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SUMMARY AND CONCLUSION

Prolonged treatment with antimicrobial agents has drastically influenced the growth of multidrug-resistant (MDR) bacterial pathogens, with an alarming rise in diseases that are challenging to cure. This unsettling scenario mainly concerns a particular class of bacteria that escapes the existing conventional antibiotics. Virulence mechanisms are currently viewed as molecular targets to develop antivirulence agents to overcome antibiotic resistance's threat to human health. These agents focus on the infection process rather than the growth of bacteria. *Pseudomonas aeruginosa* is a malevolent pathogen that infects several hosts and increases the likelihood of drug resistance to promote biofilm development. The bacterium is considered the most dreadful nosocomial pathogen and the primary cause of fatality in patients suffering from cystic fibrosis (CF).

Biofilms are bacterial cell clusters that are encased in extracellular polymeric substances that the bacteria have synthesized by itself. The generation of virulence factors and the development of biofilms are thought to be responsible for establishing resistance to pathogens. The two central quorum sensing systems in *Pseudomonas aeruginosa* are *LasI/R* and *RhlI/R*, which utilize their autoinducer (AI) molecules 3-oxo-C12-homoserine lactone and C4-homoserine lactone, respectively. Additionally, the *Pqs* system detected by *PqsR* is mediated through the signaling molecule 2-heptyl-3-hydroxy-4-quinolone. These systems are subjected to hierarchical regulation, where *LasR* regulate the *Rhl* and *Pqs* QS circuits. These interrelated cell communications systems trigger virulence factors and antibiotic resistance, demanding new strategies for treating *Pseudomonas aeruginosa* infections.

Quorum sensing disruption is one of the novel alternative strategies that has undergone extensive research. Interfering the QS network might be a promising method for decreasing bacterial virulence and improving antibiotic effectiveness, opening the door for future anti-infective therapies in medical applications. Antivirulence agents targeting the

QS circuitry and their effect on the pathogenicity of *Pseudomonas aeruginosa* have been demonstrated by *in vitro* and *in vivo* studies. Most quorum sensing inhibitors (QSIs) discovered to date are unsuitable for therapeutic development due to their cytotoxicity and undesirable pharmacological characteristics.

In recent years, *in silico* methods have been considered effective aids to traditional drug discovery processes. Diverse strategies have been developed, such as the structure-based screening for identification of QSIs and other approaches for deciphering QS signaling. Virtual screening methods based on molecular docking simulations enable the preselection of promising drug candidates from large compound libraries and allowing only a small number of potential hits to be confirmed through *in vitro* experiments.

In the present study, high throughput virtual screening (HTVS) was attempted to identify potential antagonists against LasR of *Pseudomonas aeruginosa* using Schrödinger software. The LasR inhibitors with favourable binding free energies were selected for subsequent studies. The selected inhibitors that have passed the ADMET and drug-likeness prediction were subjected to molecular dynamics simulation. The cytotoxicity of the selected LasR inhibitors was identified in human peripheral blood lymphocyte (PBL) cells. Further, the selected LasR inhibitors were validated for their antibiofilm and antiquorum sensing efficacies through *in vitro* assays. The gene expression of the essential quorum sensing regulatory genes were examined upon treatment with selected LasR inhibitors. Additionally, a reporter gene assay was used to confirm the LasR inhibitors' antagonistic potency.

The study was carried out sequentially in four phases wherein a molecular docking based virtual screening technique was used in the first phase to find inhibitors that might function as antagonists against LasR in *Pseudomonas aeruginosa.* The 3D structure of the LasR **(PDB ID: 3IX4)** was retrieved from the Protein Data Bank (PDB). The structure was processed using the Protein Preparation Wizard feature of the Schrödinger programme. The Schrödinger small molecule database was used for high throughput virtual screening. The steps involved in HTVS were structure-based virtual screening (SBVS), standard precision (SP) docking, and extra precision (XP) docking. The compounds with favourable docking scores and interactions with LasR were selected. The post-docking analysis was performed using the Prime Molecular Mechanics/Generalized Born and Surface Area (MM-GBSA) method. QikProp was used to infer the ADMET features of the chosen compounds.

