Innovare Academic Sciences

International Journal of Pharmacy and Pharmaceutical Sciences

ISSN- 0975-1491 Vol 7, Supple 1, 2015

Original Article

DESIGN, SYNTHESIS AND VALIDATION OF CqsS RECEPTOR AGONIST TO MODULATE THE QUORUM SENSING CIRCUIT OF *VIBRIO CHOLERAE*–A MOLECULAR MIMIC THERAPY

HEMA M.[1], JADAV SAIKIRAN[1], KAMLESWAR C.[1], V. SRIDHARAN[2], GURMEET KAUR[1], P. BALAMURUGAN[1], SUMANA M. N.[3], ADLINE PRINCY S.[1]*

[1]Quorum Sensing Laboratory, Centre for Research on Infectious Diseases (CRID), School of Chemical and Biotechnology, [2]School of Chemical and Biotechnology, SASTRA University, Thanjavur-613401, Tamil Nadu, India, [3]Department of Microbiology, JSS Medical College and JSS University, Mysore

Email: adlineprinzy@biotech.sastra.edu

Received: 02 May 2015 Revised and Accepted: 22 Sep 2015

ABSTRACT

Objective: Our earlier studies have characterized a compound from *Melia dubia* leaves, 4-ethyl resorcinol as an agonist of the Quorum Sensing (QS) receptor, CqsS agonist that had reverse engineered the QS circuit of *V. cholerae* from low-density to high-density state to effectively inhibit biofilm and enhance the production of protease to detach itself form the host tissue. So, the objective of this study was to synthesize structural derivatives of 4-ethyl resorcinol, to enhance its activity and to down regulate the host cell toxicity was validated.

Methods: The antimicrobial (cell-density) and anti-virulent (protease, hemolysis, stress responds and biofilm inhibition) properties of the structural derivatives of 4-ethyl resorcinol were performed.

Results: The results indicate that the compound has up surged protease expression along with a remarkable decrease in hemolytic activity at the 7th hour. The stress responds in treated culture has a higher survival rate while the bacterial cells in the control succumb to stress stating the potential of the drug to induce HCD (high cell density) condition in the LCD (low cell density) state. From CLSM analysis we state that there was significant amount of dead colonies and disrupted biofilm in the respective treated culture

Conclusion: This could possibly open up a direction to curb the bacterial biofilm formation and may also turn out to be a potent drug against treating *Vibrio cholerae* infection at an early point

Keywords: 4-Ethyl Resorcinol, *Vibrio cholerae,* Biofilm.

INTRODUCTION

Cholera is solely an intestinal diarrheal disease caused by *Vibrio cholerae,* rod-shaped, Gram-negative bacteria [1]. Antibiotics (tetracycline, doxycycline, etc.,) are useful adjuncts in treatment that shortens the infection time with the pathogen. The distinct behavioral pattern of any bacteria to their diverse milieu is an effect of the communication scheme between them known as the Quorum Sensing (QS). Chemical molecules called "Auto-Inducers" serve as the bacterial lingo. In common, most bacteria show to produce appropriate output at a high cell density (HCD) but surprisingly reverse condition is persisting in the case of *V. cholerae i.e.* under conditions of Low Cell Density (LCD) virulence factors are expressed and at HCD, repression occurs. Additionally, at HCD activation of HapA (Haemagglutinin Protease) occurs which facilitates the dissemination of the bacterial cells from the human intestinal cells. The inter-species QS System-I and intra-species QS System-II responds to signals, AI-2 and CAI-1 respectively.

The lower concentration of the auto-inducer signals leads to an inefficient binding to their cognate receptor CqsS or LuxPQ respectively. Consequently, receptors serve as kinases and initiate a cascade of mechanisms which make the Qrr1-4 to bind with mRNA transcript of HapR. (A global virulence repressor protein in *Vibrio cholerae*). [2-6] and renders the translation of HapR impossible [6]. In LCD conditions, virulence factors like Cholera toxin, Toxin co-regulated pilus, Haemolysin, Biofilm are expressed, but factors like HapA protease, σ[54]are repressed because of the absence of translation of HapR mRNA transcript [7-10]. Hence, it could be inferred that "virulence and signal concentration are inversely proportional" in the case of *Vibrio cholerae.* At HCD, the binding of CAI-1 and AI-2 to the cognate receptors CqsS and Lux P/Q occur. This binding switches them to function as kinase to phosphatase enzymes. Phosphatase activity leads to dephosphorylation of LuxO protein and brings about repression in qrr1-4 production. Qrr1-4 repression subsequently leads to a successful translation of HapR mRNA transcript. As a result, virulence factors like haemolysin, biofilm, cholera toxin, and toxin co regulated pilus are repressed and HapA protease and σ [54] are expressed at HCD conditions. Therefore, establishing QS (HCD Condition) in *Vibrio cholera* in a LCD state using either structural or functional quorum (receptor agonist) mimics molecules could be a novel approach to achieve an anti-virulent effect [11]. In this work, we had focused to increase the efficiency of an established mimic molecule 4-ethyl resorcinol derivatives, an ultimate plant-derived compound from *Melia dubia* [12].

