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FULL PAPER

A new cell-based AI-2-mediated quorum sensing interference assay in screening of LsrK-targeted inhibitors

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Abstract: Quorum sensing (QS), a bacterial communication strategy, has been recognized as one of the control mechanisms of virulence in bacteria. Thus, targeting QS offers an interesting opportunity to impair bacterial pathogenicity and develop antivirulence agents. Aiming to enhance the discovery of QS inhibitors, we developed a bioreporter *E. coli* JW5505 pET-Plsrlux and set up a cell-based assay for identifying inhibitors of autoinducer-2 (AI-2) -mediated QS. A comparative study on the performance of target-based and cell-based assay was performed. 91 compounds selected with the potential to target the ATP binding pocket of LsrK, a key enzyme in AI-2 processing, were tested in a LsrK inhibition assay providing 36 hits. The same set of compounds was tested by the AI-2-mediated QS interference assay resulting in 24 active compounds. Among those, 6 compounds were also active against LsrK whereas 18 may target other components of the pathway. Thus, the AI-2-mediated QS interference cell-based assay is an effective tool for complementing target-based assays but also as independent assay for primary screening.

Introduction

Bacterial infections are one the biggest threats to human health. [1] In fact, spontaneous mutations, combined with the general misuse of antibiotics, have now compromised the efficacy many antibiotics, exposing humans to multidrug-resistant bacteria. [2,3,4] Traditional antibiotics are bactericidal or bacteriostatic, targeting essential cellular functions which are related to bacterial growth and survival. This leads to selection of resistant bacterial subpopulations, which become the dominant population. However, the concept that infection is not just the result of the presence of pathogens but it rather depends on host-microorganism interactions is now prevailing, broadening the typology and availability of targets addressed in the development of new treatments. [5] Since virulence is the capacity of bacteria to attack the host by activating several mechanisms known as virulence factors, interfering with virulence aims to disarm the pathogens which could be then cleared by the host immune system, lowering the emergence of resistance. [6, 7, 8]

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Several antivirulence agents have been reported, targeting different processes such as toxin neutralization [, biofilm formation, and motility. [9,10, 11,12,13] However, these bacterial responses are finely regulated and very often controlled by a sophisticated process called quorum sensing (QS). [14,15,16] QS is a bacterial communication strategy, mediated by release and recognition of small molecules called autoinducers which are processed intracellularly to activate several genes involved in virulence factor production. [17] Targeting QS offers a strategy to alter bacterial gene expression, impairing simultaneously several aspects which contribute to virulence and infection. Although several QS pathways have been characterized, autoinducer-2 (AI-2) -mediated QS remains one of the most significant. (*S*)-4,5-dihydroxy-2,3-pentanedione (DPD), the precursor of AI-2, is in fact recognized and processed by both Gram-positive and -negative bacteria. [18] In enteric bacteria, *lsr* operon encodes for the Lsr transporter together with the Lsr kinase (LsrK) and the Lsr repressor (LsrR). AI-2 is internalized by lsr transporter and phosphorylated by LsrK. The phosphorylated form of AI-2 is able to bind the Lsr repressor (LsrR) enhancing the transcription of the *lsr* operon and the activation of the QS cascade. Since phosphorylation is the essential step for displacing the repressor and the activation of the pathway, LsrK has been investigated as potential target for QS inhibition with promising results. Indeed, small sets of compounds have been previously investigated against LsrK to estimate the effect of DPD modifications on the AI-2-mediated QS pathway and LsrK inhibitors have been identified. [19, 20, 21 ,22,23] However, the activity of these inhibitors in cell-based assay have been rarely reported. [22,23] Previously described cell-based assays to evaluate inhibitory effect of compounds on AI-2-mediated QS are based on β-galactosidase activity controlled by *lsr* promoter. The addition of DPD activates the *lsr* transcription leading to β-galactosidase production which can be then quantified by monitoring the formation of the colored reaction product, after addition of the substrate, ortho-nitrophenyl-β-galactoside (ONPG). [24 , 25] However, the need of several steps such as cell lysis and addition of a detection mixture, including the substrates and co-factors necessary for β-gal reaction, makes the process time-consuming and difficult to automate. Additionally, the sensitivity of absorbance-based assay is quite low and the read-out can be affected by compounds absorbing at 420 nm, used for quantifying the final product. A bioreporter strain for the evaluation of AI-2-mediated QS inhibitors based on the toxin/antitoxin system has also been reported. [26] In the construct the lethal gene *ccdB* is under control of QS responsive promoter and thus, if QS is activated, the protein is expressed causing the death of the cell whereas QS inhibitors would prevent *ccdB* transcription, resulting in a viable cell. However, toxic compounds may affect the results, thus an additional control strain is needed. Both assays require addition of external DPD to activate the pathway and, although this may offer a better control on the system, it also increases the cost and creates artificial conditions. In this study, we developed a fast, sensitive and simple cell-based assay for the identification of AI-2-mediated QS inhibitors based

