THE ROLE OF THIOCYNATE IN DEFENSE OF AIRWAY SURFACE AGAINST PSEUDOMONAS AERUGINOSA: DYSFUNCTION IN CYSTRIC FIBROSIS

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A thesis submitted to the faculty of the University of North Carolina at Chapel Hill in partial fulfillment of the requirements for the degree of Master of Science in the Department of oral biology in the School of Dentistry.

Chapel Hill 2014

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ABSTRACT

Yi Yuan: The Role of Thiocyanate in Defense of Airway Surfaces against Pseudomonas aeruginosa: Dysfunction in Cystic Fibrosis
(Under the direction of Roland.R.Arnold)

Objectives: To understand the role of thiocyanate (SCN⁻) in CF airway infection, we will determine potential antimicrobial mechanisms of SCN⁻ on P. aeruginosa and the influence of SCN⁻ on hypochlorite (HClO) -killing (a major microbicidal mechanism of inflammatory cells) on P. aeruginosa; and compare the effects of these antimicrobial reagents on mucoid and non-mucoid strains of P. aeruginosa.

Materials and Methods: The conventional strain PAO1 and its mucoidy mutant (PAM) and a mucoidy clinical isolate (C3873) of P. aeruginosa were included. Target bacteria were exposed to different treatments including H₂O₂, LPO/H₂O₂ and HClO with and without SCN⁻. Three distinct techniques were employed to examine the antimicrobial effects of these treatments including influence on planktonic growth, recovery of colony forming units and inhibition of growth on an agar surface.

Results: SCN with LPO is able to enhance the inhibitory effect of H₂O₂ on P. aeruginosa, due to conversion to the antimicrobial species OSCN. SCN can also rescue the P. aeruginosa from HClO-killing at optimal concentrations. The mucoidy character did not afford protection to P. aeruginosa from inhibition by H₂O₂, HClO or LPO/SCN/H₂O₂ system under either planktonic or biofilm condition.
**Conclusion**: SCN might play a role in airway defense by preventing *P. aeruginosa* infection. Diminished amounts of SCN− resulting from CFTR mutation may contribute to CF lung damage.
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LIST OF ABBREVIATIONS

ASL  Airway surface liquid
ATP  Adenosine triphosphate
CF   Cystic fibrosis
CFTR Cystic fibrosis transmembrane conductance regulator
DUOX Dual oxidase 2
ELF  Epithelial lining fluid
GSH  Reduced Glutathione
HCO$_3^-$ Bicarbonate
H$_2$O$_2$ Hydrogen peroxide
HClO Hypochlorite
LF   Lactoferrin
LPO  Lactoperoxidase
LPS  Lipopolysaccharide
GSSG Oxidized Glutathione
MPO  Myeloperoxidase
NADPH Nicotinamide adenine dinucleotide phosphate
$P. \text{ aeruginosa}$ $Pseudomonas \text{ aeruginosa}$
SCN$^-$ Thiocyanate
ROS  Reactive oxygen specie
CHAPTER 1: A Review of the Literature

Introduction of Cystic fibrosis and Cystic fibrosis lung infection

Cystic fibrosis (CF) is an autosomal recessive genetic disorder that affects 1 in every 2,500 individuals of Caucasian origin, making it one of the most common lethal genetic diseases affecting the Caucasian population in the United States.\(^1\)

This disease is caused by mutations of the gene that encodes the CF transmembrane conductance regulator (CFTR).\(^2\) CFTR is a multidomain integral apical membrane glycoprotein member of the ATP-binding cassette (ABC) that is required to control ion and fluid homeostasis on epithelial surfaces. Dysfunctions in the CFTR epithelial anion channel are fundamental to the etiology of CF, a disease in which all individuals clinically diagnosed have mutations in both CFTR alleles. The absence of this precisely regulated anion channel activity disrupts ionic and water homeostasis on exocrine epithelial surfaces resulting in dehydrated accumulations of macromolecules. CFTR is widely expressed by a variety of epithelial cells and thus multiple organs could be effected by mutations of CFTR, including sweat duct, airway, pancreatic duct and intestine. Different symptoms and signs could be observed from CF patients, such as salty-tasting skin, exocrine pancreatic disease, CF-related diabetes and CF lung infection. Among all the symptoms, lung infection is currently the primary cause of morbidity and mortality in CF.\(^1\)

In normally healthy airway, there are a variety of innate host defense mechanisms operating to maintain the airway in aseptic conditions, where the pathogens inhaled from the
environment can be effectively cleared. However, dysfunction of CFTR could result in airway immune defense defective, such that the patients with CF experience chronic airway infection with severe, improper inflammatory response, which eventually leads to airway obstruction, tissue destruction and failure of respiration. (3)

One of the major reasons for CF lung infections, defined as an inappropriate immune response, is that a large number of neutrophils are recruited into airway. (4) Notably, neutrophils are not the normal immune cells participating in airway host defense system. These cells release granules with numerous proteases, enzymes, and reactive oxygen species, including hydrogen peroxide (H$_2$O$_2$) and hypochlorite (HClO) which could defeat the pathogens in airway. Meanwhile, these released chemicals could also cause unrecoverable deterioration of lung function. (5) For example, a large number of proteases could increase mucus viscosity thereby leading to airway obstruction; the ROS result in airway epithelia injuries thereby exacerbating pulmonary deterioration for CF patients. Therefore, controlling of lung inflammation is considered as a promising therapeutic target in CF area. (6)

On the other hand, the unique pathogens profile in CF lung infection has drawn a lot of attention in CF research. While most of immune compromise diseases predispose to infections with a variety of pathogens, very limited species of pathogens can be identified in CF lung infections, including *Staphylococcus aureus*, *Haemophilus influenza*, *P. aeruginosa* and *Stenotrophomonas maltophilia*. (1) Notably, the pathogens in the CF lung are usually acquired in an age-dependent tendency. *S. aureus* and *H. influenza* are the most common organisms at the early stage of CF lung infection. Along with the progress of CF lung disease, *P. aeruginosa* becomes the prominent pathogen, infecting approximately 80% of the CF patients and its
conversion to a mucoidy phenotype is ultimately the primary cause of morbidity and mortality in CF. (7)

All the evidences shared in previous paragraphs suggest that a CF airway is capable of forming a very unique environment such that specific pathogens are selected to adapt to such an environment. As CFTR serves as ion channels onto epithelial cells, it has been hypothesized that the CFTR mutations cause defects in the concentrations of select ions on airway epithelial cells, and thereby lead to dehydrated airway surface layer and thickened mucus in CF lung. (8) These changes could significantly weaken the ciliary clearance of pathogens in the airways, which is an important physical barrier for maintaining the airway sterility. The failure of ciliary clearance directly leads to lung infection in CF patients. (9) However, this abnormal physiology fails to provide an explanation as to why P. aeruginosa is the predominant pathogen in CF as ciliary clearance would not be pathogen specific. This means other pathogens should also take advantage of this defective host defense. Therefore, further studies concerning the link between CFTR mutation and the predisposition of the CF lung to P. aeruginosa infection is still needed.

**Defective ions transport in CF airway**

Although CFTR was originally identified as a chloride channel, growing evidence suggest that CFTR is capable of directly or indirectly regulating the transportation of other anions, including bicarbonate (HCO$_3^-$), reduced glutathione (GSH) and thiocyanate (SCN$^-$). It has been reported that various exocrine secretions of the CF patients are deficient in GSH, HCO$_3^-$, SCN$^-$ and NO, which play a role in host immune system. For example, HCO$_3^-$ has been shown to be a critical determinant of the nature of iron binding by lactoferrin, (6) a potent antimicrobial protein of exocrine secretions and the polymorphonuclear neutrophilic leukocyte, and determines the iron scavenging (host protective) vs. bactericidal activities of LF. (10) GSH is utilized by
epithelial cells to control oxidative damage from inflammatory response, due to its potential antioxidant role that can consume reactive oxygen species from inflammatory reaction.(11) Studies showed that decreasing GSH leads to increased oxidative stress, and further alters the IL-8 gene expressions and leads to defects in bacteria killing in airway.(12) SCN− is another purported antioxidant in human body that participates in diminishing of oxidative stress from inflammation.(13) More importantly, it can serve with H2O2 as the substrate of peroxidases, including lactoperoxidase (LPO) and myeloperoxidase (MPO) to generate the antimicrobial molecule hypothiocyanite (OSCN−). (14) It also reacts non-enzymatically to convert OCl− to OSCN− serving the dual purpose of host protection and more specific antimicrobial activity.

Therefore, the overall hypothesis of our studies is that CFTR mutation interrupts the critical balance of ionic composition on the airway surface. This disruption of ionic homeostasis in the CF airway results in a unique environment that can be utilized by P. aeruginosa to colonize and thrive. The overall purpose of our studies is to delineate the potential roles and mechanisms of action of these ions in airway innate immunity, and reveal the relationship between defective of ions and the selective susceptibility of the CF lung to P. aeruginosa infection.

Introduction of Thiocyanate and its role in immune system

Thiocyanate (SCN−) is a strongly acidic pseudohalide thiolate that can reach millimolar concentrations in biological fluids, include saliva, milk, airway epithelial lining fluid (ELF) and nasal lining fluid. The original sources of SCN− in the body are from diet, but it is unclear whether SCN can be synthesized from an endogenous source of cyanide. (15) The vast majority of SCN in the human body concentrates in the secretions of exocrine glands that bathe mucosal surfaces. For example, saliva contains the highest concentration (500 –
3000µM) of SCN; nasal airway fluid has been reported to range from 100-1200µM SCN. The samples from airway epithelial lining fluid are reported to range from 30µM to 650µM. (15)

Plasma values of SCN range between 5 and 50µM, and this range is as low as 0.1-4µM in milk. It has been suggested that the higher concentrations of SCN in saliva result in more potent antimicrobial defenses required as the oral cavity is exposed directly to a hostile environment (16)

In 1962, an anti-bacterial agent was reported in saliva with activity against Lactobacillus casei (17) that was dependent on SCN. This report described that SCN can be oxidized by H₂O₂, a reaction catalyzed by the peroxidase enzyme in human body, and was responsible for antimicrobial activities in saliva. The product was later identified as hypothiocyanite (OSCN⁻). Since then, the antibacterial properties of the peroxidase/SCN/H₂O₂ have been identified in ELF, NLF, milk, and tears, and are presumed to play an important role in host immune defense at mucosal surfaces. (15)

