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Antimicrobial Photodynamic Therapy Treatment of Chronic Recurrent Sinusitis Biofilms

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Abstract

Background—Chronic recurrent sinusitis (CRS) is an inflammatory disease of the facial sinuses and nasal passages that is defined as lasting longer than 12 weeks or occurring more than 4 times per year with symptoms usually lasting more than 20 days. The National Institute for Health Statistics estimates that CRS is one of the most common chronic conditions in the United States affecting an estimated 37 million Americans. The potential etiologies of CRS include bacteria, viruses, allergies, fungi, superantigens and microbial biofilms. In clinical practice there is a significant subpopulation of patients with CRS who remain resistant to cure despite rigorous treatment regimens including surgery, allergy therapy and prolonged antibiotic therapy. The reason for treatment failure is thought to be related to the destruction of the sinus mucociliary defense by the chronic sinus infection resulting in the development of secondary antibiotic resistant microbial colonization of the sinuses and biofilm formation. Antimicrobial photodynamic therapy (aPDT) is a non-antibiotic broad spectrum antimicrobial treatment that has been demonstrated to eradicate antibiotic resistant bacteria and biofilms.

Objective—The objective of this study was to demonstrate the effectiveness of a non-invasive aPDT treatment method of eradicating antibiotic resistant biofilms/microorganisms known to cause CRS in an *in vitro* model.

Methods—Antibiotic resistant planktonic bacteria and fungi and polymicrobial biofilms of *Pseudomonas aeruginosa* and MRSA were grown on silastic sheets and treated with a methylene blue photosensitizer and 670nm non-thermal activating light. Cultures of the planktonic microorganisms and biofilms were obtained before and after light treatment to determine efficacy of planktonic bacteria and biofilm reduction.

Results—The *in vitro* CRS planktonic microorganism and biofilm study demonstrated that aPDT reduced the CRS polymicrobial biofilm by >99.9% after a single treatment.

Conclusions—aPDT can effectively treat CRS polymicrobial antibiotic resistant bacteria, fungi and biofilms both *in vivo*. Human clinical studies are currently planned to assess the safety and efficacy of this treatment for CRS.

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Keywords

chronic sinusitis; biofilms; photodynamic therapy

Introduction

Chronic recurrent sinusitis (CRS) is an inflammatory disease of the facial sinuses and nasal passages that is defined as lasting longer than 12 weeks or occurring more than 4 times per year with symptoms usually lasting more than 20 days.¹ The National Institute for Health Statistics estimates that CRS is one of the most common chronic conditions in the United States affecting an estimated 37 million Americans.² It is also estimated that CRS results in 18–22 million office visits per year and over 500,000 emergency visits per year resulting in an estimated 73 million restricted activity days with an aggregated cost of six billion dollars annually.^{3,4} CRS can present as a headache, facial pain, dental pain, breathing difficulty, purulent nasal drainage, post-nasal drip, hyposmia and/or purulence on nasal examination.¹ The potential etiologies of CRS include bacteria, viruses, allergies, fungi, superantigens, exotoxins and microbial biofilms. Importantly, CRS is also considered to be a significant factor that can exacerbate asthma, chronic lung diseases, eczema, otitis media and chronic fatigue.^{4–7} Failure to effectively treat CRS not only results in prolonged illness but can also result in significant complications including osteomyelitis of the facial bones, meningitis and brain abscesses.^{4,7}

In clinical practice there is a significant subpopulation of patients with CRS who remain resistant to cure despite rigorous treatment regimens including surgery, allergy therapy and prolonged antibiotic therapy.^{1,8–21} The reason for treatment failure is thought to be related to the destruction of the sinus mucociliary defense by the chronic sinus infection resulting in the development of secondary antibiotic resistant microbial colonization of the sinuses and biofilm formation.^{1,8–13,20} Gram-negative and Gram-positive bacteria, including *Hemophilus influenza* and *Streptococcus pneumoniae* account for 50% of clinically sampled isolates found in CRS patients.^{18–20} It is increasingly reported that methicillin resistant *Staphylococcus aureus* (MRSA) and multidrug resistant *Pseudomonas aeruginosa* are found in the clinical isolates of CRS patients and are a cause of antibiotic treatment failures.^{1,8–13} Numerous investigators have reported the presence of biofilms in the sinuses of patients with CRS and consider biofilm as a cause for the recalcitrant nature of persistent CRS.^{1,8–22} The presence of *Hemophilus influenza*, *Streptococcus pneumoniae* and *Staphylococcus aureus* biofilms have been reported to be present in patients with an unfavorable treatment outcome after aggressive antibiotic therapy and surgery for CRS.^{9,15,22} Antibiotic resistant strains of these bacteria also significantly contribute to poor clinical results with the presence of antibiotic resistant bacteria in clinical isolates as high as 30%.²³ CRS with its chronic indolent course, resistance to antibiotics and acute exacerbations has a clinical course that parallels that of other persistent biofilm related inflammatory diseases.^{17,23} Due to the failure of standard therapies to control and cure CRS, other novel non-antibiotic therapies that are able to destroy biofilms and antibiotic resistant bacteria are needed.

