The novel effect of *cis*-2-decenoic acid on biofilm producing *Pseudomonas aeruginosa*

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Abstract

Microbial biofilms are a main cause of many chronic infections and mortalities, such as dental caries, cystic fibrosis, osteoradionecrosis, urinary tract infections and native valve endocarditis. These polymeric matrices are sessile communities with different rules from those forms via known planktonic bacteria. One of the important biofilm-producing human pathogens is *Pseudomonas aeruginosa*, which causes death in the majority of people who suffer from cystic fibrosis, AIDS, burns and neutropenic cancer. To find a method for controlling the growth and resistance of *P. aeruginosa* biofilm, this study investigated the dispersion induction of this microorganism with a diffusible signal factor (DSF), *cis*-2-decenoic acid (CDA), in combination with Tobramycin as a useful antibiotic. Our findings confirmed that although CDA did not act as a dispersion inducer in this experiment, it did show an antimicrobial effect and decreased the MIC of Tobramycin. These results suggested that research on the probable new effects of DSF molecules will result in advances in the control of biofilm infections.

Introduction

Pseudomonas aeruginosa is an opportunistic and ubiquitous pathogen, responsible for many human infections such as nosocomial pneumonia, urinary tract infections (UTIs) and surgical wound or bloodstream infections.[1-4] This common Gram-negative bacterium causes death in the majority of people who suffer from cystic fibrosis, AIDS, burns and neutropenic cancer.[3] Indeed, *P. aeruginosa* is responsible for almost 10% of all hospital-acquired infections.[5] It can generate a wide range of virulence factors, including extracellular proteases or small molecule toxins like elastase, alkaline protease, exotoxin A, rhamnolipids, pyocyanin, lectins, and HCN to overcome host defenses.[4,6,7] *P. aeruginosa* can live and reproduce in various environments as a result of its great metabolic flexibility.[4] This pathogen is also resistant to the more common antibiotics because of i) its outer membrane barrier, ii) multidrug efflux pumps alongside various antibiotic-resistant genes and iii) biofilms' development of resistance to antibiotics and hosts' immune systems.[3,5] Therefore, novel treatments are urgently necessary to counter *P. aeruginosa*.

Microbial biofilms are identified as an association of microorganisms that adheres by extracellular products on a surface or at some distance further than a surface. Biofilms are widespread in different environments including food fermentors, oil-well drilling pipes and ship hulls, and are connected with many infections such as dental plaque, upper respiratory infections, peritonitis, urogenital infections and diseases related to implanted medical devices.[8] This city of microbes supplies a safe place for infection-producing organisms, because they form a barrier to host immune responses, phagocytosis and antibiotic therapies. Furthermore, the potentially pathogenic bacteria can grow on catheters, artificial joints, mechanical heart valves and other devices, releasing persistently from the developed biofilms and forming chronic infections.[9]

The variety of biofilm-producing microorganisms is fairly extensive and includes Gram-positive and Gram-negative bacteria, yeasts and fungi. Some of these public-health-related pathogens are *Staphylococcus aureus* (including methycillin-resistant strains), *Staph. epidermidis*, *Escherichia coli*, *P. aeruginosa*, *Burkholderia cepacia*, *Vibrio cholera*, *Candida parapsilosis* and *C. albicans*.[10] It has been found that biofilm forms with the virulent enhancement of such organisms. The Centers for Disease Control and Prevention (CDC) have reported that 65% of infections are caused by biofilms; similarly, the National Institutes of Health (NIH) has reported the figure as 80%.[9] These high percentages of human infections are due to the high resistance of microorganisms available in the biofilm to antimicrobial therapies.[11] It has been shown that biofilm formation in *P. aeruginosa* develops through several stages, including i) attachment of the bacterium to a surface, which is reversible, ii) the subsequent irreversible association and expansion of microcolonies and iii) growth to the last stage of development, when dispersion takes place and bacteria release into the surrounding medium.[12,13]

