

**COORDINATING GENE THERAPIES OF CYSTIC FIBROSIS AIRWAY
DISEASE WITH ANTI-INFLAMMATORY AND ANTIMICROBIAL
CATIONIC GLUCOCORTICOIDS**

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Melissa Myint

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ABSTRACT

COORDINATING GENE THERAPIES OF CYSTIC FIBROSIS AIRWAY DISEASE WITH ANTI-INFLAMMATORY AND ANTIMICROBIAL CATIONIC GLUCOCORTICOIDS

Melissa Myint

Scott L. Diamond

Certain sterol-based cationic lipids demonstrate both anti-inflammatory and antimicrobial characteristics in addition to their abilities to facilitate gene transfer. These characteristics are particularly suited for enhancing potential gene therapies of cystic fibrosis (CF) airway disease, a condition characterized by chronic infections and concomitant inflammation. Unfortunately, however, animal models of CF do not show the airway disease typically seen in humans, so *in vivo* efficacies of potential therapies are difficult to assess. In this work, three mouse models were established to evaluate adeno-associated virus (AAV) gene transfer in an infected airway environment. *Bordetella bronchiseptica* RB50 was used in a chronic, non-lethal respiratory infection model with C57BL/6 mice. Administration of an AAV vector on day 2 of this infection resulted in an approximate three-fold reduction of reporter gene expression, compared to that observed in uninfected controls. Postponement of AAV administration to day 14 resulted in an even greater (eight-fold) reduction in gene expression. Separately, *Pseudomonas aeruginosa* PAO1 was used to infect surfactant protein D or surfactant protein A knockout mice to establish acute infections. Reporter gene expression was approximately ~2.5-fold lower in these infected mice than in uninfected mice, when AAV was administered on day 2 of infection. Interestingly, when AAV administration was postponed to day 9 of infection in the surfactant protein D

knockout model, partial regain in expression was observed. In this case, infection resulted in only a two-fold (vs ~2.5-fold) reduction in gene expression. To reverse these negative effects on gene transfer, six new sterol-based cationic lipids were synthesized and characterized. Of the six lipids tested, two, budesonide spermine and disubstituted budesonide spermine, showed particularly promising *in vitro* activities and were used to establish proof-of-concept efficacy in an acute infection model. PAO1-infected mice received lipids on day 1 of infection and AAV 24 hours later on day 2. When reporter gene expression was measured one week later, only a ~1.5-1.9-fold reduction in gene expression, compared to the ~2.7-fold reduction seen with untreated controls. Results demonstrate that treatment with dual functioning anti-inflammatory, antimicrobial cationic glucocorticoids can be coordinated with CF gene therapies to restore loss of transduction efficiency caused by active infection.

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1.0 Introduction

1.1 Cystic Fibrosis

Cystic fibrosis is the most common lethal genetic disorder amongst Caucasians, affecting approximately 1 in every 2000 people [1]. The disease is caused by a single defective gene, specifically a mutated *CFTR*, which encodes for the cystic fibrosis transmembrane conductance regulator (CFTR). The CFTR is a transmembrane protein channel that is responsible for regulating liquid volumes at epithelial surfaces by transporting anions, namely chloride and bicarbonate anions, across the cell membrane as necessary. In individuals with CF, this protein is either missing or defective, resulting in osmotic imbalances that produce an unusually thick and extremely viscous mucus layer at epithelial surfaces (Figure 1.1) [2–4].

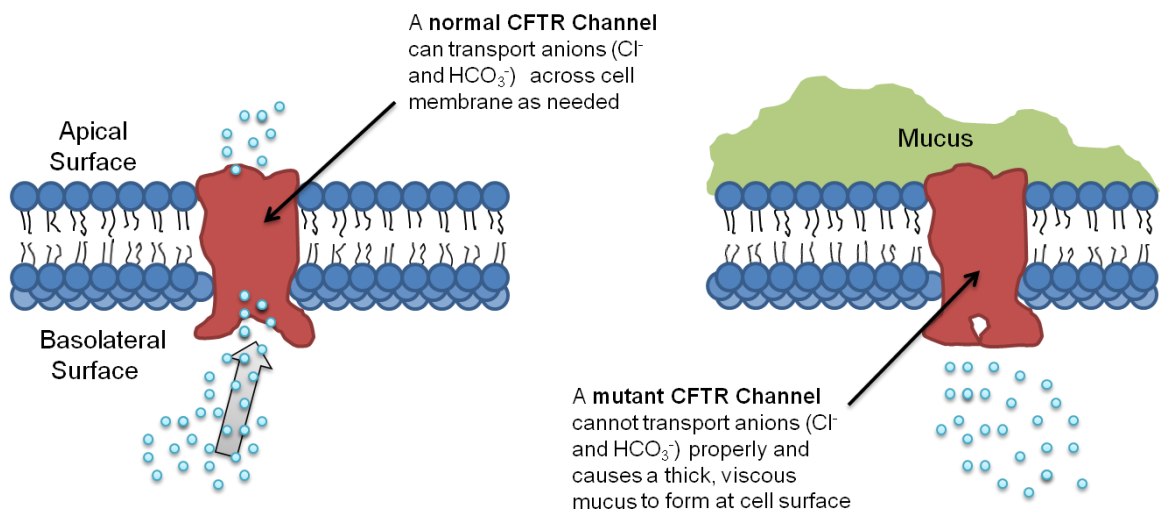


Figure 1.1: A depiction of a normal and mutant CFTR protein channel.

Adapted from [5]. Without proper transport of anions, osmotic forces cause a thick and viscous mucus to build up at the outer cell surface.

Clinical symptoms of this condition arise in several different areas of the body, including the pancreas, intestines, and reproductive organs. The most serious complications, however,

present in the upper and lower airways (Figure 1.2). Progressive respiratory disease causes 95% of all CF mortalities [6]. Due to conditions of their disease, CF patients are particularly susceptible to chronic infections by a pathogen, *Pseudomonas aeruginosa*. By the age of 17, nearly 70% of CF patients are infected with this opportunistic bacterium, and once acquired, *P. aeruginosa* is difficult to completely eradicate from the CF airway. Nearly 80% of CF adults have chronic *P. aeruginosa* infections that cannot be eliminated by antibiotic therapy [7]. The persisting infection results in ongoing inflammation and pulmonary complications that can be ultimately lethal [8].

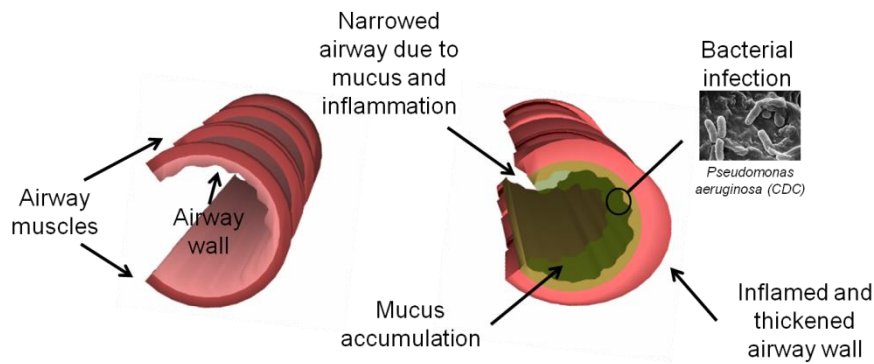


Figure 1.2: A schematic comparison of a normal and CF airway.

CF airways are characterized by narrowed and inflamed airways, which are congested with a thick and viscous mucus.

1.2 Gene Therapy for Cystic Fibrosis

Gene therapy is the use of nucleic acids to treat or prevent disease. It offers a promising therapeutic option for many genetic disorders, particularly those caused by a single defective gene, like CF. In such instances, restorative function depends on the successful delivery and expression of only a single gene [9]. Additionally, CF airway disease is especially suited for treatment by gene therapy due to the ease of accessibility of the target organ and the minimal 5-10% of normal *CFTR* expression necessary to correct the phenotype [10]. However, over 20 years has elapsed since the discovery of the *CFTR* gene, and still, no viable genetic treatment option

exists for curing or alleviating CF airway disease. Major barriers, both physical and immunological, exist as obstacles for successful gene transfer.

1.3 Barriers to Successful Pulmonary Gene Delivery and Expression

1.3.1 Extracellular Barriers

As a major interface with the external environment, the lungs and conducting airways have evolved efficient physical barriers by which to prevent penetration of any unwanted foreign particles and substances. One such barrier, mucociliary clearance, is made possible by a ciliated epithelium bathed in biphasic layer of airway fluid secretions [11,12]. The cilia are situated in the aqueous (“sol”) layer, also known as the periciliary layer, where they beat and relax. Above the sol layer sits a mucus (“gel”) layer composed of high molecular weight glycoproteins, known as mucins, linked to lipids and proteins. The physical, i.e. viscoelastic and adhesive, properties of this gel layer allow it to trap inhaled particles, while the beating the underlying cilia facilitate upward movement for removal of these unwanted substances. Other physical barriers against foreign particles include a glycocalyx that can prevent gene carriers (or vectors) from binding to appropriate cell receptors and a general scarcity of these receptors on the apical side of the epithelium [13].

Sophisticated innate and adaptive immune mechanisms of the respiratory tract also play a role in preventing successful gene transfer in the airway. Alveolar macrophages eliminate gene carriers or products either directly by phagocytosis or indirectly by their abilities to recruit other immune cells as antigen-presenting cells [14]. Other immune cells, e.g. neutrophils, dendritic cells, and lymphocytes, also can play a role in preventing successful gene transfer in the airway. These cells are responsible for initiating and facilitating non-specific inflammation as well as

cellular and humoral immune responses that can target the gene carriers and/or the gene products themselves.