The mechanism for HOSCN inhibition of bacteria is due to its capability of selective reaction with sulfhydryl groups, resulting in the oxidation of proteins to sulfenylthiocyanates (RS-SCN). When bacteria are exposed to HOSCN, the enzymes containing essential thiol groups can be inactivated due to oxidation by OSCN⁻. Since enzymes are critical for glycolysis metabolism in microbes, the growth of bacteria will be interrupted due to inactive metabolism when exposed to OSCN. (18) On the other hand, it has been reported that after treatment with OSCN, the growth of bacteria can be recovered by removing residual OSCN through washing procedures and the restoration of the essential sulfhydryls with reducing agents such as mercaptoethanol and dithiothreitol, which suggested that the inhibitory effects of OSCN are not bactericidal, and the inhibitory effect is reversible. (19)
Most of the early research on the SCN dependent peroxidase system focused on saliva and the regulation of the metabolism of the cariogenic bacteria that colonize the tooth surface. (20) Studies indicated that the concentrations of SCN and LPO in saliva were such that H$_2$O$_2$ levels were rate limiting and that the bacteria in the oral cavity produced sufficient incidental H$_2$O$_2$ during their metabolism to engage the LPO system. (21) In addition, reports showed that the combination of LPO/SCN/H$_2$O$_2$ has the capability of inhibiting oral yeast such as *Candida albicans* (22) and virus, e.g. *Herpes simplex* virus. (23) When the importance of LPO in airway bacterial clearance was first reported by Gerson *et al.* in 1999, the potential contributions of SCN in the defense of airway surfaces gained attention. Gerson *et al.* identified the LPO/SCN/H$_2$O$_2$ system in sheep airways that inhibited bacteria, and inhibition resulting from LPO could significantly impair bacterial clearance in the airways. (24) Subsequently, CFTR was reported to be responsible for transportation of SCN, and the consequent decrease in SCN concentrations in the CF airway could lead to poor bacterial clearance and eventually CF lung infections. (25)

These observations suggested that SCN could be a potential therapeutic agent for treatment of CF lung infection. However, the mechanism of inhibitory effect of LPO/SCN/H$_2$O$_2$ system on CF pathogens is still lacking. In contrast to the studies with oral pathogens, more questions remain regarding the mechanisms of the antimicrobial actions of the LPO/SCN/H$_2$O$_2$ system on potential airway pathogens. For example, most of the studies have only exposed bacteria with LPO/SCN/H$_2$O$_2$ treatment in the non-growth conditions of nutrient poor buffers. (13) In contrast, our studies focused on defining the antimicrobial effects under bacterial growth conditions. Another important question was whether the antibacterial effect of LPO/SCN/H$_2$O$_2$ system could be influenced by other molecules present in the environment of airways. As discussed in previous sections, different ions can be identified in airway surface that might
cooperate with or antagonize potential host defense factors, such that maintainence of a healthy airway may depend on the balance of different ionic components instead of one single component.

Besides the antibacterial role of SCN, the antioxidant properties of SCN might also play important roles in the maintenance of a healthy airway environment. The antioxidant properties allow SCN reaction with ROS and reactive nitrogen species (RNS) resulting from the inflammatory response in airways. SCN would also compete with chloride for peroxidase catalyzed reaction with H$_2$O$_2$, redirecting from the more host destructive HOCl to the more microbe specific HOSCN. Such reactions are capable of dampening oxidative stress in the lungs and thereby reducing the damage to lung epithelial cells. Although the mechanistic connection of abnormal CFTR function to lung infection has not been fully elucidated, the severe progressive inflammation aroused by lung infection directly leads to the failure of airway functions for CF patients. One important tissue damaging factor identified in lung inflammation in CF patients is the ROS generated by neutrophils in the CF airway.\(^3\) It has been proposed that SCN could limit the accumulation of tissue-damaging ROS such as H$_2$O$_2$ and hypochlorite (OCl-) in several ways.\(^{14}\) First, SCN can rapidly react with HClO to generate OSCN$^-$ and Cl$^-$ in a non-catalytic reaction. Second, SCN can compete with Cl$^-$ for the myeloperoxidase (MPO) catalyzed oxidation by H$_2$O$_2$ produced by neutrophils, and thus reduce the production of cytotoxic molecules, e.g. HClO in favor of the more microbe specific, OSCN$^-$.\(^{26}\) Third, lactoperoxidase (LPO) in the airway catalyzes the oxidation of SCN to OSCN, but in contrast to myeloperoxidase is enzymatically unable to use Cl$^-$. Thus this SCN dependent enzyme system consumes H$_2$O$_2$ limiting its availability for generating more host destructive species.\(^{24}\) Based on these mechanisms, SCN has been proposed as a promising reagent to protect host cell from
inflammatory damage in CF lung infections. However, the potentially protective impact of the antioxidant role of SCN on the CF lung pathogen, *P. aeruginosa* has never been systematically studied. Since antibacterial functions serve as the primary function of ROS in the inflammatory response, it is important to understand both the positive and negative impacts of antioxidant properties of SCN. Therefore, we studied whether *P.aeruginosa* can be protected from the bactericidal activities of the ROS HClO or H$_2$O$_2$ by adding SCN or perhaps redirected to the more bacteria specific OSCN.

In the present study, we examined the effects of SCN towards *P. aeruginosa*. The effects included growth inhibition from the LPO/SCN/H$_2$O$_2$ system, as well as the antioxidant role of SCN that influences bacterial killing activity of HClO. Through this part of the study, we establish a better understanding of the role of SCN serving as an important part in the host defense in the normally health airway, and how the defective SCN transport associated with CFTR mutation is related to the selective increase in susceptibility to *P. aeruginosa* infection in the CF airway.

**The adaption of P. aeruginosa in CF lung infection**

Pursuant to establishing the link between defective CFTR function and chronic *P.aeruginosa* infection in the CF airway, it is important not only to understand potential defects in innate immunity, but it is also important to understand the characteristics and particularities of the CF lung pathogens.

Although multiple microbial species are able to colonize in the CF airway, it has been widely accepted that the infection of *P. aeruginosa* is one of the most clinically important CF pathogens accounting for the vast majority of morbidity and mortality associated with this disease. The prevalence in the CF respiratory tract cultures of *P. aeruginosa* is in the range of
10%-30% by the age of five years, but the incidence rate sharply increases to 80% after the age of 18. Early acquisition of this pathogens is an indicator for worse prognosis. Detection of *P. aeruginosa* is highly associated with the rate of deterioration of lung function for CF patients. 

(27)

An important characteristic of *P. aeruginosa* that likely contributes to its successful colonization in CF lung is high degree genetic flexibility, which allows active adaptation to evolving environments especially with the pressures imposed by the various elements of the mucosal immune system. Studies have shown that even single clones of *P. aeruginosa* initially isolated from a CF lung could develop clones with numerous different gene expression profiles, suggesting that this pathogen could undergo a series of genetic mutations along with the progression of the disease.(7) This characteristic of *P. aeruginosa* suggests that there are selective pressures for optimization of the clonal types that contribute to the disease process that defines characteristic CF-associated pathology. Colonies evade immune clearance and adapt to the environment of high inflammatory stress and eventually thrive in the CF lung.

At the early stage of infection, *P. aeruginosa* isolates from CF lung present a phenotype that is non-mucoid, smooth (complete outer membrane lipopolysaccharide (LPS)), motile and relatively susceptible to antibiotics. Such phenotypes indicate that this pathogen is acquired from the environment. However, the strains isolated from chronically infected CF patients have alterations in numerous virulence factors. For example, the chronic *P. aeruginosa* CF isolates show a rough colony phenotype, which is caused by the loss of the O-side chain of LPS that is generally associated with an increase in permeability of the outer membrane and an increased susceptibility to hydrophobic molecules. (28) CF chronic *P. aeruginosa* also have lost the ability to express flagella and consequently are non-motile. The gene lasR that plays an important role
in the quorum sensing system is also inactive due to mutation in chronic *P. aeruginosa*. (29) In addition to these alterations in potential virulence factors, the metabolism of *P. aeruginosa* also undergoes a series of alterations in order to acquire nutrients for high-density growth in the CF lung. Studies have shown that *P. aeruginosa* prefers to catabolize amino acids over other carbon sources like lactate and glucose. Moreover, under the hypoxic and anaerobic conditions presumably created by chronic oxygen consumption from the excessive inflammation in the airway and possibly by limited oxygen diffusion through the alginate extracellular matrices of mucoidy phenotypes, *P. aeruginosa* is able to utilize NO$_3$/NO$_2$ as alternative electron acceptors to oxygen for respiration or can use limited fermentation of arginine as an energy source. (30)

**The conversion to mucoidy phenotype of *P. aeruginosa* in CF lung infection;**

Among all those adaptions, it is generally accepted that the adaptation to the mucoidy phenotype is the most uniquely characteristic and significant virulence determinant of chronic CF airway disease. (31) Mucoidy is the result of overproduction of the anionic exopolysaccharide alginate, a polymer of D-mannuronic and L-guluronic acid. Studies found that the expression of genes accounting for the over expression of alginate, such as MucA, MucB and MucC, has been upregulated in mucoid strains. It is believed that there is a spontaneous selection of these mutants during the process of CF lung disease, and thereby the vast majority of *P. aeruginosa* isolates from late stage of CF lung infection characteristically are of the mucoidy phenotype. After switching to the mucoidy phenotype, *P. aeruginosa* is considered to be impossible to clear by the immune system. (32) Thus, detection of mucoidy *P. aeruginosa* is considered to be associated with poor prognosis and increased lung tissue damage. In the clinic, the conversion to
mucoidy becomes a well-established indicator of failed innate immunity and chronic infection in CF lung.

It is believed that the hyperproduction of alginate promotes the persistence of *P. aeruginosa* in the CF lung, for its function as protection on the surface of *P. aeruginosa* against the environmental stresses in the CF lung. The studies indeed have shown that, when non-mucoid strains are treated with low levels of H$_2$O$_2$ or exposed to active PMNs *in vitro*, the formation of mucoid variants can be observed. (29) In addition, another study applied a mouse lung-infection model to show that anaerobiosis and lung infection can rapidly induce alginate production from non-mucoid *P. aeruginosa*. (33) However, whether the mucoid phenotype protects *P. aeruginosa* from ROS in the inflammatory environment still lacks clear evidence. In our study, we were therefore also interested in the how the LPO/SCN/H$_2$O$_2$ system influences the mucoid *P. aeruginosa*. First of all, it is unknown whether alginate production could protect a mucoid strain from the inhibitory effects of OSCN. Second, since antimicrobial function of OSCN was speculated to target the metabolism of glycolysis the production of alginate requires the synthesis of the alginate precursor guanosine diphosphate (GDP)-mannuronic acid. (18) Thus, we need to address the question concerning whether LPO/SCN/H$_2$O$_2$ system could suppress the formation of mucoidy in *P. aeruginosa*, and thereby can be applied to control the late stage of the chronic CF lung infection.

