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Liposomal N-acetylcysteine Modulates the Pathogenesis of *P. aeruginosa* Isolated from the Lungs of Cystic Fibrosis Patient

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Abstract

N-acetylcysteine (NAC) is a mucolytic agent with antimicrobial potential. We evaluated the antimicrobial activity of the free and liposomal NAC (F-NAC; L-NAC) against *Pseudomonas aeruginosa*. The minimum inhibitory concentrations (MIC), the minimum bactericidal concentrations (MBC) and the *in vitro* time kill studies of L-NAC were determined by broth-dilution method. Efficacy of the formulations on the production of *N*-acyl homoserine lactone molecules, virulence factors and motility were determined. Eradication of bacterial community within biofilms was assessed using the Calgary Biofilm Device. The L-NAC Cytotoxicity and anti-bacterial adhesion potential to human lung cells were examined using pulmonary A549 cell lines. The MIC of L-NAC was lower than the free drug (1250 mg/L and 5000 mg/L, respectively). MBC for L-NAC was 2500 mg/L compared to 5000 mg/L for F-NAC. L-NAC at 2500 mg/L killed bacteria in 2 h, whereas F-NAC exhibited the same effect at 5000 mg/L. Quorum sensing was significantly inhibited by L-NAC (P<0.001). At 1/8 MIC, L-NAC reduced the production of bacterial proteases significantly more than that of F-NAC at 1/4 MIC. L-NAC was also able to reduce the bacterial motility at eightfold lower concentration than F-NAC (P<0.001). As for biofilms, L-NAC provided 75% protection against biofilm formation, 90% reduction in the formed biofilms, and a 46% eradication effect on bacterial community within biofilms compared to treated biofilm with PBS (P<0.001). Finally, L-NAC at 2500 mg/L was safe to A549 cells, reduced bacterial adhesion by 15% compared to control (P<0.001). These data indicate that L-NAC formulation is more effective than F-NAC against *P. aeruginosa* and has the potential to improve therapeutic outcomes in CF patient.

Keywords: Biofilm; Quorum sensing; Motility; Virulence factors; Bacterial adhesion

Introduction

Cystic fibrosis (CF) is a genetic disease without any existing treatment [1]. It is the most common disorder among the Caucasians population which is caused by mutations of the CF transmembrane conductance regulator gene (CFTR) located on chromosome 7 [2]. There are more than 1900 identified CFTR mutations [3], the most prevalent mutation is caused by deletion of phenylalanine at position 508, known as ΔF508 [4]. CFTR mutations affect ion and water transport channel in epithelial cells [5] in many organs including pancreas, small intestine, liver, reproductive tract, sweat glands and respiratory tract [6]. Even though CF is known to be a multiorgan disease, pulmonary infections have the greatest role in morbidity and mortality [7] leading to death in 90% of CF patients [8]. The diminishment of chloride and water channels leads to viscous secretions and impairs mucociliary clearance [9] which allows the bacteria to multiply in the mucus and use it as an energy source [10]. *Staphylococcus aureus, Haemophilus influenzae* and *Pseudomonas aeruginosa* are the most commonly occurring bacteria in CF, where they contribute to inflammation early stages [11]. It is evident from previous studies that *P. aeruginosa* is the most predominant pathogen [12] which colonizes the lungs of 80% of adults with CF [13].

P. aeruginosa is a Gram-negative bacterium which is considered as a major opportunistic pathogen that causes infectious diseases, and is the leading cause of chronic pulmonary infections in CF. It chronically colonizes in the lungs of CF patients and causes progressive lung damages, respiratory failure and death [14]. *P. aeruginosa* depends on chemical signaling molecules to organize important activities for their survival [15]. These chemicals are known as quorum sensing (QS), which are released by the bacteria in response to an increase of bacterial cell density [16]. The most distinguished type among these signal molecules are *N*-acyl homoserine lactones (AHLs) These chemicals bind to their promoters to stimulate the formation of biofilms and virulence factors (VF) [17]. *P. aeruginosa* pathogenesis is closely associated with its motility such as swimming, swarming and twitching, which allows it to attach to different surfaces forming a biofilm [18].

P. aeruginosa grows into biofilms as a survival strategy. These biofilms are described as communities of microbial cells which are enclosed in polysaccharides matrix, proteins and nucleic acids [19]. *P. aeruginosa* forms three dimensional mushroom shaped colonies attached to the surface with the help of flagellar motility and type IV pili-mediated twitching motility [20]. Biofilms work as a shield to protect bacterial cells against the host immune system and antibiotics [21-24]. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of antibiotics are found to be 100 to 1000 fold higher for mature biofilms when compared to planktonic cells or young biofilms [25]. These communities are predominantly associated with persistence of infection as well as resistant to antibiotic; thereby making the biofilms of *P. aeruginosa* hard to control and eradicate [26].

P. aeruginosa releases several VF such as elastase, chitinase, lipase and protease [27-29]. These VF have a major effect on the alveolar epithelial permeability by damaging collagen, α-1-antitrypsin, elastin, immunoglobulins and cytokines and lead to more mucin secretion from goblet cells [29,30].

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Aggressive antibiotic therapy regimens are prescribed for CF patients from an early age to reduce bacterial infection [31] and delay an eventual chronic lung infection [8,32]. However; this strategy increases antibiotic resistance [33] which is a sign of antibiotic therapy failure [25]. It is well established that once the organism has launched a chronic infection in the bronchial tree, it cannot be eradicated [31]. Not only the bacterial load is problematic in CF, but also abnormalities in mucous secretion also have a great impact on the CF patients' health [34]. Studies have shown that abnormal mucous secretions in CF patients as well as *P. aeruginosa* biofilms formation are advantageous for bacterial survival in the host system because they limit antimicrobial penetration [26,35,36]. Mucolytic agent such as *N*-acetylcysteine (NAC) is one of the best parts of the treatment regime not only due to its mucolytic effect, but also to its ability to reduce biofilm formation [37].

NAC is L-cysteine derivative that has been known for its ability to break disulfide bonds present in mucin and to decrease the mucous viscosity as well as elasticity [38-41]. Several studies have reported the potential of NAC as an antimicrobial agent for a variety of bacteria including *Staphylococcus epidermidis, Streptococcus pneumoniae, Enterococcus faecalis, Escherichia coli* and *P. aeruginosa* [42-47]. The antimicrobial activities of NAC are believed to be due to its ability to inhibit cysteine utilization in bacteria or due to the reaction between bacterial cell proteins and NAC sulfhydryl group [23,43]. Previous studies have also reported the capability of NAC to disrupt biofilms, reduce adhesion of bacteria onto surfaces and cause a reduction of lipopolysaccharide induced lung inflammation [44,47]. However, other studies have also demonstrated that the half-life of NAC is short, approximately 2.15 h [48], and because of its high molecular weight is not able to cross cell membrane barriers [49,50]. Therefore, there is a necessity for encapsulation of NAC into a delivery system such as liposome to prolong its effects, enhance its efficacy and improve the uptake of NAC through fusion with bacterial membrane [51,52].

Liposomes are spherical nontoxic vehicles with sizes ranging from nanometers to micrometers, consisting of one or more lipid bilayers [52]. Typically they are produced from phospholipids; thereby liposomes are biocompatible and biodegradable. Liposomes are preferred for the delivery of therapeutic agents because they provide a sustained release of the drugs and reduce their toxic effects, as well as increase the bioavailability of insoluble hydrophobic drugs [53,54].

Herein, we investigated the effects of L-NAC against growth of *P. aeruginosa*; on attenuation of quorum sensing; on virulence factors such as chitinase, elastase, lipase and protease enzymes; on motility of *P. aeruginosa*; on formation of biofilms; and on *P. aeruginosa* adhesion to lung cells.