Further, to confirm the stability of the selected inhibitors in the LasR's active pocket, molecular dynamics simulation was carried out for 100 ns using Desmond. Cytotoxicity of the selected compounds was assessed in human peripheral blood lymphocyte cells using an MTT reduction assay.

Initially, the Schrödinger small molecule database (3,034,496) were screened for inhibitors against LasR through virtual screening process. 1,942,018 of their compounds were obtained as hits in the HTVS mode of docking with LasR. Standard precision (SP) docking was applied to the top 10% of the compounds from HTVS mode (19,420). The extra precision (XP) docking was applied to the top 10% of the compounds (1942) from the SP docked mode. The top 12 hits with docking scores less than -11.0 kcal/mol were chosen at the end of the virtual screening procedure. In the filtering criteria of the virtual screening process, a Gscore cutoff value of less than -11.0 kcal/mol was regarded as the threshold score. The threshold score of less than -11.0 kcal/mol was chosen based on the docking analysis of LasR inhibitors collected from the literatures. The top three hits namely CACPD2011a-0001928786 - (3-[2-(3,4-dimethoxyphenyl)-2-(1H-indol-3-yl)ethyl]-1-(2-fluorophenyl)urea), CACPD2011a-0001927437 - (3-(4-fluorophenyl)-2-[(3-methylquinoxalin-2-yl)methylsulfanyl]quinazolin-4-one), CACPD2011a-0000896051 - (2-({4- [4-(2-methoxyphenyl) piperazin-1-yl] pyrimidin-2-yl} sulfanyl) -N- (2,4,6-trimethylphenyl) acetamide) were chosen for further studies and referred to as C1, C2, and C3, respectively.

The protein-ligand interaction patterns of the selected inhibitors with LasR were compared with the autoinducer (OdDHL) and the co-crystallized ligand TP-1 (Triphenyl mimic of autoinducer). OdDHL and TP-1 demonstrated a similar hydrogen bonding pattern involving Tyr56, Asp73, Ser129, and Trp60. With the exception of C3, compounds C1 and C2 formed hydrogen bonds with three of the four active site residues. There was no hydrogen bond interaction with C3. The chosen LasR inhibitors had binding free energies (ΔG_{Bind}) ranging between -92.2 and -112.2 kcal/mol. Of the three chosen compounds, C2 had the highest binding free energy (ΔG_{Bind}), and C3 possessed the lowest binding free energy.

The pharmacokinetic profile of the chosen compounds as revealed by QikProp appeared to be good, with all of their parameters falling within the acceptable range. Root mean square deviation (RMSD), radius of gyration (R_g), root mean square fluctuation

(RMSF), intramolecular hydrogen bonds, and other parameters were used in molecular dynamics simulation study to assess the overall stability of the compounds in complex with LasR. The analysis demonstrated that the selected compounds were found to be in stable association with LasR through interaction with crucial amino acid residues in active site of LasR. The three compounds were purchased from Mcule, Hungary and Life Chemicals, Canada and used for further studies. The findings of the cytotoxicity study showed that none of the compounds were cytotoxic when tested on human peripheral blood lymphocyte cells. The percentage of cell viability, when treated with varying concentrations of compounds (125-850 μM), ranged between 70% and 90%. Thus, these findings inferred that all three selected compounds, C1, C2, and C3 are non-toxic and may effectively disrupt the LasR quorum sensing network of *Pseudomonas aeruginosa.*

The second phase was designed to assess the antibiofilm and antiquorum sensing activity of the selected LasR inhibitors through *in vitro* experiments. The minimum inhibitory concentration (MIC) of the selected compounds was determined by the method of micro broth dilution. The antibiofilm activity of the compounds was investigated using biofilm inhibition assay, eradication of preformed matured biofilm, exopolysaccharide inhibition assay, alginate inhibition assay, biofilm total protein assay and evaluation of the metabolic activity of viable cells in biofilm. A violacein inhibition assay in *Chromobacterium violaceum* and a swimming and swarming motility assay *in Pseudomonas aeruginosa PAO1* were used to measure the antiquorum sensing activities of the selected LasR inhibitors.