Therefore, we will investigate the effects of ROS on the mucoid strains of *P. aeruginosa* and compare the results with the non-mucoid *P. aeruginosa*. Two mucoid strains were selected in this study. One is a laboratory derived mucoid mutation of PAO1. The other one is a clinical isolate from the sputum of a CF patient.

**Statements of purpose, hypothesis and specific Aims:**

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This project focused on the role of one of the ions purported to be regulated by CFTR, thiocyanate (SCN) and reported to be deficient in the CF airway. This molecule has been suggested to contribute airway immune defense by reacting with H₂O₂ under catalysis by LPO to generate antimicrobial species. Moreover, it is an important antioxidant in airway that can limit oxidative stress in the airway from inflammatory response. As the SCN transportion is down-regulated by dysfunction of CFTR, it was an aim of these studies to understand whether the reduction of SCN in CF airway surface liquid could play a role in susceptibility to *P. aeruginosa* infection that finally leads to the destruction of the airway apparatus.

Three specific aims were developed to test the hypothesis that SCN plays an important role in preventing *P. aeruginosa* from establishing infection in normally healthy airway.

- **Aim 1: to determine potential antimicrobial mechanisms of SCN on *P. aeruginosa***
  If SCN contributes to the resistance of the healthy airway to *P. aeruginosa* infection, the potential antimicrobial role of SCN may be important in the early stage of CF airway infection.
  We will study the influence of SCN on the antimicrobial effects of H₂O₂ on *P. aeruginosa*.
  Moreover, spiral plating assay will be applied to understand the mechanisms of antimicrobial effects of the LPO/SCN/H₂O₂ system. Lastly we will study the influence of GSH, another important antioxidant co-exists with SCN in the healthy airway, on LPO/SCN/H₂O₂ system.

- **Aim2: To determine the influence of SCN on hypochlorite-killing of *P. aeruginosa***.
  After the initial infection of *P. aeruginosa* in CF airway, large numbers of neutrophils are recruited and release ROS against pathogens, which also directly cause host cell damage. To understand the influence of SCN on HClO, a ROS species from neutrophils is also provide more information about the role of SCN in inflammatory stage of CF lung infection. In this
experiment, *P. aeruginosa* were exposed to various concentration of HClO, and various concentrations of SCN were added to determine the influence of SCN on HClO-killing.

- **Aim3: To compare the effect of antimicrobial reagents on mucoid vs. non-mucoid strains of *P. aeruginosa***

*P. aeruginosa* generates large quantities of alginate to switch to mucoid phenotype through the progression of CF lung infection. It is proposed that the conversion to mucoidy of *P. aeruginosa* results in protection of *P. aeruginosa* from the host immune elements. Comparison of the differential susceptibilities of mucoidy and non-mucoidy strains to ROS and LPO/SCN/H2O2 system is important to gain insight into the role of SCN in the late stage of the CF lung infection. Two mucoidy strains of *P. aeruginosa* - one from mutation of PAO1, the other a CF patient isolate – will be compared with a laboratory standard smooth, non-mucoid strain. All strains will be tested for relative susceptibilities to H2O2, HClO, and LPO/SCN/H2O2 system.
CHAPTER 2: The Role of Thiocyanate in Defense of Airway Surfaces against Pseudomonas aeruginosa: Dysfunction in Cystic Fibrosis

Materials and Methods

Bacterial and reagents

PAO1: ATCC BAA47 was acquired from the American Type Culture Collection, Rockville, MD., SCN was purchased from Mallinckrodt Chemical Works (St. Louis. MO), LPO, GSH and Catalase were purchased from Sigma (St. Louis. MO), H₂O₂ was purchased from Mallinckrodt Baker. (Phillipsburg, NJ), HClO was purchased from Clorox (Oakland, CA). TSB and TSA were purchased from Becton Dickinson (Sparks, MD)

Microbial growth and storage.

Trypticase soy broth (TSB) media (3.7mL) were inoculated with the test pseudomonas strains and cultured overnight at 37°C. The following day, the cultures were streaked onto trypticase soy agar (TSA) plates for colony isolation. New cultures were inoculated from single colonies and grown overnight at 37°C. Aliquots of pure cultures were preserved by freezing a mixture of 0.1ml of bacterial culture and 0.5ml of skim milk at –80°C.

Growth curves of bacterial cultures were inoculated with ten-fold dilutions.

Bacterial cultures from isolation plates were grown overnight in TSB at 37°C. On the day of experiments, the cultures were diluted to a MacFarland standard 0.5 (~ 10⁸ CFU/mL). An aliquot of 0.1ml of the diluted bacterial culture was further diluted into 0.9ml volumes of TSB to affect a serial 10-fold dilution.
Replicate aliquots of 20µl of each dilution of the bacterial suspension were added to sterile 96 well microtiter wells as a starting inoculum, 180µl of TSB were added to each well. The microtiter plate was placed in the kinetic spectrophotometer. The temperature of the chamber inside the spectrophotometer was set constant at 37°C. Each test lasted for 24 hours, and the machine automatically measured the optical density at λ610nm every 30 min after shaking for 15 sec. The growth curves were captured by the curve fitting software Microsoft and the time required to reach an OD value of 0.2 was determined for each growth as the lag time to onset of exponential growth.

**Antimicrobial effects of H₂O₂ and LPO/SCN/H₂O₂ system**

For H₂O₂ and HClO killing, microbes were suspended to 10⁶ CFU/mL in TSB media. Killing reactions were carried out in duplicate wells of 96-well microtiter plates containing various concentrations of H₂O₂ or HClO in a volume of 200µl of TSB. Then 20µl aliquots of density adjusted bacterial suspension were added to each well of the microtiter plate. The microtiter plates and placed in spectrophotometer (SpectraMax2, Molecular Devices). The temperature of the chamber inside the spectrophotometer was maintained at 37°C. The level of inhibitory effect of each treatment is determined by onset time to exponential growth of the growth curves for each sample. The onset time is correlated with number of recoverable colony forming units for each condition.

For measuring antimicrobial effect of the LPO/SCN/H₂O₂ system, LPO powder (Sigma, St. Louis. MO) was diluted into sterile 150mM saline to prepare the working solution (10U/ml), and 10µl of this LPO solution were added to the appropriate wells of the 96-well microtiter plates, followed by 20µl of bacterial suspension. SCN was diluted into TSB media to 2mM, and various concentrations of H₂O₂ were achieved by dilution into TSB media containing 2mM SCN.
Aliquots of 200µl of H₂O₂ dilutions with 2mM SCN were then added to each test well of the microtiter plates. The inhibitory effects were determined as delays in onset time to exponential growth for each test sample and control.

**Spiral plating of microbe and measurement of colonies**

Target bacteria were adjusted using MacFarland standard to achieve a suspension of ~10⁵ CFU/mL in TSB media. H₂O₂ was serially diluted in TSB containing 2mM SCN. In the group of LPO/SCN/H₂O₂ treatment, 200µl of LPO working solution was added into tubes first, followed by the bacteria suspension. 2ml volumes of the serial dilutions of H₂O₂ were added into the mixture last. In the group of H₂O₂ treatment only, 2ml volumes of H₂O₂ dilutions were added to 200µl bacterial suspensions. 200µl bacterial suspensions were mixed with 2ml volumes of TSB as negative controls. All the mixtures were incubated at 37°C in ambient atmosphere. After 0, 2, 4, and 6 hours, 49.2µl of the mixture was spirally plated onto TSA plates using spiral plater (Spiral Systems Model DU, Microbiology International). All plates were incubated at 37°C overnight either in an ambient atmosphere or in 5% CO₂, after which the total colony forming units (CFU)/ml were counted using an automated colony counter, Protocol 3 (Microbiology International), with software designed for the purpose.

**Disk diffusion assay of effect of bacteria growth.**

Volumes of 100µl of the test bacteria suspension containing 10⁸ CFU/ml of bacteria cells were spread confluent on the surface of TSA plates of each strain by sterile cotton swaps. Blank antibiotic sensitivity paper discs (6 mm in diameter) were evenly spaced on the inoculated surface of the plates and titrated concentrations of the test reagents (7ul) were added aseptically with a micropipette to each disc. The prepared plates were incubated either in an ambient atmosphere or in 5% CO₂ at 37°C for overnight. The diameters of any zones of growth inhibition
resulting in the bacterial lawn were measured in millimeters (including disc diameter 6 mm). The experiments were performed in triplicate.
Results

Measurements of bacteria density by growth curve:

In order to determine the antimicrobial effects of the various agents on actively growing *P. aeruginosa*, we developed a method to determine the influence of a reduction in colony forming units on bacterial growth in broth cultures. The principle of the method is shown in Fig. 1. In order to establish a standard curve, the starting bacterial density was adjusted to $10^8$ CFU/mL using comparison to MacFarland standards and serial ten-fold dilutions of this bacterial suspension was used to inoculate replicate wells of a 96-well microtiter plate. The growth curve kinetics at $37^\circ$C was monitored by measuring the change in the optical density at $\lambda_{610}$ nm automatically every 30 minutes after 15 seconds of shaking. As shown in Fig. 1(b), titration of the bacterial inoculum number resulted in a prolongation in the lag phase, but did not influence the generation time, the length of time in exponential growth or the maximum bacterial density achieved. With a theoretical inoculum size of $10^0$ CFU/ml, the bacteria were able to reach stationary phase within 24h. The lag phase ends with transition to exponential growth. The size of the starting inoculum was directly proportional to the time it takes to enter log phase growth and this time to exponential growth can be uniformly captured by determining onset time to an $OD_{610}$ of 0.2 (Fig. 1(b)). Linear regression was performed between the starting inoculum size (CFU/ml) and the time to achieve this threshold OD value, i.e. the onset time. As shown in Fig. 1(c), the correlation is highly linear with a correlation coefficient of 0.9943.

The high linearity and correlation shown in Fig. 1(c) permitted the establishment of standard curves for each of the target bacteria to allow estimates of residual CFU/ml resulting from the experimental treatment protocols.
**SCN did not influence growth of bacteria by itself:**

In order to study the growth of PAO1 under the influence of LPO/SCN/H$_2$O$_2$ system, we needed to first investigate if SCN alone has any effect on the growth character of *P. aeruginosa*. A titration of concentrations (0.75 - 40mM) of SCN was added to replicate wells of the culture media prior to inoculation with PAO1. As shown in Fig. 2, after overnight growth, there were no discernible differences in the growth kinetics or onset times with concentrations of SCN to 40mM. The data from this set of experiment indicate that SCN alone does not influence *P. aeruginosa* growth.