1.3.2 Intracellular Barriers

Once past the extracellular barriers, genes carriers must then overcome additional obstacles at the intracellular level. These vectors must be able to penetrate the cell membrane, either by non-specific or receptor-mediated mechanisms. Typically these mechanisms involve endocytic pathways facilitated by clathrin-coated pits, lipid rafts, and/or direct fusion with the membrane [15]. In any case, vectors are typically internalized into endosomes, from which they must then escape before acidic changes in pH initiate the lysosomal degradation pathway [16]. Unfortunately, additional barriers exist even after endosomal release. Once in the cytosol, vectors must be able to migrate to the nucleus and penetrate the nuclear membrane for entry, all while avoiding additional opportunities for degradation by nucleases within the cytoplasm before reaching this final destination [17].

1.4 Overcoming Barriers to Gene Transfer

Multiple approaches are available to overcome these barriers to effective gene transfer. The correct choices in gene vector as well as in any pre- or co-treatments with chemical enhancers can allow desired genes to more easily by bypass host immune responses or physical barriers preventing proper delivery. Viral vectors are efficient and effective at transferring their genetic material to desired cell types but tend to be much more immunogenic than their nonviral alternatives. However, while easy to use and manufacture, nonviral gene carriers are unable to achieve the efficiencies associated with viral gene carriers. Efforts are ongoing to address the drawbacks of each type of vector.

1.4.1 Cationic Lipids as Chemical Enhancers of Gene Therapy Vectors

One such effort involves the use of cationic lipids, which are able to facilitate delivery of DNA to cells. Addition of these lipids to DNA forms complexes, known as lipoplexes, that exhibit lower overall negative charge and easier associations with cellular membranes than standalone, or naked, DNA is able to. These physiochemical changes allow lipoplexes to shield DNA from extra- and intracellular nucleases by compacting its size and concealing it within its lipid bilayers [18]. Neutral lipids, such as cholesterol or dioleoylphosphatidylethanolamine (DOPE), can be included to aid in the formation of liposomal structures [16]. DOPE is particularly useful due to its ability to fuse with other lipids at low pH, e.g. in an endosome preparing to initiate lysosomal degradation. By fusing with lipids at this stage in endosomal processing, DOPE can facilitate release of the DNA before degradation begins [19]. Additionally, cationic lipids can enhance performance of viral vectors as well. Incubation with cationic liposomes increases the gene expression efficiency, reduces the associated immunogenicity, and/or modifies the tropism of certain viral vectors [20–22].

1.5 Cationic Glucocorticoids for Improving Gene Therapies of CF

One particular category of cationic lipid has particular promise for enhancing gene therapies of CF, namely lipids that are spermine-based cationic glucocorticoids. Two such compounds, dexamethasone spermine (DS) and disubstituted dexamethasone spermine (D₂S) (Figure 1.3), were previously developed by this lab and shown to have both anti-inflammatory and antimicrobial characteristics [23,24]. This dual functionality is particularly suited for applications in CF, a disease characterized by on-going inflammation and chronic infections with opportunistic bacteria. Dual acting cationic lipids, like DS, D₂S, and similarly structured cationic glucocorticoids, could be used to facilitate better gene transfer of gene therapy vectors while also

treating the infection and hyperinflammatory environment so commonly encountered in CF patients.

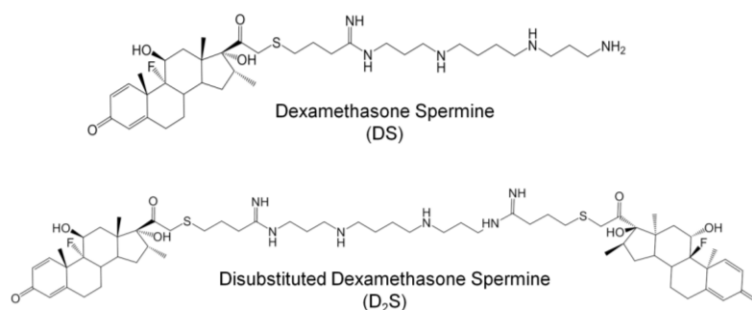


Figure 1.3: Chemical structures of DS and D₂S

1.5 Current Research Goals

DS and D₂S showed promising pharmacological activity *in vitro*, but the *in vivo* efficacy of these compounds had not been verified at the start of this work. While seemingly simple in theory to execute, this task was difficult to accomplish due to the lack of a relevant and convenient animal model in which to test potential therapeutics for CF. Mice that have been engineered to lack the *CFTR* gene do not show the airway disease typically seen in CF humans [25]. Furthermore, *P. aeruginosa*, the bacterium responsible for most chronic infections in CF patients, is not a naturally infectious pathogen of murine animals. If the bacteria are not first embedded in an artificial biofilm (i.e. embedded in beads composed of agar, agarose, or seaweed alginate) before administration to the animal, a lasting infection is unachievable [26]. Administration of free bacteria results in either rapid clearance of the organism or acute sepsis and death [27].

1.5.1 Specific Aim 1: Mouse Models of Infection for In Vivo Evaluation of Airway Gene Transfer

Consequently, the first objective of this work was to establish non-invasive and reproducible *in vivo* models of airway infection to test potential therapies relevant for CF airway

disease. Separately, the models presented here incorporate different aspects of clinical presentations of CF airway disease. Methods for one model establishes a chronic airway infection with a natural murine pathogen, while methods for the other two models establish infections that are more acute in nature but utilize *P. aeruginosa* in a physiological environment relevant for CF.

1.5.2 Specific Aim 2: Structure-activity Relationships of Novel Cationic Glucocorticoids

The second objective of this work was to investigate structure-activity relationships of similarly structured cationic glucocorticoids. DS and D₂S, while potent *in vitro*, may not be the most ideal choices for practical applications in treating CF airway disease. Preliminary studies on the effects of D₂S in rat models of arthritis demonstrated significant systemic activity of the lipid in the body [28]. While local anti-inflammatory (i.e. immunosuppressive) activity is a desirable trait for potential CF treatments, compounds with strong systemic activity could be ultimately lethal during situations involving active infections. Thus, ideal therapeutic candidates are compounds with potent local effects but limited systemic consequences. In this work, six new cationic glucocorticoids were synthesized and characterized to further understanding of how this family of compounds can be modified accordingly to achieve such desired characteristics.

1.5.3 Specific Aim 3: In Vivo Evaluation of Coordinated Gene Therapies with Anti-inflammatory and Antimicrobial Cationic Glucocorticoids during Active Airway Infections

Ultimately, the goal of this work is to coordinate gene therapies with these dual functioning cationic glucocorticoids and validate these methods as treatments for CF airway disease. The final objective of this work was to test these coordinated therapies in the animal models established as part of Specific Aim 1. Two of the novel cationic glucocorticoids developed in Specific Aim 2 showed particularly promising *in vitro* potency and were used to establish proof-of-concept efficacy *in vivo*.

2.0 *In vivo* evaluation of adeno-associated virus gene transfer in airways of mice with acute or chronic respiratory infection

2.1 Abstract

While gene therapy development for CF often involves viral-based vectors, little is known about gene transfer in the context of an infected airway. In this study, three mouse models were established to evaluate adeno-associated virus (AAV) gene transfer in such an environment. *Bordetella bronchiseptica* RB50 was used in a chronic, non-lethal respiratory infection in C57BL/6 mice. An inoculum of $\sim 10^5$ CFU allowed *B. bronchiseptica* RB50 to persist in the upper and lower respiratory tracts for at least 21 days. In this infection model administration of an AAV vector on day 2 resulted in an approximately three-fold reduction of reporter gene expression compared to that observed in uninfected controls. Postponement of AAV administration to day 14 resulted in an even greater (eight-fold) reduction of reporter gene expression, when compared to uninfected controls. In another infection model *Pseudomonas aeruginosa* PAO1 was used to infect surfactant protein D (SP-D) or surfactant protein A (SP-A) knockout (KO) mice. With an inoculum of $\sim 10^5$ CFU, infection persisted for two days in the nasal cavity of either mouse model. Reporter gene expression was approximately ~ 2.5 fold lower compared to uninfected mice. In the SP-D KO model, postponement of AAV administration to day 9 post-infection resulted in only a 2-fold reduction in reporter gene expression, when compared to expression seen in uninfected controls. These results confirm that respiratory infections, both on-going and recently resolved, decrease the efficacy of AAV-mediated gene transfer.

2.2 Introduction

Deterioration of the lung in CF patients is due to a cycle of infection by opportunistic pathogens and neutrophilic inflammation [29]. By the age of 17, nearly 70% of CF patients have *P. aeruginosa* in their sputum at levels of 10^6 - 10^8 CFU/gr sputum [30], and approximately 80% of adult CF patients are chronically infected with *P. aeruginosa* that cannot be eliminated by antibiotic therapy [7]. In a Phase II gene therapy trial with AAV-CFTR, nearly 70% of CF patients receiving the AAV2 vector were *P. aeruginosa* colonized [31]. The presence of infection was an endpoint of measurement in the trial and was not a basis for exclusion from the trial. Separately, a Phase IIB trial with AAV2-CFTR that included three doses of 10^{13} particles administered 30 days apart failed to demonstrate efficacy [32]. In this clinical trial of 102 CF subjects, the number of days of antibiotic use was considered a marker for trial monitoring. Infection in CF airways is expected during the progress of the gene therapy regimen, and about 15% of CF subjects were on antibiotics while they received aerosolized AAV2 for this study. In a clinical trial of interferon gamma-1b with 66 CF patients, sputum bacterial density was 7.1 Log_{10} CFU/gr with 80 to 90% of enrolled subjects having *P. aeruginosa* infection [33]. The sickest CF patients have chronic lung infection but the presence of *P. aeruginosa* in sputum has not been a criterion for exclusion in airway-directed gene therapy clinical trials. The failure of CF AAV gene therapy trials may be due in part to the difficulty of gene delivery to lungs that sustain and harbor a chronic disease state due to infection. Little research, however, has been done to investigate the effects of microbial infection on airway gene transfer.