MATERIALS AND METHODS

Computational studies

Homology modeling of *Vibrio cholerae* CqsS protein

The amino acid sequence of the transmembrane CAI-1 receptor protein, CqsS of *Vibrio cholerae* El tor (Uniprot Accession Number: Q9KKM66) was loaded into modeler 9v8 and searched for homology using BLAST homology search options in Schrodinger Maestro. The protein that had the maximum conserved sequences to CqsS receptor was chosen as the template and 10 models were generated independently in the modeler. The model with the least Root Mean Square Deviation (RMSD) value was selected.

Ligand and protein preparation

4 Structural derivatives of 4-Ethyl resorcinol that are to be synthesized were drawn using ACD Chemsketch. The energy minimized 3D ligand file was developed for docking using Schrödinger Lig Prep software. The modeled CqsS was prepared for docking by using protein preparation wizard of Schrödinger Maestro. Receptor grid was generated encompassing the sites with 0.375Å spacing.

Molecular docking

Docking studies were conducted using the Glide application of Schrödinger Maestro. Ligands and the prepared protein files were

imported and docked using XP precision. Glide score for all the four compounds was named and registered

Chemical synthesis

Structural derivatives of 4-Ethyl resorcinol are synthesized by condensation reactions. 4-ethyl resorcinol has two hydroxyl groups at ortho and para positions. Both individual and double replacement of these hydroxyl groups using benzyl and benzoyl groups is performed. Initially, one equivalent (500 mg) of 4-Ethyl resorcinol was added with one equivalent (618.99 mg) of benzyl bromide in the presence of potassium carbonate (600.03 mg). The mixer was set aside to shift in a magnetic stirrer for 24 h at room temperature. The product was then parted from the reaction mixer using the column chromatography. Then the same reaction was carried away with two equivalents of benzyl bromide (1.2g) to bring the double substitution product. The subsequent reactions were taken away with one equivalent of benzoyl chloride (508.58 mg) and two equivalents of benzoyl chloride (1.017g) as second starting material to generate the desired product. One equivalent of 4-ethyl resorcinol with one equivalent of benzyl bromide yielded 5-(benzyloxy)-2-ethylphenol and with two equivalents of benzyl bromide yielded 4-ethyl-1,3-phenylene) bis (Oxy) bis (methylene) dibenzene. (fig. 1 and fig. 2).

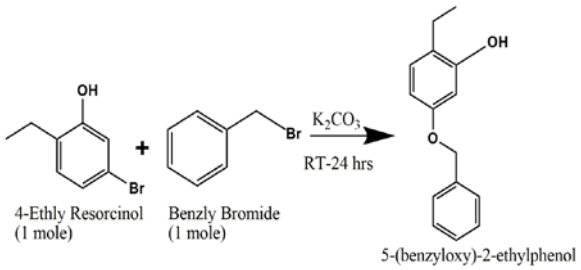


Fig. 1: Scheme for the synthesis of CqsSER-1

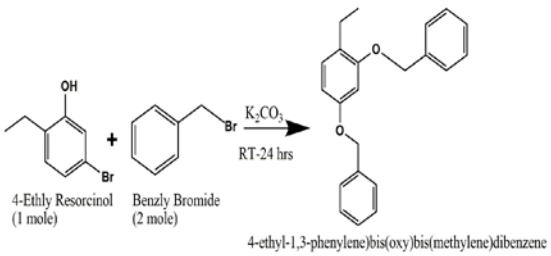


Fig. 2: Scheme for the synthesis of CqsS ER-2

One equivalent of 4-ethyl resorcinol with one equivalent of benzoyl chloride yielded 4-ethyl-3-hydroxyphenyl benzoate and two equivalents of benzoyl chloride yielded 4-ethyl-1,3-phenylene benzoate. (fig. 3 and fig. 4). Proton NMR was carried out to confirm the synthesis of compounds.