**Inhibitory effect of H$_2$O$_2$ on PAO1 is influence by bacteria density.**

As H$_2$O$_2$ is the defining substrate of the peroxidase system, in order to understand effect of LPO/SCN/H2O2 system on growth of bacteria, we investigated the influence of H$_2$O$_2$ alone on PAO1 growth. A titration of concentrations (0.10mM to 110mM) of H$_2$O$_2$ was tested against a starting inoculum of $10^7$ CFU/ml of PAO1. The data (fig. 3A) indicated that the presence of H$_2$O$_2$ in the culture media resulted in a dose dependent delay in onset time and a standard curve estimated reduction in CFU of 100 fold with a concentration of H$_2$O$_2$ of 64mM. When the initial target density of PAO1 was reduced to $10^5$ CFU/ml as a starting inoculum, there was total inhibition with H$_2$O$_2$ concentrations as low as 1.7 mM. *P. aeruginosa* produces the hydrogen peroxide-specific enzyme catalase that should afford a level of protection against exogenous H$_2$O$_2$. In order for H$_2$O$_2$ to inhibit *P. aeruginosa*, the concentration of the H$_2$O$_2$ must exceed the enzymatic activity of the the bacterial catalase. As the target density is increased, the activity of the bacterial catalase would increase reflecting an increased resistance as the population increases. This would suggest that *P. aeruginosa* would be relatively more resistant to H$_2$O$_2$
when compared to the susceptibility of a species (S. mutans) that does not make catalase. Furthermore there should not be the same effect on susceptibility by increasing target density if a ROS agent that is not a substrate of catalase (HClO) is tested for antimicrobial activity. To prove this assumption, the same two densities of P. aeruginosa were treated with HClO. No influence from bacteria density on HClO inhibition was observed. (Fig. 3b) As further evidence that the target density influence on susceptibility to H₂O₂ of P. aeruginosa is due to catalase, the bacterial density of S. mutans did not have demonstrable influence on susceptibilities to treatments with either H₂O₂ or HClO. (Figure 4c, d).

The enhanced antimicrobial activity of H₂O₂ is dependent on both LPO and SCN.

With the understanding of effect of H₂O₂ and SCN alone on PAO1 growth, we also tested how LPO/SCN/H₂O₂ system influences PAO1. First, with titrated concentrations of H₂O₂ in the culture media of PAO1, complete inhibition can be observed when 32mM or greater H₂O₂ was added. The addition of either SCN (2mM) or LPO (10U/ml) to H₂O₂ concentrations less than 32mM resulted in no additional antimicrobial activity. However, when LPO was added to the combination of SCN and H₂O₂ (6.88mM), growth was completely inhibited. This observation provides evidence that LPO/SCN/H₂O₂ system presents an inhibitory effect on PAO1 growth and can amplify the antimicrobial activity of H₂O₂ alone.

Dose dependence of H₂O₂ /LPO for growth inhibition of PAO1:

The key point of this study is to determine whether the product resulting from the LPO/SCN/H₂O₂ system is more effective than H₂O₂ alone. We thereby investigated whether this inhibitory effect of LPO/SCN/H₂O₂ system is dose-dependent on LPO concentration. In this experiment, gradient concentrations of LPO (from 10 to 0.3125U/ml) and H₂O₂ (from 6.88 to 0.78mM) were added to TSB plus 2mM SCN and 10⁶ CFU/mL of PAO1. As shown in Figure 5,
the level of inhibition was dependent on both the activity of the LPO and the concentration of H$_2$O$_2$. This observation was consistent with the generation of a new antimicrobial species, most likely OSCN, to which PAO1 was more susceptible.

**The influence of bacteria density on antimicrobial effect of LPO/SCN/H$_2$O$_2$:**

It is known that due to the catalase on the cell surface of *P. aeruginosa*, the inhibitory effect of H$_2$O$_2$ can be weaker in higher density of bacteria. To further investigate the effect of LPO/SCN/H$_2$O$_2$ system, we design the experiment to measure whether the inhibitory effect of OSCN can also be influence by density of *P. aeruginosa*.

As showed in Figure 7A, two inoculum densities of PAO1 (10$^7$ CFU/mL and 10$^6$ CFU/mL) were treated in the presence of 2mM SCN in microtiter format with H$_2$O$_2$ in two-fold dilutions added vertically, and the LPO in two-fold dilutions added laterally as a checkerboard. In the samples with lower density of *P. aeruginosa*, only H$_2$O$_2$ in 6.88mM and 3.44mM concentrations totally inhibited growth of bacteria. In the rest of the groups, we can observe that inhibitory tendency corresponded with concentration of LPO and H$_2$O$_2$, which indicated that the level of inhibition of *P. aeruginosa* is based on the concentration of OSCN generated from enzymatic oxidation of SCN by H$_2$O$_2$.

With both 1.72 mM H$_2$O$_2$ and 0.86mM H$_2$O$_2$, the same inhibitory activity can be achieved with either of the two target densities of *P. aeruginosa*. (Fig 7C) Therefore, unlike with H$_2$O$_2$ in the absence of LPO, target density did not influence the inhibitory effect of LPO/SCN/H$_2$O$_2$ system.

**Effects of treatment with the LPO/SCN/H$_2$O$_2$ system on *P. aeruginosa* on recoverable CFU/ml and colony morphology.**
The data reported in the previous sections proved antimicrobial effect of LPO/SCN/H\textsubscript{2}O\textsubscript{2} system on the CF lung pathogen, i.e. \textit{P. aeruginosa}. This inhibition was interpreted as a reduction in colony forming units resulting in an increased lag phase before detection of exponential growth as captured by onset time. Previous studies with other target bacteria indicated that OSCN reacted with targets on the bacterial surface through oxidation of essential sulfhydryls. This inhibition was to a point reversible if the modified targets could be reduced to restore function. To acquire more insight into the nature of the inhibition of the LPO system on \textit{P. aeruginosa}, the treated bacterial suspensions were sampled during the treatment period and plated to TSA to determine the number and nature of the actual recoverable colony forming units of \textit{P. aeruginosa} kinetically.

Two groups of treatments, LPO/SCN/H\textsubscript{2}O\textsubscript{2} and H\textsubscript{2}O\textsubscript{2} alone, were selected and non-treated bacteria suspensions were used as control groups. The \textit{P. aeruginosa} suspension with a concentration of 10\textsuperscript{6} CFU/mL were cultured and divided into three groups. At the time points of 0, 2, 4 and 6 hours, aliquots from each treatment group were spiral plated onto tryptic soy agar (TSA). After overnight growth at 37°C in ambient atmosphere, colonies were examined for their appearance and the quantitation of recoverable CFU/ml. The appearance and colony numbers of the sample treated by LPO/SCN/H\textsubscript{2}O\textsubscript{2} and H\textsubscript{2}O\textsubscript{2} were compared with that of the control group such that the bacterial killing and any influence on colony growth could be determined. Moreover, two different H\textsubscript{2}O\textsubscript{2} concentrations in LPO/SCN/H\textsubscript{2}O\textsubscript{2} system were tested. In the previous experiments that measured bacterial growth kinetics, the bacteria remained exposed to the antimicrobial agents throughout the analyses. In contrast, in these experiments the treatment mixture was sampled through the progression of early incubation and plated to the surface of media that did not contain antimicrobial agents. This transfer to fresh media effectively dilutes
the antimicrobial agents. Further dilution is also accomplished by the diminishing volume of the test reaction created by the spiral plating technique. In effect this approach stops the action of the agent to determine its effect on the bacterial population at the point of plating.

As shown in Figure 7(A), the sample containing only 3.44mM H$_2$O$_2$ showed no significant inhibition of bacteria growth. When the concentration of H$_2$O$_2$ is increased to 6.88mM, a one-log reduction in recoverable CFU was observed. On the other hand, the sample containing 10 U/mL of LPO, 2mM of SCN and 3.44mM H$_2$O$_2$ resulted in a two-log reduction. If the concentration of H$_2$O$_2$ is increased to 6.88mM with the same dosages of LPO and SCN, bacteria growth is entirely inhibited.

With the higher concentration of H$_2$O$_2$ (6.88mM), pictures taken from representative plates in different time point are provided in figure 7C. After two hours treatment with 6.88mM of H$_2$O$_2$, 10U/ml of LPO and 2 mM of SCN, the colonies showed a smaller size than colonies in the control group. The quantified result shown in figure 7B, where the number of bacteria with LPO/SCN/H$_2$O$_2$ group indicated a three-log reduction compared to controls. On the contrary, with 6.88mM H$_2$O$_2$ treatment there were no differences in colony size or number observed. After four hours of treatment with LPO/H$_2$O$_2$/SCN, very few colonies were recoverable. Moreover, no colonies were recovered after 6 hour in this group, suggesting that complete killing by LPO/SCN/ H$_2$O$_2$ occurs around the fourth hour. Meanwhile, at the time points of 2, 4, and 6 hours, both number and size of colonies treated with H$_2$O$_2$ in the absence of LPO did not exhibit significant difference compared to the control.

As shown in Fig. 7C, at the lower concentration of H$_2$O$_2$ (3.44 mM), H$_2$O$_2$ in the absence of LPO did also not present any discernible effect on the bacteria, for both colony characteristics and number. However, as shown in Fig. 7F, if the bacteria were treated with an equivalent
concentration of H₂O₂ in the presence of LPO and SCN for 2 hours, both small and normal size colonies were recovered on the plate. (Fig. 7F). Regarding the effect on the number of bacteria, the quantified results are provided in Fig. 7E. While the small colonies were recoverable at 10⁶ CFU/mL, the normal size colonies were present at 10⁵ CFU/mL at the two hour time point. Here the number of total recoverable CFU despite their small size are not significantly reduced compared to that of the control. After 4 hours of treatment, however small colonies cannot be found on the plate, but the normal size colonies are still recoverable at 10⁵ CFU/mL. After six hours, the number of normal size colonies increased more than ten-fold compared with that recovered at four hours. There were no small colonies in evidence.

Based on the observations in this set of experiments, we deduce that the small colonies observed in the group with LPO/SCN/H₂O₂ treatment are due to a delay in the initiation of bacterial growth. Based on the understanding of OSCN mechanism of inhibition, the treatment may interrupt the metabolism of bacterial cell, which could further inhibit the growth of bacteria. However, when the bacteria are exposed to OSCN alone for 2 hours, the inhibition is reversible as the cells are capable of recoverable colonies. In clinic, the small-colony variant of P. aeruginosa has also been observed in respiratory tract specimens from patients with CF. (34) Later, it has been identified that pel and psl gene clusters and elevated intracellular cyclic di-GMP (c-di-GMP) levels contribute to small-colony morphotype of P. aeruginosa. (35) To verify whether small colonies in the treatment of LPO/SCN/H₂O₂ is also due to inducing gene mutation, small colonies were transferred onto another TSA plate. After overnight culture, all small colonies were recovered as normal size (data not show), suggesting that the small colonies observed with treatment by the LPO/SCN/H₂O₂ system are not via inducing gene mutation.
After four hours of inhibition of metabolism, the bacteria may die (failed to recover) as a result of starvation. On the other hand, the observations of normal and small colonies with the LPO system with lower concentration of H_{2}O_{2} suggest partial inhibition from the LPO system suggesting insufficient OSCN is generated to target all the bacterial cells. While bacterial cells are attacked by OSCN and eventually die after 4 hour of treatment, a portion of the bacteria escape the antimicrobial effect of OSCN and are capable of normal growth.