The paucity of research in this area may be related to the lack of a relevant animal model necessary to study such effects. Current methods are laborious and introduce issues of mechanical and physical damage that are not naturally seen with clinical presentations of CF. This study presents three alternatives for studying gene transfer in an infected airway that are reproducible

and non-invasive. The first involves a natural murine pathogen, *Bordetella bronchiseptica* (*Bb*) RB50, to establish a chronic infection in C57BL/6 mice. *Bb* infects a number of members of the mammalian family, and in rodents, rabbits, cats, dogs, and pigs, it typically establishes an asymptomatic infection in the nasal cavity that persists indefinitely [34,35]. In the lower respiratory tract, the infection is enduring, but transient, with the bacterial load reducing to almost baseline within 45 days [35]. The second and third scenarios utilize *P. aeruginosa* PAO1 to establish an acute infection in surfactant protein D (SP-D) and surfactant protein A (SP-A) knockout mice, respectively. SP-D and SP-A are pulmonary collectins that are important in innate immunity against various bacterial and viral pathogens [36]. Decreased or lack of SP-D and SP-A has been implicated in the pathogenesis of CF airway disease [37]. When knockout mice were engineered to lack either SP-D or SP-A, they were found to have decreased ability to effectively clear *P. aeruginosa* [38]. The resulting infection was still transient lasting only a few days, but the resulting inflammatory response was exaggerated when compared to WT mice. As hyperinflammatory responses are also typical of CF patients, the SP-D and SP-A KO mice emulate key inflammatory aspects of the disease when compared to WT mice.

In this report, we employed these mouse models to study adeno-associated virus (AAV)-mediated gene transfer in infected airways. Figure 2.1 depicts the experimental design used. The presence of an on-going or even recently resolved infection decreased AAV-mediated gene transfer efficiency in the airway, validating the use of these models to optimize gene transfer for relevant airway diseases like CF.

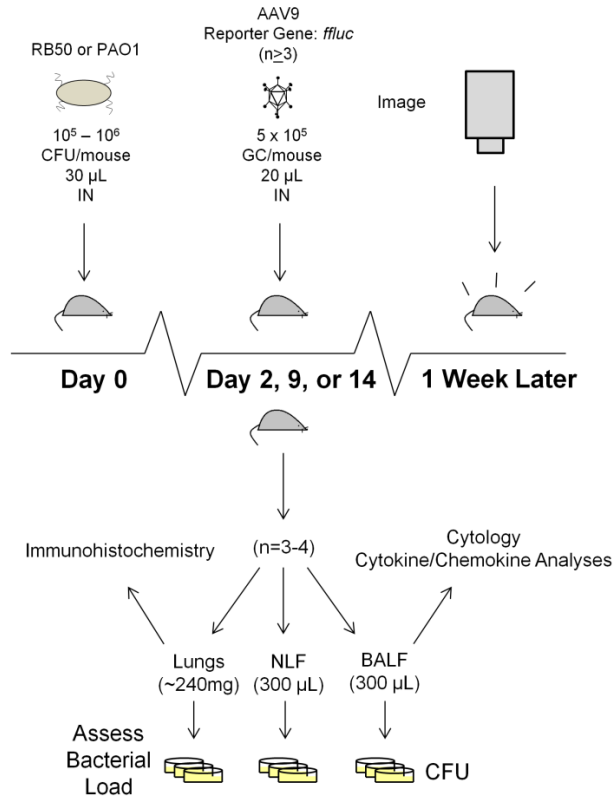


Figure 2.1: A schematic of experimental design.

Mice are i.n. infected with freshly cultured bacteria on day 0. On day 2, 9, or 14, an AAV9 vector containing a *ffluc* reporter gene is introduced to the recently infected airway via intranasal instillation. Three to four randomly selected mice are also taken on the day of AAV administration (n=3 for uninfected controls, n=4 for infected groups). Approximately every week thereafter, the remaining mice are dosed with luciferin and subjected to live imaging to evaluate *ffluc* expression. Abbreviations: AAV, adeno-associated virus; IN, intranasal; *ffluc*, firefly luciferase; NLF, nasal lavage fluid; BALF, bronchoalveolar lavage fluid.

2.3 Materials and Methods

2.3.1 Bacterial Strains and Growth Conditions

B. bordetella strain RB50 was a kind gift from Dr. Yasuhiko Irie, University of Washington, Seattle, WA. *Bb* was cultured in Stainer-Scholte (SS) broth or on Bendet Gengou (BG) blood agar (BD Diagnostic Systems, Sparks, MD) at 37°C. *P. aeruginosa* PAO1 was a kind gift from Dr. Robert Bucki, University of Pennsylvania, Philadelphia, PA. The bacterium was

cultured at 37°C in Miller's Lysogeny Broth (LB) (Mediatech, Herndon, VA) or on Pseudoseal™ Agar (cetrimide agar) from BD Diagnostic Systems.

2.3.2 AAV Preparation

The AAV9 vectors flanked with AAV2 inverted terminal repeats (ITRs) contained a firefly luciferase (ffluc) reporter gene fused to a nucleus localization sequence at the N-terminus under the transcriptional control of the cytomegalovirus (CMV)-enhanced chicken- β -actin promoter. Vectors were produced by the Penn Vector core as previously described [39].

2.3.3 Mice

C57BL/6 male mice were purchased from Charles River Laboratories (Wilmington, MA), while SP-D and SP-A knockout mice [40] were maintained in-house at the University of Pennsylvania. Mice were age-matched and used between 7 and 14 weeks of age. For all experiments, a group size of at least 3 mice was used for each experimental cohort. All animals were maintained at the Animal Facility of the Translation Research Laboratories at the University of Pennsylvania under protocols reviewed and approved by the University of Pennsylvania's Institutional Animal Care and Use Committee. Prior to all intranasal administrations, mice were anesthetized by an intraperitoneal injection of ketamine/xylazine (70/7 mg/kg) and were subsequently suspended from their dorsal incisors (hind quarters supported) for dosing.

2.3.4 Preparation of Inoculum

Bacteria were grown overnight in their appropriate liquid media at 37°C with shaking at 250 rpm. Approximately 16-18 hours later, the bacteria were diluted into fresh broth to an optical density ($\lambda=600\text{nm}$) reading of 0.1 and were allowed to grow until mid-logarithmic phase (optical density at $\lambda=600\text{nm}$ of ~ 0.5 for *Bb* RB50 and ~ 0.4 for *P. aeruginosa*). At that point, the bacteria

were harvested and washed once in phosphate-buffered saline (PBS) before being diluted into fresh PBS at the desired concentration (controlled by evaluation of optical density at 600 nm).

2.3.5 Mouse Infection Model for Evaluation of Airway Transduction

Before challenge, mice were anesthetized intraperitoneally and suspended from their dorsal incisors, as described earlier. Mice were then intranasally (i.n.) challenged with a 30- μ L bolus (delivered as two 15 μ L aliquots, one into each nostril) of *Bb* RB50 or *P. aeruginosa* PAO1 to achieve approximately 10^5 CFU/mouse. At specified time points later (i.e. 2, 9, or 14 days after infection), mice were i.n. administered 5×10^{10} genome copies (GC) of AAV vector in 20- μ L (10 μ L into each nostril).

To evaluate *ffluc* expression, mice (~20 grams) were anesthetized and suspended before 20 μ L of D-luciferin (15 mg/mL) was i.n. administered as two 10 μ L aliquots, one into each nostril. After 5 minutes, mice were imaged for 60 seconds with the IVIS Xenogen imaging system. Quantification of signal was calculated with the Living Image 2.5.1 software.

2.3.6 Evaluation of Bacterial Load

To determine bacterial load, three samples were taken from each randomly selected mouse: (a) lungs, (b) bronchoalveolar lavage fluid (BALF), and (c) nasal lavage fluid (NLF). Before the lungs were harvested, BALF was collected by instilling 500 μ L of sterile PBS through a cannula into the trachea. Fluid was collected and re-instilled into the lungs for a total of three times before being collected into a 1.5-mL tube. The lungs (~110-150mg per lung) were then excised. One lung was inflated with a 1:1 mixture of Tissue Tek® OCT and PBS, embedded in OCT in a storage cassette and flash frozen with cooled 2-methylbutane. Frozen lungs were kept at -80°C until further histological processing. The other lung was homogenized in 1.5 mL of sterile PBS for evaluation of bacterial load. For the NLF samples, mice were decapitated and a cannula

attached to a 1-mL syringe containing 300 μ L of sterile PBS was placed into the tracheal remnant. PBS was then flushed through the nasal passages and collected through the nares into a 1.5-mL tube. This recovered fluid was used to flush the nasal cavity another two times, for a total of three flushes. All samples were kept on ice until further processing.

To determine the CFU counts in each sample, solutions were diluted 10-, 100-, and 1000-fold. Ten-microliter aliquots of each dilution were spotted in duplicate on the appropriate solid selection agar for each bacterial strain (BG blood agar for *Bb* and Pseudosei™ agar for *P. aeruginosa*). Plates were incubated at 37°C for 48 hours for *Bb* RB50 and 16 hours for *P. aeruginosa*. The number of colonies at each dilution was then counted, and the concentration of CFU of each sample was back calculated from the dilution factor.

2.3.6 BALF Cell Analysis

Cytospin slides were prepared using 50 μ L of freshly isolated BALF diluted into 100 μ L of fresh PBS. After centrifugation (Shandon Cytospin 3, Thermo Fisher Scientific Inc., Waltham, MA) for 5 minutes at 1,000 rpm, cells were allowed to air dry for 10 minutes. Cells were then fixed with 10% neutral buffered formalin, washed in PBS, stained with Nuclear Fast Red (Sigma Aldrich, St. Louis, MO), dehydrated with a series of ethanols, and cleared with xylene according to standard protocols. Cells were studied and characterized using light microscopy (IX81; Olympus America Inc., Center Valley, PA).

