Hexanoic Acid Derivatives

Differently Mimics Hexanoyl Homoserine Lactone

in LuxR and CviR Quorum-Sensing Systems

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Abstract

Homoserine lactones (HSLs) are the class of bacterial signaling molecules involved in intercellular communication (quorum sensing, QS). In some species (*Vibrio fischeri, Chromobacterium violaceum* et al.) the HSLs diffuse across cell wall and bind R-type receptor protein, which activate transcription of bioluminescence, production of pigments, virulence factors and biofilm formation genes. The aim of this study is screening a set of 10 chemically synthesized hexanoic acid derivatives for their ability to interfere C6-HSL-mediated QS systems using a quantitative bioassay with LuxR protein of *V. fischeri* and CviR protein of *C. violaceum*. Our analysis has showed that 2 compounds demonstrate agonistic but not antagonistic action in LuxR system and 3 other induced same effects in CviR system. Active compounds are compared in structure with the known QS regulators. Application and development prospects of hexanoic acid derivatives as AHL-mediated QS modulators are discussed.

Keywords: quorum sensing, homoserine lactone, hexanoic acid derivatives

1. INTRODUCTION

Quorum sensing (QS) is a phenomenon of bacterial intercellular communication by means of releasing, and detecting of small signaling molecules
A large number of Gram-negative bacteria use N-acyl homoserine lactones (HSLs) as signal molecules in QS [21], which have been detected by selective receptors on the cell surface [1, 12], or diffused across the cell wall and interacted with specific cytosolic regulatory proteins [11, 7]. In the latter case there are two main genes involved in QS: I and R, where I-type gene directs the synthesis of HSL, and the R-type gene codes the transcription regulatory protein. For example, in *Vibrio fischeri* C6-oxo-HSL it is synthesized under luxI gene control and binds to the N-terminal domain of the LuxR protein [15, 16]. Then LuxR C-terminal domain interacts to the A-T-rich stretches of some genomic regions including luxCDABEG operon and activates it transcription [15], leading to bright bioluminescence of bacterial culture. Similar pathway *Chromobacterium violaceum* uses where the synthesized under cviI gene control C6-HSL binds regulatory protein CviR and activates transcription of vioABCD operon leading to occurrence of purple-colored pigment violacein [17, 22].

As many Gram-negative bacterial species control the expression of virulence factors and biofilm formation via the I/R-type QS [18, 4], this mechanism disruption is supposed to be a new strategy of antibacterial therapy [13, 8]. The offered ways of QS system interference include: (i) inhibition of HSL biosynthesis, (ii) degradation of HSL molecules by bacterial lactonases or acylases, and (iii) using small molecules to block the binding of HSL to receptor protein [9, 10, 2]. So, a variety of natural or chemically synthesized HSL analogs have been intensively investigated for agonist or antagonist activity in QS system [20]. It has been reported that the length of the side chain as well as the ring structure influence on binding of this signaling molecules to the R-type regulatory protein and modulate level of HSL-mediated expression [13, 18].

In this study we report the QS interfering activity of set from 10 chemically synthesized hexanoic acid derivatives, having same with C6-HSL carbon chain but structurally different headgroups. Its agonistic and antagonistic activity was tested in quantitative bioassays with LuxR protein of *V. fischeri* and CviR protein of *C. violaceum*.