It is recognized that the production of virulence factors, development of biofilm and exchange of DNA in *P. aeruginosa* are controlled by a quorum-sensing (QS) system. QS is a global regulatory signal transduction mechanism based on the production of intercellular small molecules called *autoinducers*.[4,7] This system allows bacteria to develop collective decisions with the mediation of autoinducers, and manages different biological functions like virulence, bacterial conjugation, bioluminescence and biofilm formation.[1,2] Gram-negative bacteria usually use N-acyl homoserine lactones (AHLs) as autoinducers.[1,14] When these signaling molecules reach a specific concentration, which is dependent on the bacterium population, they attach to each other and activate related transcriptional factors in a cascade of regulatory procedures.[2,14] Of the three stages of biofilm formation, the last step – dispersion – is the most important because it provides a mechanism to manage the growth and resistance of biofilms, especially in household, medical and industrial locations. Research to discover the regulatory signals responsible for dispersion has resulted in the discovery of different chemical substances (such as NaCl, monochloramine and high concentrations of urea), chelators (such as EDTA), surfactants (such as sodium dodecyl sulfate and Tween 20) and lysozymal enzymes, in addition to a number of antimicrobial factors. Research

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shows that the addition of such chemicals to the mixed *P. aeruginosa* and *Klebsiella pneumoniae* biofilms causes the elimination of some of the surface proteins and release of the infection-causing cells from the biofilms. Moreover, other studies have shown that factors such as a sudden rise in organic carbon, a rapid decrease in oxygen, starvation, the presence of prophages and nitric oxide may trigger the dispersion of aggregated bacteria.[12]

In the last decade, the determination of the QS molecule responsible for biofilm dispersion in the cell-cell communications has been researched extensively. Davies and Marques suggested that *cis*-2-decenoic acid (CDA),[12] the unsaturated fatty acid formed by *P. aeruginosa* in biofilm societies, is the mediator of the dispersion response in biofilms of both Gram-negative (including *P. aeruginosa*) and Gram-positive bacteria. However, when employing this signaling molecule, the resistance of microorganisms to antimicrobial therapy could be inverted as a result of the cells' transition from biofilm to the planktonic position. Therefore, the use of a dispersion agent before or along with antimicrobial agents will increase the effectiveness of these therapies by disrupting persistent biofilms.

In our present study, we investigated the effect of CDA as a dispersion inducer in combination with Tobramycin (a common antibiotic in *P. aeruginosa* infections) to determine whether it would increase the efficacy of this antimicrobial agent.

Materials and Methods

Chemicals and materials

P. aeruginosa ATCC 15442 was employed (American Type Culture Collection). All tests were performed in soybean casein digest broth (agar) and Mueller-Hinton broth (agar) from Himedia (India). Tobramycin and CDA were purchased from Sigma (USA). 2,3,5-triphenyltetrazolium chloride (TTC) was ordered from Merck (Germany). Statistical analysis was performed based on One-way ANOVA followed by Dunnett's t-test using SPSS software (version 16.0) at a significance level of 0.01.

Antibacterial activity of tobramycin

A growth culture of *P. aeruginosa* (24 h at 37°C) on soybean casein digest agar (SCDA) was adjusted to 10^6 CFU/mL with sterile normal saline (NS) (0.9%). Different concentrations of Tobramycin (0.5-16 µg/mL) were prepared in Mueller-Hinton broth (MHB) by two-fold serial dilution. 180 µL of each concentration was placed in a 96-well culture plate along with 20 µL bacterial suspension (10^6 CFU/mL). Each test was performed in triplicate. Wells with only culture media and only test bacteria inoculated in MHB comprised the negative and positive control wells, respectively.

After 24 h incubation of the plate at 37°C, the bacterial growth was evaluated in each well with the addition of 20 µL of the colorimetric indicator 2,3,5-triphenyltetrazolium chloride (TTC) (5 mg/mL). Thereafter, the plates were incubated again (1 hour at 37°C). The minimum inhibitory concentration (MIC) was the lowest concentration of Tobramycin without any color changes. Minimum bactericidal

Table 1. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of Tobramycin with and without *cis*-2-decenoic acid (CDA).