2.3.7 Lung Immunohistochemistry

Immunofluorescence staining was performed on frozen lung sections. Sections were fixed in acetone at -20°C for 7 min, air dried, blocked in 1% donkey serum in PBS for 20 min, and incubated with primary rat antibodies diluted in blocking buffer against CD8 (clone 53-6.7, BD Biosciences; 1:20), CD4 (clone RM4-5, BD Biosciences; 1:20), and Mac-2 (clone M3/38,

Cedarlane; 1:200) for 45 min. After washing in PBS sections were stained with secondary donkey antibodies labeled with FITC or TRITC (Jackson ImmunoResearch Laboratories) for 30 min, washed in PBS, and mounted in Vectashield containing DAPI (Vector Laboratories). For detection of neutrophils, cryosections were fixed in 4% paraformaldehyde in PBS for 10 min. Sections were then permeabilized and blocked in 0.2% Triton containing 1% donkey serum for 30 min and stained with a rabbit antibody against myeloperoxidase (Abcam) followed by secondary antibodies and washing steps as described above.

2.3.8 BALF Cytokine Analysis

A multiplex bead assay based on the Luminex technology was used to measure cytokine/chemokine levels in BALF samples that were collected from mice as described earlier. A 25-panel multi-analyte cytokine/chemokine kit (Millipore, MA) was used according to the manufacturer's instructions. Briefly, 25 μ L of samples were incubated overnight at 4°C with capture beads against G-CSF, GM-CSF, IFN- γ , TNF- α , RANTES, IL-1 α , IL-1 β , IL-2, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12p40, IL-12p70, IL-13, IL-15, IL-17, IP-10, KC, MCP-1, MIP-1 α , MIP-1 β , and MIP-2 on a rocking platform. The following day beads were washed using a hand held magnetic block (Millipore, MA) and stained with biotin antibodies at room temperature for one hour. The beads were further stained for 30 min using streptavidin detection antibodies before a series of final washes. Beads were read on a Luminex 200 instrument (Luminex, TX) and levels determined by regressing against a 7-point standard curve (Bioplex Manager, Biorad).

2.3.9 Statistical Analysis

Statistical analysis was performed using Microsoft Office Excel 2007 (Microsoft, Redmond, WA). The Student's *t*-Test was used to determine significance of differences between two groups.

2.4 Results

To evaluate the effects of a chronic bacterial infection on gene transfer in the airway, C57BL/6 mice were i.n. challenged with approximately 10^5 CFU of *Bb* RB50 or sterile PBS. Mice were tested for evidence of infection through day 21 post-infection, and in all RB50-infected mice, bacteria were detected at significant levels (10^4 - 10^7 CFU) in both the nasal cavity and lung throughout the duration of the study (Figure 2.2A). All samples from mice receiving only sterile PBS showed no evidence of RB50 at all time points tested (data not shown). On day 2 or day 14 of infection, mice were given an intranasal dose of AAV9.*ffluc*. One week after AAV administration, mice were subjected to live whole animal luminescent imaging. At both time points, the luminescent signal in the nasal cavity of the infected mice was significantly lower ($P < 0.01$, Student's *t*-Test) than that observed in the nasal cavity of the uninfected mice (Figure 2.2, B and C). It is worthwhile to note that the loss in expression was greater (eightfold, Figure 2.2C) when AAV administration was delayed to day 14 of infection. The significant difference in *ffluc* expression in the nasal cavity between uninfected mice and infected mice receiving AAV on day 14 was sustained for 50 days after AAV delivery (Figure 2.3).

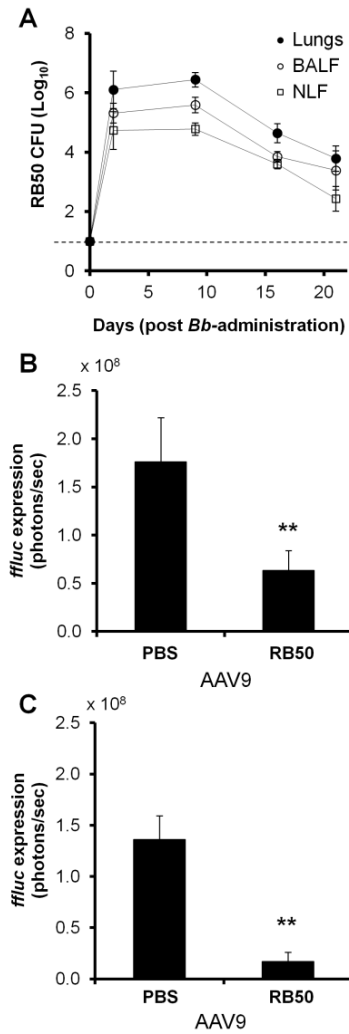


Figure 2.2: Effects of a sustained RB50 airway infection on AAV9-mediated transduction in C57BL/6 mice. (A) RB50 colonization of C57BL/6 mouse airway. Approximately 10^5 CFU of bacteria were delivered i.n. in 30 μ L. Bacterial colonization was analyzed through harvest of lungs, BALF, and NLF from three to four randomly selected mice at specified time points over the course of 21 days. The plots show means $\pm$ standard deviations for n=4-11 mice tested at each time point in four different experiments. Samples taken from uninfected mice were clear of RB50 at all time points. Dashed line denotes detection limit. Abbreviations: BALF, bronchoalveolar lavage fluid; NLF, nasal lavage fluid. (B and C) AAV9-mediated transduction in wild-type C57BL/6 mice recently infected with RB50. Two or fourteen days after receiving intranasal inoculations of either sterile PBS or ca. 105 CFU of *B. bronchiseptica*, mice were i.n. dosed with AAV9.fLuc. Mice were later assessed for gene expression one week later: (B) mice that received AAV on day 2 of infection and (C) mice that received AAV on day 14 of infection. Plots show quantification of luminescence from mouse nasal cavity. Results are presented as means, and error bars represent the standard deviation for the six mice imaged at each time point. Significantly lower signal was observed from the nasal cavities of infected mice (**P < 0.01, Student's t-Test, n=6).

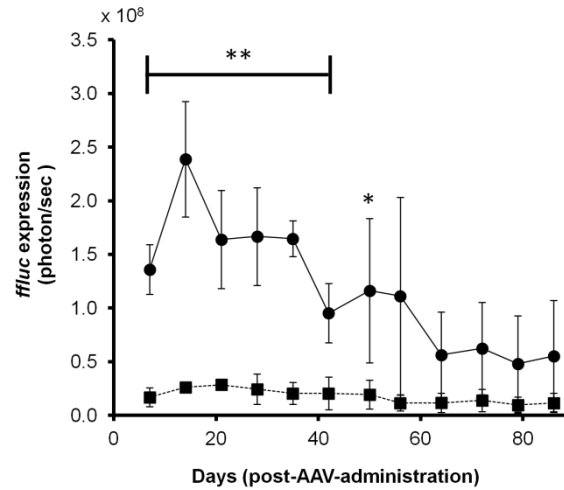


Figure 2.3: Long-term AAV9 gene expression in nasal cavities of C57BL/6 mice previously infected with RB50. Animals were transduced with AAV9.*ffluc* on day 14 of a *Bb* infection and imaged for *ffluc* expression every week until day 86 post-AAV administration. *Bb*-infected mice show significantly lower nasal cavity luminescence than that observed from uninfected controls through day 50 (** $P < 0.01$, * $P < 0.05$, Student's *t*-test, $n = 6$ at day 7, $n = 3$ for all other time points). ●— PBS, AAV9, -■- *Bb*, AAV9.

To determine whether a transient acute infection would have a similar effect, *P.*

aeruginosa PAO1 was used to infect SP-D knockout mice. Mice were i.n. challenged with approximately 10^5 CFU of PAO1 and control mice given sterile PBS. On day 2 or day 9 post-infection, a representative sampling of mice (uninfected $n = 3$, infected $n = 4$) was taken to determine the status of the infection in the lungs and nose at the time of AAV administration (Figure 2.4, A and B). Samples from uninfected mice showed no evidence of PAO1 at both time points tested (data not shown). On day 2, PAO1 was only found in the nasal cavity at relatively low levels (~ 80 CFU). Lung and BALF samples isolated from the lower respiratory tract showed no evidence of infection. By day 9, the majority of mice had cleared the bacteria from their airways. However, despite the limited presence of bacteria in the nasal cavity, *ffluc* expression was still significantly lower ($P < 0.01$, Student's *t*-Test) in the noses of infected mice in both cases (Figure 2.4, D and E). Consistent observations were seen at subsequent time points through to day 14 post-transduction (Figure 2.5, A-B).