Materials and Methods

Chemicals

Dipalmitoylphosphatidylcholine (DPPC) was obtained from Avanti Polar Lipids, Inc. (Alabaster, Alabama, USA). *N*-Acetylcysteine (NAC), Triton X-100, Trypan Blue, Chitin azure, elastin-Congo Red and all other chemicals were purchased from Sigma-Aldrich Co. (Oakville, ON, Canada). Mueller Hinton Agar (MHA), Trypsin EDTA, penicillin/streptomycin, Dulbecco's modified Eagle's medium (DMEM) and Dulbecco's phosphate buffered saline (DPBS), Cell Titer-Blue® Cell viability assay kit and Chloroform were purchased from Fisher Scientific (Ottawa, ON, Canada). Lysogeny broth and Luria Bertani (LB) agar were from (Becton Dickinson Microbiology Systems, Oakville, ON, Canada). Cationic Mueller Hinton broth (CMHB) and Crystal violet (CV) were purchased from BD (Mississauga, ON, Canada).

Microorganisms

The mucoid and non-mucoid clinical isolates of *P. aeruginosa* (PA-13572, PA-5, PA-M13641-2, PA-7, PA-13639-2, PA-3, PA-M13640, PA-11, PA-12, PA-M13639-1, PA-48913, PA-M26250, PA-1, PA-48912-2, PA-48912-1, PA-8 and PA-13641-1) were isolated from the sputum of CF patients and obtained from Clinical Microbiology Laboratory of Sudbury Regional Hospital (Sudbury, ON, Canada). The American Type Culture Collection of *P. aeruginosa* (ATCC 25619, ATCC 27853 and ATCC 10145) strains were purchased from PML Microbiologicals (Mississauga, ON, Canada). *Pseudomonas aeruginosa* strain (PAO1) was generously donated by Dr. R.E.W. Hancock (University of British Columbia). Bacteria were preserved at −80°C in Mueller–Hinton broth containing glycerol at a final concentration of 10% (v/v). *Agrobacterium tumefaciens* strain A136 (pCF218) (pCF372) (Ti^-) was used as the biosensor for the detection of AHLs and cultured in Lysogeny broth at 30°C.

Cell culture

A549 human lung carcinoma epithelial cells were obtained from the American Type Culture Collection (ATCC CCL-185, Manassas, USA) and cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. The cells were grown to 85% confluence in 5% CO_2 at 37°C and maintained using traditional cell culture techniques. For viability and adhesion studies cells were treated with non-supplemented DMEM media to assure accuracy of results.

Preparation of L-NAC

NAC was incorporated into liposomes containing a mixture of DPPC and NAC in a 1:1 molar ratio and prepared by the dehydration–rehydration method as described previously [55]. DPPC were dissolved in chloroform in a 50 mL round-bottomed flask and dried at 45°C with a rotary evaporator (BuchiRotavapor R 205). The lipid film was then hydrated with 1 mL of 10 mg/mL NAC stock solution and subsequently sonicated (model 500 Dismembrator, Fisher Scientific) for 5 min (cycles of 40s on and 20s off). Once multilamellar vesicles were formed, the liposome mixture was freeze-dried overnight as reported previously [56] and un encapsulated drug was separated by centrifugation at 28000×g (Thermo IEC, IEC multi-RF, Thermo Fisher Scientific, Canada). After rehydration of liposomes, liposomal vesicle size and the polydispersity index (PI) were determined with Submicron Particle Sizer (Nicomp Model 270, Santa Barbara, CA). The liposomal size was found to have mean diameter 200 nm. The PI of the liposomes was 0.85 ± 0.04. The encapsulation efficiency of NAC in DPPC-liposomes was 37 ± 1.5% after rehydration of liposomes. The encapsulation efficiency (E.E.) was calculated as follows:

E. E. = (Released NAC Conc. / Initial NAC Conc.)×100

The liposomes were shown to release 20% and 25% of its content in NAC after 24h incubation in PBS (4°C) and 37°C respectively. When incubated at 37°C in rat bronchoalveolar lavage an plasma, the liposomes, release respectively 30% and 40% of its content in NAC.

The NAC concentration was detected by spectrophotometry and the absorbance was measured at 412 nm

Antimicrobial activity of free and liposomal NAC

To test the antimicrobial activity of liposomal NAC on *P. aeruginosa* the MICs and MBCs were evaluated. The MICs and MBCs of all 21

strains of *P. aeruginosa* used in this study were determined by using the micro-broth dilution technique. Serial dilutions of F-NAC and L-NAC at (10000, 5000, 2500, 1250, 630, 310, 160, 80 and 40 mg/L) were prepared. In addition, bacteria were exposed to empty liposomes as well as NAC to test the effect of empty liposomes and NAC without encapsulation. Overnight cultures of bacteria suspensions in cationic Mueller Hinton broth (CMHB) were then added to reach a final concentration of 0.5 McFarland standards to each F-NAC and L-NAC concentrations [57,58]. Samples containing bacterial culture without the drug were included as positive controls. *P. aeruginosa* strains (PAO1, ATCC 25619, ATCC 27853, ATCC 10145PA-13572, PA-5, PA-M13641-2, PA-7, PA-13639-2, PA-3, PA-M13640, PA-11, PA-12, PA-M13639-1, PA-48913, PA-M26250, PA-1, PA-48912-2, PA-48912-1, PA-8 and PA-13641-1) were used. The MIC of F-NAC and L-NAC that prevented bacterial growth in the cultures were inspected after incubation at 37°C for 24 h and the MICs were recorded. The lowest concentrations of either F-NAC or L-NAC that resulted in more than 99.9% reduction in bacterial growth were recorded as MBCs [52]. All samples were prepared in triplicates and three separate experiments were performed.

Growth of PA-13572 in the presence of L-NAC at sub-MIC

The effect of F-NAC and L-NAC on the bacterial growth at sub-inhibitory concentrations was evaluated on clinically isolated *P. aeruginosa* strain (PA-13572) from CF patients. The clinically isolated PA-13572 growth was monitored for 24 h. A final concentration of 1.5×10^8 colony forming units (CFU)/mL was mixed with F-NAC and L-NAC at sub-MICs (1/2 and 1/4) and incubated at 37°C with rapid shaking. At time 1, 2, 3, 4, 6, 8, 12 and 24 h, turbidity was measured at OD_{600} [59]. The experiment was repeated three times in triplicates.

Time kill study

Time kill study was conducted to evaluate the microbial reduction by liposomal NAC compared to free NAC in regard to time length. In time kill experiments clinical isolate of *P. aeruginosa* (PA-13572) in a final concentration between 6 and 8 $\log_{10}$ CFU/mL was cultured in test tubes containing CMHB with either F-NAC or L-NAC at final concentration of 5000, 2500 and 1250 mg/L or with no drug as a control. These cultures were then incubated in a shaker incubator at 37°C for 2, 4, 6 and 24 h. At the end of each time period, tenfold serial dilutions were prepared with PBS (pH=7.4) and 100 µL samples were plated onto Mueller Hinton agar (MHA) plates in triplicate. The CFU at each time period were then counted after 18 h incubation at 37°C. Plates with 30–300 colonies were used for CFU counts as reported by Rukholm et al. [52]. Three separate experiments were performed in triplicate.