### 2. MATERIALS AND METHODS

#### 2.1. Signaling Compounds

The HSL used in this study, N-hexanoyl-L-homoserine lactone (C6-HSL) was obtained from Cayman Chemical Company (Michigan, USA). A series of hexanoic acid derivatives (HADs) were synthesized by standard organic procedures and purchased from Enamine Ltd. (Kiev, Ukraine). These compounds have same N-acyl chain (Fig. 1) but varied rings (R) consisting from 7-15 carbon, 0-1 nitrogen, 0-4 oxygen, 0-1 sulphur, 0-1 chlorine, and 5-11 hydrogen atoms. Stock 0.01 M/ml solutions of C6-HSL and HADs were prepared in 96 % ethanol.
2.2. Bacterial Strains and Culture Conditions

A reporter vector pAL103, previously constructed by A. Lindsay and B. M. M. Ahmer [14], has been used in the study. It utilizes the transcription regulatory luxR-gene of \( V. \ fischeri \) and a corresponding QS-controlled promoter placed upstream of \( luxCDABE \) operon. Additionally, to avoid potential complications arising from host HSL-detecting \( sdiA \) gene, reporter construction has been transferred in \( E. \ coli \ sdiA \) mutant. Received strain \( E. \ coli \ JLD271, \ pAL103; \ luxR^+ \ luxI^- \ luxCDABE; \ TetR \ p15A \) origin, strongly activates in the presence of HSLs showing dramatic growth of bioluminescence level.

The \( Chromobacterium \ violaceum \) NCTC 13274 was acquired from the National Collection of Type Cultures (Health Protection Agency, UK). This mini-Tn5 mutant of wild-type strain \( C. \ violaceum \) ATCC 31532 has insertion in \( cviI \) gene, and is unable to synthesize own \( C_6\)-HSL, but retains the ability to respond on exogenous one [17]. Therefore \( C. \ violaceum \) NCTC 13274 is colourless without HSLs, but produces purple pigment violacein in its presence.

2.3. Luminescence Assays

\( E. \ coli \ JLD271, \ pAL103; \ luxR^+ \ luxI\_luxCDABE; \ TetR \ p15A \) origin, were grown on Luria - Bertani (LB) agar supplemented with appropriate antibiotic at 37°C for 18 h. Before experiment a biomass was transferred to LB-broth, grew up 3 h to optical density 0.5, and then was diluted to 1:10 in fresh LB broth.
containing investigated compounds or its mixtures. For luxR activation we used C6-HSL, as well as HADs at concentrations from 0.5 nM to 250 µM. While testing of HADs antagonistic activity the response to their mix with C6-HSL optimal induction concentration 0.5 µM has been studied. The samples were poured into a 96-well microplate with non-transparent side walls, and a luminometer LM 01T (Immunotech, Prague, Czech Republic) was used for dynamic luminescence measurements at 37°C for 120 min. Bioluminescence of reporter strain was estimated in relative light units (RLU). Activation or inhibition effects were evaluated for determining 50% effective concentrations (EC50), where zero level – bioluminescence without compounds and 100 % - in presence of 0.5 µM C6-HSL.

2.4. Violacein Assays

C. violaceurn NCTC 13274 was grown in LB broth with glucose (10 mg/ml) and yeast extract (10 mg/ml) at 30°C for 24 h then was used in the agar plate or liquid medium assays. For the plate assay a 5 ml of kindled LB agar was inoculated with 100 µl of C. violaceum culture, and poured onto the Petri dish with previously prepared layer (10 ml) of LB-agar. For agonistic activity this system was sufficient, and for antagonistic activity has been supplemented with 1 µM C6-HSL in the top layer. On 20 µl of C6-HSL and HADs stock solutions have been pipetted into 3 mm diameter wells punched in the solidified agar with a sterile cork borer. Equal amount of 96 % ethanol was used as control. Assay plates were incubated overnight at 30°C for 24-48 h, then examined for the violacein synthesis. QS activation was detected by a purple-colored halo on the bacterial lawn around the wells, and QS inhibition by colorless, but viable halo in a purple background. For the liquid medium assay a series of HADs (0.06 nM to 250 µM ) and their mix with 5 nM C6-HSL were provided to the LB broth. On 20 µl of C. violaceum culture was inoculated into the 1 ml of LB broth with or without tested compounds, and incubated at 30°C for 24-48 h. Violacein extraction was carried by drying, ethanol addition, vortexing, and centrifugation at 13000 rpm for 5 min. The upper phase containing the violacein was analysed at 620 nm wavelength (OD620) in 96 well flat transparent bottom microplates using a Uniplan reader (Pikon ZAO, Moscow, Russia). The EC50 value of violacein activation or inhibition in the individual experiments was calibrated with respect to the zero level – OD620 without compounds and 100 % - in presence of 5 nM C6-HSL.