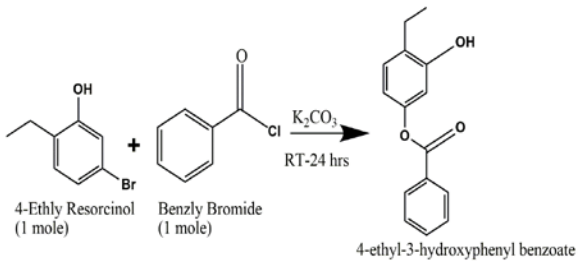


Fig. 3: Scheme for the synthesis of CqsS ER-3

Bacterial survival and growth assay

Fresh inoculum of *Vibrio cholerae* O1 MTCC 3906 collected from Microbial Type Culture Collection Centre, Chandigarh in Luria Broth was prepared at the dilution rate of 1:100 from an overnight culture. The number of colonies at the exponential stage was determined by plating the samples from the inoculum on LB agar plates. The culture at the exponential stage was then seeded in a microliter plate with each well supplemented with varying concentration (2, 4, 6, 8, 10, 50, 100, 120, 140, 150µg/ml) of the four test compounds in triplicates. The optical density was observed at 600 nm to observe the bacterial growth at the time of seeding in the microtiter plate. The plate was then left for 24 h to examine the bactericidal effect of the test compounds. After 24 h optical density were observed at 600 nm to calculate the bacterial growth and at the same hour plating of samples was carried out on LB agar plates from the samples taken from each well to determine the number of colonies survived. The Gompertz growth curve equation percentage of bacterial survival was used for analysis [13].

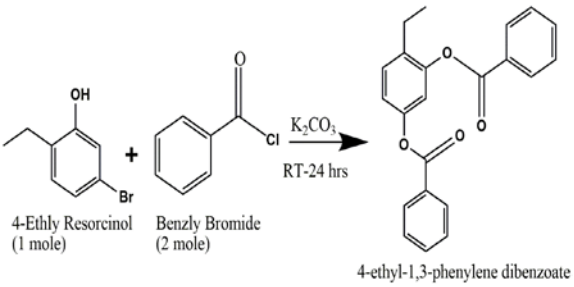


Fig. 4: Scheme for the synthesis of CqsS ER-4

Biofilm inhibition assay

The assay was carried out as described in Ravichandiran V*et al.*[14]. In this assay, 10 µl of the *Vibrio cholerae* O1 MTCC 3906 culture was added to the wells in a 96-well microtitre plate along with 100 µl of LB medium and 100 µl of varying concentrations (2, 4, 6, 8, 10, 50, 100, 120, 140, 150µg/ml) of all the four compounds in triplicates. The plate was incubated at room temperature for 24 h. Then planktonic cells were washed off. Further the step was followed by washing the wells twice with de-ionized water. 210 µl of 0.1% Crystal Violet (w/v) was added to the wells and a short incubation time of 10 min was given. The dye was discarded and the wells were again washed twice with de-ionized water. Finally, the crystal violet adhering to the biofilm was solubilized in 210 µl of Dimethyl Sulphoxide (DMSO). Optical Density (OD) was measured at 595 nm in Bio Rad i-Mark microplate reader. A graph between percentages of inhibitions versus the various concentrations of these compounds were plotted to calculate the minimum biofilm inhibitory (MBIC).

Biofilm eradication assay

In this assay, 100 µl of *Vibrio cholerae* O1 MTCC 3906 culture was added to a 96-well microtitre plate having 100 µl of LB medium and incubated for 24 h at room temperature. Plank tonic cells were removed from all wells and the respective wells were treated with the compounds at varying concentrations (2, 4, 6, 8, 10, 50, 100, 120, 140, 150µg/ml) and incubated for 30 min at room temperature. The assay was carried out in triplicates. The minimum biofilm eradication (MBEC) was calculated and a graph was plotted between percentage eradication versus the various concentrations of the compounds.

Anti-virulent assays

Proteolysis assay

Azocasein assay was employed for quantitative estimation of protease as described by Hasegawa H*et al.*[15]. The cell supernatants of *Vibrio cholerae* O1 MTCC 3906 of both treated and untreated with two concentrations of drug (EC_{50} and EC_{90}), native CAI-1 and 4-Ethyl resorcinol were obtained by centrifugation the culture tubes at 2600rpm/10 min/4 °C. 200 µl of the supernatant was incubated with 800 µl of Azocasein for 30 min at 37 °C. For this volume, 1200 µl of 1% Tri Chloro Acetic Acid (TCA) was added to arrest the enzymatic reaction. Ice incubation for 30 min was done

and it was centrifuged at 13000rpm/5 min. To 1600 µl of this supernatant, 400 µl of 1.8N NaOH was added. Optical density was measured at 420 nm against the blank (Azocasein+TCA+NaOH). One unit of protease activity is defined as the amount of enzyme required to digest 1 mg of azocasein in 1 min.

$$\% \text{ proteolysis} = \frac{\text{Absorbance (Sample)} - \text{Absorbance (Control)}}{\text{Absorbance (Control)}}$$

