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Gentian Violet and Ferric Ammonium Citrate Disrupt *Pseudomonas Aeruginosa* Biofilms

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Abstract

Objective/Hypothesis: Bacterial biofilms are resistant to antibiotics and may contribute to persistent infections including chronic otitis media and cholesteatoma. Discovery of substances to disrupt biofilms is necessary to treat these chronic infections. Gentian violet (GV) and ferric ammonium citrate (FAC) were tested against *Pseudomonas aeruginosa* biofilms to determine if either substance can reduce biofilm volume.

Study Design: The biofilm volume and planktonic growth of PAO1 and otopathogenic *P. aeruginosa* (OPPA8) isolated from an infected cholesteatoma was measured in the presence of GV or FAC.

Methods: OPPA8 and PAO1 expressing a green fluorescent protein plasmid (pMRP9-1) was inoculated into a glass flow chamber. Biofilms were grown under low flow conditions for 48 hours and subsequently exposed to either GV or FAC for an additional 24 hours. Biofilm formation was visualized by confocal laser microscopy and biofilm volume was assayed by measuring fluorescence. Planktonic cultures were grown under standard conditions with GV or FAC. Statistical analysis was performed by Student *t* test and one-way ANOVA.

Results: GV reduced PAO1 and OPPA8 biofilm volume ($P < .01$). GV delayed the onset and rate of logarithmic growth in both strains. FAC reduced OPPA8 biofilm volume ($P < .01$), but did not effect of PAO1 biofilms. FAC had no effect on planktonic growth.

Conclusions: The efficacy of GV in disrupting biofilms in vitro suggests that it may disrupt biofilms in vivo. The effect of FAC on *Pseudomonas aeruginosa* biofilms is strain dependent. Strain differences in response to increasing iron concentration and biofilm morphology stress the importance of studying clinically isolated strains in testing antibiofilm agents.

Keywords

Biofilm; *Pseudomonas aeruginosa*; gentian violet; ferric ammonium citrate; inhibitor

INTRODUCTION

Chronic otologic infections are often refractory to treatment, despite appropriate systemic and local antibiotics. Treatment of the infection followed by a prompt recurrence is also seen commonly in the middle and external ear. The recalcitrant nature of chronic otitis media with and without cholesteatoma has lead many investigators to study the role of bacterial biofilms in perpetuating these diseases. Microbiological and histological evidence of bacterial biofilms in chronic otitis media and cholesteatoma support this contention.¹⁻³

Resistance to both antibiotics and host defenses are a hallmark of microbial biofilms.⁴ Biofilms are organized communities of microorganisms encased in an extracellular matrix and adherent to a surface.⁵ How the biofilm phenotype conveys increased antibiotic resistance is not fully understood. Distinct gene expression profiles, inter-cellular communication and altered metabolic activity within microenvironments have all been implicated in biofilm antibiotic resistance. The mechanisms by which antibiotic resistance is established in a biofilm may be numerous and vary with the composition of the biofilm itself. Physical removal or disruption of the biofilm is effective, but not always clinically feasible.⁴ Discovering substances that inhibit biofilm formation and/or disrupt established biofilms is essential as biofilms are estimated to account for 65% of human bacterial infections.⁶

Pseudomonas aeruginosa (PA) biofilms were established in flow chambers and exposed to gentian violet (GV) or ferric ammonium citrate (FAC) to determine if either substance inhibits and/or disrupts biofilms. PA is a commonly isolated organism from chronic ear infections.³ Our finding that otopathogenic *Pseudomonas aeruginosa* (OPPA8) isolated from infected cholesteatomas are competent bio-film formers further support the role of PA biofilms in chronic ear disease.³ GV is an antimicrobial dye used against bacteria, fungi, and