3. RESULTS AND DISCUSSION

Screening individual HADs molecules for their ability to activate or inhibit LuxR protein of V. fischeri was conducted using E. coli JLD271, pAL103; luxR+ luxI_luxCDABE; TetR p15A origin. In this quantitative bioassay the expression of luxCDABE gene cassette (encoding bacterial luciferase and fatty acid reductase) is under control of the HSL-regulated promoter, allowing rapid screening for LuxR
agonists or antagonists by measuring the level of bioluminescent response. For a natural induction of LuxR system we used exogenous C₆-HSL at concentration from 0.5 nM to 250 µM, shown the EC₅₀ activation value as 60 nM.

Results of HADs agonistic activity screening are shown in the Figure 2. One weak agonist (compound 1) and one strong agonist (compound 2) were present in the library. The weak agonist is identified as hexanoylamino-4-phenylthiophene-3-carboxylic acid, and strong one as hexanoic acid (1-chloro-9, 10-dioxo9, 10-dihydro-anthracen-2-yl)-amide, which caused a bioluminescence induction in 17.5 % and 64.0 % from C₆-HSL in equal concentration 62.5 µM. Although both compounds have exhibited detectable levels of agonistic activity, their EC₅₀ activation value is > 250 µM and 31.3 µM respectively (Table 1), that is up to 500-fold more than for natural signal. Interestingly, compound 2 has had small structural similarity with previously described two cyclic derivatives based on a phenothiazine scaffold that were capable of modulating the *Vibrio harveyi* HSL and AI-2 regulated bioluminescence [6]. But contrary to these molecules operating as selective QS inhibitors, no HADs antagonistic activity in LuxR quantitative bioassay with C₆-HSL has been observed (data not shown).

![Figure 2](image_url)

**Figure 2.** Dynamics of *E. coli* JLD271, pAL103; *luxR+ luxI_luxCDABE* luminescence level after induction with natural signal (C₆-HSL) and hexanoic acid derivatives.

For testing a HADs library to interact CviR regulatory protein the agar plate and liquid medium assays with *C. violaceum* ATCC 31532 have been used. This strain has insertion in cviI gene, therefore is unable to synthesize C₆-HSL, but is inducible by similar molecules with N-acyl side chains from C4 to C8 in length. By virtue of these features, *C. violaceum* ATCC 31532 can be used as biosensor to HSLs and mimic compounds also as quorum sensing inhibitors in mixes with C₆-HSL.
Table 1. EC50 activation values of hexanoic acid derivatives in quantitative bioassays with LuxR protein of \textit{V. fischeri} and CviR protein of \textit{C. violaceum}.