Antimicrobial-Photodynamic Therapy (aPDT)

The use of PDT is extensively reported in the literature to be safe and effective for the photodestruction of various microorganisms. The PDT induced effect has been reported by numerous investigators to be target specific to only those organisms that have absorbed the photosensitizer and are exposed to a specific wavelength of light.^{25,29–34} Recently, PDT has been more comprehensively studied as a potential alternative to conventional antibiotic therapy as antibiotic resistant strains of bacteria become more prevalent. It has been reported

in the literature by us, as well as by other investigators, that PDT is equally effective against normal strains and antibiotic resistant strains of bacteria.^{24–28,29–35} Furthermore, there is no evidence of bacterial photoresistance occurring after repeated PDT treatment cycles.^{30,33} Many different types of photosensitizers and light sources have been investigated.³⁰ Methylene blue (MB) and other phenothiazines have been used extensively as photosensitizer agents in numerous investigations, including ours, due to its lack of toxicity and well known photoreactive behavior. MB has been shown to be effective in eradicating both gram-positive and gram-negative bacteria.^{24–28,30–33,35–37} MB is a phenothiazinium salt that has a strong absorption at wavelengths longer than 620 nm. The absorbance peak of MB is at 664 nm and its optical extinction coefficient is $81600 \text{ M}^{-1}\text{cm}^{-1}$. The photoactivity of MB results in two types of photooxidations: 1. The direct reaction between the photoexcited dye and substrate by hydrogen abstraction or electron transfer creating different active radical products and 2. The direct reaction between the photoexcited dye in triplet state and molecular oxygen producing singlet oxygen. Both kinds of active generated products are strong oxidizers and cause cellular damage, membrane lysis and protein inactivation. MB has a high quantum yield of the triplet state formation ($\sim T = 0.52 - 0.58$) and a high yield of the singlet oxygen generation (0.2 at pH 5 and 0.94 at pH 9).^{38,39}

The photodynamic mechanism of bacterial and fungal cell destruction is by perforation of the cell membrane or wall by PDT induced singlet oxygen and oxygen radicals thereby allowing the dye to be further translocated into the cell. Once there, the photodynamic photosensitizer, in its new sites, photodamages inner organelles and induces cell death.⁴⁰ Importantly, the PDT mechanism of microbial cell death is completely different from that of oral and systemic antimicrobial agents. Therefore, it is effective against antibiotic resistant bacteria.

MB Photosensitizer Toxicity and Safety

MB has been used in medical practice for over 100 years. As a result, the toxicity of MB in humans has been widely studied and well documented including an extensive study by the National Toxicology Program.^{41–43} MB has been approved by the FDA for intravenous (IV) administration for various applications at doses greater than 1 mg/kg without toxicity in the human.^{44,45} MB is also prescribed in oral doses of 65–130 mg, 3 times daily, or in single daily doses of 100–300 mg.^{44,45} FDA approved clinical uses of MB are numerous and include the treatment of ifosfamide encephalopathy, methemoglobinemia, urolithiasis, and cyanide poisoning.^{44,45} These FDA approved uses of MB are at concentrations that are one hundred-fold greater than the concentration of MB proposed in this study. Furthermore, it has been reported in the literature that when MB is photoactivated, no toxic byproducts are produced.^{46–52} MB mediated PDT for the photoablation of microorganisms has been reported to be used safely and effectively in the human for dental applications without any reports of mucosal tissue toxicity or harmful effects.^{53–55}

Materials and Methods

In vitro studies were performed to demonstrate the efficacy of MB PDT to eradicate antibiotic resistant planktonic gram positive and gram negative bacteria and candida as well as polymicrobial antibiotic resistant biofilms as are commonly found in the chronic sinusitis.

