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Fructose furoic acid ester: An effective quorum sensing inhibitor against uropathogenic *Escherichia coli*

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ABSTRACT

Uropathogenic *Escherichia coli* (UPEC) are the most common cause of UTI, accounting for more than 90% infections in the normal and unobstructed urinary tracts. Multi-drug resistance (MDR) is an emerging threat to the mankind and hence, there is an urge to develop alternative therapies. Targeting quorum sensing (QS), a cell-cell communication process regulates various biofilm and virulence factors would be a most promising alternate which curbs the pathogenesis without killing the bacteria, unlike antibiotics. SdiA, a quorum regulator is well-known to control the behavioural changes of UPEC in establishing biofilm and virulence. Therefore, we have hypothesized that the SdiA-selective inhibitors derived from the plant, *Melia dubia* using the molecular docking would be a remarkable therapeutic candidate to down regulate the UPEC biofilm and virulence phenotypes. In this study, we have designed, synthesized and characterized the fructose-furoic acid ester by NMR and ESI-MS. *In vitro* studies revealed that the QSI-MD selectively inhibits UPEC adherence and confocal laser scanning microscopy (CLSM) analysis showed the effectiveness of QSI-MD to inhibit the UPEC biofilm. Genetic studies using qRT-PCR revealed the down-regulation of quorum sensing regulated genes (*fimA, csgA, espA*). Based on the findings, we could propose that the QSI-MD could possibly act through SdiA and show target-specific inhibition of biofilm and virulence. It is notable that more than 70 bacterial species execute their communication through the SdiA homologues (LuxIR system). Hence, the QSI-MD could be further developed as a broad-spectrum anti-infective drug.

1. Introduction

Urinary tract infections (UTIs) beset millions of individuals annually and remain rising as a remarkable health burden globally [1]. The key causative agents of UTIs are the gram-negative, rod-shaped bacteria, uropathogenic *Escherichia coli* (UPEC). These are adhesive and motile, with an inclination to form biofilms within the urinary tract including the kidneys, bladder, and ureters [2]. UTIs are partially cured with antibiotics and recur repeatedly [3]. This attribute is due to the capability of UPEC to invade, replicate and persist inside the host epithelial cells. The biological intricacy pooled with a dreadful upsurge in multi-drug resistance (MDR) emphasizes the need for alternatives towards the treatment of UTI [4,5].

SdiA is a LuxR homologue and a well-known transcriptional activator of UPEC to detect the exogenous N-acyl-homoserine lactone (AHL) and mediate inter-species communication to establish virulence and biofilm phenotypes [6]. Hacking the inter-species communication would be a robust strategy to target the crucial functions to infection, like biofilm formation and virulence factors expressions as it is an ultimate pre-requisite for the pathogens to evade the host [7–10]. Being remarkable alternative to antibiotics, quorum sensing inhibitory approaches have numerous potentials to increase the gamut of bacterial targets, safeguarding the host's internal microbiome and also exert less selective pressure that may prohibit the development of bacterial resistance [11–14].

It is reported that the compounds derived from algae and plant sources have potential anti-quorum sensing activity against a wide range of pathogenic bacterial strains [15–18]. Researchers have identified several quorum sensing inhibitors from natural and synthetic sources that are capable of effectively reduce quorum sensing regulated biofilm formation and virulence [19–23]. Moreover, researchers have made an attempt to investigate the efficiency of quorum sensing

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inhibitors *in vivo* through animal models by experimentally induced infection [24–28]. A furanone based quorum-sensing inhibitor, from *Delisea pulchra* was found to suppress the swarming of *Escherichia coli* but did not have any effect on its growth rate [29]. It has been found that 7-hydroxyindole decreased the biofilm formation of the enterohemorrhagic *Escherichia coli* O157:H7 (EHEC) (27-fold) and also decreased *E. coli* K-12 biofilm formation (8-fold) without showing any influence on its growth [30].