**Blocking by Catalase or GSH of the inhibitory effects of LPO/SCN/H_{2}O_{2} system.**

Glutathione (GSH) is another important antioxidant in ELF in lungs besides SCN. The transportation into extracellular of GSH has been shown to directly involve CFTR, and pronounced depletion in ELF of lung has been observed in CF patients. In the normally healthy airway, GSH is present in high concentrations in the epithelial lining fluid. Together with other ions in ELF on the surface of epithelial cell, it is presumed to play an important role in host immune defense in the lung. Due to its antioxidant role, that can reduce amount of oxidant like H_{2}O_{2}, we were interested in its influence on the effect of LPO/SCN/H_{2}O_{2} on CF pathogens.

In this set of experiments, two combinations of LPO/SCN/H_{2}O_{2} were selected. One group with 6.88mM H_{2}O_{2} results in total inhibition of PAO1 growth in TSB and the other combination with 3.44mM H_{2}O_{2} resulted in partial inhibition of PAO1 growth. To this combination, either GSH to 128mM or catalase to 100U/mL were added at different times during bacterial treatment to include immediately following addition of the target bacteria or after one or 2 hours of treatment. As shown in Fig. 8, with the higher concentrations of H_{2}O_{2} in the LPO system, initially adding GSH could rescue 10\% of the recoverable CFU. At this time point, adding catalase fully rescued the bacteria from inhibition by the LPO system. However, if either the GSH or catalase were added following one hour of treatment, less than 10^2 CFU/ml bacteria
were recovered; after 2 hours, no rescue could be demonstrated. Similar phenomena were observed in LPO/SCN/H₂O₂ with 3.44 mM H₂O₂. That substantial blocking can be observed if adding GSH or catalase immediately with LPO/SCN/H₂O₂, and the effect decreased significantly suggests that the rescue of GSH is possibly from overwhelming H₂O₂ and prevent the reaction of LPO/SCN/H₂O₂ system, instead of clearing the product OSCN or reversing its effect in this system. It would seem important in future studies to examine intermediate time intervals between initial and one hour for the blocking/rescue effects of GSH and catalase.

**Influence of SCN on hypochlorite-killing on P. aeruginosa.**

Besides playing a role in the LPO/SCN/H₂O₂ system to inhibit pathogens in the host, another important property of SCN that contributes to host protection is to reduce the amount of HClO in human body through antioxidant potential. This property is particularly valuable for the treatment of CF lung disease, considering lung damage is purportedly mainly caused by the exaggerated inflammatory response, and that HClO is likely one of the main tissue-noxious molecules generated in this response. Studies have shown that by adding extra SCN, more host cell can be protected from HOCl damage since SCN non-enzymatically reduces HClO and converting it to tissue-innocuous molecules (i.e. OSCN and Cl).

However, a question remained in the previous studies, i.e. whether adding SCN could also decrease the antibacterial effect of HClO such that the innate immune defenses in the airway are compromised. To address this question, we compared the inhibitory effect of HClO with and without adding SCN. Specifically, in one group, only HClO was added to coinubate with the samples containing *P. aeruginosa* with an initial density of 10⁶ CFU/mL. As shown in Figure 3A, when the concentration of HClO exceed 9.75 mM, the growth of PAO1 is completely inhibited; three logs of inhibition can be observed when the concentration of HClO reached 4.875mM.
Next, titrated concentrations of SCN were added into bacteria culture media containing HClO. As shown in Fig. 3A, substantial protection can be observed for the groups with the HClO concentrations are 9.75mM and 4.875 mM. In both groups, adding 2.5mM SCN resulted in the best protection for PAO1 to resist HClO killing. As shown in Fig. 3B, with 9.75mM HClO in the sample, which resulted in complete inhibition of P. aeruginosa, 2.5mM SCN rescued more than $10^4$ CFU/mL bacteria. An interesting observation is that, when the SCN concentration was increased, the effect of protection on bacteria growth tended to decrease. In the 9.75mM HClO group, adding 20mM and 10mM SCN, only $10^3$ CFU/mL bacteria were rescued. The same tendency occurred in 4.875mM HClO group, which only provide partial inhibition on P. aeruginosa. The highest level of protection occurred during the co-incubation with 2.5mM SCN, where approximately $10^6$ CFU/ml bacteria were detected. On the contrary, only $10^3$ CFU/ml bacteria were detected at 4.875 mM HClO. However, with 20mM SCN adding to 4.875 mM, the bacteria number is 10 fold less than that for 2.5mM SCN.

These results suggest that not only does SCN protect host cell from the killing due to HClO, but SCN is also able to rescue pathogens from HClO killing. However, the protection decreases when amount of SCN increases to a certain level, presumably due to the extra OSCN generated from oxidized SCN that can inhibit bacteria growth.

**Susceptibility of mucoid strain of P. aeruginosa to oxidant stress.**

A unique character of CF lung infection is that P. aeruginosa is the dominate pathogen in more than 80% of the patients, while most of other immune compromised diseases are often infected by various pathogens. Although the exact reason for P. aeruginosa to dominate in CF lung is under debated, several studies have shown that this pathogen undergoes several genetic
transformation along with the progress of CF lung infection, and it is generally accepted that the genetic flexibility of \textit{P. aeruginosa} plays an important role in its ability to dominate in the CF airway. Among different genetic change, the conversion to mucoidy of \textit{P. aeruginosa} is considered as the most characteristic and important adaptation.

Current speculation regarding the purpose for conversion of \textit{P. aeruginosa} to over-production of alginate to form mucoidy strains is to protect against stress from inflammation in the CF lung, like reactive oxygen species, oxidant molecule; however, there have been no studies demonstrating an association between alginate production and ROS resistance. Thus, we applied our method of measurement of inhibition of bacteria growth to compare the susceptibility of mucoid and non-mucoid strain to H$_2$O$_2$, Hclo, and LPO/SCN/ H$_2$O$_2$ system. Three strains were chose in this comparison. One mucoid strain PaM is a laboratory-derived mutant of PAO1, the other mucoid strain C3873 is a clinical sputum isolate from a CF patient. Initially, the correlations between onset time to exponential growth vs. initial inoculum size were established for both mucoidy strains as had been done for PAO1. The influence of log$_{10}$ titration starting inoculum on growth curves are shown in Figure 10 A and B As with PAO1, the influence of starting inoculum density on time to onset of exponential growth of both mucoidy strains were highly linear with correlation coefficients of 0.9986 and 0.9997 respectively.

Next, the three strains of \textit{P.aeruginosa} were exposed to four different treatments: H$_2$O$_2$ alone, H$_2$O$_2$ with LPO and SCN, HClO alone, and HClO with SCN. First of all, as showed in Figure 9A the results showed that PAO1 was not totally inhibited until the concentration of H$_2$O$_2$ reached 64mM; however, there was total inhibition of the two mucoidy with H$_2$O$_2$ concentrations as low as 4 mM. These results indicated that the non-mucoidy strain of \textit{P. aeruginosa} was actually more resistant to H$_2$O$_2$, suggesting that the over-production of alginate
does not serve to protect against reactive oxygen species. Moreover, PAO1 proved more resistant to the LPO system than the two mucoid strains. While LPO system with only 2mM $H_2O_2$ resulted in total inhibition of PAM and C3837, 8mM $H_2O_2$ was required in the LPO system for the inhibition of PAO1. (Fig. 9B) This observation also suggested that, in contrast to what was observed with PAO1, the LPO/SCN/ $H_2O_2$ system did not enhance the inhibition of the mucoidy strains compare to $H_2O_2$ alone.

When treated with HOCl, total inhibition of all three strains was observed when HOCl was increased to 8mM, which suggested that both mucoidy and non-mucoidy strains of *P. aeruginosa* showed similar susceptibility profiles to this ROS. We also tested the influence of SCN on HClO killing. After 20 mM of SCN addition to titrated concentrations of HClO, the protection from SCN in all three strains was observed and there were no significant differences in susceptibility between the mucoid and non-mucoid strains.

**Effect of LPO/SCN/ $H_2O_2$ on mucoidy forming of *P. aeruginosa***

It is known that conversion of *P. aeruginosa* from non-mucoid to mucoid phenotype is attributable to overproduction of alginate. The alginate biosynthetic pathway requires guanosine diphosphate-mannuronic acid. Since it is speculated that LPO/SCN/ $H_2O_2$ may inhibit bacteria growth by interruption of carbohydrate metabolism, it was the purpose of these studies to determine if LPO/SCN/ $H_2O_2$ influenced the producing of alginate by mucoidy isolates of *P. aeruginosa*.

In this experiment, we performed spiral plate assay to understand the effect of LPO/SCN/$H_2O_2$ system on the two mucoidy strains. Similarly, small colonies are observed after two hours of treatment of LPO/SCN/$H_2O_2$, (Fig. 10 & 11). The small colonies can also grow bigger with mucoid phenotype later, indicating that the alginate generation was not suppressed by the LPO
system. Moreover, colonies did not showed up on plates after 4 hours of treatment of LPO/SCN/H₂O₂, suggesting that the mucoid phenotype did not influence the anti-microbial kinetic curves of LPO/SCN/ H₂O₂. Therefore, we further proved that conversion to mucoidy did not protect P. aeruginosa from inhibition of the LPO system.

**Comparisons of susceptibilities of mucoidy vs. non-mucoidy strains of P. aeruginosa by disc diffusion assay.**

Besides mucoid conversion on P. aeruginosa, studies also showed that biofilm formation is another important characteristic in later stage of CF lung infection. It is evident that mucoidy P. aeruginosa colonize in the CF airway as a biofilm rather than in planktonic form and that the alginate contributes to the biofilm structure. Therefore, we also investigated whether mucoidy strains cultured on a fixed agar surface emulating a biofilm would have demonstrably different susceptibilities to antimicrobial molecules like H₂O₂ and HClO compared to that of planktonic targets.