on luminescence. Using this assay, we performed screening of a 91-compound library, originally designed as gyrase B inhibitors targeting its ATP binding site [26] and thus endowed with capability to target also LsrK ATP binding pocket. The screening against LsrK in target-based functional assay led to the identification of 29 active compounds, with an IC_{50} ranging from 8 to 147 μM. Among those, 6 showed activity in the bioreporter strain, impairing the response to QS as result of LsrK inhibition. Additionally, 18 compounds, which did not display any activity against LsrK, were found active in the bioreporter strain, as result of the inhibition of other components of the pathway, proving the effectiveness of our bioreporter strain as a new tool for discovery of QS inhibitors.

Results and Discussion

Screening against LsrK

Compound library synthetized as ATP-competitive gyrase B inhibitors, was used as a starting point. This library contained more than 800 compounds out of which 91 structurally diverse compounds were selected visually to cover the whole chemical space of the library and were tested against LsrK at 100 μM using a kinase inhibition assay. [27] Compounds showing an inhibition ≥ 70 % were selected for dose-response experiments (Table 1, Figure S1).

Table 1. IC_{50} values for the 29 positive hits selected by primary screening against LsrK. Data points represent means ± SD from two independent experiments (n=4).

Compound	IC_{50} (μM)
UL-01	26 ± 1
UL-02	76 ± 6
UL-03	28± 3
UL-04	17± 2
UL-05	18± 2
UL-06	147±12
UL-07	54± 2
UL-09	78± 1
UL-10	63± 11
UL-11	36 ± 5
UL-12	33 ± 3
UL-13	18 ± 1
UL-14	32 ± 2
UL-15	11 ± 0.1
UL-16	40 ± 2
UL-17	8 ± 1
UL-18	39 ± 2
UL-19	29 ± 2
UL-20	83 ± 8
UL-21	15 ± 1
UL-22	27 ± 4
UL-23	40 ± 3
UL-24	21 ± 2
UL-25	45 ± 6
UL-26	10 ± 1
UL-27	31 ± 1
UL-28	51 ± 4
UL-29	59 ± 2.5
UL-30	24 ± 4

Cell-based AI-2-mediated QS interference assay

Previously reported cell-based AI-2-mediated QS interference assays present some limitations due to the complexity and length of the protocol together with low sensitivity and potential interference by toxic compounds. Aiming to enforce the discovery of antivirulence agents based on AI-2-mediated QS inhibition, we designed a new plasmid pET-Plsrlux which contains the bacterial luciferase operon *luxABCDE* under control of *lsr*, a QS responsive promoter, and developed a new bioreporter assay which allows rapid identification of QS inhibitors by luminescence detection technology. Thus, when AI-2-mediated QS is activated, this leads to *lux* expression and production of light whereas, in the presence of QS inhibitors, no light will be detected. The plasmid was constructed by replacing the PesaR, a QS responsive promoter induced by 3-oxo-hexanoyl-homoserine lactone, with the lsr promoter in the pET-PesaRlux plasmid and the new construct was transformed into single-gene knock-out *E. coli* JW5503 which, due to efflux pump defect, is unable to expel xenobiotics from the cell. [28, 29] Since extrusion by the efflux pump system is one of the main obstacles for the discovery of new antibacterial agents and even limits the identification of target-active compounds to be further optimized, we considered the reduced efflux activity of the strain as an advantage to identify candidate molecules which can be then further optimized. The strain is able to synthetize its own DPD contributing to more physiological conditions, reduced cost and complexity of the assay. To decrease the rate of false positives, luminescence and turbidity to assess QS inhibition and cell density, respectively, can be monitored in parallel to ensure that the reduced luminescence results from QS inhibition is not due to bacterial toxicity.