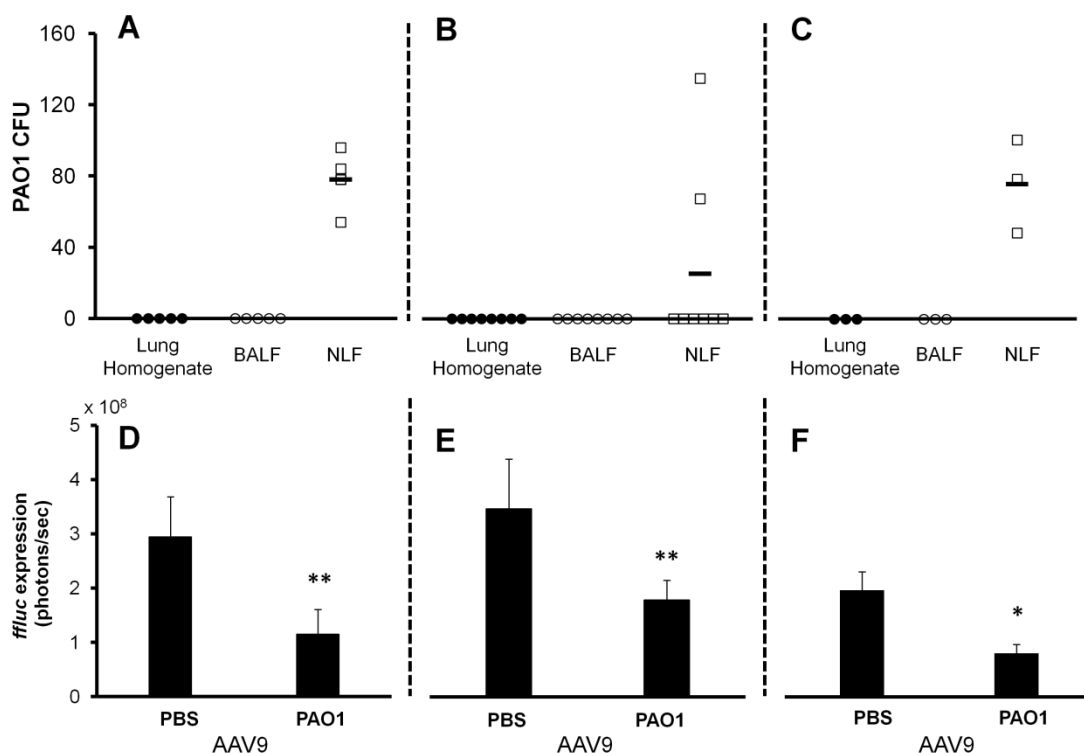


Figure 2.4: Effects of a transient *P. aeruginosa* PAO1 airway infection on AAV9-mediated transduction in SP-D or SP-A KO mice.

(Top, A-C) PAO1 colonization of SP-D or SP-A KO mouse airway. Approximately 10^5 CFU of bacteria were delivered i.n. in 30 μ L. Bacterial colonization was analyzed through harvest of lungs, BALF, and NLF from three or four randomly selected mice at specified time points: (A) SP-D KO mice at day 2 post-infection, (B) in SP-D KO mice at day 9 post-infection, and (C) in SP-A KO mice at day 2 after infection. Plots show means $\pm$ standard deviations for $n=5-7$ mice tested at each time point in three different experiments for SP-D KO mice and $n=3$ mice from a single experiment for SP-A KO mice. Samples taken concurrently from uninfected mice were clear of PAO1 at all time points tested. Abbreviations: BALF, bronchoalveolar lavage fluid; NLF, nasal lavage fluid. (Bottom, D-F) AAV9-mediated transduction in SP-D or SP-A KO mice recently infected with PAO1. Two or nine days after being challenged with sterile PBS or approximately 10^5 CFU of *P. aeruginosa* PAO1, mice were i.n. dosed with AAV9-ffIuc. One week later, mice were imaged for gene expression: (D) SP-D KO mice that received AAV on day 2 of infection, (E) SP-D KO mice that received AAV on day 9 of infection, and (F) SP-A KO mice that received AAV on day 2 of infection. Plots show quantification of luminescence from mouse nasal cavity. Results are presented as means, and error bars represent the standard deviation for the mice imaged at each time point. Significantly lower signal was observed from the nasal cavities of infected mice (** $P < 0.01$, * $P < 0.05$, Student's *t*-Test, uninfected $n=6$, infected SP-D KO mice receiving AAV on Day 2 $n=11$, infected mice SP-D KO receiving AAV on Day 9 $n=8$, $n=7$ for both SP-A KO mice groups).

To determine whether infection would have similar effects on gene expression in mice lacking SP-A, the SP-A KO mice were i.n. challenged with $\sim 10^5$ CFU of PAO1 or sterile PBS. On day 2 post-infection, mice (uninfected $n=3$, infected $n=4$) were peeled to mice determine the

status of infection in the SP-A nasal and lung airways at the time of AAV administration (Figure 2.4C). As observed in the SP-D KO mice, bacteria were only found in the nasal cavity at relatively low levels (~80 CFU) on day 2 post-infection, and samples from uninfected mice showed no evidence of PAO1 (data not shown). Additionally, similar trends on reporter gene expression were also observed; *ffluc* expression was significantly lower ($P<0.05$, Student's *t*-Test) in the nasal cavity of infected mice (Figure 2.4F). Consistent observations were seen at subsequent time points through day 21 post-transduction (Figure 2.5C).

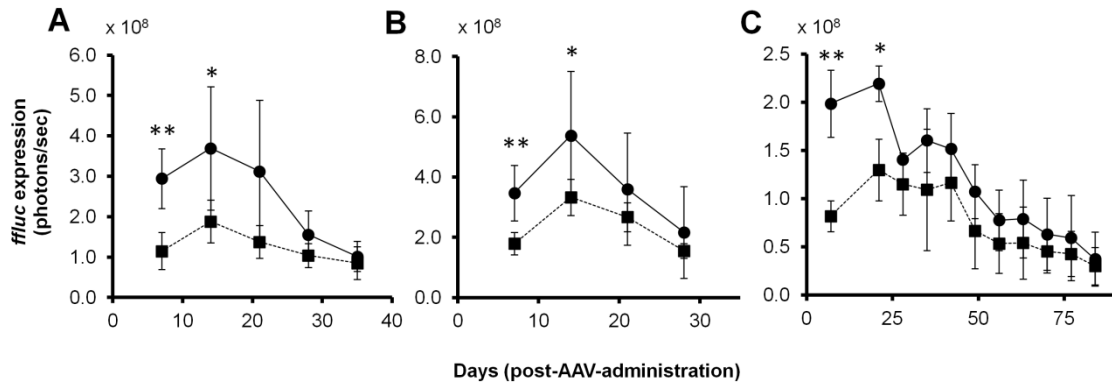


Figure 2.5: Long-term AAV9 gene expression in nasal cavities of SP-D or SP-A KO mice previously infected with PAO1.

(A) SP-D KO mice transduced on day 2 of infection. (B) SP-D KO mice transduced on day 9 of infection. (C) SP-A KO mice transduced on day 2 of infection. Mice were imaged every week until the specified day post-AAV administration. PAO1-infected mice show significantly lower nasal cavity luminescence than that observed from uninfected mice through the first two time points in each experiment (** $P<0.01$, * $P<0.05$, Student's *t*-Test, for (A): Day 7 uninfected $n=6$, Day 7 infected $n=10$, for all other time points uninfected $n=3$ and infected $n=6$; for (B): Day 7 uninfected $n=6$, Day 7 infected $n=8$, for all other time points uninfected $n=3$ and infected $n=4$; for (C): Day 7 uninfected $n=7$, Day 7 infected $n=7$, for all other time points: uninfected $n=3$ and infected $n=4$). ●— PBS, AAV9, —■— PAO1, AAV9.

The immune response at the time of AAV administration was then characterized to provide insights into the observed mechanisms. Cytology on fresh BALF samples indicated that actively infected mice were characterized by substantial neutrophil infiltration on day 2 of infection (Figures 2.6R, 2.7R, 2.8J). Samples from uninfected mice only showed evidence of alveolar macrophages (Figures 2.6Q, 2.7Q, 2.8I). Interestingly, uninfected SP-D KO mice had a

small number of neutrophils in their BALF samples in addition to macrophages that were characterized by a foamy and/or multi-nucleated appearance (Figure 2.7Q). By day 9 post-PAO1 infection, many of the neutrophils had band, rather than segmented, nuclei (Figure 2.7T), indicative of a shift towards immature precursors.

Histology on frozen lung sections showed similar trends (Figures 2.6-2.8). Day 2 of infection was characterized by significant neutrophil and macrophage infiltration for the *Bb* chronic infection and the PAO1 acute infection in the SP-A KO model (Figures 2.6 and 2.8, E-H). Day 2 of infection was also characterized by an elevated CD8 T cell population in the SP-A KO / PAO1 model (Figure 2.8G). Interestingly, in the SP-D KO / PAO1 acute infection model, neutrophils and macrophages were present in the lungs of uninfected mice (Figure 2.7, A and B). We also observed small numbers of CD8 T cells in the uninfected lung and these increased slightly with infection. By day 9 post-infection with PAO1, lung samples from the SP-D KO mice showed little to no differences between uninfected and infected groups for all cell types detected (Figure 2.7N). Day 14 of *Bb* infection in C57BL/6 mice, on the other hand, was characterized by elevated neutrophil and macrophage populations (Figure 2.6, M and N). Additionally, day 14 samples from infected lungs also showed a larger presence of CD4 T cells but not CD8 T cells (Figure 2.6, O and P).

Analysis of cytokine/chemokine levels in BALF samples isolated from a representative sampling of mice (typically uninfected n=3, infected n=4) at the time of AAV administration demonstrated the most notable differences on day 2 of infection for all infection models (Figure 2.9). Of the 25 analytes measured, nine of them (GM-CSF, IL-2, IL-4, IL-5, IL-7, IL-9, IL-12p40, IL-13 and IL-15) showed no differences between groups in any of the infection models used in this study (data not shown). The remaining 16 (G-CSF, IFN- γ , IL-1 α , IL-1 β , IL-6, IL-10, IL-12p70, IL-17, IP-10, KC, MCP-1, MIP-1 γ , MIP-1 β , MIP-2, TNF- α , and RANTES) displayed

substantial differences between uninfected and infected groups on day 2 post-infection in at least one of the infection models tested (Figure 2.9). Determination of statistical significance for differences observed in the SP-A KO / PAO1 model was limited by the number of samples available for analysis (n=2 for each group). At the later time points tested, i.e. at day 14 after *Bb* infection and at day 9 after PAO1 infection, many of the elevated analytes (i.e., G-CSF, IFN- γ , IL-1 β , IL-6, IL-10, IL-12p70, IP-10, MCP-1, and MIP-1 γ) returned to baseline. The remaining analytes, IL-1 α , IL-17, KC, MIP-1 β , MIP-2, TNF- α , and RANTES, were still elevated in samples obtained from *Bb*-infected mice. In the SP-D KO / PAO1 model, all cytokines and chemokines levels had returned to baseline by day 9 post-infection.