Initially, two-fold serial dilution of the compounds with concentrations that ranged between 1.95 and 4000 μM was performed in LB broth to determine the minimum inhibitory concentration (MIC). The results demonstrated that the MIC of C1 was 1000 μM (43.3 μg/ml), that of C2 was 1000 μM (42.8 μg/ml), and that of C3 was 500 μM (23.8 μg/ml). The compounds at their subinhibitory concentrations (½ MIC and ¼ MIC) were taken for subsequent studies. The impact of the chosen compounds to prevent the biofilm development was measured using the method of crystal violet staining. The compounds C1, C2, and C3 inhibited biofilm formation more than 80% at their two tested concentrations.

The disruption potential of the selected LasR inhibitors on the preformed matured biofilm of *Pseudomonas aeruginosa PAO1* was observed by measuring their absorbance at

570 nm. The results revealed a dose-dependent disruption of matured biofilm with an inhibition percentage of 84%, 75%, and 81% at ½ MIC for C1, C2, and C3, respectively. The biofilm was reduced by 78%, 74%, and 76% for C1, C2, and C3, respectively when treated with ¼ MIC. The impact of the LasR inhibitors on the production of exopolysaccharides (EPS) was measured. The outcomes suggested that treatment with ½ MIC and ¼ MIC reduced the generation of EPS. The production of EPS was decreased by greater than 50% on treatment with both ½ and ¼ MIC doses of compounds.

The efficiency of the chosen compounds in inhibiting *Pseudomonas aeruginosa PAO1's* ability to produce alginate revealed that the compounds C1, C2, and C3 reduced alginate production at ½ MIC up to 64%, 60%, and 54%, respectively. In the case of ¼ MIC, alginate production was decreased by 46% for all three compounds. The amount of total protein in biofilm was estimated by Lowry's method. In comparison to untreated control, the concentration of extractable protein in samples treated with ½ and ¼ MIC of C1, C2, and C3 was significantly reduced. MTT reduction assay was carried out to observe the metabolic activity of viable biofilm cells. The findings revealed that after treatment with half-MIC and one-fourth MIC of the compounds, the percentage of metabolically active cells in the biofilm was only between 40% and 45%.

The capacity of the selected compounds to retard violacein pigment production in the QS biomarker strain *Chromobacterium violaceum* allowed us to determine their antiquorum sensing activity. All three compounds exhibited an explicit, opaque halo around the well which strongly suggested that they possessed potent antiquorum sensing capacity. To further validate the antiquorum sensing potencies of the compounds, the flagellar motilities of *Pseudomonas aeruginosa PAO1* were investigated. The swimming and swarming motility of *Pseudomonas aeruginosa PAO1* was shrunk by 9-20% and 81-87% on treating with compounds at their tested concentrations. The results inferred that the three compounds profoundly affect swarming motility compared to swimming motility. Thus, the results of phase II indicated that the selected LasR inhibitors possessed potent antibiofilm and antiquorum sensing activity against *Pseudomonas aeruginosa.*

In third phase of the study, the influence of compounds on the quorum sensing mediated secretion of virulence factors in *Pseudomonas aeruginosa* were evaluated. The virulence factors namely pyocyanin, rhamnolipid, *lasB* elastase, total protease, alkaline protease, and lipase, were examined. The change in the appearance and structural

organization of the biofilm on treatment with selected compounds was analyzed using FESEM.