In our previous studies [31–33], we had screened inhibitors from *Melia dubia* extracts targeting the quorum regulator SdiA and experimentally reported the quorum sensing inhibition activity against UPEC. The SdiA, a LuxR homologue and a native transcriptional regulator of uropathogenic *E. coli* are known to establish interspecies communication in response to signals like indole, AHL and Autoinducer-2 (AI-2) [34]. The structural analysis revealed that the protein, SdiA have two domains, the amino acids 1–171 contribute the autoinducer (AI) binding domain [6] whereas the aminoacid residues, 197–216 has the helix-turn-helix DNA binding domain [35]. The C-terminus deletion removes the DNA binding domain of SdiA without any effect on the ligand binding domain are well characterized to enhance the control of SdiA over biofilm formation [36]. Based on molecular docking studies, we found that fructose-furoic acid ester (QSI-MD) effectively interact the molecular target protein, SdiA. Hence, in this study, the compound fructose-furoic acid ester (QSI-MD) was synthesized and investigated for its therapeutic potential to inhibit the SdiA selective quorum sensing inhibition in UPEC.

2. Material and methods

2.1. Docking studies

The QSI-MD and C_8HSL were drawn using ACD ChemSketch and the energy minimized 3D ligand file was prepared using Schrödinger LigPrep module. The three-dimensional structure of SdiA was retrieved from Protein Data Bank (PDB: 2AVX). Further, SdiA structure was prepared by using the protein preparation wizard.

The docking was performed using GLIDE (Grid Based Ligand Docking with Energetics) module in Schrodinger suite. Grid files were generated using the C_8HSL, the native ligand to the center of both the boxes. The compounds were subjected to flexible docking using the pre computed grid files. The pose that have the potential GScore (Glide Score) was saved and only the best scoring pose was analysed further.

2.2. Chemical synthesis of fructose-furoic acid ester (QSI-MD)

The synthesis of fructose-furoic acid ester (QSI-MD) was summarized in the reaction scheme (Fig. 2a). The compound was synthesized in two steps from Diacetone-D-fructose. The first step was the esterification of furoic acid with Diacetone-D-fructose in dry dichloromethane. Furoic acid (775 mg, 6.92 mmol) was stirred in 50 ml of anhydrous CH_2Cl_2 taken in a 100 ml round bottom flask. Diacetone-D-fructose (1 g, 3.84 mmol) and 4-Dimethylaminopyridine (DMAP) (470 mg, 3.84 mmol) were added subsequently to the above solution. The temperature of the reaction mixture was lowered to ~5 °C using an ice bath. The solution of Dicyclohexylcarbodiimide (DCC) (1 g, 5 mmol) in 30 ml of anhydrous CH_2Cl_2 was then added slowly for a period of 10–15 min. After the addition, the reaction mixture was kept at room temperature and stirred for 4 h and monitored its completion by thin layer chromatography (solvent system: CH_2Cl_2/CH_3OH in 8:2 ratios). At the end of the reaction, the solid by product, Dicyclohexyl urea (DHU), was removed by vacuum filtration. The filtrate was further washed with 10% Na_2CO_3 (3 × 50 ml), 10% HCl (3 × 50 ml) and with brine solution (2 × 75 ml) and dried with anhydrous Na_2SO_4, and concentrated under reduced pressure and a colorless oily residue was formed. Further, the residue was taken to synthesize the fructose-furoic acid ester. A mixture of crude diacetone fructose-furoic acid ester (250 mg, 0.018 mmol) and 70% acetic acid (4 ml) were mixed and stirred in a 50 ml round bottom flask. The reaction mixture was heated at reflux under nitrogen atmosphere for 2 h. The clear solution formed was concentrated under reduced pressure and then dried in a high vacuum atmosphere. Column chromatographic purification (silica gel, methylene chloride/methanol) was performed to obtain the product, fructose–furoic acid as a pale white solid, 0.8 g (17%). All the synthesized compounds were purified through silica gel flash column chromatography, and then their chemical structures were confirmed by ^{1}H NMR, ^{13}C NMR and ESI-MS.