QS molecules production and β-galactosidase activities

We examined weather L-NAC or free NAC have quorum sensing inhibitory (QSI) effect, therefore, PA-13572 was grown overnight and (OD_{600}=0.13) bacterial solution was prepared in a 100 mL flasks. When the bacterial cell density doubled to an OD_{600}=0.26, subinhibitory concentrations of F-NAC and L-NAC were added to the bacterial suspension (1/16–1/4 the MICs). These flasks were then incubated at 37°C in shaker incubator rotating for 24 h. After 24 h, bacterial-NAC mixtures were centrifuged at 16,000×g for 20 min at 4°C and the supernatants were then filter sterilized (0.22 µM) as reported previously [60]. LB agar was prepared and cooled to 45°C, and β-D-galactopyranoside (20 mg/mL in dimethylformaldehyde) with *Agrobacterium tumefaciens* strain A136 cells equal to a density of 10^6 CFU/mL was added to the agar. This mixture was poured into Petri dishes, holes were punctured in the agar using a vacuumed device and 80 µl from treated bacteria and control supernatants were added to the holes. The plates were then incubated for 48 h at 30°C. AHL production was detected as blue-green pigment and documented by a digital camera. To quantify the level of AHLs in the bacterial supernatant, the ability of *P. aeruginosa* AHL signaling molecules to activate the release of β-galactosidase in *A. tumefaciens* (A136) was examined in this experiment as reported previously [61]. This bioassay was carried out starting with tubes containing 4 mL of A136 and 1 mL of supernatant incubated in a 30°C water bath for 5 h with rotation at 100 rpm. After incubation, the bacterial cell densities were read at OD_{600} and then centrifuged at 16,000×g for 15 min at 4°C. Supernatants were discarded and pellets were resuspended in 4 mL of Z buffer (0.06 M $Na_2HPO_4.7H_2O$, 0.04 M $NaH_2PO_4.H_2O$, 0.01 M KCl, 0.001 M $MgSO_4.7H_2O$, 0.05 M β-mercaptoethanol, pH 7.0). These cells were then permeabilized by adding a solution of 200 µl of chloroform and 100 µl of 0.1% sodium dodecyl sulphate. Then, 0.4 mL of *O*-nitrophenol-β-D-galactopyranoside (4 mg/mL in PBS) was added to the samples prior incubation. Samples were then incubated again until the development of yellow color and the time was recorded. To stop the reaction, 1 mL of 1M Na_2CO_3 was added. Optical density of the reaction samples were measured at 420 and 550 nm. Miller units of β-galactosidase were calculated as $[(1000 \times A_{420nm}) - (1.75 \times A_{550nm})] / (time \times volume \times A_{600nm})$ as described previously [62].

Production of PA-13572 virulence factors in the presence of L-NAC

To determine the effect of L-NAC on virulence factors, PA-13572 filtered supernatant was prepared as mentioned previously in the QS assay. For the chitinase assay, 1 mL of the filtered supernatant was mixed with 1 mL PBS and 5 mg of insoluble chitin azure and incubated at 37°C for 24 h with 200 rpm agitation. The samples were then centrifuged at 16,000×g for 10 min at 4°C in order to remove the insoluble chitin azure, absorbance was measured at OD_{290}. For the elastase assay, the filtered supernatant (1 mL) was mixed with 1 mL PBS and 20 mg of the insoluble elastin-congo red, and incubated with 200 rpm agitation for 24 h at 37°C. The absorbance was measured at OD_{495} after the insoluble elastin-congo red was removed by centrifugation at 16,000×g for 10 min at 4°C [29]. For lipase assay, 0.6 mL of the filtered supernatant was mixed with 0.6 mL 10% Tween 20 in Tris-buffer saline, 2 mL H_2O and 0.1 mL of 1 mol/L $CaCl_2$ and incubated with 200 rpm agitation at 37°C for 24 h. Turbidity of the samples were then measured at OD_{400} [59]. These experiments were repeated three times in triplicate and data was normalized by dividing the optical density by the cell density (OD_{600}). For the protease assay, Petri dishes were prepared with 2% agar containing 3% skimmed milk, 100 µl of the filtered supernatants were added to the well of the dishes and incubated at 37°C for 48 h. The clear zones were then measured [29]. The experiment was repeated three times in triplicates.

Effects of L-NAC on PA-13572 motility

The effect of L-NAC on PA-13572 motility was examined in the presence and absence of the sub-inhibitory concentrations of both F-NAC and L-NAC. Mueller–Hinton broth was prepared with 1.0% agar for twitching, 0.5% agar for swarming and 0.3% for swimming motility. For the twitching experiment, 1 mL of the bacteria OD_{600}=0.13 was stabbed into the agar-plastic interface, point inoculated on the surface for the swarming examination and stabbed into the center of agar for the swimming motility. Petri dishes were then incubated at 37°C for 12 h for swarming and swimming and diameters were measured. Twitching samples were incubated for 24 h, agar removed

and Petri dish was stained with 1% CV after it was air dried and pattern was measured [29,59,63]. These experiments were repeated three times in triplicate.

Antibiofilms activities

Biofilms experiments were assessed to determine whether the presence of L-NAC at sub-MICs (1/2–1/64 the MIC) could prevent the formation of biofilms on surfaces, while the biofilm reduction experiments allowed determination of whether L-NAC at sub-MICs (1/2-1/32 the MIC), MIC and 2×MIC could reduce young structured biofilms. Using a surface binding assay, biofilm formation was assessed in 96-well plates with F-NAC, L-NAC or without treatment as control. For the prevention experiment, PA-13572 (1:100 dilution of the 0.5 McFarland standards) were grown in the presence of F-NAC and L-NAC at sub-MICs for 18 h at 37°C and unadhered bacteria were washed off with PBS. Attached bacteria were stained with 1% CV, dissolved with 95% ethanol and the absorbance was read at OD_{590} using a spectrophotometer. The experiment was repeated three times in triplicate. For biofilm reduction, bacterial biofilms were allowed to structure on the surface for 18 h, unadhered bacteria were removed and structured biofilms were then incubated for an additional 18 h with F-NAC and L-NAC before measuring biofilm formation [29]. Effect of L-NAC on *P. aeruginosa* within mature biofilms was also tested as described previously [59]. Calgary Biofilm Device plates (CBD; MBEC Biofilms Technology Ltd., Calgary, Alta., Canada) were used for growing mature biofilms in this experiment. 24 mL of the PA-13572 strain (1.5×10^6 CFU/mL) was added to the Calgary biofilm device plates. This plate was then incubated in an incubator shaker rotating at 50 rpm at 37°C for 96 h to ensure equal distribution of medium in the troughs and adherence of bacteria to the pegs. Medium was replaced with fresh broth every 24 h to remove unattached bacteria. On the fifth day, biofilms formed on the pegs were rinsed with medium two times. For positive control, 3 pegs were removed with a sterile forceps and placed into a microcentrifuge tubes containing 1 mL PBS, which was then sonicated to detach bacterial biofilms from pegs into the PBS. The solution was then serially diluted (tenfold) and 100 µl of each dilution was plated on MHA plates and incubated for 24 h at 37°C for CFU counts. The remaining pegs were then submerged into a 96-well plate containing 200 µl of 10-fold dilutions of F-NAC and L-NAC. The plate was incubated for another 24 h at 37°C, peg lids were washed, pegs were removed and transferred to microcentrifuge as mentioned above, and CFU were counted after incubation for 24 h at 37°C. This experiment was repeated three times in triplicates.

Toxicity of L-NAC

Using traditional cell culture techniques, human lung carcinoma epithelial cell line A549 were grown to 85% confluence in 5% CO_2 at 37°C and maintained in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin. At confluence, cells were seeded into 24-well plates at a density of 2×10^4 cells/well and allowed to adhere overnight. On the next day, media was discarded and cells were washed with PBS to remove any traces of FBS and antibiotic. F-NAC and L-NAC were prepared in non-supplemented media at a serial dilution of 2500, 1250, 630, 310, 160, 80 and 40 mg/L, and added to the adhered cells. Untreated cells (non-supplemented media, no NAC added) were used as control. Plates were then incubated at 37°C in 5% CO_2 for 24 h. The wells were then washed twice with DMEM and exposed to a mixture of 500 µl of media and 100 µl of resazurin. Cells were then incubated in the dark overnight at 37°C in 5% CO_2. Solution were then transferred to cuvettes, absorbance was read at 570 nm using 600 nm as a reference wavelength. For the blank, a mixture of 500 µl media and 100 µl cell titer blue dye was used [64]. This experiment was repeated three times in triplicates.