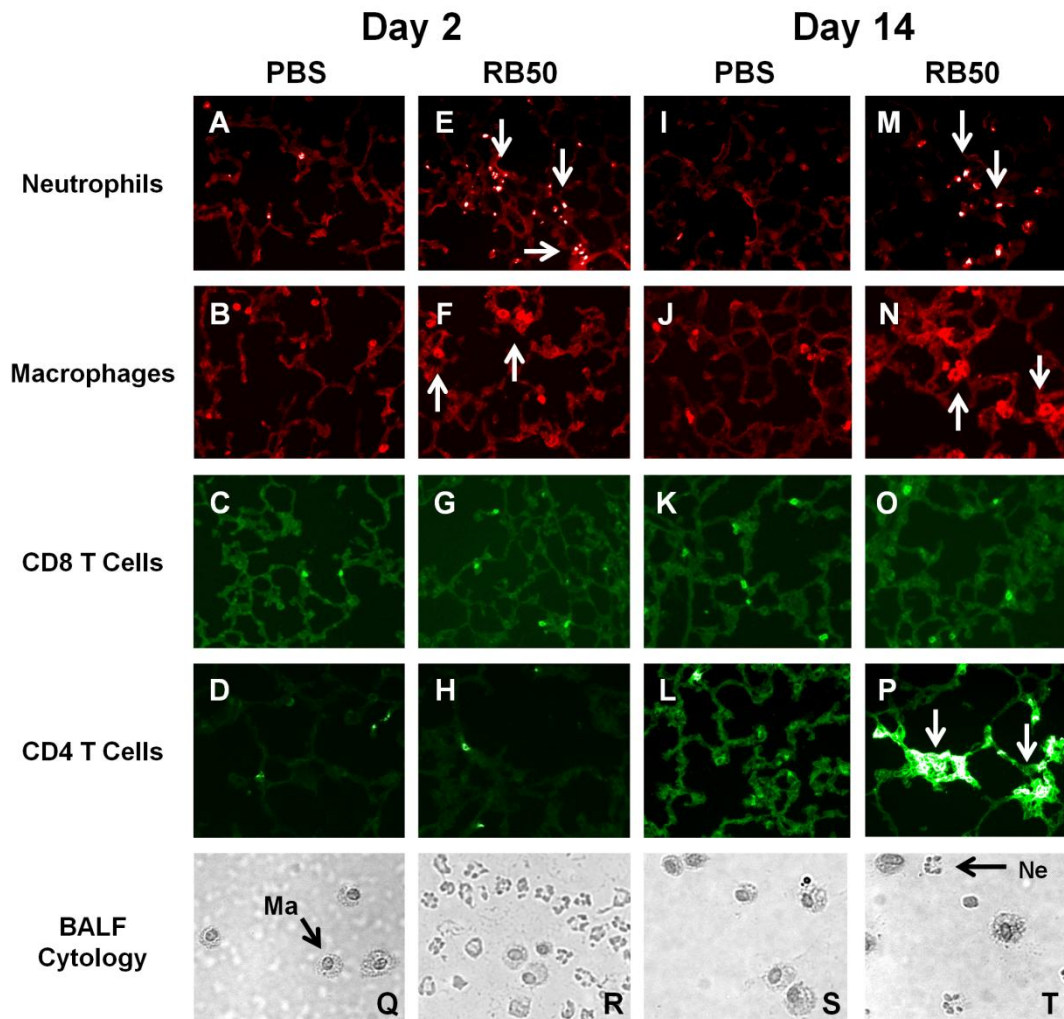


Figure 2.6: Characterization of immune response at time of AAV administration in *Bb* chronic infection model. (A-P), Lung histology of C57BL/6 mice transduced at specified time points during *Bb* airway infection. Lungs were harvested on the day of AAV administration and sectioned and stained with antibodies against antigens for the specified cell type. Representative images are shown here: sections from (A-D) uninfected mice on day 2, (E-H) infected mice on day 2, (I-L) uninfected mice on day 14, and (M-P) infected mice on day 14. Notable neutrophil and macrophage infiltration was observed in samples from infected lungs. Day 9 showed an elevated CD4 T cell population as well. Arrows indicate evidence of elevated cell populations. Magnification, x 20. (Q-T) Cytospin preparations of cells recovered from fresh BALF samples taken on day of AAV administration: (Q,S) samples taken from uninfected mice on day 2 and day 14, respectively, (R,T) samples taken from infected mice on day 2 and day 14, respectively. Representative macrophages (Ma) and neutrophils (Ne) are indicated in the figure. Samples from uninfected mice showed only macrophages, while samples from infected mice showed a prominent neutrophil presence in addition to the macrophage cell population at both time points.

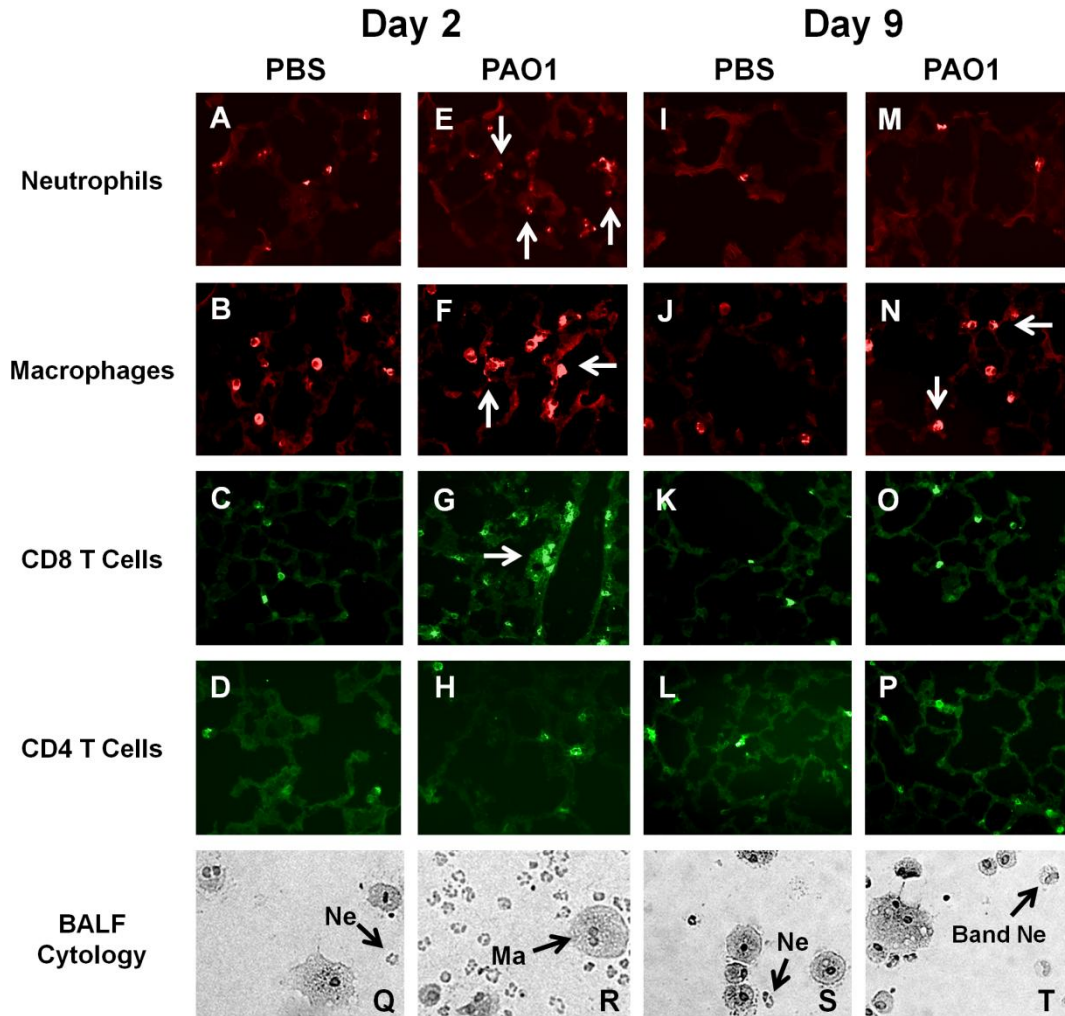


Figure 2.7: Characterization of immune response at time of AAV administration in *SP-D KO* / *PAO1* acute infection model.

(A-P), Lung histology of *SP-D KO* mice transduced at specified time points during *PAO1* airway infection. Lungs were harvested on the day of AAV administration and sectioned and stained with antibodies against antigens for the specified cell type. Representative images are shown here: sections from (A-D) uninfected mice on day 2, (E-H) infected mice on day 2, (I-L) uninfected mice on day 9, and (M-P) infected mice on day 9. Day 2 samples from uninfected mice displayed evidence of neutrophils, macrophages, and CD8 T cells. Infected samples at this time point showed slightly elevated levels of the same cell types, as indicated by the arrows. By day 9, only macrophages remain slightly elevated. Magnification, x 20. (Q-T) Cytospin preparations of cells recovered from fresh BALF samples taken on day of AAV administration: (Q,S) samples taken from uninfected mice on day 2 and day 9, respectively, (R,T) samples taken from infected mice on day 2 and day 9, respectively. Representative macrophages (Ma), neutrophils (Ne), and band neutrophils (band Ne) are indicated in the figure. Day 2 samples from uninfected mice showed macrophages and a small neutrophilic presence. Infected samples at this same time point displayed a much larger neutrophils population. By day 9, both macrophages and neutrophils were still seen. However, neutrophils on day 9 had band (instead of segmented) nuclei, indicative a shift towards immature precursors.