protozoa.⁷ Topical application of GV for the treatment of external otitis, another common PA infection, has been used in otology for many years.⁸ GV is used to stain bacterial biofilm for rapid microtiter plate analysis; however, no study has examined its efficacy in disrupting biofilms.³ FAC is an iron salt.⁹ Both excess iron^{9,10} and iron restriction^{11,12} have been implicated in reducing PA biofilms. Differences in PA strains, growth conditions and biofilm assay technique may contribute to this observed difference. In this study, we utilized a well characterized method for biofilm growth, flow chambers and both a laboratory strain, PAO1 and a clinically isolated strain, OPPA8 to test the efficacy of increased iron salts and GV to reduce PA biofilm volume. The hypothesis that GV and FAC can inhibit biofilms *in vivo* is supported if these substances reduced the total bacteria volume of OPPA and PAO1 biofilms in flow chambers.

MATERIALS AND METHODS

Bacterial Strains and Plasmids

Two strains of PA, (OPPA 8) originally isolated from an infected cholesteatoma and PAO1, a standard laboratory strain, were used throughout.³ Stock cultures were maintained as frozen glycerol (20% w/v) preparations at -80°C .

Transformation of OPPA

Plasmid pMRP9–1 containing a Green Fluorescent Protein (GFP) cassette in pUCP-18 was kindly donated by E.P. Greenberg (University of Washington, Seattle, WA). This plasmid was introduced into each PA strain by electroporation as previously described.³ Briefly, bacteria were washed in 1 mmol/L MOPS in 15% glycerol three times and resuspended 100 μL of 1 mmol/L MOPS in 15% glycerol. The bacterial suspension was mixed with 100 ng of plasmid DNA in a Gene Pulser Cuvette (BioRad, Hercules, CA), electroporated at 1800 V in an Electroporator 2510 (Eppendorf, Westbury, CT), incubated in LB at 37° and 250 rpm for 1 hour, and plated on to selective media (carbenicillin at 300 $\mu\text{g}/\text{mL}$) (Sigma, St. Louis, MO).

Biofilm Flow-Cell Chamber Experiments

A single PA colony was used to inoculate 5 mL of Lucia-Bertani (LB) supplemented with carbenicillin (300 $\mu\text{g}/\text{mL}$) and grown for 16 hours at 37°C with agitation (250 rpm). An aliquot of the culture was then diluted to an OD_{600} of 0.7 in fresh LB and inserted into a flow cell (BST Corp. FC 91). The flow cell was incubated for 2 hours without flow. Flow was then initiated at a constant rate of 2.45 mL/H with a peristaltic pump (Watson Marlow 205U, Falmouth, Cornwall, England), which corresponds to laminar flow ($R_e = 1.082$). After 48 hours of continuous flow in LB, the media was changed to LB supplemented with differing concentration of GV or FAC and allowed to flow through the flow cells for an additional 24 hours. Concentrations of GV tested were 12.2, 122, and 1225 $\mu\text{mol}/\text{L}$. Concentrations of FAC tested were 100 and 250 $\mu\text{mol}/\text{L}$. Control flow cells were exposed to LB for 72 hours. All flow cells underwent image analysis 72 hours after initial inoculation. All experiments were performed at room temperature in triplicate.

Confocal scanning laser microscopy (CSLM, Bio-Rad, Radiance 2000 MP) interfaced with a Nikon TE300 inverted micro-scope at 60× water immersion magnification was used to visualize the living GFP fluorescence emitting PA after laser excitation at 488 nm. Dead PA cells were stained with propidium iodide and fluorescence was captured after laser excitation at 490 nm. Image acquisition was performed at three points within the capillary, roughly corresponding to the proximal, middle, and distal end of the capillary.

Z-series of optical sections were reconstructed into three-dimensional images by Lasersnap 2000 Software, and imported into the image analysis software package, Volocity, Version 3.1 (Improvision, Lexington MA). The spatial profile of organisms was estimated by measuring the volume of the fluorescent signal in a series of three-channel optical sections by using simple thresholding. A fixed threshold value was used for all image stacks.