Pseudomonas aeruginosa PAO1 modify the host immune response using redox mediated pyocyanin and biosurfactant rhamnolipid. All three compounds had inhibited the pyocyanin and rhamnolipid production above 50% level at their ½ and ¼ MIC dosages. These results proved the antiquorum sensing potential of the compounds. Protease and lipase plays a major role in the pathogenesis of *Pseudomonas aeruginosa*. In the present work, the selected compounds were able to quench more than 50% of total protease, alkaline protease and lipase activity. The other important virulence factor, elastase, which are crucial during infections, was quantified for its relative gene expression by quantitative real-time reverse transcription PCR (qRT-PCR). The findings demonstrated that the compounds significantly downregulated the expression of *lasB* which in turn will lead to the reduced elastase activity. The FESEM micrograph of the compounds treated samples portrayed disintegrated biofilm, whereas the untreated sample had well organized biofilm architecture. Thus, the results of phase III further confirmed the antibiofilm and antiquorum sensing potential of the selected compounds.

In the fourth phase of the study, qRT-PCR was carried out to quantify the relative gene expression of the quorum sensing regulatory genes, namely *lasI, lasR, rhlI, rhlR, pqsA,* and *pqsR.* Further, the antagonistic efficiency of the compounds at the transcriptional level was evaluated using an *in vitro* β-galactosidase reporter gene assay. *Escherichia coli* DH5α cells harboring plasmid pKDT17 were utilized, in which the LasR gene is linked to a translational fusion of *lasB::lacZ.* The expression of LasR was measured as the amount of β-galactosidase enzyme activity.

From the results of phase IV, it was seen that the compounds at their half-MIC downregulated the quorum sensing regulatory genes, specifically *lasI, lasR, rhlI, rhlR, pqsA,* and *pqsR.* In particular, C1 noticeably lowered the expression levels of all quorum sensing regulatory genes compared to C2 and C3. β-galactosidase reporter gene assay inferred that in the presence of acyl homoserine lactone (AHL), there was a decrease in the β-galactosidase enzyme production with an increase in the concentration of compounds. The LasR inhibitors drastically reduced the activity of β-galactosidase at 100 nM concentration. The percent reduction was found to be 85%, 92%, and 79%, for C1, C2, and C3, respectively. The results of reporter gene assay further confirmed that antiquorum

sensing ability of the compounds are due to their interference in binding of AHL to the LasR receptor.

The results of the *in vitro* antibiofilm and antiquorum sensing activity validated the antagonistic potential of the three compounds CACPD2011a-0001928786 - (3-[2-(3,4-dimethoxyphenyl)-2-(1H-indol-3-yl)ethyl]-1-(2-fluorophenyl)urea), CACPD2011a 0001927437 - (3-(4-fluorophenyl)-2-[(3-methylquinoxalin-2-yl)methylsulfanyl]quinazolin-4-one), CACPD2011a-0000896051 - (2-({4-[4-(2-methoxyphenyl)piperazin-1-yl]pyrimidin-2-yl}sulfanyl)-N-(2,4,6-trimethylphenyl) acetamide) for LasR screened through HTVS. Among the three compounds, compound C1 was found to have a greater antibiofilm and antiquorum sensing potential compared to the other compounds C2 and C3. Thus, the present research work helped to identify three compounds that could be used as drug leads for designing effective quorum sensing inhibitors to treat *Pseudomonas aeruginosa* and its related biofilm ailments in the near future.

Recommendations for future studies

- The metabolomic and transcriptomic profiles of *Pseudomonas aeruginosa* biofilms can be integrated and studied to understand the significant regulatory pathways associated with its infections.
- The ability of the screened compounds to retard the biofilm development can be studied in *in vivo* model systems.
- The selected compounds can be used as coating agents to develop multifunctional biocompatible medical implants with improved anti-infective potential and to overcome implant-associated infections.
- The effect of the selected compounds in combination with existing antibiotics can be studied to enhance antibiotic sensitivity in *Pseudomonas aeruginosa*.