In this experiment, we applied the disc diffusion assay to mimic the biofilm condition of P. aeruginosa. Varying concentrations of H₂O₂ and HClO were titrated to blank filter paper disks placed on TSA plates inoculated with the three different strains of P.aeruginosa. After overnight culture, the sizes of the zones of inhibition were measured as a diameter to estimate susceptibility of each strain. Specifically, two cultural conditions were applied to the clinical strain, C3873. Based on our previous studies, aerobic chamber with 5% CO₂ resulted in exaggerated production of alginate on C3873; whereas, growth using nitrate respiration in an anaerobic atmosphere containing 5% CO₂ suppressed alginate production. Here we utilized aerobic growth on TSA plates containing 1% NO₃, which our preliminary data determined suppresses the alginate
production from the clinical mucoidy strain. These two cultural circumstances allowed comparisons of the susceptibilities of the target strains grown under the two conditions.

In Fig. 12A and B, we observed smaller zones of inhibition by H₂O₂ with PAO1 compared to that with the two mucoidy strains consistent with the planktonic data suggesting that PAO1 is more resistant to H₂O₂ inhibition. Furthermore, there was no evidence that either increased (5% CO₂) or decreased (1% NO₃) alginate production altered susceptibility to either HClO or H₂O₂ under conditions that mimic biofilm conditions. (Fig. 12C and D)
Discussion

In the normally healthy human body, there are two general mechanisms that can be utilized to eliminate the inhaled bacteria in airway (36): the mucociliary escalator physically removes inhaled particles, and the soluble antimicrobial proteins of the secretions that bathe the airway surfaces such as secretory antibodies or innate defense proteins including defensin, lysozyme, lactoferrin and lactoperoxidase that exert both bacteriostatic or bactericidal effects. When considering the relationships between CFTR deficiency/dysfunction and susceptibilities to lung infection, hypotheses involving both airway defense mechanisms have been suggested.

The “dehydration hypothesis” (37) proposes that the loss of normal ionic transport to airway surface attributable to CFTR mutation results in insufficient amounts of salt and fluid leads to dehydration of the airway surface liquid and a thickening of the surface mucus layer. This abnormal, thick mucus contains higher concentrations of mucins and can collapse onto the epithelium, thus the cilia on the surface of epithelium fail to work properly to eliminate the pathogenic challenges. The failure of ciliary clearance is considered as the first step of CF lung infection. Up to now, most of the data supporting this theory is based on air interface human bronchial epithelial cell culture system. Studies on this system showed that when small volume of liquid is added to normal airway culture, the epithelium is able to maintain an ASL height volume of 7μm. In contrast, CF airway epithelial culture surface absorbed the liquid rapidly and fail to maintain this height (3μm) and the liquid trapped by cilia that have collapsed onto airway surfaces.(8)

A limitation of this theory is that only well-differentiated ciliated epithelial cell cultures were examined in studies. In the human body, the airway system also includes submucosal
glands, which are the primary source of the exocrine secretions that bathe the airway surfaces. These glands consist of both proximal mucous and distal serous epithelial acini and play an important role in secreting and transport of ions. (38) In the mouse model of cystic fibrosis lung, normal ASL homeostasis and mucus clearance have been observed.(39) Another limitation of the “dehydration hypothesis” is that it failed to explain the unique pathogenic profile of CF lung infection. It is widely accepted that CF lung infection has different microbial species compare to other lung infection. Moreover, one single pathogen, P. aeruginosa, predominates in the late stages of CF lung disease. However, the dehydration hypothesis considers the major defense mechanism that protect airway as the mechanical clearance by mucus clearance system, which is not bacteria specific. (1) This means all species would be expected to be able to take some advantage of the failure of mucus clearance to colonize in CF lung. Therefore, the role of ASL and mucus clearance in CF airway is still under discussion.

On the other hand, the other hypothesis, normally named “high salt hypothesis”, suggested that in the normally healthy airway, the CFTR is responsible for maintaining relatively lower level of NaCl solutions on airway surface. The mutated CFTR leads to high salt environment on the airway surface, like in sweat duct, which diminish the efficient activity of secreted polyionic antimicrobial substances on airway surface. (40, 41). This theory emphasizes the role of innate immune proteins by the submucosal glands in keeping airway sterile. It is also believed that serous cells require CFTR for secretion of the antimicrobial-rich fluid elaborated by submucosal glands in response to pathogens. (42) Therefore, in CF airway, deficient secretion of antimicrobial molecules from submucosal glands is followed by infection and inflammation.
The central hypothesis of our project is that the ionic composition of the secretions on the airway surface is critical to the maintenance of homeostasis in a healthy airway. The deficient CFTR in CF patients results in improper concentrations of selected ions necessary to the function of critical soluble innate defense factors. CF lung infection is characterized by a dominating pathogen, *P. aeruginosa*, which is highly associated with disease progress, and mainly drive to determine the morbidity, clinical deterioration and mortality. (43) This is very different from most genetically determined diseases, since normally large variety of pathogenic species could be found in immune compromise subjects. It is also known that *P aeruginosa* is highly genetically flexible and able to actively adapt to the inflammatory, stressful environment in CF lung. (32) Therefore, instead of attributing the CF lung disease to dysfunctional mucus clearance, we speculate that the abnormal ionic composition of secretion in CF airway caused by deficient CFTR creates a unique environment that *P. aeruginosa* can take advantage of.

Secretions of submucosal glands in the airway contain soluble antimicrobial components, and they play an important role in host immune system. Lactoferrin (6), lactoperoxidase (LPO), lysozyme (44), mucins and secretory IgA (S-IgA) are among the principal antimicrobial proteins found in exocrine secretions that bathe mucosal surfaces including the conducting airways and the oral cavity. The antimicrobial function of those proteins are depended on or regulated by different molecules and anions that delivered by glandular ductal epithelial cells or by the ciliated epithelium lining the airways. For example, the amount of bicarbonate (HCO$_3^-$) can influence the ability of LF of eliminating bacterial by determining the ironing binding site (10); also, the most common reactive oxygen species, hydrogen peroxide, is synthesized by the NADPH oxidase family member dual oxidase 2 (DUOX 2) expressed by ductal epithelial cells. (45). Besides, anions like GSH and SCN are important antioxidant in airway that can diminish
the host cell damage from inflammatory response. (46). Although recognized as a Cl channel initially, increasingly studies have shown evidence that all those molecule above are transported or regulated by CFTR (47) Therefore, further studies are needed for understanding the link between CF lung infection and abnormal molecules in secretions caused by CFTR mutations.

In my thesis project, we focused on one of the anions, SCN, which have been shown decreased in amount in CF airway due to the CFTR mutation. We investigated its potential influence on the clearance of *P. aeruginosa* in the healthy airway, and how CFTR dysfunction contributes to the infection progression of *P. aeruginosa* in the CF airway, including its influence on the the conversion of of this pathogen to the mucoidy phenotype characteristic of the CF pathogen.

SCN has been proposed to play important roles in human host defense systems from two different perspectives. (15) First, SCN is the preferred substrate for peroxidase catalyzed reaction with H$_2$O$_2$ to produce HOSCN, a potent antimicrobial agent. The other role of SCN in host protection as an antioxidant. It provides the protection on host cell in the inflammatory environment via reducing the accumulation of tissue –damaging species from inflammatory response, such as H$_2$O$_2$ and OCl$^-$, and generate tissue innocuous OSCN. (14)

In the first part of my project, we proved the antimicrobial effect of LPO/SCN/ H$_2$O$_2$ system. By showing that SCN itself did not influence the growth of *P. aeruginosa*, and the combination of either H$_2$O$_2$ plus SCN or H$_2$O$_2$ plus LPO did not influence the inhibitory effects from H$_2$O$_2$ on *P. aeruginosa*, we showed total inhibition can be observed when three components together with 8 fold lower concentration of H$_2$O$_2$. In the next step, we showed the inhibition efficiency is dose-dependent on both H$_2$O$_2$ and LPO. These results suggested that with LPO,
SCN, and H₂O₂ all together can generate new component to inhibit the growth of *P. aeruginosa*, rather than just enhance the effect of inhibition from H₂O₂.

Lactoperoxidase is a peroxidase that was first identified in cow’s milk. The activity of LPO/SCN/H₂O₂ system has since been identified in sheep airway (24) and the deficiency in SCN associated with CFTR dysfunction has been suggested as compromising the antimicrobial and anti-inflammatory functions of the LPO system in the CF airway. Studies also showed that all three components are concentrated enough to give active antimicrobial effect. The DUOX system of ductal epithelial cells has been shown to be a source of H₂O₂ in the secretions of airway surfaces. Although H₂O₂ itself can damage bacterial cells directly, the levels of H₂O₂ encountered in airway are likely too limited (10⁻⁶M) for this function to be effective.(48)

Moreover, our data showed that antibacterial effects of H₂O₂ are largely influenced by target numbers of *P. aeruginosa*, due to catalase on the bacterial membrane. Thus, it is suggested that LPO/SCN/ H₂O₂ work together against the *P. aeruginosa*. Moreover, our experiments demonstrated that the antimicrobial effects of OSCN is not influenced by the density of *P. aeruginosa*.

In addition to the antimicrobial effect of LPO/SCN/H₂O₂ system, we also studied the mechanism of inhibition of this system on *P. aeruginosa*. It has been found that OSCN suppresses the growth of oral pathogens by oxidation of essential thiol groups on glycolytic enzymes, disrupting the critical metabolic process of glycolysis. (49) Another interesting finding is that inhibition by OSCN is reversible as some bacteria are capable of regenerating their thiol groups after removing the residual oxidant either by exogenous reducing agents or through bacterial generation of reducing equivalents. (19) Therefore, the antimicrobial effects of OSCN in this situation would be considered as bacteriostatic. However, the characteristic of effect of
LPO/SCN/ H₂O₂ system on the CF pathogen, *P. aeruginosa*, has never been well studied, though several papers described the LPO/SCN/ H₂O₂ system as having bactericidal activity in the airway. (50)

Based on our experiment, the *P. aeruginosa* when plated to agar surface after two hours treatment with LPO/SCN/ H₂O₂ yielded small colonies after overnight growth compared to controls. These colonies eventually grew to full size and were recovered to normal growth rate after transferring to a new plate. This suggested that the inhibition by OSCN of *P. aeruginosa* was also due at least initially (before two hours of treatment) to bacteriostasis, rather than causing any irreversible damage to the cell. Furthermore, the small colonies but not the normal size colonies disappeared by four hours of LPO/SCN/ H₂O₂ treatment, suggesting that *P. aeruginosa* starved after interruption of metabolism. Another possibility is that the LPO system caused cell damage on *P. aeruginosa*, but bacterial cells are able to repair some of the damaged cells within only two hours, while the damage becomes irreversible after four hours.