Hemolysis assay

The procedure was a modified version of the assay carried by Fagerlund A *et al.*[16]. 10 ml of sheep blood was centrifuged at 2400rpm for 5 min. The pellet obtained was washed twice with 10 ml Phosphate Buffer Saline (PBS). 10 µl of this erythrocyte suspension was incubated with the cell supernatants of *Vibrio cholerae* O1 MTCC 3906 (untreated-control) and treated with two effective concentrations of the drug (EC_{50} and EC_{90}), native CAI-1 and 4-Ethyl resorcinol (that was previously obtained by centrifugation of the cell culture sample at 12400rpm/10 min/20 °C) for 1 hour at 37ºC. Finally, the incubated sample was centrifuged at 2400 rpm/5 min. The optical density of the supernatant obtained was read at 540 nm using 1 % Triton X as the positive control. Water along with the erythrocyte suspension was considered to be the blank. % Haemolysis was calculated using the following formula:

$$\% \text{ Haemolysis} = \frac{\text{Absorbance (Sample)} - (\text{Absorbance Blank}}{\text{Absorbance (Positive Control)}}$$

Stress response assay

The assay was based on stress response of the *Vibrio cholerae* to H_2O_2 [17]. 15 mM H_2O_2 was incubated with *Vibrio cholerae* O1 MTCC 3906 both untreated and treated groups with two concentrations of drug (EC_{50} and EC_{90}), native CAI-1, 4-Ethyl resorcinol for various time intervals (30 min & 60 min).

The percentage survival counts were calculated based on time zero and indicated time points plate counts. The experiment was repeated for LCD (7^{th} hour sample), HCD (15^{th} hour sample) and crude CAI-1 treated. The stress response profile was evaluated based on plotting a graph of percentage survival versus time for culture samples treated with various moieties. The increased stress response correspond to the production of RpoS (RNA Polymerase Sigma Factor–σ[54]) which in turn is activated by Hap R. The assays were done in order to confirm the binding of CAI-1 mimic molecule. to activate the production of Hap R and reverse engineer the quorum sensing circuit of *Vibrio cholerae* from LCD to HCD state.

Cell cytotoxic studies

Hep-G2 cells were seeded in 48 well plates at a seeding density of 15,000 cells/well. The cells were checked for its confluence, once it has attained 70% of confluence, the $MBIC_{50}$ (10µg/ml) and $2XMBIC_{50}$ (20µg/ml) concentrations of the synthesized drug was added. After 24 h of incubation, the supernatant was used for LDH (Lactate Dehydrogenase) assay.

Lactate dehydrogenase (LDH) assay

Cytotoxicity of the synthesized drug at its varying concentration was determined by lactate dehydrogenase (LDH) assay. The assay determined the release of cytoplasmic lactate dehydrogenase into the cytosol due to the leakage of the damaged cells. After 24 h of incubation, 50 µl of culture medium was collected and incubated with the reaction mixture consisting of NAD^+(50 µl), lactate (50 µl) and phosphate buffer (0.2 M) pH 7.4. The absorbance was measured at 340 nm.

Confocal laser scanning microscopy analysis of biofilm

The overnight culture of the *Vibrio cholerae* O1 MTCC 3906 was diluted 1:100 in a fresh medium of TSB. The cover slips were placed in sterile 96-well plates and diluted culture was added in the cover slip placed in the wells. A well with the diluted culture without drug as a negative control, with 4-Ethyl resorcinol as positive control and the test wells with diluted culture were supplemented with two different concentrations of the compounds ($EC_{50 \text{ and }} EC_{90}$). After the biofilms were grown for 18h, the suspension was aspirated and removed carefully. The biofilm was rinsed delicately in 0.9% NaCl solution. Stock solutions of fluorescein isothiocyanate (5 mg/ml) and ethidium bromide (1.25 mg/ml) were prepared beforehand. 5 µl each of the dyes were mixed with 1 ml of cold 0.9% NaCl solution to obtain the working solution. The biofilm was dyed with 5 µl of the working solution of the dyes for 10 min, and then the excess dye was removed by washing with 0.9% NaCl. The cover-slip was then dried for 2 min at ambient temperature and then fixed using 50 µl of toluene. Confocal imaging was performed at wavelengths around 500 nm using Olympus Confocal Laser Scanning Microscope to obtain the live/dead imaging [18].

RESULTS

Docking results

Molecular docking studies demonstrated the high binding affinity (Least G Score) for 4-Ethyl Resorcinol (Positive control) and its derivatives (CqsS ER-1, CqsS ER-2, CqsS ER-3, CqsS ER-4) towards the CqsS receptor model. It has been proved already that both 4-ethyl resorcinol and native CAI-1 binds in the same binding pocket in CqsS. (His 73 residues). Interaction pattern of these ligands with CqsS receptor (table 1) showed that among all four ligands CqsS ER-1 had higher a binding affinity and made similar interactions with His73 residue as in the case of native CAI-1 and 4-Ethyl resorcinol.

