Biochemical and Biophysical Research Communications 382 (2009) 153–156

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

A new phenothiazine structural scaffold as inhibitors of bacterial quorum sensing in *Vibrio harveyi*

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ARTICLE INFO

Article history:
Received 20 February 2009
Available online 4 March 2009

Keywords:
MM32
Autoinducer-1
Autoinducer-2

ABSTRACT

Quorum sensing has attracted much attention due to its involvement in pathologically relevant events such as biofilm formation, virulence factor production, and sporulation. Inhibitors of quorum sensing are important research tools and potential therapeutic agents. In this paper, we describe a phenothiazine structural scaffold as a new type of quorum sensing inhibitors with IC_{50} values in the single digit micro molar range in *Vibrio harveyi*.

Introduction

Bacteria use quorum sensing to coordinate community-wide behaviors such as biofilm formation [1] and virulence factor production [2]. Such processes are mediated by the secretion and detection of small molecules called autoinducers [3,4]. There are several types of autoinducers such as acyl homoserine lactones (AHLs/AI-1) [5], autoinducing peptides (AIPs) [6,7], and autoinducer-2 (AI-2) [8], each of which has unique functions. Because of the important roles that quorum sensing plays in pathologically relevant events, it is conceivable that inhibitors of quorum sensing could have therapeutic applications. Along this line, there have been a large number of reports on bacterial quorum sensing inhibitors, especially on AHL- and AIP-mediated quorum sensing [9–16]. Our lab has also reported several inhibitors [17–20].

Among all the autoinducers in quorum sensing, AI-2 is the only one which exists in both Gram-positive and Gram-negative bacteria. We are especially interested in searching for new AI-2 inhibitors because of the limited activities in this area [9,21–30]. In our studies, *Vibrio harveyi* is used as the model organism because of its ability to emit luminescence upon quorum sensing and the fact that it is a pathogen of marine fish and invertebrates, particularly penaeid shrimp [31,32]. Both AI-1 and AI-2 quorum sensing pathways exist in *V. harveyi*. AI-2 has several forms. For example, boric acid complex A (Fig. 1) is the active form of AI-2 in *V. harveyi* quorum sensing [33–35]. In *V. harveyi*, the AI-1 molecule is 3-hydroxy-C_4-HSL (B) (Fig. 1). In this paper, we described a new structural scaffold that shows inhibitory effect on both AI-1 and AI-2 mediated quorum sensing pathways in *V. harveyi*.

Materials and methods

Reagents. All phenothiazine analogs were from commercial sources. The MM32 (#BAA-1121) and BB886 (ATCC# BAA-1118) strains of *V. harveyi* was purchased from ATCC. DPD was synthesized following literature procedures [36].

MM32 tests (*AI-2 inhibition*). Experiments were conducted by following literature procedures [19,20,33]. Briefly, 5 μM of DPD was used for quorum sensing induction. DMSO concentration was strictly controlled below 1% as described earlier because of its inhibitory effect on quorum sensing in *V. harveyi*. Iron ($FeCl_3$) concentration was kept at 1.2 mM [37].

BB886 tests (*AI-1 inhibition*). The BB886 mutant strain of *V. harveyi* was tested in a similar way as the MM32 mutant. The only difference was that 5% (v/v) cell free culture was used as the source of AI-1.

Cell growth studies. Bacterial doubling time was determined by following literature procedures [18–20].

Results and discussion

For the initial studies, we were interested in looking for new small molecule scaffolds that exhibit inhibitory effects on the AI-2 pathway in *V. harveyi*. In doing so, we randomly screened a large number of compounds against the MM32 mutant, which lacks the LuxS enzyme needed for producing DPD and the LuxN receptor needed to respond to AI-1 [38]. The screening was conducted by

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doi:10.1016/j.bbrc.2009.02.157

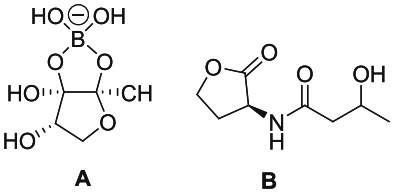

Fig. 1. Structures of AI-2 (A) and AI-1 (B) active in *V. harveyi*.

following published procedures using a microplate reader by observing the effect of the test compounds on the luminescence intensity of the bacteria [33,39].

Among all the compounds screened, phenothiazine (Fig. 2) showed potent inhibitory effect in this whole cell assay with an IC_{50} of about 7 μM in MM32. Fig. 3 shows the concentration-dependent luminescence intensity changes resulting from phenothiazine addition to the MM32 strain of *V. harveyi*.