Planktonic Experiments

A single colony of each PA strain was used to inoculate 5 mL of LB media and incubated overnight at 37°C with agitation (250 rpm). A 10- μ L aliquot of the overnight culture was added to 10 mL of fresh LB broth containing either GV or FAC. Concentrations of GV tested were 12.2, 122, and 1225 μ mol/L. Concentrations of FAC tested were 100 and 250 μ mol/L. OD₆₀₀ was then measured at 1, 2, 3, 4, 6, 9, 15, and 24 hours after dilution and compared with control planktonic growth in LB only.

Statistical Analysis

Statistical analysis was performed using Sigma Stat, version 3.0 by *t* test and one-way ANOVA ($\alpha = 0.05$) (SPSS Science, Chicago, IL).

RESULTS

Effects of GV on PA Biofilms

Both OPPA8 and PAO1 grew robust biofilms in the flow chamber system (Fig. 1). A GFP-plasmid was inserted into OPPA8 and PAO1, allowing detection of live bacteria by fluorescence. By CSLM, PAO1 grew in the characteristic pattern with a lawn of bacterial growth on the surface, punctuated by mushroom projections.¹³ OPPA8 grew more uniformly with a dense lawn of bacterial growth and fewer projections. At 48 and 72 hours after inoculation, dense fluorescence was seen with OPPA8 and PAO1. Total fluorescence continued to increase between 48 and 72 hours after inoculation, suggesting that both PAO1 and OPPA8 biofilms were in a growth phase.

GV disrupted and inhibited OPPA8 and PAO1 bio-film growth in flow chambers. GV concentrations of 1225 and 122 μ mol/L significantly reduced bacterial volume in OPPA8 biofilms ($P < .01$). The lowest concentration of GV (12.2 μ mol/L) demonstrated a measurable, but not statistically significant decrease in bacterial volume. After 24 hours of continuous exposure to the highest tested concentration of GV (1225 μ mol/L), little fluorescence was detected in OPPA8, indicating few to no live PA were present. Additionally no dead bacteria were detected by propidium iodine testing. The effect of GV on OPPA8 biofilms was dose-dependent ($P < .05$). Decreasing concentrations of GV were

associated with increased GFP detection, suggesting either biofilm persistence or regrowth (Fig. 1). Interestingly, GV concentrations at 122 and 12.2 $\mu\text{mol/L}$ were associated with increased numbers of detectable dead bacteria by propidium iodine staining. No propidium iodine staining for dead bacteria was seen in control cultures.

GV also disrupted PAO1 biofilms, but less effectively than against OPPA8. Similar to OPPA8, PAO1 biofilms exposed to GV concentration at 1225 $\mu\text{mol/L}$ demonstrated little to no detectable fluorescence ($P < .01$). GV concentrations of 122 and 12.2 $\mu\text{mol/L}$ were associated with a measurable, but not significant decrease in PAO1 volume. In comparison with OPPA8, propidium iodine staining of dead PAO1 was low to none.

Effect of FAC on PA Biofilms

FAC disrupted OPPA8 biofilms, but had little effect on established PAO1 biofilms. FAC at 100 and 250 $\mu\text{mol/L}$ significantly decreased the fluorescence of OPPA8 bio-films after 24 hours of exposure ($P < .03$). Additionally, dead bacteria were detected by propidium iodine staining in the OPPA8 cultures after exposure to FAC at 100 and 250 $\mu\text{mol/L}$. In contrast, FAC at 100 and 250 $\mu\text{mol/L}$ did not statistically reduce the bacterial volume of established PAO1 biofilms (Fig. 2).