	MIC (µg/mL)	MBC (µg/mL)
Tobramycin	2	2
Tobramycin and CDA (70 µM)	1	2

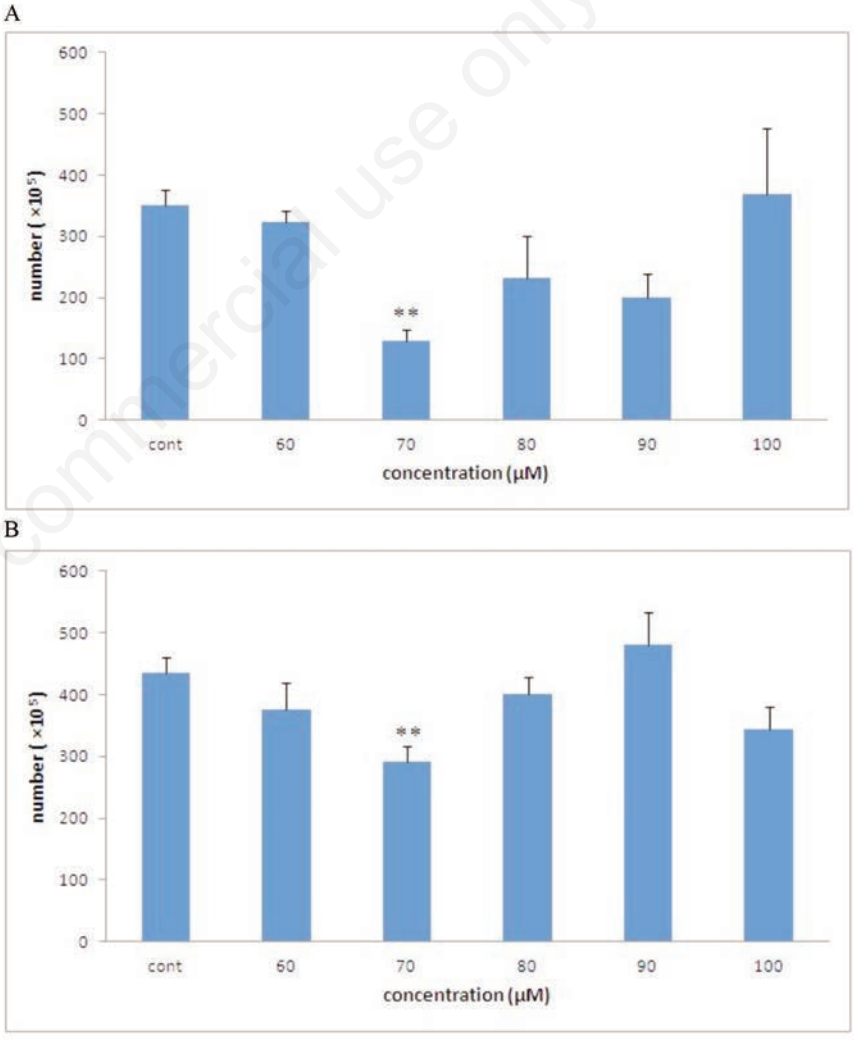

Figure 1. The effect of increasing concentrations of *cis*-2-decenoic acid (CDA) on planktonic cells of biofilm producer *Pseudomonas aeruginosa*: A) CDA was added initially B) CDA was added just at the last 8h. The results are the average of three replicates. Error bars of each concentration were represented by one standard deviation. Dunnett test was performed for all CDA samples (60, 70, 80, 90 and 100 µM) and compared to control (first column) at P<0.01.**

concentration (MBC) was also studied by cultivating 20 µL of no-color-change on the surface of plates containing Mueller-Hinton agar (MHA).

Cis-2-decenoic acid effect on dispersion of biofilm cells

Addition of cis-2-decenoic acid from the first level

To find out the appropriate quantity of CDA in bacterial dispersion, different concentrations were prepared in MHB containing 2.5% glucose and inoculated in a 96-well culture plate. 20 µL of bacterial suspension (10^6 CFU/mL) was added to each well. The test was repeated three times for each concentration. Negative and positive controls were also included.

The plate was then incubated for 96 h and the mixture in the wells was replaced after 24 h for the first day, every 12 h in the second and third day and every 8 h in the fourth day. Thereafter, the bulk-liquid of each well was removed completely, serially diluted and cultured on SCDA. Biofilms attached to the well were also removed by fully scratching the well surface in the presence of 100 µL sterile NS and transmitted separately to SCDA containing plates. All plates were incubated at 37°C (24 h). Dispersion induction was assessed by counting colonies obtained from the bulk-liquid and biofilm portion of each well.

Addition of cis-2-decenoic acid in the final 8 h

This experiment was also repeated in the presence of different concentrations of CDA, added to the wells only in the final 8 h of media replacement. Other conditions were as before. Similarly, the resulting colonies from each well were counted.

Antibacterial activity of *cis*-2-decenoic acid in combination with tobramycin

The most effective concentration of CDA was analyzed in combination with Tobramycin. The test stages were shown to be the same as in the MIC determination. Briefly, the resulting MIC of Tobramycin was prepared again and various concentrations of the antibiotic were provided according to the two-fold serial dilution method. From each concentration, 180 µL was inoculated in a 96-well culture plate to which was added a 20 µL bacterial suspension (10^6 CFU/mL).