In an effort to achieve some initial understanding of the structure–activity relationship, 14 additional phenothiazine analogs were tested against the MM32 strain of *V. harveyi* (Fig. 4). Surprisingly, closely related analogs such as **2** and **3** did not show any inhibitory effects. Such results indicate that the tolerance for structural modifications on the aryl ring is low. Oxidizing the sulfur to the sulfone moiety (**4**) and replacing the phenothiazine nitrogen by an oxygen atom (**5**) all resulted in loss of activities. So did the elimination of the third ring accompanied by the conversion of the aniline group to an amide (**12**, **13**).

The screening of the 14 additional analogs yielded compounds **6**, **7**, **8**, **10**, **11**, **14** and **15** as having some inhibitory effect (Table 1). Consequently, we determined the IC_{50} values of these compounds. Among them, only compounds **14** (IC_{50} 47.1 ± 4.6 μM) and **15** (IC_{50} 61.3 ± 15.6 μM) showed IC_{50} lower than 100 μM. It is interesting to note that the phenothiazine analog (**15**) with an ionizable side chain group (amino) showed modest inhibitory activities. Such results may suggest a beneficial role in binding for a positively charged side chain functional group.

In order to gain some initial understanding of the inhibition mechanism, we also tested the inhibitory effects of phenothiazine and its analogs for their AI-1 inhibition by using the BB886 strain of *V. harveyi*, which lacks the LuxP receptor required for AI-2 response. To our surprise, all those that showed some inhibitory effect on AI-2-mediated quorum sensing also showed inhibitory

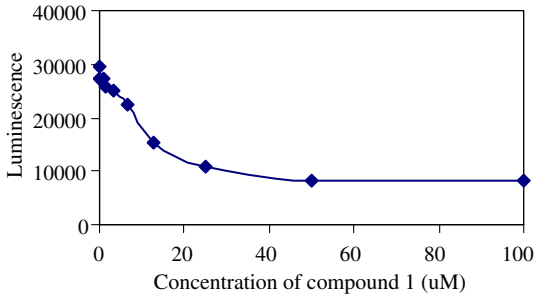

Fig. 3. Concentration-dependent inhibition of *V. harveyi* luminescence by phenothiazine (**1**): IC_{50} = 7.4 ± 3.5 μM.

effects on the AI-1 pathway. Furthermore, it seems that the inhibitory effect is stronger against AI-1. For example, the IC_{50} value of compound **7** is over 100 μM against AI-2 and 17 μM against AI-1 (Table 1). For compounds **10**, the IC_{50} value is over 100 μM against AI-2 and 23 μM against AI-1. Among these 8 compounds, seven compounds (**6**, **7**, **8**, **10**, **11** **14**, and **15**) showed good to modest selectivity towards AI-1; and phenothiazine itself seems to be about equally active against both. Table 1 shows the IC_{50} values of these compounds against different *V. harveyi* strains.

With these compounds that showed inhibitory effects, we also examined their effect on bacterial growth to eliminate cytotoxicity as reflected in retarded bacterial growth as the reason for the observed reduced bioluminescence production. In this assay, only compounds **1** and **10** did not exhibit significant inhibition of bacterial growth when compared with the control group (no compound) based on bacterial doubling time (Blank: 70 min; **1**, 72 min; **10**, 80 min). Therefore, only these two compounds can be considered as quorum sensing inhibitors. The observed luminescence intensity decrease by the other compounds may or may not be due to quorum sensing inhibition because of the complications posed by the observed retardation in growth.

As discussed above, compound **10** is selective against AI-1-mediated quorum sensing while compound **1** is equally active against both AI-1 and AI-2. It is not clear why **1** could inhibit both pathways and what the mechanism(s) is. It is interesting to note that two other reported AI-2 inhibitors also showed similar promiscuity [9,24]. Studying the underlying biological reasons behind the observed inhibition promiscuity might lead to important new understanding in quorum sensing research.