Effect of GV and FAC on PA Planktonic Growth

GV reduced the growth of planktonic cultures of OPPA8 and PAO1 whereas FAC demonstrated no effect on either strain. GV at 1225 $\mu\text{mol/L}$ delayed the onset of logarithmic expansion in planktonic cultures of OPPA8 and PAO1 and slowed the rate of expansion in these cultures. GV at 1225 $\mu\text{mol/L}$ delayed the initiation of logarithmic growth for 9 hours in OPPA8 and for 15 hours in PAO1. Both strains exhibited a decreased rate of expansion in response to GV at 1225 $\mu\text{mol/L}$. GV at 122 $\mu\text{mol/L}$ slowed the rate of growth for both OPPA8 and PAO1, but only delayed the onset of logarithmic growth in PAO1 by 4 hours. GV at 12.2 $\mu\text{mol/L}$ had no effect on OPPA8 or PAO1 planktonic growth. FAC at 100 and 250 $\mu\text{mol/L}$ had no effect on planktonic growth of either OPPA8 or PAO1 (Fig. 3).

DISCUSSION

The characteristic feature of the bacterial biofilm phenotype is increased resistance to antibiotics and host defenses, making eradication clinically challenging.^{4,5} Physical removal or disruption of the biofilm is effective, but can be limited secondary to the clinical situation.⁴ In some cases, surgical debridement combined with appropriate antimicrobial therapy is not effective in preventing recurrence of the infection. Adjuvant therapies to inhibit and disrupt biofilm formation are necessary to comprehensively treat these chronic infections. Discovery of antibiofilm agents has proceeded along two principal strategies: testing antimicrobial agents against biofilms and studying substances that alter the local environment and intercellular signaling to inhibit biofilm growth. In the present study, we employ both strategies by examining a known antimicrobial agent, GV and testing the effect of increasing iron concentrations with FAC.

Gentian violet, an antimicrobial dye, has been used in microbiology and medicine for 100 years.⁷ Hans Gram initially described the use of gentian or crystal violet for the staining of

bacteria in 1883 and Hinton in 1925 first described the use of intravenous GV to treat an extreme case of sepsis.¹⁴ GV has been utilized as an antifungal, antibacterial, and antihelminthic agent.⁷ Endotracheal tubes, central venous catheters, and urinary catheters impregnated with gentian violet (gentian violet/chlorhexidine) are shown to reduce bacterial adherence and prolong antimicrobial durability in vitro.^{15,16}

In our flow chamber system, GV is effective at disrupting PA biofilms and inducing bacterial death. It is significant that minimal detectable bacteria volume was seen after a 24-hour exposure of GV at 1225 $\mu\text{mol/L}$ to an established growing PA biofilm. This concentration is equivalent to 0.1% GV, less than most clinical application of the agent (1% gentian violet).⁷ The detection of dead bacteria by propidium iodine staining suggests that bio-film disruption may reveal dead bacteria within the extra-cellular matrix and/or GV is also capable of antimicrobial activity. Strain differences between the clinically isolated OPPA8 and laboratory PAO1 were seen. OPPA8 biofilms were more sensitive than PAO1 to GV as OPPA8 biofilms were disrupted at lower concentration and increased bacterial death was detected by propidium iodine staining. In contrast, PAO1 in planktonic cultures was more sensitive to GV. This contrast between efficacy in biofilm and planktonic states suggests that GV has activity against the biofilm and individual bacteria and the biofilm disruption is not solely because of individual bacterial death.

GV is thought to act by disassociating into a positive cation that can freely permeate cell walls and subsequently bind to the target organism DNA.⁷ Evidence also suggests that GV dissipates the bacterial membrane potential by inducing permeability.¹⁷ This interaction with negatively charged bacterial components such as lipopolysaccharide, peptidoglycan, and DNA has important implications in bacterial biofilms. Biofilm extracellular matrix is principally composed of these substances.¹⁸ The mechanism by which GV is able to permeate and disrupt cell membranes and DNA in intact bacteria may also serve to disrupt the extracellular matrix of biofilms. A photodynamic action of gentian violet, apparently mediated by a free-radical mechanism, has recently been described in bacteria and in the protozoan *T. cruzi*.⁷ The ability of GV to disrupt both individual planktonic bacteria as well as biofilm is supported by our findings.