ADME property

Absorption, Distribution Metabolism, Excretion are the four basic criteria that influence the drug levels and their kinetics of exposure to the tissues. This greatly influences the pharmacological activity of the drug. ADME results obtained from all the four compounds were tabulated (table 1). Rule of five evaluates drug likeness of a chemical compound with some pharmacological activity and its ability to be an orally active drug for human being. According to this rule a drug to be orally active should not have more than one violation of the following criteria. Not more than 5 donors and 10 hydrogen bond acceptors. The molecular mass of the compound should be less than 500 daltons and the octanol-water partition coefficient log *P* not greater than 5. It was found that all ligands except CqsS ER-2 showed to be cooperative for drug-likeness property.

Proton NMR

Synthesized compounds were confirmed using proton NMR. Samples were dissolved in $CDCl_3$ for analysis by proton nuclear magnetic resonance. The NMR results for all the four compounds are as follows.

CqsS ER1-5-(benzyloxy)-2-ethylphenol

Yield 79%, ^{1}H–NMR ($CDCl_3$), 300Hz): δ: 1.23 (t, *J* = 7.5 Hz, 3H), 2.57 (q, *J* = 7.5, 2H), 4.28 (s, 1H), 5.02 (s, 2H), 6.45 (d, *J* = 2.4 Hz, 1H), 6.25 (dd, *J* = 8.4Hz, 2.4 Hz, 1H), 7.03 (d, *J* = 8.4 Hz, 1H), 7.32-7.43 (m, 5H)

CqsS ER2-4-ethyl-1,3-phenylene)bis(oxy)bis(methylene) dibenzene

Yield 65%, ^{1}H–NMR ($CDCl_3$), 300Hz): δ: 1.20 (t, *J* = 7.5 Hz, 3H), 2.65 (q, *J* = 7.5, 2H), 5.03 (s, 2H), 5.03 (s, 2H), 6.53 (dd, *J* = 8.4 Hz, 2.4 Hz, 1H), 6.6 (d, *J* = 2.4 Hz, 1H), 7.07 (d, *J* = 8.4 Hz, 1H), 7.32-7.45 (m, 10H)

CqsS ER3-4-ethyl-3-hydroxyphenyl benzoate

Yield 84%, ^{1}H–NMR ($CDCl_3$), 300Hz): δ: 1.24 (t, *J* = 7.5 Hz, 3H), 2.63 (q, *J* = 7.5, 2H), 5.11 (s, 2H), 6.67 (d, *J* = 2.1 Hz, 1H), 6.73 (dd, *J* = 8.4 Hz, 2.7 Hz, 1H), 7.16 (d, *J* = 8.4 Hz, 1H), 7.50 (t, *J* = 7.5Hz, 2H) 7.64 (t, *J* = 7.3Hz, 1H) 3.19 (d, *J*= 7.5 Hz, 2H).

CqsS ER4-4-ethyl-1,3-phenylene dibenzoate

Yield 73%, ^{1}H–NMR ($CDCl_3$), 300Hz): δ: 1.23 (t, *J* = 7.5 Hz, 3H), 2.64 (q, *J* = 7.5, 2H), 7.11-7.14 (m, 2H), 7.36 (d, *J* = 9 Hz, 1H), 7.48-7.56 (m, 4H), 7.61-7.66 (m, 2H), 8.18-8.24 (m, 4H).

Bacterial growth and survival

The CqsS agonist effect over *Vibrio cholerae* growth and survival was carried out to optimize the minimum inhibitory concentrations. At concentrations below the NIC, growth occurs at a pace equal to the control and the Gompertz model was used to ascertain the MIC and

NIC values. The increasing concentration of all four CqsS agonist showed considerable decline in their growth. Further the NIC and MIC values obtained from the graph were tabulated (table 2).

Biofilm Inhibitory and Biofilm Eradication

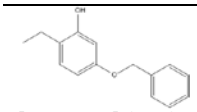
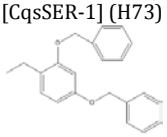
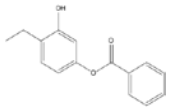
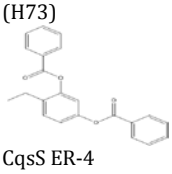
The CqsS agonists were tested with varying concentrations for their biofilm inhibitory and eradication capacity. The $MBIC_{50}$ and $MBEC_{50}$ values for all four CqsS agonist were tabulated (table 3).

These results suggest that the biofilm eradication concentration is much lower than the bactericidal concentration of the compounds CqsS ER-1, CqsS ER-3, CqsS ER-4. This proposes that these compounds have high potential in eradicating the biofilm at a much lower concentration by altering the quorum sensing pathway.