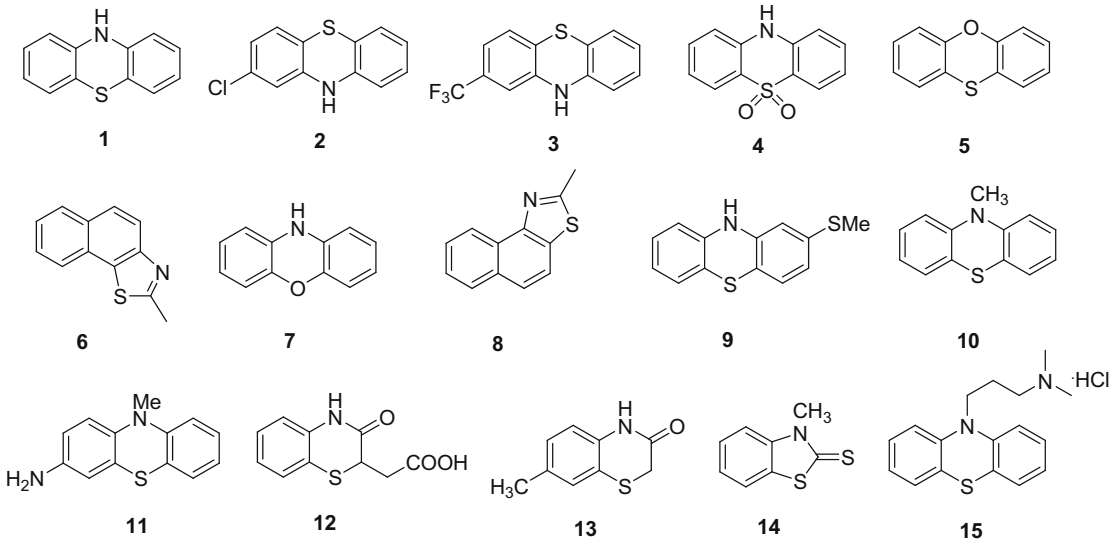

Fig. 2. Phenothiazine and its analogs.

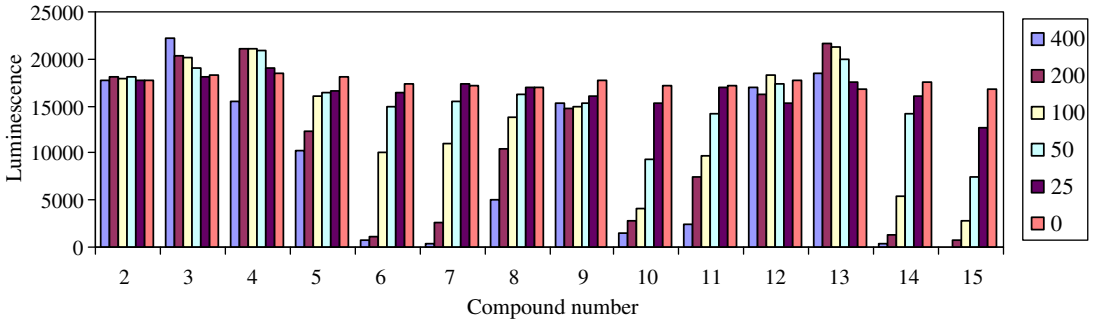

Fig. 4. Screening results for compounds **2**–**15**.

Table 1
IC_{50} values of 8 compounds against MM32 (AI-2) and BB886 (AI-1) strains of *V. harveyi*.*

Compound No.	**1**	**6**	**7**	**8**
IC_{50} (MM32)	7.4 ± 3.5	>100	>100	>100
Compound No.	**10**	**11**	**14**	**15**
IC_{50} (MM32)	>100	>100	47.1 ± 4.6	61.3 ± 15.6
Compound No.	**1**	**6**	**7**	**8**
IC_{50} (BB886)	4.6 ± 1.1	30.8 ± 17.1	17.2 ± 8.7	71.3 ± 26.9
Compound No.	**10**	**11**	**14**	**15**
IC_{50} (BB886)	22.7 ± 4.1	41.7 ± 10.5	23.9 ± 16.0	19.2 ± 2.8

* Results were based on triplicate experiments.

Conclusion

In this study, a novel structural scaffold, phenothiazine, has been found to afford inhibitors of both AI-1 and AI-2 quorum sensing in *V. harveyi*. Such information can be used for the further development of novel inhibitors for research and possibly antimicrobial applications. Understanding the inhibition promiscuity observed in this new class of compounds as well as in previously reported inhibitors [9,24] might lead to new revelations as to the intricate interplays of various biological processes in *V. harveyi* and possibly other bacteria.

Acknowledgments

Financial support from the Molecular Basis of Disease program at GSU, Georgia Cancer Coalition, and Georgia Research Alliance is gratefully acknowledged.

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