Potential clinical applications of GV to disrupt bio-films are evolving. GV is used to treat chronic otitis externa and mastoid bowl infections. The efficacy of topical GV in chronic draining ears has also been reported.¹⁹ The flow chamber system provides a constant flow of GV to the biofilm, suggesting reapplication of GV may be necessary for PA biofilm eradication if used primarily. However as an adjuvant therapy to surgical debridement, GV can act against both residual biofilm and planktonic bacteria, preventing infection recurrence. The physical disruption and debulking of a biofilm matrix during surgery can reduce the bacterial burden, allow for increased exposure of bacteria to GV, and enhance the efficacy of GV. At present, the translation of our in vitro findings to clinical application of GV is speculative. Further studies demonstrating GV disruption of biofilms with multiple organisms and in vivo biofilms is needed.

Although safety concerns regarding GV in the middle and inner ear are warranted, most evidence suggests that GV is well tolerated. Acute limited systemic exposure has shown

limited side effects. Additionally infusion into the pleural and mediastinal cavities has not demonstrated any significant side effects in limited case reports.²⁰ However, 1% GV in the middle ear was associated with symptoms of vestibular disturbance in the guinea pigs as well as histological findings consistent with inflammation.⁸ GV is commonly used in chronic otorrhea with associated tympanic membrane perforation without known vestibular complications. Further studies examining the effect of GV in the middle and inner ear inflammation is necessary. Additionally GV concentrations that are effective against PA biofilms may be lower than the tested toxic concentrations with fewer toxicities.

Unlike GV, FAC has few side effects and is commonly used for food preservation and iron supplementation.²¹ The effect of extracellular iron concentration on PA bio-films is unclear with evidence supporting decreased bio-film formation in response to both high- and low-iron concentrations. Yang et al. found that increasing iron concentrations decreased DNA release and biofilm structural development in flow chambers.¹⁰ In contrast, Patriquin et al. demonstrated low-iron concentrations resulting in decreased biofilm mass.¹² Additionally, Singh et al. found that increasing doses of lactoferrin, a binder of free iron, inhibited biofilm formation.¹¹

Using a rapid microtiter plate analysis to screen greater than 4000 substances, Musk et al found that FAC inhibits and disrupts PA14 biofilms.⁹ This study did utilize flow chambers to support the efficacy of FAC in disruption PA14 biofilms, but analysis of total biofilm volume was not performed. However, our findings demonstrate that different strains of PA may respond differently to increased iron conditions. When exposed to 100 and 250 $\mu\text{mol/L}$ of FAC, PAO1 biofilm mass was not significantly different. In contrast, OPPA8 had a robust decreased in biofilm mass after exposure to either concentration, supporting the findings seen with PA14. It is unclear why this stark contrast in response to FAC exists between PAO1 and OPPA8. A significant amount of genomic diversity exists between PA strains, suggesting that differences in iron metabolism and requirement between the strains explain this response. It may also reflect phenotypic changes resulting from adaptation within the cholesteatoma micro-environment which is different from a wound infection or cystic fibrosis. Our control studies also support a morphological difference between the biofilms formed by PAO1 and OPPA8. PAO1 biofilms in flow chambers formed mushroom structures whereas OPPA8 biofilms had fewer projections and a more dense lawn of bacteria. The biofilm structure seen in OPPA8 may be less mature than PAO1 or more amenable to FAC inhibition and disruption. Our findings suggest the study of clinical strains in addition to laboratory strains is vital in regards to biofilm inhibition. The strain dependent differences in biofilm disruption seen in response to FAC reflect our clinical experience with PA infections. PA infections vary significantly in the response to treatment, suggesting that multiple therapies may be necessary for the eradication of the infection.