In vitro effects of L-NAC on bacterial adhesion to human lung cells

The effects of L-NAC on PA-13572 adhesion to A549 cells were studied using two different procedures

Treatment of bacteria with F-NAC and L-NAC prior incubation with A549: In this experiment bacteria were exposed to F-NAC or L-NAC prior to incubation with A549 cells as described previously [64]. Briefly, A549 cells were grown to 85% confluence in 5% CO_2 at 37°C and maintained in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin, cells were then washed twice with PBS and incubated with non-supplemented media for 2 h prior to addition of bacteria. Bacteria (PA-13572) were grown over night in a shaker incubator at 37°C and adjusted to 0.5 McFarland standards in non-supplemented DMEM to avoid any interaction. Bacterial aliquots were incubated in a shaker incubator for 3 h at 37°C with F-NAC and L-NAC in final concentrations of 2500, 1250, 630, 310 and 160 mg/L. Treated bacteria were added onto cell monolayers for 3 h at 37°C supplemented with 5% CO_2. Non treated cells were also incubated to be used as positive control. After incubation, cells were washed five times with PBS to remove any non-adhered bacteria. Using a sterilized cell lifter (Costar, Fisher Scientific) cells were removed and re-suspended in PBS, 100 µl of each sample was plated on MHA plates in triplicates. Agar plates were then incubated for 24 h at 37°C. Bacterial colonies were counted, to determine the number of adhered bacteria.

A549 treatment with F-NAC and L-NAC prior exposure to bacteria: In this experiment A549 cells were grown as mentioned previously, and then they were treated with F-NAC and L-NAC in final concentrations of 2500, 1250, 630, 310 and 160 mg/L for 3 h prior to bacterial exposure. After incubation cells were washed twice with non-supplemented media to remove any traces of drug. PA-13572 were grown over night in a shaker incubator at 37°C and adjusted to 0.5 McFarland standards in non-supplemented DMEM. Bacterial aliquots were added to the cells monolayers and incubated for another 3 h to allow bacteria to adhere. The same procedures were applied to evaluate bacterial adhesion to the lung cells. Both experiments were repeated three times in triplicate.

Data analysis

The data were represented as mean ± S.E.M. of three separate experiments. All groups were compared by one-way ANOVA method using GraphPad Prism. Probability (*P*) values of $^*P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$ were reported as statistically significant.

Results

Antimicrobial activity of free and liposomal NAC

The antimicrobial activity of liposomal NAC on *P. aeruginosa* was evaluated. MIC and MBC values of F-NAC and L-NAC against clinical isolates and laboratory strains of *P. aeruginosa* are shown in Table 1. The MIC values for L-NAC were significantly lower than similar concentrations of F-NAC for the clinically isolated strains of *P. aeruginosa* (PA-13572, PA-5, PA-M13641-2, PA-3, PA-11, PA-12, PA-48913, PA-M26250, PA-1 and PA-48912-2) which presented an MIC of 1250 mg/L for L-NAC verses 5000 mg/L for F-NAC. However, there were no differences in the MIC between F-NAC and L-NAC on *P. aeruginosa* strains (PAO1, ATTC 25619, ATTC 27853, ATTC 10145

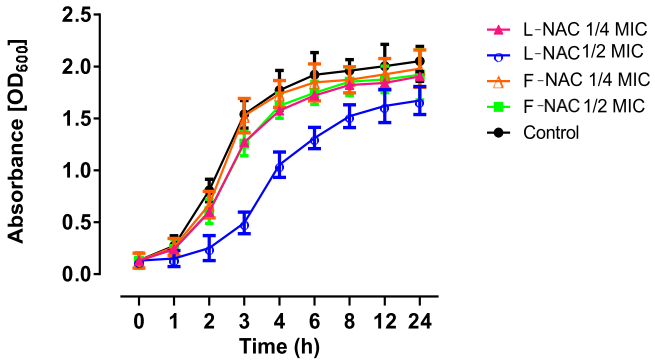

Figure 1: Growth curve of *P. aeruginosa* PA-13572. *P. aeruginosa* was exposed to media without antibiotics (**filled circles**), in the presence of sub-inhibitory concentrations of 1/2 MIC of L-NAC [625 mg/L] (open circle), 1/4 MIC of L-NAC [312.5 mg/L] (filled triangle), 1/2 MIC of F-NAC [2500 mg/L] (filled **squares**), or 1/4 MIC of F-NAC [1250 mg/L] (open triangle).The experiment was performed three times with means shown.

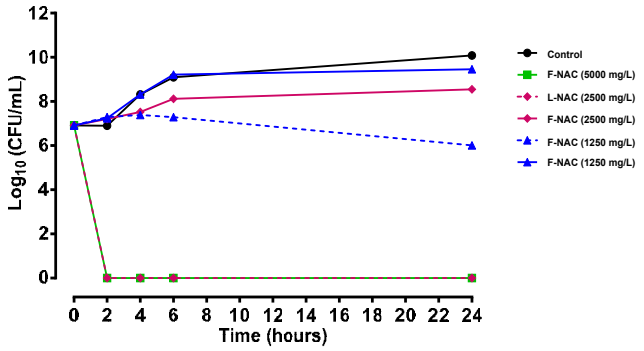

Figure 2: Time kill curves for PA-13572. *P. aeruginosa* exposed to 5000, 2500 and 1250 mg/L of free NAC as well as 2500 and 1250 of liposomal NAC for time periods of 2, 4, 6 and 24h. After each time period samples were diluted and then cultured on Petri dishes, these dishes were incubated at 37°C for 18 h, CFU were counted. The results represented the mean ± S.E.M of three independent experiments in triplicates.

PA-7, PA-13639-2, PA-M13640, PA-M13639-1, PA-48912-1, PA-8 and PA-13641-1) where they all exhibited an MIC of 5000 mg/L. The MBC values of F-NAC for all strains were either equal to or two times higher the MBC for L-NAC. The MIC values of DPPC and NAC were not different than F-NAC and empty liposomes had no antimicrobial activity.

Growth of PA-13572 in the presence of L-NAC at sub-MIC

The effect of F-NAC and L-NAC on the bacterial growth at sub-inhibitory concentrations was evaluated on several clinically isolated *P. aeruginosa* from CF (data not shown), then we chose one strain (PA-13572), which was affected mostly by NAC and had a significant different between the F-NAC and L-NAC to be used as a model of *P. aeruginosa* for the rest of the experiments. The growth curves for PA-13572 grown in presence or absence of sub-MICs (1/4–1/2 the MICs) for both F-NAC and L-NAC were determined to concentrations that do not inhibit bacterial growth (Figure 1). Since the sub-MICs 1/4 did not reduce the growth of bacteria compared to the control, all the experiments that involve QS, VF and bacterial motility were done using concentrations of 1/16-1/4 the MICs of F-NAC or L-NAC.

Time kill study

In this study a maximum of 24 h exposure of PA-13572 to 5000, 2500 and 1250 mg/L of F-NAC or L-NAC was studied. The results of the time kill curves for clinically isolated PA-13572 *P. aeruginosa* are shown in Figure 2. At 5000 mg/L of F-NAC was able to eradicate bacterial growth after 2 h. However, at 2500 mg/L liposomal *N*-acetylcysteine was completely able to eradicate bacteria at 2 h, whereas free *N*-acetylcysteine at 2500 mg/L did not affect the bacterial growth throughout the study. At 1250 mg/L, L-NAC demonstrated a large drop (4 $\log_{10}$ CFU/mL) in colony counts after 24 h, whereas F-NAC did show bactericidal effect.