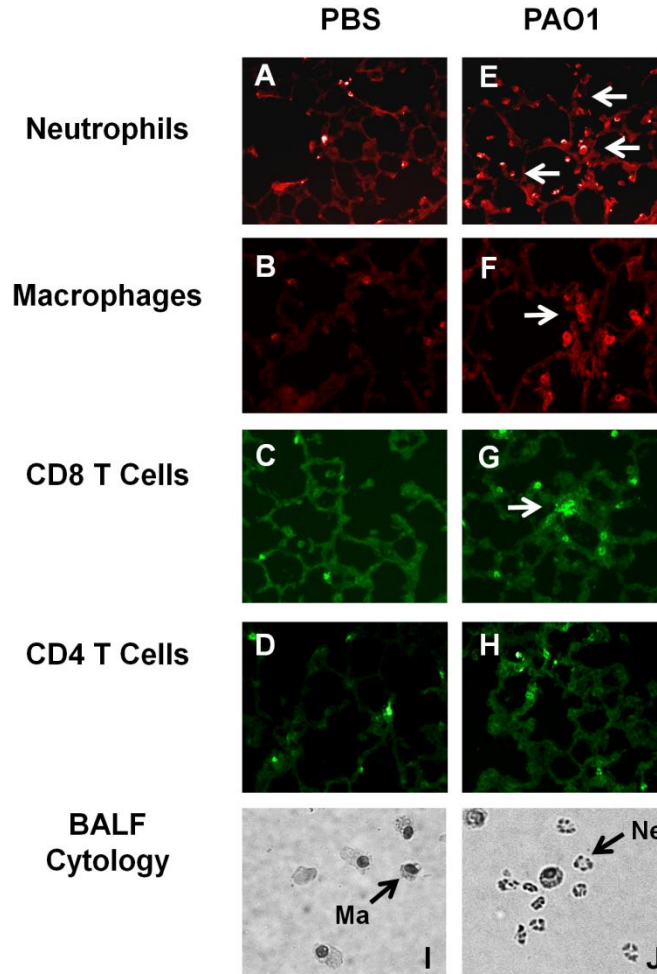


Figure 2.8: Characterization of immune response at time of AAV administration in *SP-A KO* / *PAO1* acute infection model.

(A-H), Lung histology of *SP-A KO* mice transduced on day 2 during *PAO1* airway infection. Lungs were harvested on the day of AAV administration and sectioned and stained with antibodies against antigens for the specified cell type. Representative images are shown here: sections from **(A-D)** uninfected mice and **(E-H)** infected mice. On day 2, elevated levels of neutrophils, macrophages, and CD8 T cells were evident in samples from infected lungs (see arrows). Magnification, x 20. **(I-J)** Cytospin preparations of cells recovered from fresh BALF samples taken on day of AAV administration: **(I)** samples taken from uninfected mice and **(J)** samples taken from infected mice. Representative macrophages (Ma) and neutrophils (Ne) are indicated in the figure. Samples from uninfected mice showed only macrophages, while infected samples showed obvious neutrophil infiltration in addition to the macrophage cell population.

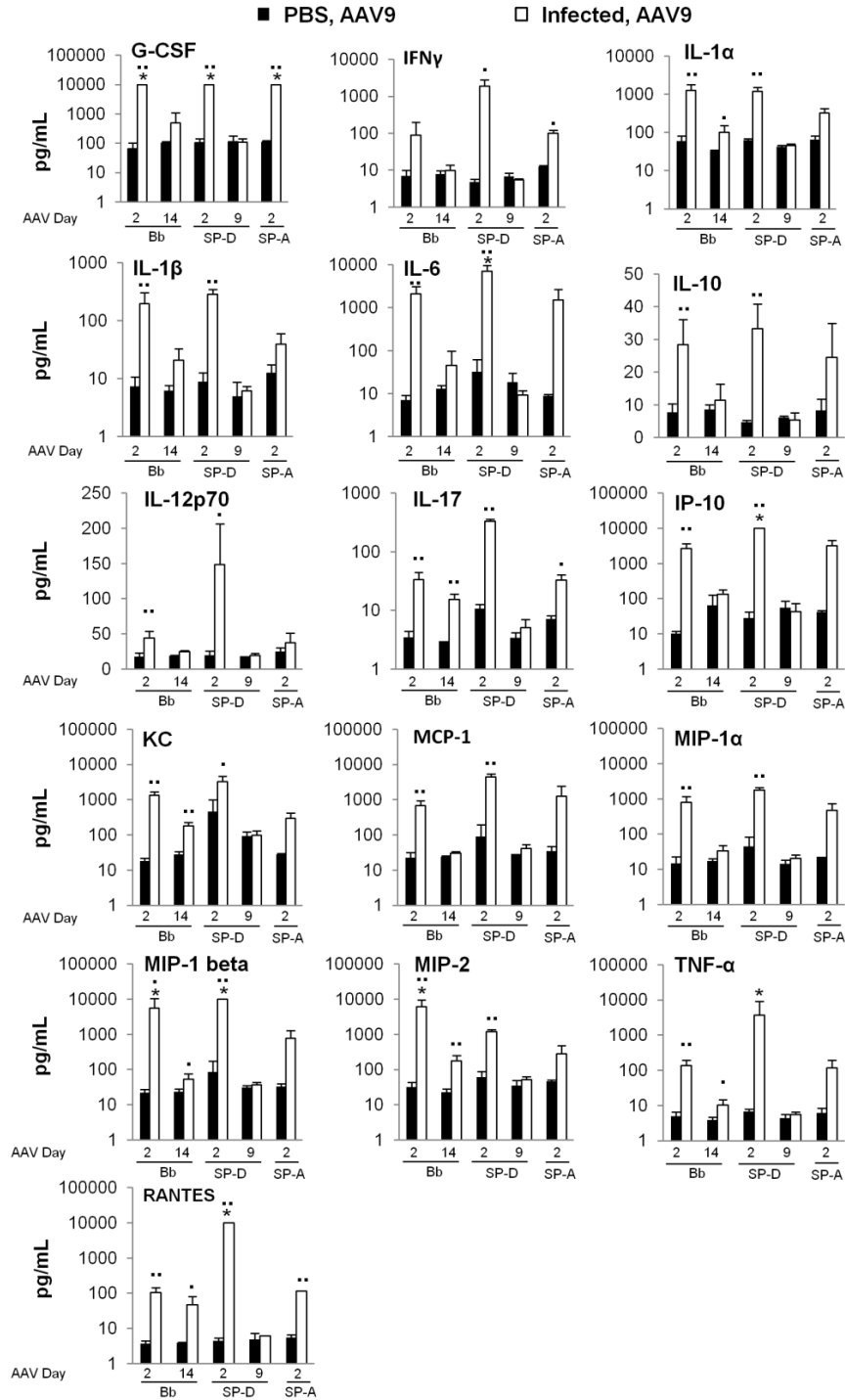


Figure 2.9: BALF cytokine/chemokine levels at time of AAV administration.

Cytokine/chemokine levels were determined using a Milliplex 25-plex premixed magnetic mouse cytokine/chemokine array and Luminex bead reader according to the manufacturer's instructions. Single asterisk (*) indicates samples that exceeded the upper limit of detection of the assay, approximately 10,000 pg/mL. Results are presented as means, and error bars represent the standard deviation (** $P < 0.01$, * $P < 0.05$, Student's *t*-Test, uninfected $n = 2-5$, infected $n = 3-7$ for all groups except SP-A KO infected mice where $n = 2$ for both uninfected and infected mice).

2.5 Discussion

CF airway disease remains an ideal candidate for treatment by gene therapy, despite its limited success seen so far. Individuals with CF are characterized by chronic respiratory infections that are primarily responsible for the high morbidity and early mortality rates associated with the disease. However, limited research has been done to determine the effects of active airway infections on pulmonary gene transfer. The lack of research on this topic is not surprising as no convenient animal model exists to study such effects. Three different mouse models were established in this study to remedy this issue. The first involved creating a chronic infection in C57BL/6 mice with a natural murine pathogen, *B. bronchiseptica* RB50. The second and third models utilized PAO1 to establish an acute, but persistent, infection in SP-D and, separately, SP-A KO mice, respectively.

When an AAV vector was administered to the airways of infected mice on day 2 post-infection, transduction efficiency was similarly reduced in all mouse models, despite remarkable differences in the duration and intensity of all infection models. The approximate 3-fold reduction in gene expression was seen regardless of several additional factors, including the bacterium used, the strain of mouse used, or the bacterial load at the time of AAV administration. This suggests that immune response at the time of vector administration was the main cause for the observed reduction in gene transfer efficiency.

Characterization of these immune responses at day 2 of infection showed that the responses in each infection model were generally similar to each other. Analyses showed that day 2 inflammatory responses were dominated by macrophage and neutrophil infiltration, the latter of which is characteristic of the infected CF lung [29]. However, in the SP-D KO / PAO1 acute

infection model, elevated levels of macrophages and neutrophils were present even in uninfected lung samples, suggesting that their presence alone is insufficient to reduce gene transfer.

While immune responses at day 2 were generally similar between all infection models studied, one striking difference observed was between the chronic and transient acute infection models. In the SP-D and SP-A KO / PAO1 infection models, CD8 T cell activation/recruitment was observed in the infected lungs. In the lungs of *Bb*-infected mice, the numbers of CD8 T cells were not elevated compared to uninfected mice. Despite this apparent difference, we observed a similar level of reduction in gene expression in all three infection models, suggesting that CD8 T cells are not directly involved in the reduction of gene transfer.