E. coli JW5503 pET-Plsrlux was grown overnight and diluted into fresh LB. The day of the assay bacteria were grown at 30°C until exponential phase and then centrifuged to remove the supernatant. This step is essential for the success of the assay to ensure the removal of the DPD produced during the incubation time which may otherwise interfere with the assay. To select the optimal bacterial inoculum for the assay, 3 concentrations were evaluated: $5*10^5$ CFU/ml, $5*10^6$ CFU/ml, $5*10^7$ CFU/ml (Table S2). Due to its ability to fully repress the transcription of *lsr* operon, glucose was used as a positive control [30]. Bacteria were then incubated up to 7 hours, with and without 2% glucose, and luminescence and absorbance was measured every hour. For each time point signal/background (S/B) and Z´ factor, quality parameters commonly used to determine assay quality in screening assay development, were calculated to select the optimal conditions (Table S2). For S/B, which is defined as the ratio of the maximum signal to the minimum signal, values ≥ 2 were considered acceptable whereas for Z´ values, which takes into account not only the averages but also their variability, values ≥ 0.5 were pursued. [31]

In case of lowest CFU/ml concentration acceptable S/B values were reached after 5 h but the Z´ factor remained ≤ 0 indicating high signal variability. Similar situation was observed for $5*10^6$ CFU/ml concentration where, despite the good separation already observed after 1 h incubation, the Z´ value reached an acceptable value of 0.5 only at 5 h, starting then to decrease. At the highest tested concentration, good separation was observed after 1 h incubation and an excellent Z´ value of 0.92 was observed at 3-h time point. Aiming to obtain the best performance in the shortest

possible incubation time, $5*10^7$ CFU/ml and 3h incubation were selected as optimal assay conditions. Additionally, the Z´ factor remained acceptable up to 7 hours, ensuring the high stability of the assay.

DMSO tolerance of the assay was also evaluated. At concentrations above 2% DMSO significantly inhibits the luminescence and, at concentrations ≥ 4%, it also dramatically affects the growth, thus DMSO concentrations were kept below 2% in the experiments (Figure S2).

Screening against *E. coli* JW5503 pET-Plsrlux

The set of 91 compounds was tested against *E. coli* JW5503 pET-Plsrlux to verify the activity of the positive hits against LsrK in cellular context and to evaluate simultaneously the potential of the compounds to affect other targets involved in the same pathway. 74 among 91 compounds showed QS inhibition ≥ 50%. Inhibition of growth was evaluated in parallel to ensure that the reduced luminescence is the result of QS inhibition and not due to compound toxicity or antibacterial activity. Indeed, among the 74 active compounds, 44 showed a growth inhibition ≥40 %, thus the observed decrease of the luminescence was consequence of bacterial death (Table S3).

Combined analysis of QS inhibition and growth inhibition led to the selection of 24 compounds with QS inhibition ≥ 80% and growth inhibition ≤ 40 % for dose-response studies (Table 2, Figure S3).

Table 2. IC_{50} values for QS inhibition for the positive hits selected by primary screening on *E. coli* JW5503 pET-Plsrlux. Data points represent means ± SD from two independent experiments (n=3).