QS molecules production and β-galactosidase activities

In Figure 3, the production of AHL molecules of *P. aeruginosa* were confirmed by the blue-green pigmentation around the edges of the wells in LB agar. The maximum blue-green color was present by the positive control (where bacteria were incubated with no NAC). It appears that both F-NAC and L-NAC reduced AHL production, but they did not prevent the production completely. However, L-NAC reduced AHL production at sub-inhibitory concentrations that were

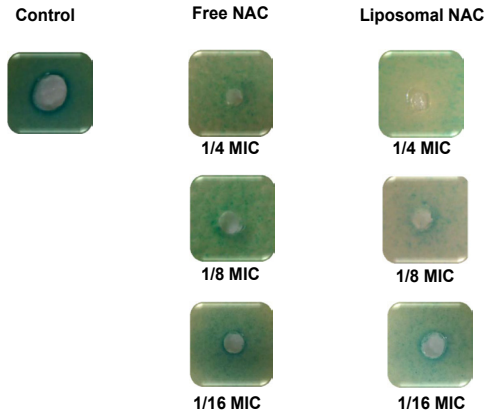

Figure 3: Sub-MICs effects on QS. The production of AHL molecules was observed after 48 h incubation at 30°C in the presence of sub-MICs (1/16-1/4) concentration of both free and liposomal NAC.

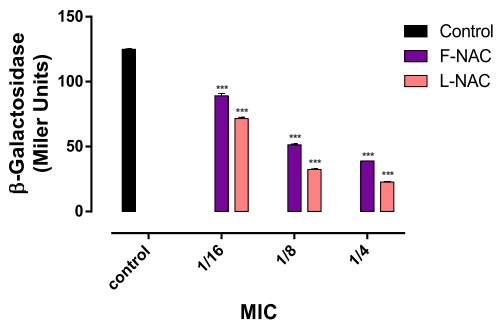

Figure 4: Sub-MICs effects on QS by β-galactosidase activity**:** PA-13572 samples were treated with 1/16, 1/8 and 1/4 MICs of both F-NAC and L-NAC, supernatants were collected and incubated with *A. tumefaciens* (A136) and β-galactosidase activities were calculated in miller units for three independent experiments each were prepared in triplicates. These data represents the mean ± S.E.M. for the three experiments, *P* values were considered significant when compared to control: ***$P<0.001$.

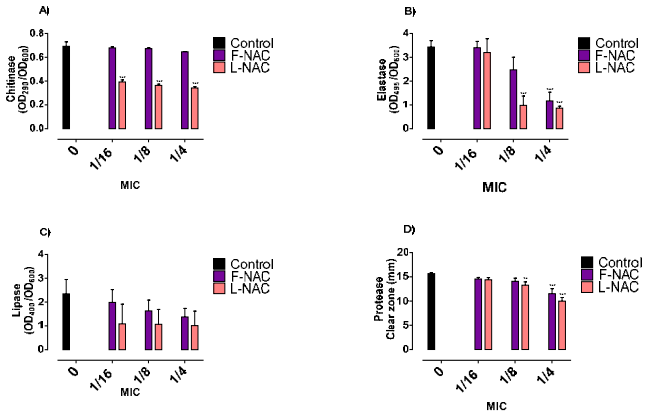

Figure 5: The production of virulence factors in the presence of L-NAC. Subinhibitory concentrations of both F-NAC & L-NAC were added to PA-13572 and incubated for 24 h. The supernatants were tested for **(A)** chitinase, **(B)** elastase, **(C)** lipase, and **(D)** protease. *P* values were considered significant when compared to control *** $P<0.001$, ** $P<0.01$. All experiments were repeated 3 times in triplicates.

4 times lower than F-NAC. At 1/4 MIC of L-NAC, the color intensity was pale blue compared to 1/4 MIC of F-NAC. At 1/8 MIC of F-NAC, the production of blue pigment ring around the edge was darker than 1/8 MIC of L-NAC. The levels of β-galactosidase activity in response to AHL indicated decreasing levels of AHL signaling molecules released from *P. aeruginosa* cells exposed L-NAC compared to F-NAC (Figure 4). For example, exposing bacteria to F-NAC at 1/4 MIC resulted in 38.94 ± 0.06 Miller unit, whereas L-NAC significantly reduced AHL signaling molecules, based on β-galactosidase activity, to 22.7 ± 0.41 Miller unit compared 124.9 ± 0.34 Miller unit resulted in control. L-NAC at 1/8 MIC reduced β-galactosidase activity to 32.49 ± 0.39 Miller unit, whereas F-NAC only exhibited reduction of β-galactosidase activity to 51.32 ± 0.63 Miller unit. L-NAC was significantly more active in reducing AHL production than F-NAC at all subinhibitory concentrations ($P<0.001$).

Production of PA-13572 virulence factors in the presence of L-NAC

The effects of F-NAC or L-NAC at 1/4–1/16 the MIC on *P. aeruginosa* virulence factors including chitinase, elastase, lipase and protease were evaluated. F-NAC did not reduce the production of chitinase at all MICs compared to control (Figure 5A). On the other hand, L-NAC was able to reduce the level of chitinase significantly at all concentrations compared to control ($P<0.001$), (Figure 5A). L-NAC was able to reduce the level of elastase significantly at 1/4 MIC compared to control. Furthermore, elastase activity was reduced significantly by L-NAC at concentration eightfold lower than F-NAC (1/8 *versus* 1/4) ($P<0.001$) (Figure 5B). Although, F-NAC and L-NAC reduced the level of lipase, the reduction was not significant. However, L-NAC was more effective at concentration fourfold lower than the F-NAC at all subinhibitory concentrations compared to control (Figure 5C). For protease, L-NAC was able to reduce protease at a concentration fourfold lower than F-NAC (1/4 L-NAC *versus* 1/4 F-NAC) (Figure 5D). Furthermore, L-NAC at concentration 16-fold lower than F-NAC, exhibited a significant reduction in protease activity (1/8 L-NAC *versus* 1/4 F-NAC) ($P<0.01$).

Effects of L-NAC on PA-13572 motility

We examined bacterial motility, including twitching, swimming and swarming in the presence of subinhibitory concentrations of either free or liposomal *N*-acetylcysteine. F-NAC at 1/4 MIC did not reduce swarming of *P. aeruginosa* PA-13572 significantly compared to control (Figure 6A). However, L-NAC at 1/8 MIC reduced *P. aeruginosa*

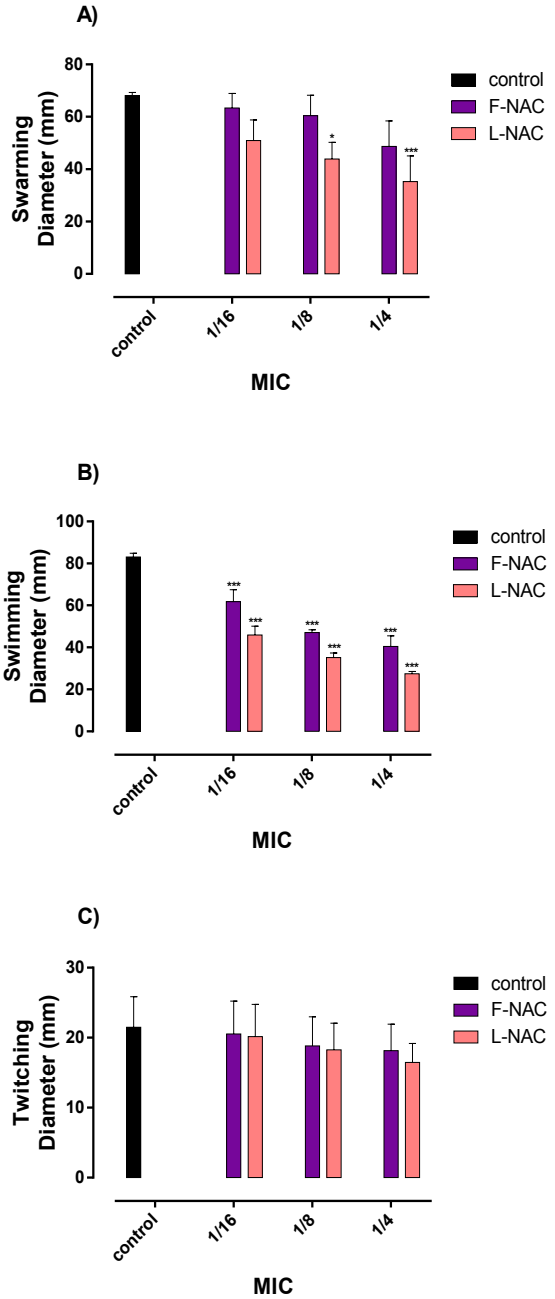

Figure 6: The effects of L-NAC on *Pseudomonas aeruginosa* motility. Sub-inhibitory concentration (1/16, 1/8 and 1/4) of both formulations (F-NAC & L-NAC) was added to different concentrations of agarose plates and motility was examined. **(A)** Swarming (0.5% agarose w/v), **(B)** swimming (0.3% agarose w/v) and **(C)** twitching (1% agarose w/v). *P* values were considered significant when compared to control *** $P<0.001$, ** $P<0.01$, * $P<0.05$. All experiments were repeated 3 times in triplicates.

