasR protein. The docking protocol was standardized by performing the re-docking of bound ligands in order to get the correct binding pose of HLC and OHN as found in their co-crystallized states. HLC was able to get dock inside the active site of protein with almost identical binding pose (GLIDE score of -8.06) as compared to its co-crystallized x-ray structure. The best scored docked conformer of HLC with its crystallized protein showed superimposition with the RMSD of 0.03 (Fig. 3). This protocol was acceptable and was further utilized to dock other sets of ligands. Similarly, the OHN was re-docked (GLIDE Score = -7.52) in the active site of LasR receptor for validating the docking protocol and found suitable to use the protocol for docking albendazole inside its active site pocket.

The 3D structure of albendazole in SDF format was taken from the PubMed library. Further, the structure was prepared for docking by using Ligprep module of Maestro interface, which was optimized by using the OPLS 2005 force field. The optimized structure was docked inside the active site, where 10 different docked conformers of albendazole were generated. The best-docked conformer of albendazole showed a GLIDE score of -6.89. Fig. 4 gives the binding model of albendazole inside the active site of CviR protein along with the key interactions. Although, the amide hydrogen (2.07 Å) and ring hydrogen (2.49 Å) of albendazole were engaged in the formation of a bifurcated hydrogen bond with Asp97 (1.89 Å), however, in HLC the amide hydrogen involved in the hydrogen bond formation. Secondly, imidazole ring nitrogen also forms hydrogen bonding interaction with Trp84 (2.35 Å), thus it plays the function of lactone moiety of HLC (Fig. 4). The benzene ring that is attached to imidazole ring, showed a hydrophobic interaction with Tyr80 residue.
and involved in π-π stacking. Similarly, in the active site pocket of LasR receptor, albendazole showed hydrogen bonding interaction with Tyr56 (2.06 Å) and Ser129 (2.41 Å). Both these amino acids act as hydrogen bonding donors as shown in Fig. 4. The presence of albendazole in the hydrophobic pocket of CviR and LasR receptors demonstrated the presence of hydrophobic interactions that were formed by benzimidazole framework and hydrophobic amino acid residues. Thus, the molecular docking studies have established the potential of the structural framework of albendazole to quench the mechanism of QS. Table 1 provides the values of docking scores of different compounds against CviR and LasR receptors.

**Biological studies**

Based on the results obtained from the molecular docking analysis, the experimental studies were designed. Albendazole was purchased from Indo-AM and then various in vitro assays were performed to evaluate the presence of QSI activity in this drug.

**QSI activity on CviR**

The QSI activity was checked using CV12472 bio-monitor strain. Albendazole was found to show the QSI activity as indicated by the zone of inhibition of violacein production without any effect on the growth of the indicator organism (Fig. 5).

**QSI activity on LasR**

The QSI activity of albendazole on lasR gene expression was checked using bioassay strain and based on P. aeruginosa LasR receptor i.e. plasB-gfp (ASV). The decreasing concentrations of albendazole were increased the GFP expression as indicated by the dose-response assay plot. This finding confirmed the QSI activity of albendazole against LasR without any significant effect on the growth of the microorganism (Figs. 6 and 7).

**Table 1. Docking scores of different compounds on CviR and LasR receptor**

<table>
<thead>
<tr>
<th>Molecular formula</th>
<th>Inhibitor/Autoinducer</th>
<th>Docking score CviR Protein (3QP5)</th>
<th>Docking score LasR Protein (3IX3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C14H16ClNO4</td>
<td>Chlorolactone (HLC)</td>
<td>-8.06</td>
<td>--</td>
</tr>
<tr>
<td>C10H15NO</td>
<td>C6-HSL</td>
<td>-6.54</td>
<td>--</td>
</tr>
<tr>
<td>C16H27NO4</td>
<td>(OHN)</td>
<td>--</td>
<td>-7.52</td>
</tr>
<tr>
<td>C12H15N3O2S</td>
<td>4-nitro pyridine oxide (NPO)</td>
<td>-5.90</td>
<td>--</td>
</tr>
<tr>
<td>C5H4N2O3S</td>
<td>Albendazole</td>
<td>-6.89</td>
<td>-7.14</td>
</tr>
</tbody>
</table>

**Fig. 3.** Docking pose of positive control chlorolactone (HLC) in the active site of ligand CviR.

**Fig. 4.** Binding model of albendazole and its hydrogen bonding interactions (blue dashed lines) with (A) CviR (PDB ID: 3QP5) and (B) LasR (PDB ID: 3IX3).
Effect of albendazole on Pseudomonas aeruginosa biofilms

The bacterial cells that were remained on the microscopic coverslips were considered as \textit{plasBgf} (ASV) biofilms. And yet the planktonic cells were removed every six hours of growth, whereas the old broth was replaced with fresh broth. On the confocal image analysis of the biofilms, it was found that the control biofilm was formed as a dense mat of green fluorescence bacteria with only 0.01% dead cells (Fig. 8A). The film that was treated with albendazole alone, was found with few partial death of cells. This result implies the biofilm clearance (Fig. 8D-E). The biofilm treated with both albendazole and tobramycin showed an enhanced cell death in the region of biofilm clearance, clearly indicating that at sub-MIC concentration albendazole has acted as QS inhibitor (Fig. 8G-H). It is supported by the fact its sub-MIC concentration is only clearing biofilm with partial cell death. Thus, it is properly permeable for tobramycin to complete cell death in the penetrated area (Fig. 8G-H). Hence, it indicates that albendazole can act as a potent QS inhibitor and can be used with tobramycin in adjunct therapy to curb the pathogenesis of \textit{P. aeruginosa}.