<table>
<thead>
<tr>
<th>№ of compound</th>
<th>Formal name</th>
<th>EC 50</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Hexanoylamino-4-phenyl-thiophene-3-carboxylic acid</td>
<td>&gt;250 µM</td>
</tr>
<tr>
<td>2</td>
<td>Hexanoic acid (1-chloro-9, 10-dioxo9, 10-dihydro-anthracen-2-yl)-amide</td>
<td>31.25 µM</td>
</tr>
<tr>
<td>3</td>
<td>2-Hexanoylamino-5,6-dihydro-4H-cyclopenta[b]thiophene-3-carboxylic acid amide</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>Hexanoic acid (5-chloro-2-methyl-phenyl)-amide</td>
<td>0 250 µM</td>
</tr>
<tr>
<td>5</td>
<td>2-Hexanoylamino-5,6-dihydro-4H-cyclopenta[b]thiophene-3-carboxylic acid</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>Hexanoic acid benzo[1,3]dioxol-5-yl amide</td>
<td>0 46.9 µM</td>
</tr>
<tr>
<td>7</td>
<td>Hexanoic acid (3-cyano-4,5,6,7-tetrahydro-benzo[b]thiophen-2-yl)-amide</td>
<td>0</td>
</tr>
<tr>
<td>8</td>
<td>Hexanoic acid (4,5-digydro-thiazol-2-yl)-amide</td>
<td>0 62.5 µM</td>
</tr>
<tr>
<td>9</td>
<td>Hexanoic acid (4-phenyl-theazol-2-yl)-amide</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td>7-Hexanoylamino-2,3-dihydro-benzo[1,4]-dioxine-6-carboxylic acid</td>
<td>0</td>
</tr>
</tbody>
</table>

In particular, in liquid medium bioassay this compound has shown the higher induction of violacein production (EC50 activation value has been 0.5 nM), explained by CviR affinity to C6-HSL in comparison with LuxR (see above) preferring C6-oxo-HSL.

Results of HADs agonistic activity screening on agar plate and in liquid medium assays are shown in the Figure 3. Three active compounds (4, 6, and 8) have been identified in the library, being not identical ones in LuxR bioassay. A headgroup of compound 4 (hexanoic acid (5-chloro-2-methyl-phenyl)-amide) was similarity \(p\)-Cl substituent molecule, previously tested for their ability to interfere HSL regulated QS mechanism in \textit{Pseudomonas aeruginosa} [24]. But in contrary this analogue showing poorest LasR-driven QS inhibition, a compound 4 was a weaker agonist in a CviR assay (EC50 activation value about 250 µM). A new strong agonist (compound 6) is identified as hexanoic acid benzo[1,3]dioxol-5-yl amide, which have induced violacein production with EC50 activation value 46.9 µM (Table 1). Interestingly, compound 6 has had structural similarity with piperonal (1,3-benzodioxole-5-carbaldehyde) which is a minor natural component of the vanilla extract, a widely used spice and flavour. This fact will be corresponded with results [5] suggesting that extract of \textit{Vanilla planifolia} beans...
Hexanoic acid derivatives

significantly modulate violacein production in a concentration-dependent manner. However, contrary to this observation we did not detect the expressed QS inhibition. A compound 8 (hexanoic acid (4,5-dihydro-thiazol-2-yl)-amide) was the most structurally similar to HSLs analogues, and showed intermediate activity inducing violacein production on agar plate and in liquid medium assays with EC50 activation value 62.5 µM. All HADs operated in concentrations more than on 1000-fold exceeding HSLs, and no antagonistic activity in CviR quantitative bioassay with C6-HSL was observed again (data not shown).

![Image of induction of violacein synthesis in C. violaceum NCTC 13274 plate (left) and liquid medium (right) assays by hexanoic acid derivatives.]

Figure 3.

Our identification of mimics that structurally unrelated to the natural QS signal but can substitute for C6-HSL by interacting with LuxR protein of V. fischeri or CviR protein of C. violaceum, is of interest for several reasons. First, our evidence indicates that the headgroup (rings structure) is important for molecule species selectivity. It has been shown that 2 compounds found in the library are agonistic in LuxR system but does not activate CviR, and 3 other are specific to CviR system but are inactive in LuxR. Thus, HADs found in the library are the useful tool for investigation of bacterial signaling, and differentiation of various HSL-binding regulatory proteins. Secondly, although HADs exhibited detectable levels of agonist activity, their antagonistic activity is absent. It indicates that the side chain is crucial for antagonistic activity, and varying this parameter will possibly develop two-faced compounds, which work as QS agonist and antagonists. This suggestion provides a strategy for rational structure-based design of small molecules targeting R-family of quorum-sensing proteins.
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