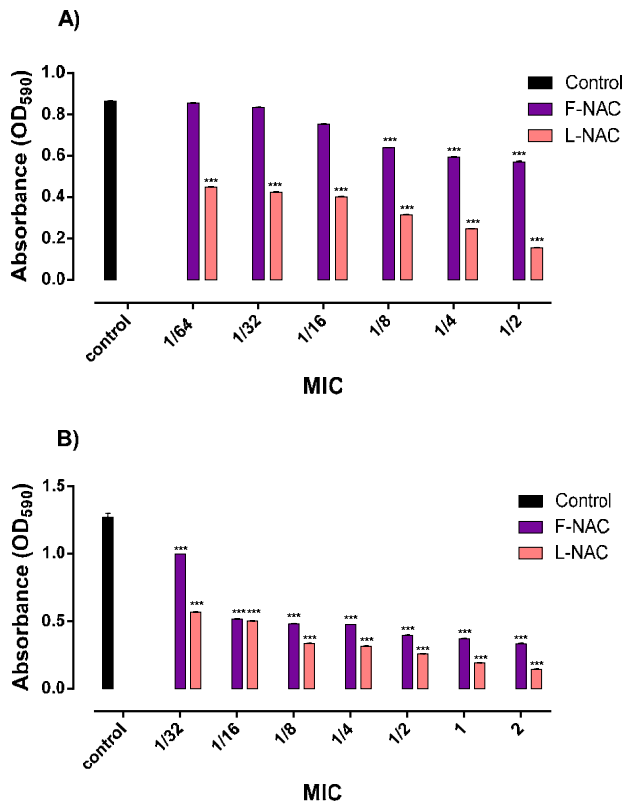

Figure 7: Prevention of biofilm formation: **(A)** and biofilm reduction (1-day-old biofilms) **(B)** in the presence of free or liposomal NAC was examined. At the prevention of biofilm formation experiment, PA-13572 were grown in 96-well plates at 37°C for 18 h in the presence of different concentrations (1/2–1/64 the MIC) of both F- and L-NAC. Nonattached bacteria were washed with PBS while attached bacteria were stained with CV and absorbance read at 590 nm was measured. For the biofilm reduction, PA-13572 were grown as above without any treatment then treated with sub-MIC or concentration above MICs F- and L- NAC then incubation for another 18 h. Same washing and staining steps were applied and absorbance was measured as described above. All experiments were repeated three times in triplicate. *P* values were considered significant when compared to control *** $P<0.001$.

swarming significantly ($P<0.05$) compared to control. Furthermore, the reduction of bacterial swarming was more significant at 1/4 MIC of L-NAC ($P<0.001$) (Figure 6A). On the other hand, swimming was reduced by all concentrations for both F-NAC and L-NAC compared to control (Figure 6B). However, L-NAC at concentration eightfold lower than F-NAC was able to significantly reduce swimming (1/16 L-NAC *versus* 1/8 F-NAC) ($P<0.001$). L-NAC at 1/8 MIC also reduced the swimming pattern of *P. aeruginosa* to 35 ± 2.2 mm, whereas F-NAC at concentration fourfold higher than L-NAC reduced *P. aeruginosa* swimming to 40 ± 5.2 mm (1/8 L-NAC *versus* 1/4 F-NAC). F-NAC and L-NAC did not affect *P. aeruginosa* twitching significantly. However, L-NAC at 1/4 MIC exhibited 16.5 ± 2.7 mm compared to 21.5 ± 4.4 mm of control, whereas F-NAC exhibited 18.2 ± 3.8 mm (Figure 6C).

Anti-biofilms activities

Polystyrene 96-well plates were used to study the prevention and reduction of *P. aeruginosa* biofilm complexes. There was no biofilm formed at the MIC, due to killing of the bacteria (data not shown) and a gradual prevention of biofilm formation no the surface was noticed when bacteria were incubated with concentration below the MIC (1/64-1/2) of F-NAC and L-NAC. L-NAC at all tested concentrations were able to reduced attachment of biofilm onto plastic surfaces, while only three concentrations of F-NAC (1/8-1/2 MIC) were able to reduce the formation compared to control (Figure 7A). However, L-NAC was more significant in preventing formation of the biofilm. For instance, L-NAC at concentration 128-fold lower than F-NAC was able to reduce the biofilm formation significantly compared to the free formulation (1/64 L-NAC *versus* 1/2 F-NAC) ($P<0.001$). The reduction effects of F-NAC and L-NAC on young structured biofilms are depicted in (Figure 7B). Both formulations had a significant effect on reducing young structured biofilm; however, liposomal NAC was more significant in biofilm reduction. For example, L-NAC at concentration 16-fold lower than F-NAC (1/8 L-NAC *versus* 1/2 F-NAC) was able to reduce young biofilm structure significantly than the free formulation ($P<0.01$). L-NAC at 1/4 MIC was more significant in reducing young biofilm compared to 1 MIC of F-NAC ($P<0.01$). The L-NAC and F-NAC activities against *P. aeruginosa* within mature biofilm community are shown in Figure 8. L-NAC was significantly better than F-NAC in reducing bacterial counts within mature biofilms compared to control and free formulation ($P<0.001$). For instance, L-NAC at 2500 mg/L was able to reduce bacterial community significantly compared to F-NAC at 2500 mg/L ($P<0.001$). L-NAC at 630 mg/L was more significant in reducing bacterial counts compared to control and free formulation ($P<0.001$).

Toxicity of L-NAC

L-NAC formulation had no toxicity effect on A549 cells. However, a slight drop of cells viability was noticed when cells were treated with 630, 1250, 2500 and 5000 mg/L of F-NAC, where viability was 98 ± 0.31%, 98 ± 0.05%, 98 ± 0.05% and 97 ± 0.61% respectively. Cells were maintained in full viability (100% viable) at all concentrations when treated with L-NAC (Table 2).

In vitro effects of L-NAC on bacterial adhesion to human lung cells

Treatment of bacteria with F-NAC and L-NAC prior incubation with A549: The bacterial colony counts when bacteria were treated with 2500, 1250, 630, 310 and 160 mg/L concentrations of both F-NAC and L-NAC are represented in Figure 9A. Bacterial adhesion to lung cells were gradually reduced as the concentrations increased, L-NAC was significantly able to reduce *P. aeruginosa* adhesion at all concentrations

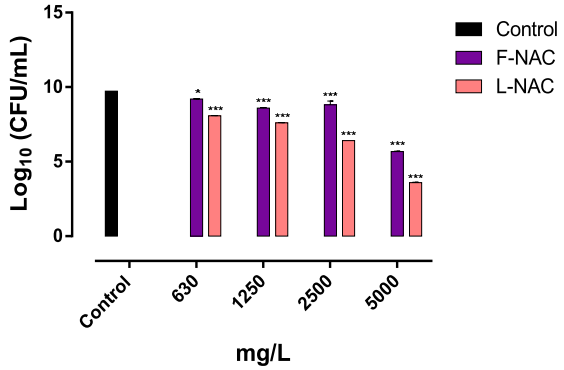

Figure 8: Liposomal NAC antibiofilm effect on PA-13572 L-NAC was able to eliminate mature biofilms (6-day-old biofilms) significantly, *P* values were considered significant when compared to control ***$P<0.001$, **$P<0.01$, *$P<0.05$. All experiments were repeated 3 times in triplicates.