Selection of the most credible compound

In accordance with the docking and *in vitro* studies, the effect of all four CqsS agonist on *Vibrio cholerae* survival and biofilm eradication results 'CqsS ER-1' was chosen as the best. It had a higher binding affinity (-3.80) and was found to bind to the same binding pocket where the native CAI-1 and the proven mimic molecule 4-ethyl resorcinol binds (His 73 residue). Its biofilm inhibitory concentration was also found to be within the non-inhibiting concentration which proves that this compound was not lethal but suppresses the biofilm formation. The CqsS ER-1 was further taken to characterize its mode of action as a CqsS agonist to reverse the LCD (low cell density state) to HCD (High Cell Density) state.

Table 1: Structure, interaction pattern and ADME property of the CqsS agonists

Compound	Rule Of Five	Molecular weight (daltons)	Toxicity Predicition			Log P
			QPlog HERG	QPlog BB	Qplog Khsa	
 [CqsSER-1] (H73)	Positive	214.263	Positive	Positive	Positive	4.09
 CqsS ER-2 (Y75)	Positive	318.415	Positive	Positive	Positive	6.15
 CqsS ER-3 (H73)	Positive	242.274	Positive	Positive	Positive	4.03
 CqsS ER-4 (Y75)	Positive	346.382	Positive	Positive	Positive	5.91

- Toxic free behavior

Table 2: The non-inhibitory (NIC) and minimum inhibitory (MIC) concentration of CqsS agonist on growth of *V. cholerae*

Compound	MNIC (µg/ml)	MIC (µg/ml)
CqsS ER-1	21.48	332.1
CqsS ER-2	6.94	8.30
CqsS ER-3	5.27	83.98
CqsS ER-4	40.34	114.1

Table 3: The minimum biofilm inhibition (MBIC) and eradication (MBEC) concentration of the CqsS agonist

Compound	$MBIC_{50}$ (µg/ml)	$MBEC_{50}$ (µg/ml)
CqsS ER-1	10	6
CqsS ER-2	8	8
CqsS ER-3	6	8
CqsS ER-4	4	50

Protease assay

Protease assays were carried out to confirm the activity of the cell signaling mimic molecule 'CqsS ER-1' to reverse the LCD state to HCD state. Protease assay was performed with the two concentrations of the compound ($MBEC_{50}$, $MBEC_{90}$) and with native CAI-1 & 4-ethyl resorcinol as positive controls. Results (fig. 5) indicate that the compound has up surged protease expression.

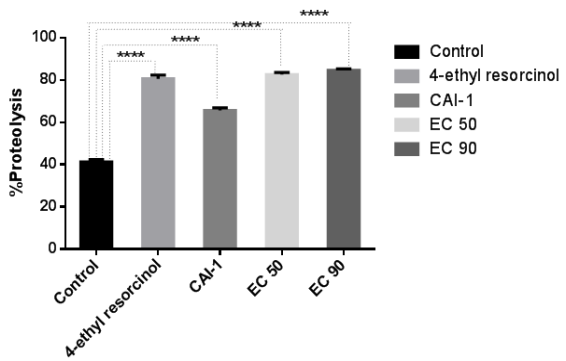


Fig. 5: Effect of CqsS agonist (EC_{50} and EC_{90}) on reversing the phosphate flow to turn on the protease production. (Positive Control-4-Ethyl resorcinol; native CAI-1)

The graph was plotted in accordance with the values obtained from two ways Anova and the asterisk in the graph shows the significance. This is a characteristic attribute of a high cell density condition as there is a dephosphorylation of LuxO protein, which brings about repression in qrr1-4 production. Qrr1-4 repression subsequently leads to a successful translation of HapR mRNA transcript and as a result, HapA protease was expressed.

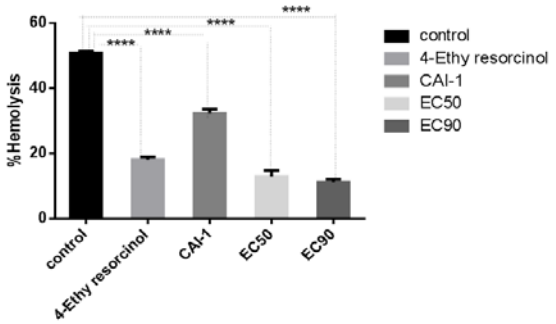


Fig. 6: Effect of CqsS agonist (EC_{50} and EC_{90}) on reversing the phosphate flow to turn off the hemolysin production. (Positive Control-4-Ethyl resorcinol; native CAI-1)

Hemolytic activity

Under LCD conditions (7th hour from inoculation), haemolysin is expressed because of the absence of translation of HapR mRNA transcript. Hemolytic assay was carried out with the two concentrations of the compound ($MBEC_{50}$, $MBEC_{90}$) and with native CAI-1 and 4-ethyl resorcinol as positive controls. The results in fig. **6** suggest that there is a remarkable decrease in the haemolytic activity at the 7th hour of growth which is proposed to be the LCD state. This again clearly shows that the LCD conditions had been reversed to HCD state. The graph was plotted in accordance with the values obtained from two way Anova and the * in the graph shows the significance.