The MIC was determined after 24 h incubation of the plate at 37°C by addition of TTC, and the MBC was defined by cultivation of no-color-change cultures.

Results

The antibacterial activity of Tobramycin against the tested bacteria is presented in Table 1, which showed that in this case, the MIC is equal to the MBC.

In the next step, CDA efficacy was studied in two ways. Firstly, CDA was added from beginning of the experiment to the bacterial culture in low levels (2.5, 5, and 10 nM). Table 2 summarizes the average numbers of colony-forming units (CFU) resulting from the upper phase of formed biofilms. The results indicated that the employed concentrations of CDA were not enough to disperse bacteria from biofilm polymeric matrices. Therefore, upper concentrations (60, 70, 80, 90 and 100 µM) were prepared and the experiment was repeated the same as previous. The results were presented in Table 3. Once more, no effect was observed compared with the positive control. However, the average number of microbial colonies in the well containing 70 µM CDA decreased significantly ($P<0.01$) in the upper phase (Figure 1A).

Secondly, CDA was added just in the final 8 h of incubation period. The other stages of the test were completed as before. Again, no significant difference in the cell dispersion was achieved, but the average number of colonies in the upper phase decreased considerably ($P<0.01$) in the presence of 70 µM CDA (Table 3 and Figure 1B).

Figure 2. The structure of *cis*-2-decenoic acid.

Table 2. Average number of CFUs of *Pseudomonas aeruginosa* in presence of *cis*-2-decenoic Acid (CDA) at concentrations of 2.5, 5, and 10 nM.

	CDA added initially		CDA added in the last 8 h	
CDA conc. (nM)	Average N. of planktonic cells ($\times10^5$)	Average No. of biofilm cells ($\times10^5$)	Average No. of planktonic cells ($\times10^5$)	Average No. of biofilm cells ($\times10^5$)
0	380	230	360	235
2.5	420	250	220	290
5	320	277	170	270
10	260	119	350	125

Table 3. Average number of CFUs of *Pseudomonas aeruginosa* in presence of *cis*-2-decenoic Acid (CDA) at concentrations of 60, 70, 80, 90 and 100 µM.

	CDA added initially		CDA added in the last 8 h	
CDA conc. (nM)	Average N. of planktonic cells ($\times10^5$)	Average No. of biofilm cells ($\times10^5$)	Average No. of planktonic cells ($\times10^5$)	Average No. of biofilm cells ($\times10^5$)
0	350	170	435	330
60	323	180	375	353
70	130	147	292	315
80	233	212	400	335
90	200	430	480	410
100	370	400	345	361

To investigate the possible antimicrobial effect of CDA, the concentrations of 70 µM were tested in combination of Tobramycin. As shown in Table 1, CDA reduced the MIC of Tobramycin to half of its previous value. However, the MBC of this antibiotic remained unchanged.

Discussion

Tobramycin, an aminoglycoside antibiotic derived from *Streptomyces tenebrarius*, operates through disrupting protein synthesis. This mechanism results in enhancement of the cell membrane's permeability, increasing disruption of the microorganism envelope and finally the death of the bacteria. It is confirmed that Tobramycin acts against Gram-negative bacteria such as *P. aeruginosa* and has bactericidal effects at concentrations identical to or somewhat greater than those of MIC.[15,16]

It has been confirmed that unlike planktonic cells the microorganisms within biofilms are not susceptible to the antibiotics.[17] This resistance disappeared after the alteration of biofilm cells to a planktonic growth condition. The inherent tolerance of microbial biofilms to antibiotics has resulted in the troubles with their eradication and control of patients with such infections.[18] For example, antibiotic therapy in cystic fibrosis patients with *P. aeruginosa* pneumonia often provides somewhat relief from symptoms, but it cannot treat the infection.[19] The reason for this variation in antibiotic susceptibility between the same cells in planktonic and biofilm forms may possibly be due to differences in the antibiotic diffusion or complex alterations in the microbial physiology of the biofilm.[18]