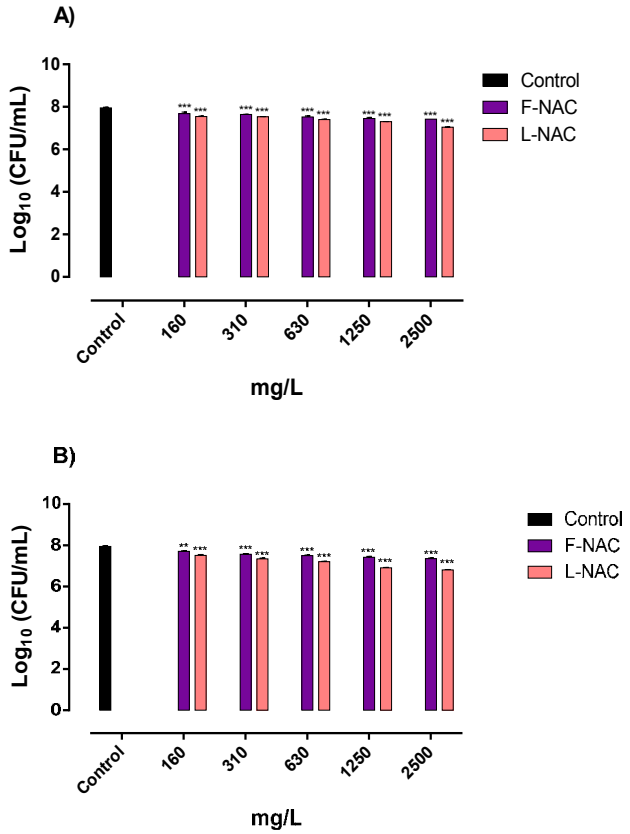

Figure 9: Bacterial adhesion to lung cells, **(A)** bacteria were pretreated with different concentrations of F/L-NAC, and then were added to A549 monolayers. Columns present the CFU counts when bacteria were treated. **(B)** A549 cells were treated with different concentrations of F/L-NAC and then bacteria were added to the cells. Columns here present the CFU counts when cells were treated. Data are the mean ± S.E.M. of three independent studies. *P* values were considered significant when compared to control *** $P<0.001$, ** $P<0.01$, * $P<0.05$. All experiments were repeated 3 times.

compared to control ($P<0.001$). However, L-NAC at 2500 mg/L was more significant in reducing bacterial adhesion to lung cells by one log compared to F-NAC at 2500 mg/L ($P<0.001$).

A549 treatment with F-NAC and L-NAC prior exposure to bacteria: The bacterial colony counts when lung cells were pre-treated with 2500, 1250, 630, 310 and 160 mg/L F-NAC and L-NAC prior exposure to bacteria are represented in Figure 9B. All L-NAC concentrations were significantly effective against bacterial adhesion ($P<0.001$) compared to control. L-NAC at the concentration of 2500 mg/L was able to reduce adhesion by more than one log (1.14) compared to the same concentration of F-NAC.

Discussion

P. aeruginosa is the most prevalent bacteria in CF; despite the use of antibiotic therapies it remains to be the most challenging bacterial infection linked to poor clinical outcomes due to the high rate of antibiotic resistance [14]. Hence, other drugs with possible antimicrobial activities are being tested for the aim of improving health status of CF patients.

NAC is one of these new promising therapies, where it has been established that NAC can enhance the efficacies of some antibiotics such as carbenicillin and ticarcillin against *P. aeruginosa* [65]. It was also reported that NAC is able to inhibit the growth of both Gram-positive and Gram-negative bacteria [47], have a potential to decrease biofilm formation and reduce bacterial infection [66].

In this *in vitro* study we extensively tested the antimicrobial effects of L-NAC alone on the clinically isolated PA-13572. We demonstrated that liposomes were capable of enhancing NAC's antimicrobial activities against the resistant strain of *P. aeruginosa* virulence factors and biofilm. To analyze the antimicrobial activity of L-NAC against resistance *P. aeruginosa*, we initially validated their effectiveness against planktonic bacteria (Table 1). The results from the MIC and MBC experiments showed that the L-NAC was more effective than the F-NAC against more than 57% of the strains involved in this study. The MIC of F-NAC was found to be 5000 mg/L in most cases. This is in agreement with results found by Abbas et al. [23] who reported 2500-5000 mg/L for MIC values. Our L-NAC formulation was highly effective against *P. aeruginosa* strains, where MIC values for L-NAC were reduced by twofold compared to F-NAC. Our MBC values for F-NAC were between 5000 to >10000 mg/L, which are consistent to the result reported by Abd El-Aziz et al. [66] who tested *P. aeruginosa* isolate and found an MBC of >8000 mg/L. The MBCs for F-NAC were in a range of 2500 to >5000 mg/L, however, L-NAC were more effective in reducing the MBC values by twofold to fourfold compared to the free form of NAC. The different in MICs and MBCs between F-NAC and L-NAC might be due to liposomes fusion with the outer membrane of *P. aeruginosa*; which leads to higher concentration of NAC inside the bacterial cell, therefore; more effective results [67].

To confirm our present MIC and MBC findings, time kill assays were performed (Figure 2). This was also carried out to confirm the ability of L-NAC to eliminate *P. aeruginosa* growth *in vitro*. Even though both F-NAC and L-NAC were able to kill bacterial after only 2 h exposure; L-NAC killed bacteria with concentration twofold less than F-NAC (2500 mg/L *versus* 5000 mg/L), this confirms our MBC values for both F-NAC and L-NAC. Despite that bacterial proliferation was expected for the 1×MICs for F-NAC, F-NAC exhibited a bactericidal effect at 1 x MIC due to the fact that MIC and MBC values for F-NAC were equal to 5000 mg/L for the bacterial strain used in this study (PA-13572). However, the L-NAC at 1250 mg/L inhibited bacterial growth after 24 h of incubation with 4 log reduction in bacterial growth compared to control and 3 log reduction compared to the same concentration of F-NAC. Moreover, previous study reported the bactericidal effect of F-NAC on a number of bacterial strains and showed that NAC at 80000 mg/L was bactericidal on time kill study, whereas L-NAC in our study was bactericidal at concentration 32-fold lower than the reported concentration [37]. These antibacterial activities of NAC may be due to the fact that it acts competitively to inhibit amino acid (cysteine) utilization or, by reacting with bacterial cell proteins using the sulfhydryl group [43,65].

To evaluate whether L-NAC was able to control infection, the effectiveness of L-NAC to inhibit *P. aeruginosa*'s QS was determined. The QSI effects were investigated by monitoring the production of AHLs, which were attenuated by L-NAC at concentration four times lower than the free NAC in qualitative (Figure 3) and quantitative studies (Figure 4). Cugini et al. [68] have explained NAC's ability to inhibit QS in a very interesting study. They have stated that reactive oxygen species (ROS) are responsible for activating the QS pathway, thereby NAC was able to block QS pathway by suppressing hydrogen peroxide and RhIR activity (a transcriptional regulator for LasR (the master QS regulator). It was also able to stop the production of pyocyanin (one of

Bacterial strains	Free N-Acetylcysteine (mg/L)		Liposomal N-Acetylcysteine (mg/L)	
	MIC	MBC	MIC	MBC
PA ATCC 25619	>10000	>10000	>500	5000
PA 13572	5000	5000	1250	2500
PA 5	5000	5000	1250	2500
PA 13641-2 (M)	5000	5000	1250	2500
PA 7	5000	10000	5000	>5000
PA 13639-2	5000	10000	5000	>5000
PA 3	5000	5000	1250	2500
PA ATCC 27853	5000	10000	5000	5000
PA 13640 (M)	5000	>10000	5000	>5000
PA 11	5000	5000	1250	2500
PA ATCC 10145	5000	>10000	5000	>5000
PA 12	5000	5000	1250	2500
PA 13639-1 (M)	5000	>10000	5000	>5000
PA 48913	5000	5000	1250	2500
PA 26250 (M)	5000	5000	1250	2500
PA 1	5000	5000	1250	2500
PA 48912-2	5000	5000	1250	2500
PA 48912-1	5000	10000	5000	5000
PA 8	5000	>10000	5000	5000
PA 13641-1	5000	10000	5000	5000
PAO1	5000	10000	5000	5000

Table 1: *In vitro* activities of free and liposomal NAC against *P. aeruginosa* strains.