Discussion

The unmet demand for anti-virulence agents with the aim of treating infectious diseases is highly desired. The growing awareness regarding the relationship between QS inhibition and antimicrobial action has led the scientific community to work on the compounds that have the potential for inhibition of the mechanism of QS in pathogenic bacteria.\textsuperscript{17,18} The various bio-monitor organisms have been used for screening the QSI activity. Among all the organisms, \textit{P. aeruginosa} is extensively studied as a model system that can be used for evaluating the QS and biofilm inhibition.

The research on the development of QS inhibitors is largely focused on the identification of structural homologues of autoinducer ligands of QS proteins. Various QS inhibitors belonging to different chemical classes have been discovered by screening libraries of the chemical and natural compounds.\textsuperscript{19, 20} Use of synthetic methodologies is also adopted as a tool for the development of QS inhibitors.\textsuperscript{21} However, some of the compounds prove to be effective in treating \textit{P. aeruginosa} infection in both in vitro and in vivo studies.\textsuperscript{22-24} Despite the fact that multiple leads as QS inhibitors had already been tested, yet the scientific literature supports only three verified clinical trials as listed in the ClinicalTrials.gov database.\textsuperscript{25} This situation is mainly due to the toxicity and pharmacokinetic issues that can be associated with QS inhibitors, so far. Garlic extract is the only QS inhibitor which has been tested in humans.\textsuperscript{26, 27} But the results were not found to be statistically significant, so the improvements were planned. In the following study, ajoene, the active constituent of garlic extract, was synthesized and tested, however, it was found to be less active alone in in vitro models and have shown poor response in in vivo murine model of infection.\textsuperscript{28} Thus, despite all the efforts made in the development of QS inhibitors, the clinical application of QS-based therapeutics remains as an imaginative approach yet.

The main aim of this study was to find out a potential QS inhibitor as an anti-virulence agent using the alternative strategy. Thus, a new application (anti-QS activity) of the already used drug was explored.

Taking into account the QS potential of anthelmintic drug niclosamide (an amide derivative) and its structural features,\textsuperscript{18} another anthelmintic drug albendazole which is an amide ester and contains a benzimidazole framework is selected for investigations. The literature also supports the anti-QS activity of benzimidazole backbone.\textsuperscript{29} Hence, evaluation of QS inhibiting potential of albendazole is worth exploring. Initially, the molecular docking studies...
of albendazole in the active site pocket of CviR and LasR receptors was carried out. It was found that this drug indeed showed interaction with key amino acid residues in the active site of the target proteins. Further, to prove this finding, the biological activity of albendazole was also analyzed which confirms QSI activity. Further, the anti-biofilm activity of albendazole on biofilms of \textit{P. aeruginosa} strain \textit{plasB-gfp} (ASV) was also estimated. The confocal images of biofilms have been clearly demonstrated the regions of clearance in albendazole treated \textit{P. aeruginosa} biofilms (Fig. 8). The frequency of resistance in \textit{P. aeruginosa} isolates to tobramycin, which is an aminoglycoside-based antibiotic is found to be high nearly 30% owing to overexpression of efflux pumps. The biological activity of combined therapeutics (tobramycin with albendazole) was also evaluated and found to be effective more than that of albendazole alone which is also marked by cell death. Hence, it is proven that albendazole show anti-QS activity in sub-MIC concentration and its effectiveness increases to many folds if given in as a complex with an antibiotic.

\textbf{Conclusion}

Development of bacterial-resistant strains has exposed the human population to the large variety of bacterial infections. The QS mechanism in bacteria is involved in the expression of various virulence factors and biofilm formation. Many drug discovery scientists working in the respective field are developing synthetic/natural QS-inhibiting receptor proteins. The compounds that developed so far are associated with the toxicity and pharmacokinetics issues, which have become huge obstacles in their clinical application. Thus, in this study, a contemporary approach was utilized to identify a new perspective for an old drug by keeping in mind its extended advantages.

To summarize the results, the molecular docking studies of albendazole showed that the active site of CviR and LasR protein are perfectly in close contact with this drug. The binding model revealed that the drug can bind with the similar fashion and form the key interactions as that of bound ligands. Further, we have performed the bioassay of albendazole on CviR and LasR receptor-based systems for evaluating its potential as an anti-QS drug. Among a large number of network of QS proteins, LasR had a vital role in sensing mechanism in \textit{P. aeruginosa}. The QSI activity was confirmed by performing dose response assay against LasR on basis of the \textit{lasB-gfp} bioassays. It was also found that this drug can inhibit the virulence gene expression, with restricted exopolysaccharide and biofilm production in \textit{P. aeruginosa}. Further, it can also be used in the conjugate therapy with tobramycin to show the combined effect of antimicrobial even for drug-resistant strains. In this way, the anti-QS and antibiotic activity of the respective drugs acts in a synergistic manner and thus increased the activity of many folds causing cell death of resistant strain, too. Albendazole is already an FDA approved drug and there will not be a question on the safety profile of this molecule. Hence, it can be interested as a candidate drug in the anti-pathogenic modalities. Here, we highlighted the translational importance of this study in terms of its real-life application. Therefore, albendazole can be utilized as an anti-virulence drug to solve the problem of persisters generation.
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Ethical approval

Not applicable.

Competing interests

The authors alone are responsible for the content and writing of the paper.
Quorum Sensing inhibitors