Stress response assay

Vibrio cholerae O1 MTCC 3906 was subjected to stress, in addition to15 mM H_2O_2. At HCD state successful translation of HapR mRNA transcript results in the production of HapR protease, as well as 'σ[54]'the stress response factor. The stress response factor helps in survival of bacteria under the stress conditions. The results (fig. **7**) obtained suggest that the bacterial cultures seeded with the compound (CqsS ER-1) has a higher survival rate while the bacterial cells in the control succumb to stress stating the potential of the drug to induce HCD condition in the LCD state. The treatment with 4-Ethyl resorcinol and native CAI-1 is used as positive controls in the experiment.

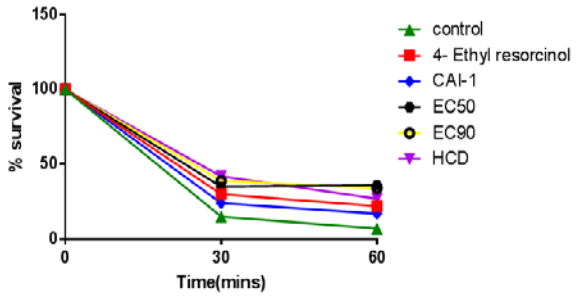


Fig. 7: Effect of CqsS agonist (EC_{50} and EC_{90}) on responding to stress induced with H_2O_2. (Positive Control-4-Ethyl resorcinol; native CAI-1)

Cell Cytotoxicity studies

The LDH assay was carried out to determine the viability of cells by the concentration of LDH observed in the medium in addition the drug, CqsS ER-1. The LDH concentration in the medium without drug was taken as the control. It was found that the concentration values for control (0.501 IU/l) and the two drug concentrations (EC_{50}= 0.4630 IU/l and EC_{90}= 0.4630 IU/l) were almost same. This shows that the compounds did not involve in cell lysis stating that the compound is non-toxic at both concentrations.

CLSM analysis of biofilm

The CLSM analysis of *Vibrio cholerae* biofilm on treatment with 4-Ethyl resorcinol (Positive control) significant amount of dead colonies and disrupted biofilm were witnessed. Whereas, more dead colonies and highly inhibited biofilm was observed on treatment with the drug, CqsS ER-1 (EC_{50} and EC_{90}). It was also observed that the effect of lower concentration CqsS ER-1 did not show any significant effect as remained to be close to the positive control (fig. 8).

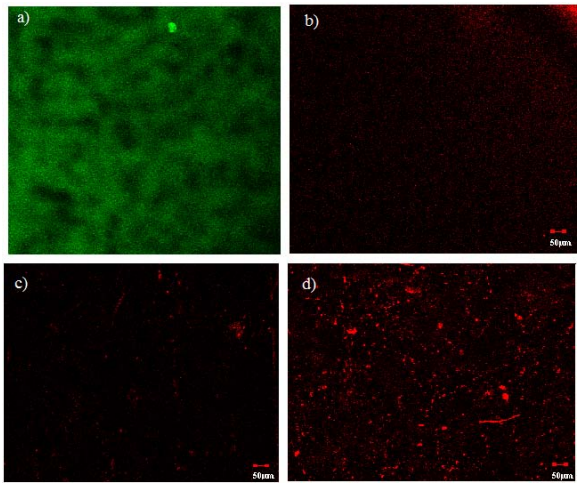


Fig. 8: CLSM analysis of the CqsS agonist (EC_{50} and EC_{90}) on the biofilm phenotype towards reversing the High Cell Density (HCD) to Low Cell Density (LCD) state. a. Negative Control; b. Positive Control-4-Ethyl resorcinol; c. CqsS-1 (EC_{50}); d. CqsS-1 (EC_{90})

DISCUSSION

The pathogen, *Vibrio cholerae* prominent to show biofilm-based infection to increase its resistance pattern over conventional antimicrobial agents and to evade the host intestinal system and the infection often turns out to be untreatable. As an alternative to conventional antibiotic therapy and to control multidrug resistance, we have established quorum sensing HCD Condition in *Vibrio cholerae* in a LCD state using the structural derivatives of a proven auto-inducer structural or functional mimic's molecule known as 4-ethyl resorcinol. Our *in vitro* data has proven that the chosen compound yielded results in a positive manner, establishing the potential of the drug. As early hours of growth i.e., at the LCD state where usually virulence is expressed in *Vibrio cholerae* bacterial cultures seeded with the compound showed significant changes in their behavior. Protease and stress response was considerably high, where as the production of hemolysin was repressed which is the characteristic nature of HCD state [19]. The biofilm inhibitory concentration of the drug is below the non-inhibiting concentration value, as a consequence of which the bacteria are not killed, whereas their virulence factors are repressed making it easy for the immune system to control the infection.