It is also important to note that OSCN may not be the only product from LPO/SCN/ H₂O₂ system. It has been speculated that when H₂O₂ /SCN ratio are high enough, the OSCN can be further oxidized to O₂SCN and O₃SCN. (51) It has been suggested that O₂SCN and O₃SCN can produce irreversible damage to bacterial cells. In our experiment, there is certainly the possibility that O₂SCN and O₃SCN may contribute to the lethal effect of the LPO system on *P. aeruginosa*.

Although there is good evidence that OSCN is capable of inhibiting bacterial metabolism through oxidation of essential sulfhydryls of target enzymes, there is another aspect worth considering. It has been suggested that SCN is associated with large, positive entropies, and is considered a chaotropic ions. Chaotropic ions are defined as favoring the transfer of apolar groups to water. Moreover, studies have suggested that such chaotropes can weaken the
hydrophobic bonds of membranes and multi-component enzymes and increase the water solubility of particulate proteins. (52) Based on this theory, the antimicrobial effect of OSCN may also come from the chaotropic prosperity which leads to damage of the bacterial member. In theory the single negative charge spread over the increased molecular diameter of OSCN vs. SCN would result in more potent chaotropic activities for the oxidized species of SCN.

In my thesis, we also looked at the antioxidant role of SCN. In most studies, SCN is proposed as a host beneficial molecule in CF lung inflammation, due to its capability of limiting accumulation of harmful inflammatory factors including HClO and H2O2. As the neutrophil is major inflammatory cell recruited to the CF airway surface, large amount of HClO are generated by MPO/Cl/ H2O2 resulting from the activation of neutrophils, which is responsible for host cell injury and contributes to failure of respiration in CF patients. (53). Xu Yanping et. al have showed that adding SCN protects the lung epithelial cell lining against injuries caused by MPO activity, and also proved the protection is due to SCN inhibited OCl production by MPO. (14)

However, the fact should not be ignore is that the main purpose of neutrophils to generate HClO is to kill invading pathogens in our body. Therefore, one question arises when considering SCN as a potential therapeutic for reducing HClO from MPO system in order to limit host damage: whether adding SCN also protects the pathogen from the MPO system thus providing the window of opportunity to infect the CF lung. From our experiments, we showed that with 2.5mM SCN, P. aeruginosa is almost fully rescued from total inhibition with 9.75mM HClO. Interestingly, the number of bacterial cells rescued from SCN was decreased when SCN concentration went higher. Our explanation towards this phenomenon is that that when SCN reacts with HClO, the antimicrobial species OSCN is generated. When SCN increased to sufficient concentration the OSCN levels were adequate to achieve inhibition of growth of P.
*aeruginosa*. In situ it would be expected that the HClO would not be the only source for OSCN would also be generated by the peroxidase dependent oxidation by H$_2$O$_2$.

Therefore it seems that SCN can reduce the concentration of cytotoxic HClO to generate a more microbe specific, tissue-innocuous species, OSCN, serving the dual function of antimicrobial and antiinflammatory. While the antimicrobial activity of OSCN would be interpreted as being greater than of H$_2$O$_2$, it is evident that this activity is less potent than that of HClO. Thus, we believe that caution should be exercised when considering adding SCN as a therapeutic molecule to CF patient.

Another important concept in our hypothesis is that the exocrine secretions of the airway submucosal glands serve to deliver critical innate defenses to the healthy airway surface. These defense mechanisms are attributable not to one molecule but to a combination of interdependent factors that are dependent on a variety of ions for maintenance of a healthy homeostasis to limit potential pathogens from establishing an infection in the airway.

Although several studies have suggested that SCN may be beneficial in the airway, both in antimicrobial and antioxidant roles, none of the studies consider the possibility that the effects of SCN might be influence by other molecules in the airway surface liquid. However, it should be noted that SCN does not exist alone in the airway, but coexists with all the other molecules in ASL (36). Thus, in order to investigate the role of SCN in airway immune system, it is important to understand the interactions of SCN with other molecules in the secretions of submucosal glands and how they are modified by the ciliated epithelial surfaces that they bathe. It is hard to define the interaction between SCN and all the other molecules present in on the airway surfaces. Therefore, in my thesis project, we began to look at GSH, an important tripeptide that is also delivered to the airway surface liquid. Normally, GSH is synthesized and
functions inside human cells and serve as antioxidant that can prevent damage caused by reactive oxygen species such as free radicals and H$_2$O$_2$. However, on the airway GSH is secreted through the apical surface of the cell to the airway surface liquid by a CFTR dependent mechanism. (11) It is suggested that the function of GSH in airway is also limiting accumulation of reactive oxygen species. (54) Consistent with the role of CFTR in glutathione transport, the amount of GSH is also diminished in the CF airway due presumably to absence or dysfunction of CFTR.

As discussed before, SCN is considered as an antimicrobial molecule, because it is able to be oxidized by LPO with H$_2$O$_2$ to generate OSCN, which is able to inhibit growth of bacteria. However, when GSH and SCN exist together in the secretion, it is possible that GSH limits the generation of OSCN owing to its ability to consume H$_2$O$_2$ as a substrate for GSH-peroxidase (12). Another possibility is that GSH can directly reduce amount of OSCN due to its antioxidant potential to prevent antimicrobial function of the LPO system either by blocking its activity or direct reaction with OSCN.

Based on our results, it is clear that when LPO, SCN, H$_2$O$_2$ and GSH are present together, the initial antimicrobial activities of the LPO system on *P. aeruginosa* can be dampened. However, if LPO/SCN/ H$_2$O$_2$ system is allowed to react with the bacterial target for a period of time before the addition of GSH, the antimicrobial effects cannot be reversed. Therefore, these studies imply that the GSH can decrease the antimicrobial function of LPO/SCN/ H$_2$O$_2$ system only by blocking the enzymatic activity, but not by reversing the OSCN oxidized sulfhydryls. Considering the high concentrations of GSH in epithelial lining fluid in the health airway, it is important to consider its influence on any potential antimicrobial functions of SCN in normally healthy airways.
The last part in this thesis project, investigated the antimicrobial activities of SCN against CF characteristic mucoidy strains of *P. aeruginosa*. As discussed before, chronic infection of the cystic fibrosis lung by *P. aeruginosa* is the major cause of morbidity and mortality among CF patients. (27) Due to the high selective pressure of inflamed CF airway, from the initial colonization to establish a chronic infection, *P. aeruginosa* undergoes significant genetic changes to adapt to this environment. It has been shown that, at the younger ages of CF patients, the majority of *P. aeruginosa* strains infecting are acquired from the environment and undergo characteristic adaptations in the progression to the late stages of CF infection, such as excessive production of extracellular alginate, loss of the ability to produce LPS O-side chains, loss the color of colony, loss of motility and modifications in quorum sensing molecules. (32)

Among those changes, one of the most prominent virulence determinants in the chronic CF airway disease is the conversion to the mucoidy phenotype characterized by alginate production. Clinical studies showed that the most profound lung function deline occurs when *P. aeruginosa* transitions to this mucoidy phenotype, and this change can be observed in more than 80% of late stage CF lung infections. (31) It is believed that the mucoidy characteristic aids the persistence of *P. aeruginosa* in the CF lung, and it has been considered the hallmark of chronic progression of infection in the CF lung.

The environment of the CF lung presents an escalating variety of stressors, including high concentrations of reactive oxygen and nitrogen species, associated oxygen limitations, nutrient limitations and locally and systemically delivered antibiotics. It is believed that alginate production is a strategy for *P. aeruginosa* creates an extracellular biosphere environment that provides a barrier to such stressors. Indeed, studies have showed that by consistently treated with low levels of H2O2, or cultured in anaerobic growth conditions, *P. aeruginosa* can be induced
from the smooth, non-mucoid wild type to the mucoidy phenotype. (29, 33) However, we did not find any studies that indicated that excessive alginate production would afford protection of this pathogen against H$_2$O$_2$ or other reactive oxygen species. There is data that indicate that the transition to the mucoidy phenotype is associated with upregulation of genes such as catalase, superoxide dismutase and flavoheme protein that would be assumed to afford protection from the activities of ROS and RNS.(33)

In our studies, by comparing the titrations of reactive oxygen species HClO and H$_2$O$_2$ give totally inhibition on mucoid vs. non-mucoid strains of _P. aeruginosa_, we did not observed increased resistance of the alginate producing strains to the antimicrobial effects of either HClO or H$_2$O$_2$. which suggested that mucoidy did not afford protection of _P.aeruginosa_. In contrast, we observed that wild type _P.aeruginosa_ required higher concentrations of H$_2$O$_2$ to get total inhibition than the other two tested mucoidy strains. Therefore, from our studies, we did not see mucoidy function as protection for _P. aeruginosa_ against inhibition from reactive oxygen species. Still, there are other potential advantages of mucoidy formation that need to be considered. It is suggested that alginate promotes the biofilm formation of _P. aeruginosa_ and helps the pathogen establish integration into the thick mucus layer characteristic of the CF airway. (55) Further, alginate keeps phagocytes from entering the biofilms and gaining the direct access likely required for bacterial killing by these innate defense cells.(56).

Another observation in our study was that due to the higher sensitivity of the mucoidy strains of _P. aeruginosa_, the LPO system did not show significantly higher inhibitory effects when compared to that with treatment with H$_2$O$_2$ alone. Therefore, it is possible that LPO/SCN/H$_2$O$_2$ may play an important role in clearing the initial environmentally challenged _P. aeruginosa_ that protects the normally healthy airway in contrast to the failure in the compromised CF lung.
In the CF lung there would be no SCN to convert ineffective concentrations of H₂O₂ to the more effective OSCN species necessary to resolve the challenge with the H₂O₂ resistant environmental P. aeruginosa. Consider the alginate are form the main structure of P. aeruginosa biofilm, and mucoidy strains often exist in biofilm structures, we also utilized a disk diffusion assay on an agar surface to mimic the surface growth of the biofilm structure. These approaches failed to reveal any protection against either H₂O₂ or HClO that could be attributed to alginate production even under exaggerated alginate production induced by a 5% CO₂ atmosphere compared to the alginate suppressive conditions of 1% NO₃ observed with the clinical isolate C3873.

In summary, our current data confirmed the antimicrobial potential of SCN against P. aeruginosa in the airway. On the one hand, it is clear that the presence of LPO and SCN at optimal concentrations can significantly enhance the antimicrobial activity of H₂O₂ against relevant environmental isolates of P. aeruginosa through generation of OSCN. Thiocyanate is also capable of reducing the less discriminating but apparently more potent anti-P. aeruginosa activity of HClO. The chemistry of this interaction should result in conversion of the HClO to HOSCN and NaCl that should reduce the oxidative damage to the host while generating the microbe specific antimicrobial species of OSCN. Presumably this would be an advantage to the host as the bacteria are effectively growing in the presence of massive numbers of HClO generating neutrophils in the CF airway. Furthermore, there is no evidence that the normally healthy airway is dependent on the neutrophil or its HClO product for defense against P. aeruginosa challenge. It seems more likely that the soluble content of the exocrine secretions that bathe these surfaces provide antimicrobial activity necessary to prevent P. aeruginosa colonization and growth. The current studies indicate that LPO and its SCN substrate are capable of inhibiting environmental P. aeruginosa at concentrations that may be found in exocrine
secretions such that OSCN may make important contributions to the defense against this pathogen. By extension, deficiencies in SCN in the CF airway surface liquid may predispose to the unique susceptibility to infection with this opportunistic pathogen.
FIGURES

Figure 1 Measurement of bacteria density by onset time in growth curve.