2.3. Biofilm inhibition assay

Initially, the minimum biofilm inhibitory concentration (MBIC) was determined by crystal violet method as described by O'Toole and Colter, 1998 [37]. Briefly, 100 µl inoculum of 1:200 (v/v) diluted overnight culture of *E. coli* QSL/S4, a multi-drug resistant clinical isolate received from Dr. KAP Viswanatham Govt. Medical Hospital, Trichy, Tamil Nadu, India was plated onto 96-well polystyrene microtitre plate containing Luria Bertani (LB) broth. Varying concentrations of the test compound were added, in triplicates. Furthermore, the exogenous AHL (C_8HSL) was added to the culture medium. After 18 h, the planktonic cells were removed. PBS wash was done twice and the cells were fixed using 100 µl of 99% methanol. Then, 150 µl of 0.2% crystal violet was used to stain the biofilm cells for 20 min. Excess stain was removed by washing under slow-flowing cold tap water and the plates were air dried. Further to the air dried plates, 150 µl of 33% acetic acid was added to elute the bound crystal violet and the optical reading was read in an ELISA plate reader (BioRad i-Mark, Japan) at 595 nm. Based on MBIC (12 µg/ml), the inhibitory concentrations taken for further experiments were as follows; low dose (LD, 6 µg/ml), medium dose (MD, 12 µg/ml) and high dose (HD, 18 µg/ml). A known antiquorum compound, furanone was taken as positive control (10 µg/ml) for comparison [29]. Bacterial culture without the test compound was considered as negative control. The biofilm inhibitory effect was seen against the various strains of *E. coli* such as *E. coli* MTCC 729, *E. coli* QSL/S4, *E. coli ΔsdiA* (*SdiA* null strain, BW25113/pCA24N::-Chloramphenicol) and *E. coli SdiA*$^+$ (*SdiA* positive strain, BW25113/pCA24N-SdiA::Chloramphenicol) in the presence of the SdiA native ligand, C_8HSL [29]. *E. coli* strains *ΔsdiA* and *SdiA*$^+$ were generously gifted by Prof. Thomas K wood. All the strains were cultured in LB broth and incubated at 37 °C for 18 h to reach a OD of ~0.4 containing 10^6–10^7 cfu/ml. Biofilm assays were carried out in 96-well polystyrene microtitre plate using the crystal violet method as described earlier [37]. Based on the Optical density (OD) measured against bacterial biofilms, strains were classified into various categories such as no biofilm producers, weak, moderate and strong biofilm producers. Briefly, the cut-off OD (ODc) was calculated as three standard deviations above the mean OD of the negative control. Strains were classified as follows: OD ≤ ODc = no biofilm producer, ODc < OD ≤ (2 × ODc) = weak biofilm producer, (2 × ODc) < OD = (4 × ODc) = moderate biofilm producer and (4 × ODc) < OD = strong biofilm producer [37].

2.4. Biofilm inhibition assay by confocal laser scanning microscopy (CLSM)

Static biofilms were grown over glass cover slips (1: 100 diluted culture of *E. coli* inoculated in LB broth and incubated overnight at 37 °C in stationary condition) in 8-well cell culture plates either with (6.12 and 18 µg/ml) or without the addition of QSI-MD in the presence of the SdiA native ligand, C_8HSL. The developed biofilms were washed twice to remove loosely bound cells and stained with concanavalin A-fluorescein isothiocyanate (FITC-ConA) for 15 min. Cells were washed twice in PBS to remove the excess stains and the adhered cells was analyzed using CLSM (Olympus, FV1000, Japan) with the excitation and emission wavelength set at 488 and 520 nm respectively [38–40].