CONCLUSION

The quorum quenching activity of CqsS ER-1 CqsS ER-1 possibly works through quorum sensing mechanism and prevents the biofilm formation. This work could open up a way to control the bacterial biofilm formation and ultimately aims at suppressing the virulence factors and pathogenicity during the early hours of an infection. In a synergistic approach with antibiotics, these compounds may prove to be a potent drug against *Vibrio cholerae* infection

ACKNOWLEDGEMENT

We sincerely acknowledge the management of SASTRA University for their support and encouragement throughout this project. The authors are also grateful to the TRR–in-house funding scheme of SASTRA University.

CONFLICT OF INTERESTS

The Authors declare that we have no conflict of interest.

REFERENCES

1. Trucksis M, Michalski J, Deng YK, Kaper JB. The *Vibrio cholerae* genome contains two unique circular chromosomes. Proc Natl Acad Sci 1998;95:14464-9.
2. Ng WL, Perez L, Cong J, Semmelhack MF, Bassler BL. Broad spectrum pro-Quorum-Sensing molecules as inhibitors of virulence in vibrios. PLoS Pathog 2012;8:e1002767.
3. Miller MB, Skorupski K, Lenz DH, Taylor RK, Bassler BL. Parallel quorum sensing systems converge to regulate virulence in *Vibrio cholerae*. Cell 2002;110:303-14.
4. Ng WL. Probing bacterial transmembrane histidine kinase receptor–ligand interactions with natural and synthetic molecules. Proc Natl Acad Sci 2010;107:5575-80.
5. Wei Y, Ng WL, Cong J, Bassler BL. Ligand and antagonist driven regulation of the *Vibrio cholerae* quorum-sensing receptor CqsS. Mol Microbiol 2012;83:1095-108.
6. Lenz DH, Miller MB, Zhu J, Kulkarni RV, Bassler BL. CsrA and three redundant small RNAs regulate quorum sensing in *vibrio cholerae*. Mol Microbiol 2005;58:1186-202.
7. Waters CM, Lu W, Rabinowitz JD, Bassler BL. Quorum sensing controls biofilm formation in *vibrio cholerae* through modulation of cyclic di-GMP levels and repression of vps T. J Bacteriol 2008;190:2527-36.
8. Hammer BK, Bassler BL. Quorum sensing controls biofilm formation in *vibrio cholerae*. Mol Microbiol 2003;50:101-4.
9. Tsou AM, Zhu J. Quorum sensing negatively regulates hemolysin transcriptionally and posttranslationally in *vibrio cholerae*. Infect Immun 2010;78:461-7.
10. Finkelstein RA, Boesman-Finkelstein M, Chang Y, Häse C. *Vibrio cholerae* hemagglutinin/protease, colonial variation, virulence, and detachment. Infect Immun 1992;60:472-8.
11. Hema M, Balasubramanian S, Princy SA. Meddling vibrio cholerae murmurs: a neoteric advancement in cholera research. Indian J Microbiol 2015;55:121-30.
12. Srikkanth Balasubramanian, Hema M, Santhosh RS, Karthi Shanmugam, Adline Princy S. Molecular characterization of cholera autoinducer-1 (CAI-1) mimic as a potent CqsS receptor agonist. Biotechnology-An Indian Journal 2014;9:56-66.
13. Zwietering M, Jongenburger I, Rombouts F, Van't Riet K. Modeling of the bacterial growth curve. Appl Environ Microbiol 1990;56:1875-81.
14. Ravichandiran V, Shanmugam K, Anupama K, Thomas S, Princy A. Structure-based virtual screening for plant-derived SdiA-selective ligands as potential antivirulent agents against uropathogenic *Escherichia coli*. Eur J Med Chem 2012;48:200-5.
15. Hasegawa H, Gharaibeh DN, Lind EJ, Hase CC. Virulence of metalloproteases produced by *Vibrio species* on Pacific oyster Crassostrea gigas larvae. Dis Aquat Org 2009;85:123-31.
16. Fagerlund A. SinR controls enterotoxin expression in *Bacillus thuringiensis* biofilms. PloS One 2014;9:e87532.
17. Joelsson A, Kan B, Zhu J. Quorum sensing enhances the stress response in *Vibrio cholerae.* Appl Environ Microbiol 2007;73:3742-6.
18. Netuschil L, Reich E, Unteregger G, Sculean A, Brecx M. A pilot study of confocal laser scanning microscopy for the assessment of undisturbed dental plaque vitality and topography. Arch Oral Biol 1998;43:277-85.
19. Hema M, Karthi S, Adline Princy S. Design of LuxO based inhibitors to reverse engineer the genetic circuit of *Vibrio* cholera- an anti-virulent cholera therapy. J Chem Pharm Res 2015;7:1544-52.