Compound	IC_{50} (μM)
UL-87	34 ± 12
UL-54	45 ± 4
UL-31	12 ± 1
UL-81	24± 3
UL-32	7± 0.2
UL-41	53± 8
UL-19	1± 0.1
UL-03	0.6± 0.1
UL-21	0.4 ±0.2
UL-36	2± 0.7
UL-05	10± 0.4
UL-67	2± 0.2
UL-55	34± 0.5
UL-53	31± 1
UL-69	0.6± 0.1
UL-73	30 ±1
UL-48	96± 2
UL-76	1± 0.1
UL-09	17 ±2
UL-78	6 ±1
UL-90	8± 0.2
UL-83	39± 6
UL-84	2± 0.1
UL-06	2 ±0.2

The QS inhibition activity of UL-03, UL-05, UL-06, UL-09, UL-19 and UL-21, which were identified as LsrK inhibitors, was confirmed in the cell-based assay. Moreover, the most potent LsrK inhibitors UL-03 and UL-21 were docked to the LsrK crystal structure (PDB entry: 5YA2) to predict their possible binding mode in the ATP-binding site.[32] Based on docking calculations both inhibitors can form a salt bridge with Arg319 side chain and several hydrophobic interactions with Tyr341, Thr342 and Trp435 (Figure S4) in the LsrK active site. The discrepancy observed between the IC_{50} determined by cell-based assay and the IC_{50} determined by target-based assay may be attributed to structural differences in the binding pocket region between the LsrK from *S. typhimurium*, used for the target-based assay, and the LsrK from *E. coli*, used for the cell-based assay, which may differently impact the interaction with small molecules. [33] UL-03 and UL-21 were the most effective compounds with an IC_{50} in the submicromolar range (Figure 1). Additionally, 18 hits were found which may target other elements in the pathway such as the *lsr* transporter or repressor.

Unfortunately, when tested on *E. coli* ATCC255922, a strain with regular efflux pump activity, which was transformed with the same construct pET-Plsrlux, the set of hits selected against *E. coli* JW5503 pET-Plsrlux did not show significant QS inhibition highlighting efflux issues.

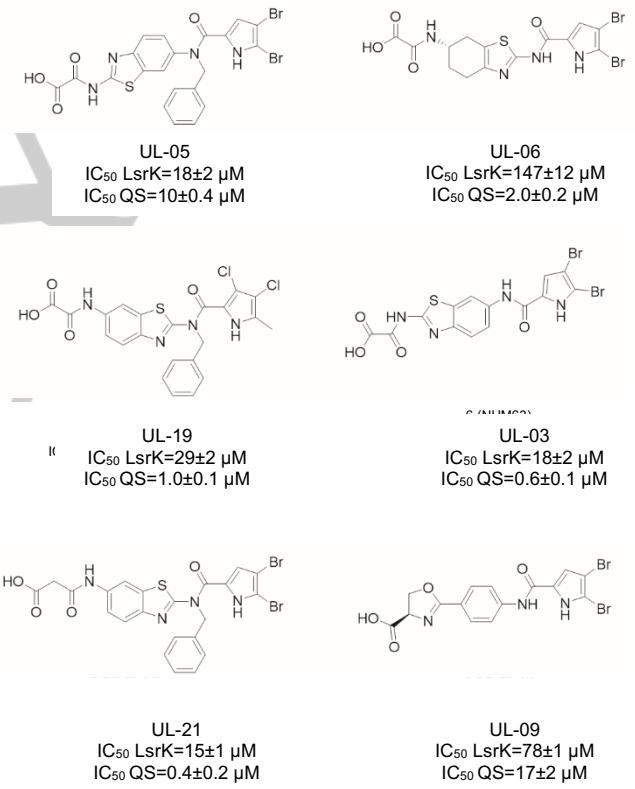

Figure 1. Structures of hit compounds active against LsrK and in the AI-2-mediated QS interference assay.

Conclusions

To support the discovery of new QS inhibitors, we developed a new AI-2-mediated QS interference assay based on the detection of luciferase produced in *E. coli* JW5503 pET-Plsrlux. The assay was validated by screening a library of 91 compounds originally designed as ATP-competitive gyrase B inhibitors endowed with capability to target also ATP binding pocket of LsrK, a key enzyme in AI-2-mediated QS. [27] The set of compounds was tested on a

LsrK inhibition assay and on the cell-based AI-2-mediated QS interference assay and the obtained hits were compared.
Six compounds, which were active against LsrK, also inhibit the QS activation in the cell-based assay. The cell-based AI-2-mediated QS interference assay also provided 18 additional hits which did not show any activity against LsrK and may target other members of the *lsr* pathway. More in-depth study will be needed to identify the mode of action and a multidisciplinary effort will be required to understand and develop the potential of these molecules as precursors of a new class of antivirulence drugs. However, the new developed assay proved its effectiveness as complementary assay to confirm the activity of hits selected by target-based screening and as a primary tool for cell-based screening of compound libraries to facilitate the identification of new scaffolds to be used as inhibitors of AI-2- mediated QS.