In vitro Study of MB (Methylene Blue) Mediated PDT of Various Pathogenic Bacteria and Fungi Associated with Chronic Recurrent Sinusitis

A study was performed to evaluate the effectiveness of MB PDT in photoeradication of various pathogenic planktonic bacteria and fungi. Clinical isolates of drug-resistant *C.*

albicans (patient iso.), *C. albicans* 14053, *E. coli* 35218, *E. faecalis* 51229, *H. influenzae* (patient iso.), *K. pneumoniae* 13882, *P. aeruginosa* 27853, *S. marcescens* (patient iso.), *S. aureus* 29213, *S. pneumoniae* 49619 were used in this study. The day prior to the experiment, fresh bacterial cultures were grown overnight on tryptic soy agar (Remel, Lenexa, KS) at 37°C in a TempCon incubator (Baxter, Deerfield, IL). On the day of the experiment, the cultures were removed and the bacteria were suspended in 1× phosphate buffered saline (PBS) at a concentration of approximately 1.5×10^8 cells/mL (McFarland Standard of 0.5) of each bacterium using a CrystalSpec nephelometer (BD Diagnostic Systems, Sparks, MD).

Methylene blue trihydrate 98.5% (Sigma-Aldrich, St. Louis, MO) was prepared by dissolving it into sterile PBS. MB concentrations of 50–400 µg/mL were used. The bacterial solution and methylene blue (MB) were mixed and incubated for 30 seconds. Fifty microliters of this solution was pipetted into a test tube for light activation. Light was provided by a DD4 diode laser (Miravant, Santa Barbara, CA) outputting light at 664 nm with a 400-µm microlens fiber (PDT Systems, Santa Barbara, CA) at a dose rate 100 mW/cm² and a light dose of 30 or 40 J/cm². The microlens fiber was positioned 2 cm from the test tube bottom so that the light spot size matched the 1.27 cm tube diameter.

After treatment a cotton-tipped swab (Puritan, Guilford, ME) was dipped into the suspension, placed in 1 mL of PBS, the stick broken off, and then vortexed for 3–5 seconds. These samples were plated for qualitative growth onto TSA blood agar plates. All TSA blood plates were grown at 37°C for 48 hours. Results were visually scored using the following qualitative scoring system: 0 = no colonies, 1 = 1–5 colonies, 2 = 6–100 colonies, 3 = 101–300 colonies, 4 = 301+ colonies. The results demonstrate that MB PDT is highly effective in the photoeradication of pathogenic *Candida* and various antibiotic resistant gram positive and gram negative bacteria that are commonly associated with chronic recurrent sinusitis (Table 1).

***In vitro* Study of MB (Methylene Blue) Mediated PDT of Antibiotic-Resistant Multi-Specie Bacterial Biofilms**

Clinical isolates of multidrug-resistant *Pseudomonas aeruginosa* and methicillin-resistant *Staphylococcus aureus* were used in this study. Overnight tryptic soy broth (Remel, Lenexa, KS) cultures were centrifuged for 15 minutes at 5,000 RPM. The supernatants were removed and the cells washed and resuspended in 1× phosphate buffered saline (PBS) by alternate rounds of centrifugation for 2 minutes at 12.1xg and resuspension. Resulting bacterial suspensions were diluted with PBS to obtain approximately 1.5×10^8 cells/mL of each bacterium measured using a CrystalSpec nephelometer (BD Diagnostic Systems, Sparks, MD). The suspensions were then combined. The FC 270 dual-flow cell system (BioSurface Technologies Corp., Bozeman, MT) was assembled as described and validated by Leung et al and others.^{56,57} Biofilms were grown on 5.5×7.5×0.1 cm silicone sheeting (Invotec International, Jacksonville, FL) cut into circular 1.27 cm discs. Flow cells were inoculated with 500 µL of the bacterial suspension and the bacteria were allowed to adhere to the disc surfaces for one hour. Next, tryptic soy broth was pumped through the flow cells at a constant rate of 30 mL h⁻¹ for 24 hours using a peristaltic pump (Control Company, Friendswood, TX). The presence of biofilm growth on representative discs was confirmed using confocal microscopy.

Discs were removed from the wells and gently washed thrice with PBS to remove non-adherent bacteria. Next, the discs were placed in the empty wells of a tissue culture plate and covered with 100 µL of the photosensitizer for 5 minutes in the dark. The photosensitizer consisted of either 300 or 500 µg/mL of methylene blue trihydrate 98% (Sigma-Aldrich, St.

Louis, MO) dissolved in sterile PBS. After the dark incubation, the discs were removed, gently washed with PBS, and placed into different wells for irradiation. The microlens fiber was positioned at a distance from the disc so that the light spot size matched the disc size. Discs treated with 300 µg/mL were exposed to 664 nm light at a dose rate of 150 mW/cm² and a light dose of 60 J/cm² for 400 seconds. The discs belonging to the 500 µg/mL group were exposed at a dose rate of 76 mW/cm² and two light doses of 55 J/cm² separated by a 5 minutes dark time between each light dose. Control groups of no treatment, light alone and MB alone were also performed

After treatment the discs were cultured by making an X-like pattern across the surface using a swab, placing the swab in 1 mL of PBS, breaking off the stick, and vigorously vortexed. Samples were serially diluted in PBS and spirally plated using an Autoplate 4000 (Spiral Biotech, Norwood, MA) onto tryptic soy agar with 5% sheeps blood (Remel). Plates were incubated at 37°C for 48 hours. The colonies were counted by a Protos automated counter (Synoptics, Frederick, MD) to determine the total number of viable bacteria and expressed as CFU.