The present study is limited by the use of flow chambers. Although an established and commonly utilized method for studying PA biofilms, the flow chamber conditions are artificial and do not necessarily reflect the conditions in the ear or any chronic infection. Biofilms are highly plastic and briskly respond to changes in local environment. Animal modeling or clinical correlation is necessary to truly judge the efficacy of any antibiofilm agent.

CONCLUSION

GV disrupts and inhibits PA biofilms in flow chambers. GV also has some efficacy against planktonic PA. The efficacy of GV in vitro suggests that it may disrupt biofilms in vivo. The effect of FAC on PA biofilms is strain dependent. FAC is effective at disrupting OPBA8 biofilms, but has little effect on PAO1 biofilms. Strain differences in response to increasing iron concentration and GV as well as the observed morphology of biofilm itself stress the importance of studying both clinically isolated and laboratory strains in testing antibiofilm agents.

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Fig. 1.

P. aeruginosa biofilms exposed to Gentian Violet. OPPA8 and PAO1 strains expressing green fluorescent protein were grown in flow chambers for 48 hours and subsequently exposed to 1225, 122 and 12.2 $\mu\text{mol/L}$ of gentian violet for an additional 24 hours. Confocal laser microscopy was utilized to create three-dimensional reconstructions of the PA biofilms. Representative images of the three-dimensional biofilm reconstructions are shown. GV concentrations of 1225 and 122 $\mu\text{mol/L}$ significantly reduced bacterial volume in OPPA8 biofilms. The lowest concentration of GV (12.2 $\mu\text{mol/L}$) demonstrated a measurable, but not statistically significant decrease in bacterial volume. Similar to OPPA8, PAO1 biofilms exposed to GV concentration at 1225 $\mu\text{mol/L}$ demonstrated little to no detectable fluorescence. GV concentrations of 122 and 12.2 $\mu\text{mol/L}$ were associated with a measurable, but not significant decrease in PAO1 volume. Propidium iodine staining of dead bacteria (red) was only seen in OPPA8 biofilms exposed to GV.

Fig. 2.

P. aeruginosa biofilms exposed to ferric ammonium citrate. GFP expressing OPPA8 and PAO1 strains were grown in flow chambers for 48 hours and subsequently exposed to 100 and 250 $\mu\text{mol/L}$ of ferric ammonium citrate for an additional 24 hours. Confocal laser microscopy was utilized to create three-dimensional reconstructions of the PA biofilms. Representative images of the three-dimensional biofilm reconstructions are shown. FAC at 100 and 250 $\mu\text{mol/L}$ significantly decreased the fluorescence of OPPA8 biofilms. Propidium iodide staining of dead bacteria was seen in the OPPA8 cultures after exposure to FAC. In

contrast, FAC at 100 and 250 $\mu\text{mol/L}$ did not statistically reduce the bacterial volume of established PAO1 biofilms.

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Fig. 3.

Effect of gentian violet and ferric ammonium citrate on PAO1 planktonic cultures. OPPA8 and PAO1 planktonic cultures were initiated in LB at 37°C and 250 rpm. GV at 1225, 122, and 12.2 μ mol/L and FAC at 100 and 250 μ mol/L were added to cultures. OD₆₀₀ was then measured at 1, 2, 3, 4, 6, 9, 15, and 24 hours after dilution and compared with control planktonic growth in LB only. GV at 1225 μ mol/L delayed the onset of logarithmic expansion and slowed the rate of expansion of OPPA8 and PAO1 planktonic cultures. GV at 122 μ mol/L slowed the rate of growth for both OPPA8 and PAO1, but only delayed the onset of logarithmic growth in PAO1. GV at 12.2 μ mol/L had no effect on OPPA8 or PAO1 planktonic growth. FAC at 100 and 250 μ mol/L had no effect on planktonic growth of either OPPA8 or PAO1.