Experimental section

Materials

E. coli MET1158 was donated by Prof. Karina Xavier (Instituto Gulbenkian de Ciência, Portugal). [30] *E. coli* LW7 pLW11 was provided by Prof. William Bentley (University of Meriland, USA). *E. coli* JW5503 and *E. coli* ATCC 25922 were obtained from the NBRP-*E. coli* collection at the National Institute of Genetics (NIG, Japan) and the American Type Culture Collection (ATCC) via Microbiologics Inc. (St.cloud, MN), respectively. [29] The plasmid pET-PesaRlux was provided by Dr. Collins (Rensselaer Polytechnic Institute, NY, USA) and obtained through Addgene (Watertown, Massachusetts, USA). [28, 34]
For the enzymatic assay, DPD and Kinase-Glo Max Luminescent kinase assay were acquired from Carbosynth (Compton, Berkshire, UK) and Promega Corp. (Madison, WI, USA), respectively.
Materials for cloning including restriction enzymes (XhoI, BamHI-HF), Q5® High-Fidelity PCR Kit and T4 ligase were purchased from New England Biolabs (Ipswich, Massachusetts, USA). Subcloning Efficiency™ DH5α™ Competent Cells were obtained from Thermo Fisher Scientific (Waltham, MA, USA). All other reagents were purchased from Sigma (USA). Plates were purchased from Greiner Bio One (Kremsmünster, Austria) for the LsrK inhibition assay, and from PerkinElmer (Waltham, MA, USA) for the cell-based AI-2-mediated QS interference assay.

LsrK overexpression and purification

LsrK from *S. typhimurium* was overexpressed in *E. coli* MET1158 [*E. coli*, amp resistance, BL21 (DE3) luxS - , with pMET1144 (lsrK - His in pET21b), and purified by affinity chromatography as previously described. [23]

Primary screening against LsrK and dose-response assay

91 compounds, dissolved in DMSO, were tested at a final concentration of 100 µM. The assay was performed with 300 nM LsrK, 100 µM ATP and 300 µM DPD in assay buffer (25 mM TEA, pH 7.4, 200 µM $MgCl_2$ and 0.1 mg/ml BSA) using the Kinase-Glo Max Luminescent kinase assay as previously reported. [23] Positive hits selected by primary screening were tested in a dose-response assay following the same protocol.

Docking

A library of UL-03 and UL-21 conformers was generated using OMEGA software (Release 3.1.0.3, OpenEye Scientific Software, Inc., Santa Fe, NM, USA; www.eyesopen.com) using default settings. [35] For docking with FRED software (Release 3.3.0.3, OpenEye Scientific Software, Inc., Santa Fe, NM, USA; www.eyesopen.com), LsrK ATP-binding site (PDB entry: 5YA2) was prepared using MAKE RECEPTOR (Release 3.3.0.3, OpenEye Scientific Software, Inc., Santa Fe, NM, USA; www.eyesopen.com). [36,37, 38] The grid box around the ADP bound in the crystal structure was generated automatically and was not adjusted. This resulted in a box with the following dimensions: 16.00 Å *18.00 Å * 18.33 Å and the volume of 5280 $Å^3$. For "Cavity detection" slow and effective "Molecular" method was used for detection of binding sites. Inner and outer contours of the grid box were also calculated automatically using "Balanced" settings for "Site Shape Potential" calculation. The inner contours were disabled and no docking constraints were defined. The OMEGA-generated library of UL-03 and UL-21 conformers was then docked in the prepared binding site. Ten docking poses were inspected visually and the highest ranked docking pose was used for the presentation in Figure S4.