Statistics

Results from the untreated and treatment groups were logarithmically transformed and compared using a two-tailed unequal variance Students T-test at a significance level of $p < 0.05$.

Results

Both treatment groups showed statistically significant reductions ($*p < 0.05$) (Figure 1). Light was delivered to the 300 µg/mL MB treated biofilms at a dose rate of 150 mW/cm² using a light dose of 60 J/cm². Light was delivered to the 500 µg/mL MB treated biofilms at a dose rate of 76 mW/cm² using two light doses of 55 J/cm² with a 5-minute pause between light doses. The control groups of light alone and MB alone demonstrated no statistical decrease in CFU compared to the untreated control (results not shown on Figure 1).

Group	Mean Log Reduction	Sample Size (n)	Group Comparison (P-value)
300 µg/mL MB	6.67	63	$p < 0.005$ (0.004793216)
500 µg/mL MB	7.39	47	

Two-tailed unequal variances Students T-test demonstrating a significant difference between the 300 and 500 µg/mL treatment groups.

Discussion

CRS is one of the most common chronic conditions in the United States affecting an estimated 37 million Americans²⁹ and a significant number of patients with CRS remain resistant to cure despite rigorous treatment regimens including surgery, allergy therapy and prolonged antibiotic therapy.^{3-17,33} One of the major causes of recalcitrant CRS is the polymicrobial biofilm colonization of the sinuses resulting in a local inflammatory response, edema and ultimate infection. Due to the failure of standard therapies to control and cure CRS, other novel non-antibiotic therapies that are able to destroy biofilms and antibiotic resistant bacteria and fungi which contribute to the chronic recurrent nature of CRS are needed.

The present studies demonstrate that MB PDT is highly effective in the photoeradication of pathogenic *Candida* and various antibiotic resistant gram positive and gram negative bacteria that are commonly associated with chronic recurrent sinusitis. Importantly, the in

vitro biofilm studies demonstrate a greater than 6.5 log reduction of antibiotic-resistant multi-specie bacterial biofilms after a single PDT treatment. Using a higher MB concentration and lower light parameters achieved greater than 7 logs of bacteria kill using two PDT light treatments. These results demonstrate that MB PDT results in a significant reduction in antibiotic-resistant multi-specie bacterial biofilms that are commonly found in CRS. These studies therefore indicate that MB PDT may be an effective treatment method for the control or eradication of antibiotic resistant bacteria, fungi and biofilms that are a major contributing cause of CRS. Further studies to evaluate the effectiveness of this non-invasive treatment of CRS are ongoing including a planned human clinical trial.

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Figure 1. Results from treating an antibiotic-resistant multi-specie biofilm with 300 and 500 µg/mL MB based PDT.

Table 1
Photokilling of different microorganisms using methylene blue - mediated photodynamic therapy.

Organism	Methylene Blue (µg/mL)	Power ^a (mW)	Dose Rate (mW/cm ²)	Light Dose (J/cm ²)	Treatment	
					No Light	Light
<i>C. albicans</i> (patient iso.)	100	113	100	40	N/a	2
	400	"	"	"	N/a	0
<i>C. albicans</i> 14053	100	113	100	30	N/a	1 (3)
	400	"	"	"	N/a	0
<i>E. coli</i> 35218	50	113	100	30	N/a	2
	100	"	"	"	N/a	0
<i>E. faecalis</i> 51229	50	113	100	30	N/a	0
<i>H. influenzae</i> (patient iso.)	50	113	100	30	N/a	0
<i>K. pneumoniae</i> 13882	50	113	100	30	N/a	0
<i>P. aeruginosa</i> 27853	100	113	100	40	N/a	4
	300	"	"	"	N/a	0
<i>S. marcescens</i> (patient iso.)	50	113	100	30	N/a	0
<i>S. aureus</i> 29213	50	113	100	30	N/a	1
	100	"	"	"	N/a	0
<i>S. pneumoniae</i> 49619	50	113	100	30	N/a	0

^aWavelength = 664 nm.

^bN/a = Not applicable.

^cQualitative score: 0 = no growth, 1 = 1–5 colonies, 2 = 6–100 colonies, 3 = 101–300 colonies, 4 = 301+ colonies, () = number of colonies.