A) Growth curves of *P. aeruginosa* in broth cultures with gradient initial density. $20\mu$L of *P. aeruginosa* culture media
were added into 200 µl TSA media, the starting bacterial density was adjusted to 10⁸ CFU/mL using comparison to MacFarland standards serial ten-fold dilutions of this bacterial suspension was used. B) The time growth curve takes to enter log phase growth and this time to exponential growth can be uniformly captured by determining onset time to an OD₆₅₀nm of 0.2 (red line). C) Linear regression was performed between the starting inoculum size (CFU/ml) and the time to achieve this threshold OD value, i.e. the onset time.

Figure 2 SCN did not influence growth of bacteria by itself:

SCN did not influence growth of bacteria by itself: A titration of concentrations (0.75 - 40mM) of SCN was added to replicate wells of the culture media prior to inoculation with PAO1(10⁸ CFU/ml) The effect of SCN on microbes were measured by relative bacteria number indicated by onset time for each sample.
Figure 3  Inhibitory effect of H$_2$O$_2$ on PAO1 is influence by bacteria density.

A) 

![Graph](image)

**Inhibitory effect of H$_2$O$_2$ on PAO1 is influence by bacteria density.**

*P. aeruginosa* (A) and *S. mutans* (B) with two different starting inoculum of $10^7$ CFU/ml (blue) and $10^5$ CFU/ml (red) of were exposed to a titration of concentrations (0, 10mM to 110mM) of H$_2$O$_2$ for overnight culture. Higher density of *P. aeruginosa* showed higher resistant to H$_2$O$_2$ killing. Different susceptibility to to H$_2$O$_2$ killing did not been observed in different density of *S. mutans.*
Figure 4 The enhanced antimicrobial activity of \( \text{H}_2\text{O}_2 \) is dependent on both LPO and SCN

The enhanced antimicrobial activity of \( \text{H}_2\text{O}_2 \) is dependent on both LPO and SCN

10\(^7\) CFU/ml \( P.\text{ aeruginosa} \) were exposed to a titration of concentrations (0. 10mM to 110mM) \( \text{H}_2\text{O}_2 \) alone (blue), plus only SCN (orange), plus only LPO (yellow) or SCN plus LPO (grey).

Total inhibition can be observed with 6.88mM \( \text{H}_2\text{O}_2 \) with LPO plus SCN group compared to \( \text{H}_2\text{O}_2 \) alone. No significant difference can be observed with the other two groups (\( \text{H}_2\text{O}_2 \) with SCN, \( \text{H}_2\text{O}_2 \) with LPO)
Figure 5 Dose dependence of $\text{H}_2\text{O}_2$/LPO for growth inhibition of PAO1

A)

B)

C)

Dose dependence of $\text{H}_2\text{O}_2$/LPO for growth inhibition of PAO1: $10^7$ CFU/ml *P. aeruginosa* were exposure to four concentrations (from 6.88 to 0.78mM) of $\text{H}_2\text{O}_2$ and ten concentrations (from 10 to 0.3125U/ml) of LPO. TSB Plus 2mM SCN media was used to dilute all the reagents. A) Growth curves of *P. aeruginosa* in all the treatment. B) Growth curves with *P. aeruginosa* with same concentration of LPO (5u/ml) and SCN (2mM), and four different concentration of $\text{H}_2\text{O}_2$. C) Growth curve with *P. aeruginosa* with same concentrations of $\text{H}_2\text{O}_2$ (3.44mM) and
SCN (2mM) and 10 group of LPO. The level of inhibition was dependent on both the activity of the LPO and the concentration of H$_2$O$_2$.

**Figure 6** Antimicrobial effect of LPO/SCN/ H$_2$O$_2$ did not been influence by target density
Antimicrobial effect of LPO/SCN/ H\textsubscript{2}O\textsubscript{2} did not been influence by target density two inoculum densities of PAO1 (10\textsuperscript{7} CFU/mL and 10\textsuperscript{6} CFU/mL) were treated in the presence of 2mM SCN in microtiter format with H\textsubscript{2}O\textsubscript{2} in two-fold dilutions added vertically, and the LPO in two-fold dilutions added laterally as a checkerboard. No LPO were set up as control group. Growth curves (A) of all the samples were acquired. The efficiency of inhibition of were estimated as onset time in each group. In 10\textsuperscript{6}CFU/mL of P. aeruginosa, 6.88mM and 3.44mM H\textsubscript{2}O\textsubscript{2} alone can totally inhibit growth of bacteria.(B). With both 1.72 mM H\textsubscript{2}O\textsubscript{2} and 0.86mM H\textsubscript{2}O\textsubscript{2}, the same inhibitory activity can be achieved with either of the two target densities of P. aeruginosa. (C)

Figure 7 Investigate of mechanism of antimicrobial effect of LPO/SCN/ H\textsubscript{2}O\textsubscript{2} system on P. aeruginosa by spiral plating assay
A)

B)
C)

<table>
<thead>
<tr>
<th></th>
<th>LPO/SCN/ H₂O₂</th>
<th>H₂O₂/SCN</th>
<th>CONTROL</th>
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**Investigate of mechanism of antimicrobial effect of LPO/SCN/H₂O₂ system on *P. aeruginosa* by spiral plating assay.** A) Two concentrations (3.44mM and 6.88mM) of H₂O₂ were selected in this experiment in order to acquire the mechanism of both total inhibition and partial inhibition from LPO/SCN/ H₂O₂ system. 3.44mM H₂O₂ (B) and 6.88mM H₂O₂ applied in two groups of treatments, LPO/SCN/H₂O₂ and H₂O₂ alone in spiral plating assay. Non-treated bacteria suspensions were used as control groups. The *P. aeruginosa* suspension with a concentration of 10⁶ CFU/mL were cultured and divided into three groups. At the time points of 0, 2, 4 and 6 hours, aliquots from each treatment group were spiral plated onto tryptic soy agar
(TSA). After overnight growth at 37°C in ambient atmosphere, colonies were examined for their appearance and the quantitation of recoverable CFU/ml.

**Figure 8 Blocking by Catalase or GSH of the inhibitory effects of LPO/SCN/ H2O2 system.**

![Graph showing relative bacteria number](image)

**Blocking by Catalase or GSH of the inhibitory effects of LPO/SCN/ H2O2 system.** One group with 6.88mM H2O2 results in total inhibition of PAO1 growth in TSB and the other combination with 3.44mM H2O2 resulted in partial inhibition of PAO1 growth. To this combination either GSH to 128mM or catalase to 100U/mL were added at different times after during bacterial treatment to include immediately following addition of the target bacteria or after one or 2 hours of treatment. That substantial blocking can be observed if adding GSH or catalase immediately with LPO/SCN/ H2O2.

**Figure 9 Influence of SCN on hypochlorite -killing on PAO1.**

A)
Influence of SCN on hypochlorite-killing on PAO1.

Inoculum densities of $10^6$ CFU/mL PAO1 were treated in the presence of 20mM SCN in microtiter format with $\text{H}_2\text{O}_2$ in two-fold dilutions added vertically, 128mM HClO and the in two-fold dilutions added laterally as a checkerboard. The group with only HClO was set as control group. When the concentration of HClO exceed 9.75 mM, the growth of PAO1 is completely inhibited; three logs of inhibition can be observed when the concentration of HClO reaches 4.875mM. (A) In both groups, adding 2.5mM SCN resulted in the best protection for PAO1 to resist HCLO killing. (B)
Figure 10 Susceptibility of mucoid strain of *P. aeruginosa* to oxidant stress.

A)

PoM standard curve

\[ y = 5 \times 10^9 e^{-3 \times 10^{-4}x} \]

\[ R^2 = 0.9986 \]

B)

c3783standard curve

\[ y = 5 \times 10^9 e^{-3 \times 10^{-4}x} \]

\[ R^2 = 0.9986 \]

C)
Susceptibility of mucoid strain of *P. aeruginosa* to oxidant stress. The correlation between onset time in growth curve and initial density in bacteria sample were also measured in both mucoid strain PAM (A) and C3873(B), both strain showed highly linear, and the correlation coefficient is 0.9986 and 0.9997. Three strains of *P. aeruginosa* were exposed to four different group of treatment: H₂O₂ alone(C), H₂O₂ with LPO and SCN(D), and HClO alone(E).

**Figure 11 Effect of LPO/SCN/H₂O₂ system on mucoidy forming of P. aeruginosa.**

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<th>SCN/H₂O₂</th>
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<td>PAM</td>
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Effect of LPO/SCN/ H₂O₂ system on mucoidy forming of *P. aeruginosa*.

6.88mM H₂O₂, 10 U/mL, 2mM SCN were incubated with PaM (A) and C3783 (B) to exam the effect of LPO/SCN/ H₂O₂ system on generation of alginate in mucoid strain of *P. aeruginosa*. H₂O₂ alone and pure bacteria samples were used as control groups. After 2 and four hours of treatment, 5μL culture were took to do spiral plating. The result indicated that small colonies can also be found in mucoid strain after 2 hours of LPO/SCN/ H₂O₂ treatment.
Figure 12 Susceptibility of mucoid and unmucoid strain of *P. aeruginosa* by disc diffusion assay.

A)

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B)

![Graph](image5.png)

H$_2$O$_2$ (mM) vs. Zone of inhibition (mm) for different concentrations of H$_2$O$_2$. The graph shows the inhibition zones for different strains under varying H$_2$O$_2$ concentrations.

- **h2o2-pam**
- **h2o2-pao1**
- **h2o2-m-co2**
- **h2o2-m-no3**
Susceptibility of mucoid and unmucoid strain of *P. aeruginosa* by disc diffusion assay. Disc diffusion assay were applied to mimic the biofilm condition of *P. aeruginosa*. Varies concentration of H$_2$O$_2$ (A, B) and HClO (C, D) were added to filter paper disks and placed on TSA plates with three different strain of *P. aeruginosa*. Here we also applied a TSA plate with 1% NO3, which can suppress the alginate production from c3837 strain. After overnight culture, the size of ring were measured to estimate susceptibility of each strains.
REFERENCE