Plasmid construction

The plasmid pLW11 containing lsr promoter was extracted from *E. coli* LW7 and used as a template for the amplification by PCR of the lsr promoter with the following primers:

lsp_F : TATCTCGAGGCGACCTGTTCTTCTTCACACATT
lsp_R: TATGGATCCTCGATGCCTTTCAGGACATTG

The primer lspF introduced a restriction site for XhoI. The PCR product was digested with XhoI and BamHI and cloned into XhoI and BamHI digested pET-PesaRlux [28] using T4 ligase. The reaction product was transformed into Subcloning Efficiency™ DH5α™ Competent Cell according to manufacturer´s instructions and then transferred into *E. coli* JW5503 or *E. coli* ATCC 25922 competent cells. [39] *E. coli* JW5503 pET-Plsrlux was grown overnight in LB supplemented with 25 µg/ml kanamycin and 100 µg/ml ampicillin and luminescence was measured to confirm the functionality of the construct by Varioskan LUX plate reader.

Cell-based AI-2-mediated QS interference assay protocol

E. coli JW5503 pET-Plsrlux was grown overnight in LB supplemented with 25 µg/ml kanamycin and 100 µg/ml ampicillin. The following morning bacteria were diluted 1:100 into fresh LB supplemented with antibiotics and grown at 30 °C until exponential phase (2.2-3.5 McFarland unit) [24]. Bacteria were centrifuged at 3000xg for 10 minutes and the pellet was resuspended into fresh LB. A suspension containing $1*10^8$ CFU/ml was prepared and 50 µl were added to the plate. 50 µl of LB for negative control or 50 µl of LB supplemented with 2% glucose for positive control were also added and the plate was incubated at 37 °C under shaking for 3 hours. Luminescence and Abs600 were recorded with Varioskan LUX plate reader.

Compound screening

91 compounds were tested on the bioreporter strain at final concentration of 100 µM. Assay was performed as described above. Luminescence and Abs600 were measured and compounds showing an inhibition of luminescence ≥80% and an inhibition of growth ≤ 40 % were selected for dose-response experiment. Positive hits were also tested against *E. coli* ATCC 25922 pET-Plsrlux according to the described protocol.

Data analysis

During the assay development, optimization and validation, the assay performance was evaluated by calculating quality parameters typically used in HTS, Z' and S/B according to the following equations: [31]

$$Z' = 1 - (3*\sigma M + 3*\sigma m)/(|\mu M - \mu m|)$$

$$S/B = \mu M/\mu m$$

In the equations μM and σM represent, respectively, the average and the standard deviation for the maximum signal, given by the wells containing only bacteria. μm and σm represent average and standard deviation for the minimum signal, from wells containing bacteria and 2% glucose. Good quality assays are indicated by Z' factor > 0.5.
QS and growth inhibition % for each test compound was determined according to the following equations:

Inhibition (%) = 100* {1- [(X-Xm)/(XM-Xm)]}

In the equation, X represents the detected luminescence or absorbance value from a sample, while XM and Xm are respectively the average of the detected luminescence or absorbance value for the maximum and minimum controls.

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Conflict of interest

The authors declare no conflict of interest.

Keywords: antivirulence • Gram-negative bacteria • bioreporter • LsrK • quorum sensing

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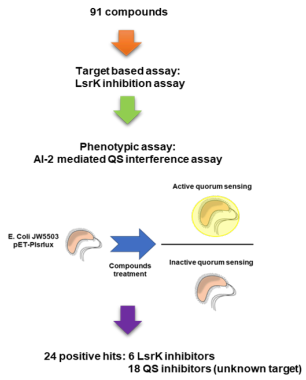

Exploring quorum sensing inhibition: Target- and bioreporter-based approaches were utilized in parallel to screen 91 compounds targeted to the ATP binding pocket of LsrK, a kinase that functions in the autoinducer-2 pathway. Six compounds active against LsrK inhibited also the QS activation in the novel *E. coli* JW5503 pET-Plsrlux bioreporter. In addition, the cell-based set up provided 18 additional hit compounds with unknown target.

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