Numerous studies have defined different fatty acids as signaling molecules – named diffusible signal factors (DSF) – in bacterial communication.[12,20-24] For example, eight fatty acids that aid translocation of the bacteria and communication between *P. aeruginosa* cells have been separated from *Stenotrophomonas maltophila.*[23,25] Another fatty acid, *cis*-2-dodecenoic acid, facilitates the communication between *B. cenocepacia* and *C. albicans*. Therefore it seems that DSFs, like AHLs, have a special role in cell-cell interaction.[23]

One of these DSFs is CDA (Figure 2); a dispersion autoinducer which has been demonstrated that is effective in biofilm dispersion of not only *P. aeruginosa*, but also *Streptococcus mutans*, *E. coli, K. pneumoniae*, *Proteus mirabilis*, *Strep. pyogenes*, *Bacillus subtilis*, *Staph. aureus,* and the yeast *C. albicans.*[23] Since Davies and Marques suggested that the minimum concentration of CDA for dispersion induction of biofilms formed by *P. aeruginosa* PAO1 was 2.5 nM,[12] this concentration was selected as the lowest concentration, and the effect of the molecule was evaluated at 2.5, 5 and 10 nM.

In this experiment, CDA efficacy was investigated in two different ways: addition from the first level and only for an eight hour period. We hypothesized this might result in cell dispersion and reduce biofilm formation. As a result, the planktonic bacterial density would increase. At the same time, biofilm cells would decrease in contrast to the positive control. These two requisites were considered for selecting an appropriate concentration of CDA.

As the two declared requisites, compared with the positive control, were not satisfied in different concentrations of CDA (Table 2), the test was repeated with higher quantities. Again, no dispersing effect was detected in the presence of various concentrations of CDA but surprisingly, the microbial colonies in the well containing 70 µM CDA (either from the first level or just in the final 8 hours of incubation period), decreased significantly ($P<0.01$) in the upper phase (Table 3 and Figure 1).

In this experiment, plate-counting was used instead of measurements of released cells' optical density (OD), which is used by Davies and Marques.[12] Plate-counting is a good method for the study of bacterial growth. It is also useful when examining the effects of different factors on bacterial cultures. We should note that OD measurement techniques cannot determine the size of bacterial colonies. Thus plate-counting will remain an essential approach for validating new techniques to study growth factors.[26] These findings are also different from the observations of Davies and Marques,[12] who reported that CDA was capable of dispersion induction in established biofilm micro-colonies after being added exogenously to *P. aeruginosa* PAO1 biofilms at the native concentration of 2.5 µM. They also believed that the *cis* isomer of 2-decenoic acid was active in the range of 1.0 nM to 10 mM. Additionally, according to their findings, exposure of *P. aeruginosa* PAO1 biofilms with dispersion inducer at the final hour of incubation resulted in complete micro-colony disaggregation.[12] The results of colony-counting showed no difference between the tests and positive control. Furthermore, the addition of CDA from the first or in the final 8 h of incubation cannot induce dispersion.

The diversity in the current study's results may be the result of strain variations in *P. aeruginosa.* In fact, inter-species genome diversity could be the reason for these different responses. However, this diversity in decenoic-acid activity has been seen before in another decenoic-acid derivative, 10-Hydroxy-2-decenoic acid. In an experiment, this unsaturated fatty acid, which is extracted from royal jelly, caused an effective reduction in the adherence of *Streptococcus mutans,*[27] the common flora of mouth and the major etiological cause of dental caries,[28] while in another study this molecule showed antimicrobial effects on some microorganisms, including *Lactobacillus brevis* and *Pediococcus damnosus.*[29] In our experiment, concentrations higher than 100 µM were not tested.

As shown in Table 1, CDA decreased the MIC of Tobramycin to 50% at the critical concentration of 70 µM. The other prepared concentrations of this signaling factor were not tested in combination with Tobramycin. The reason for this antibacterial outcome in the presence of only 70 µM CDA could not be determined, but some kinds of antiseptics, such as alcohol, act in the same way. More investigations are necessary to find out whether CDA has dispersion or antimicrobial effects on different strains of *P. aeruginosa*.

Conclusions

The results of the current work enhance knowledge about DSFs' role in *P. aeruginosa* and provided different effects for CDA. In other words, our findings indicated that CDA, which performs as a dispersion inducer in *P. aeruginosa* PAO1, has antibacterial properties against another strain, *P. aeruginosa* ATCC 15442. These outcomes can facilitate the development of new methods to increase the effectiveness of Tobramycin against *P. aeruginosa,* especially in cystic-fibrosis patients.

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