	Cell viability (%)		
Concentration (mg/L)	Control	Free NAC	Lipo NAC
0	100 ± 0.00	100 ± 0.00	100 ± 0.00
160	-	100 ± 0.00	100 ± 0.00
310	-	100 ± 0.00	100 ± 0.00
630	-	98 ± 0.31	100 ± 0.00
1250	-	98 ± 0.05	100 ± 0.00
2500	-	98 ± 0.02	100 ± 0.00
5000	-	97 ± 0.61	100 ± 0.00

Table 2: Cell viability of epithelial lung cells exposed to free or liposomal NAC.

many toxins produced by *P. aeruginosa* that is stimulated by hydrogen peroxide). They also suggested that ROS might stimulate VF production so NAC might decrease *P. aeruginosa* virulence by decreasing the ROS level or by preventing the activation of this regulatory pathway [68]. Since the AHL production plays an important role in regulating VF [29], reduction in AHL signaling molecules results in decreasing of VF secreted by bacterial cells [69]. In this study we demonstrated that encapsulation of NAC into liposomes resulted in improving the efficacy of NAC in inhibiting VF, where L-NAC was able to reduce chitinase, elastase and protease significantly (P<0.001) at sub-MICs. This support the hypothesis stated by Cugini et al. [68] that ROS stimulates the production of VF and since NAC is able to suppress the level of ROS, it was able to decrease the level of VF produced by *P. aeruginosa*.

Usually *P. aeruginosa* resist antibiotic treatment when it attaches to respiratory epithelial cells using their pili and flagella motility by forming biofilms [70]. Multiple pili generally exist on the surface of *P. aeruginosa* as small filamentous and they are responsible of rapid colonization of the airway. Flagella complex also presents on the surface of *P. aeruginosa* and play a major role in its pathogenesis where they are the main motile for Gram-negative bacteria and considered to be very immunogenic [71]. When PA-13572 was inoculated on the agar, type IV pili designated as "twitching" was not significantly inhibited (Figure 6). However, flagellum were attenuated significantly by L-NAC at 1/4 MIC (compared to the same MIC of F-NAC), designated as "swarming (P<0.01) and swimming (P<0.001)". Since attenuation of motility is valuable in moderating biofilm formation and bacterial adhesion to lung cells, NAC was evaluated in terms of its ability to prevent biofilm formation (biofilm attachment to plates), biofilm reduction and eradication of bacteria within mature biofilm. NAC was able to prevent *P. aeruginosa* biofilm formation at concentration as low as 1/64 the MIC for both F-NAC and L-NAC (Figure 7A), both were significantly reduced compared to control; and L-NAC was significantly better than F-NAC when compared to the same group (P<0.001). Concentrations above MIC were used for the aim of studying the biofilm reduction effects of L-NAC on young biofilms, where biofilms usually develop more resistance to the formulation compared to planktonic cells (Figure 7B). We demonstrated 92% less biofilm (1-day-old PA-13572 biofilms) at 2 MICs (2500 mg/L) of L-NAC (P<0.001). Our finding demonstrated higher biofilm inhibition compared to other investigator, Abd El-Aziz et al. [66] where they got 67.12% less biofilms at 2000 mg/L NAC. Mature (6-day-old) biofilms were grown on polystyrene pegs of the CBD plate to investigate eradication effects of L-NAC on more resistance form of biofilms. We have established (Figure 8) that L-NAC was able to reduce bacterial counts in mature biofilms by 46% at 2 times the MBC (5000 mg/L). Quah et al. [42] have reported that they needed 3 times the MBC of NAC to produce the maximum biofilm reduction for *E. faecalis*. The enhance delivery of NAC might be due to penetration of liposomal formulation into biofilms structure. A study reported that amikacin encapsulated in 300 nm liposomes consist of DPPC and cholesterol diffused more easily than 1 μm liposomes through biofilms structure [72]. Liposomes used for this study was 200 nm in size, which offers better penetration of liposomal NAC into biofilms. Once NAC released into biofilms, it might be interfered with exopolysaccharides (EPS) production, which is considered to be one of the most important components in biofilms, by two possible ways. Either that NAC's sulfhydryl group disrupt the disulfide bonds of the bacterial enzymes responsible of EPS production, or due to NAC's antioxidant properties

which indirectly effects bacterial cell metabolism and EPS production [46,47].

To investigate the anti-adhesive capability of L-NAC, we examined the effects of this formulation on cell viability. Human lung cancer cells A549 maintain 100% viability when exposed to all L-NAC concentrations used (Table 2). On the other hand, when exposed to F-NAC cells were less viable. This might be due to the ability of liposomes to reduce toxicity of drugs by decreasing their availability within the cell membrane; thus moderates cell toxicity [73]. Then we studied the ability of L-NAC to reduce bacterial adhesion to lung cells (A549) *in vitro*. L-NAC reduced bacterial adhesion to A549 monolayers significantly in dose-dependent manners compared to F-NAC (Figure 9). This support our motility experiments finding were flagella, which are responsible of *P. aeruginosa* adhesion to epithelial cells, were significantly reduced by L-NAC. We also have noticed that pretreatment of cells with L-NAC (Figure 9B) gave better protection to lung cells against bacterial adhesion compared to the results of the presence of NAC during growth of bacteria (Figure 9A). This might be due to the ability of NAC to increase the antioxidant lung cell defenses by elevating the levels of glutathione, an important antioxidant, and lowering the number of inflammatory cells [74,75]. We only could find few studies representing the effects of NAC on bacterial adhesion to epithelial cells; one study was by Riise et al. [76] where they have established that NAC was able to reduce adhesion of *H. influenzae* to oropharyngeal epithelial cells by 50% when bacteria were incubated with 20 mg/mL. Another study represented by Zheng et al. [77] established 54% less attachment of *M. catarrhalis* to pharyngeal epithelial cells when cells where treated with 10 mg/mL of NAC, and they found no effects on the attachment when *M. catarrhalis* were treated with NAC. However, we could come with agreement with both studies that NAC proved to possess antiadhesive effects.

The bacteriostatic effect of NAC is still relatively unknown; more studies on the bacterial responses to NAC may open doors to the use of this compound as an anti-biofilm agent in the future. In this study we demonstrated dramatic effects of NAC on biofilms and attachment of bacteria, this might be related to the –SH group which react with the bacterial proteins' disulfide bond causing irreversible damage to the bacterial proteins that are responsible for the metabolism and growth [78]. Another possibility is the ability of NAC to reduce EPS, a major component in biofilm, which leads to the changing characters of the bacterial surface and texture therefore unable *P. aeruginosa* to grow in large microcolonies [47] and to anchor to surfaces [79,80].

In conclusion, our results revealed the great antimicrobial effects of NAC on *P. aeruginosa*. Liposomes increase the therapeutic efficacy of NAC when encapsulated. This liposomal formulation may serve as a new strategy for the treatment of the serious problem of biofilm formation and bacterial resistance to antibiotics after conducting *in vivo* animal models and clinical trials to demonstrate the efficacy and safety of our formulation in patients.

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