When AAV administration was delayed to a later time point post-infection, different effects on AAV-mediated gene expression were seen between the *Bb* chronic infection and the SP-D KO / PAO1 acute infection models. In the *Bb* chronic infection model, postponement of AAV administration to day 14 post-infection resulted in a significant (8-fold, $P < 0.01$, Student's *t*-Test) reduction in gene expression when compared to the expression seen in uninfected controls. In the SP-D KO / PAO1 acute infection model, however, delay of AAV administration to day 9 post-infection resulted in a ~2-fold reduction. The notable differences observed in the immune response at these time points include: (1) elevated levels of macrophages, segmented neutrophils, and CD4 T cells in *Bb*-infected mice but not in PAO1-infected mice and (2) elevated levels of IL-1 α , IL-17, KC, MIP-1 β , MIP-2, TNF- α , and RANTES in *Bb*-infected mice but not PAO1-infected mice. While PAO1-infected SP-D mice did display elevated levels of neutrophils in their BALF samples, many of these cells showed a band, rather than a segmented, nucleus, indicative of immature neutrophilic precursors. Taken together, these results indicate that activated, mature neutrophils and CD4 T cells may be responsible for causing significant reduction in gene expression observed in the *Bb*-infected mice. In particular, the presence of the CD4 T cells may

be playing the more significant role, as that cell type was not present at the earlier AAV administration time point (day 2 post-infection), which reduced gene expression by only ~3-fold.

Additionally, it is worthwhile to note that one of the elevated cytokines on day 14 of the chronic infection, IL-17, is produced by CD4 T cells and has been implicated in the stimulation of airway mucin gene expression [41]. This could suggest the development of an enhanced mucus barrier by day 14 of the chronic infection. Such a phenomenon could explain the significant loss in gene transfer efficiency at this later time point for AAV administration. The enhanced mucus layer would prevent transduction by hindering normal transport of vector to the target cells.

In several studies, AAV vector administrations targeting liver have proven to be safe and rarely elicit an immune response, even when challenged with an adenovirus expressing a similar transgene [42,43]. However, co-administration of TLR ligands, including CpG-containing ODNs, along with antigen-specific T cells or adenoviruses led to loss of liver transgene expression [42,43]. It is likely that in our models, the inflammatory microenvironment and cytokine release profile following bacterial infections may have resulted in the breaking of immune tolerance to AAV particles and/or their gene products. Hence, the bacterial presence in our infection models likely serves as a form of adjuvant, triggering sufficient immunity to the AAV vector and/or to the ffluc product to result in a diminished expression or visualization thereof.

In each of our models, free bacteria were administered to the nares of healthy mice to facilitate a prolonged infection of the respiratory tract. The administration of free bacteria contrasts with the established practice of artificially embedding bacteria in agar beads before depositing them into the airway through a tracheal incision. The methods presented here are thus simpler and less invasive. Additionally, our methods do not limit the infection to the conducting airways, which is not the case with methods involving embedded beads. Due to their size, the

beads create a mechanical block to the bronchi, resulting not only in limited bacterial access to the respiratory airways but also in increased morbidity, unwanted lung damage, and potentially even collapse or closure of the lung [44].

To the best of our knowledge, this the first study to examine the effects of an active, naturally occurring respiratory infection on AAV airway gene transfer. Our findings clearly show that the efficiency of AAV-mediated gene transfer in the airway is negatively impacted by the presence of an on-going or recently resolved bacterial infection. These results underscore the importance of considering such circumstances when developing potential genetic treatments for relevant diseases, like CF and potentially other airway diseases such as asthma. The methods validated here provide simple and minimally invasive techniques that will allow this type of infected environment to be more easily accounted for.

3.0 Synthesis and structure-activity relationships of novel cationic lipids with anti-inflammatory and antimicrobial activities

3.1 Abstract

Certain membrane-active cationic steroids are known to also possess both anti-inflammatory and antimicrobial properties. This combined functionality is particularly relevant for potential therapies of infections associated with elevated tissue damage, e.g. cystic fibrosis airway disease, a condition characterized by chronic bacterial infections and ongoing inflammation. In this study, six novel cationic glucocorticoids were synthesized using beclomethasone, budesonide, and flumethasone. Products were either monosubstituted or disubstituted, containing one or two steroidal groups, respectively. *In vitro* evaluation of biological activities demonstrated dual anti-inflammatory and antimicrobial properties with limited cytotoxicity for all synthesized compounds. Budesonide-derived compounds showed the highest degree of both glucocorticoid and antimicrobial properties within their respective mono- and disubstituted categories. Structure-activity analyses revealed that activity was generally related to the potency of the parent glucocorticoid. Taken together, these data indicate that these types of dual acting cationic lipids can be synthesized with the appropriate starting steroid to tailor activities as desired.

3.2 Introduction

Pathophysiological symptoms of CF airway disease are cyclic in intensity, with the more intense periods termed “exacerbations.” Such exacerbations are associated with high bacterial (usually, *P. aeruginosa*) concentrations in the airway sputum, and aggressive antibiotic treatment is typically required to improve lung function [45,46]. However, while early *P. aeruginosa* infections are susceptible to common anti-pseudomonal antibiotics (e.g. β -lactam antibiotics, aminoglycosides, and fluoroquinolones), later infections are more difficult to treat as antibiotic resistance emerges with the patient’s age [8]. Resistance develops under the pressure of continued heavy use of antibiotics and is linked to increased occurrence of hypermutable *P. aeruginosa* isolates [47]. The emergence of these isolates is associated with the ongoing oxidative stress caused by the chronic polymorphonuclear leukocyte inflammation typical of many CF patients [48]. Thus, a novel antibiotic with a dual anti-inflammatory function may prove useful for treating and preventing additional exacerbations of CF airway disease.

Previous cationic sterol-based cationic lipids synthesized by our laboratory, dexamethasone spermine (DS)[23] and disubstituted dexamethasone spermine (D₂S)[49], exhibited dual antimicrobial and anti-inflammatory functions. This study presents the synthesis and characterization of six new cationic steroids of similar structure to further understanding of this family of compounds. Instead of dexamethasone (dex), the six novel lipids presented (Figure 3.1) here contain other glucocorticoids (GCs) as the steroidal side groups: beclomethasone in BeS and Be₂S, budesonide in BuS and Bu₂S, and flumethasone in FS and F₂S. These cationic lipids were the result of linking a polyamine, i.e. spermine, to the 21-OH position of each steroid. Products from this linkage reaction were either monosubstituted or disubstituted, with linkages occurring at one or both terminal amino groups of spermine, respectively. Compounds were

evaluated for glucocorticoid, antimicrobial, and DNA lipofection activities, as well as for any potential cytotoxic effects on mammalian cells.

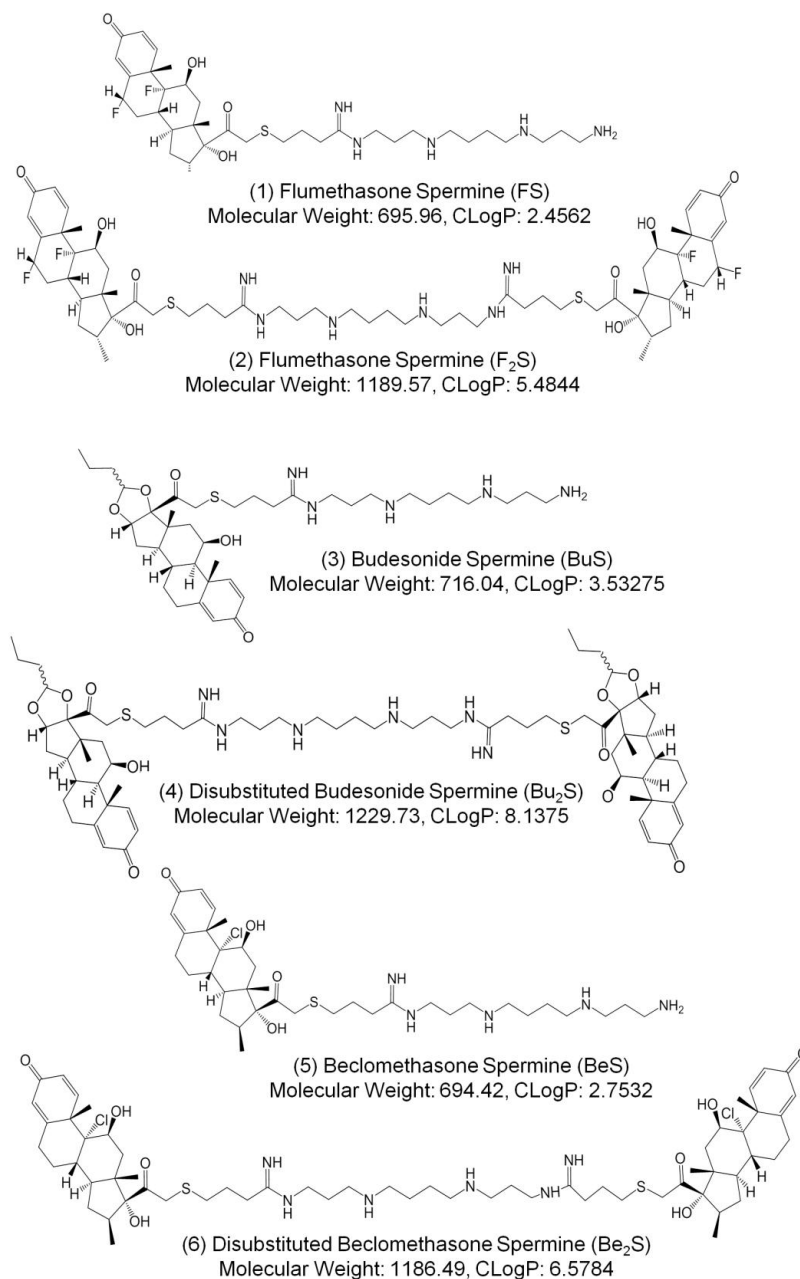


Figure 3.1: Structures, molecular weights, and CLogP values of FS, F₂S, BuS, Bu₂S, BeS, and Be₂S.

3.3 Materials and Methods

3.3.1 *Synthesis of Beclomethasone-21-mesylate and Flumethasone-21-mesylate*

A 125-mL Erlenmeyer flask was filled with 1 g of the desired GC, 3.2 mL of anhydrous pyridine, and 240 μ L of methanesulfonyl chloride. The flask was kept at 0°C with an ice bath and stirred continuously with a magnetic stir bar. After 1 hour, an additional 160 μ L of methanesulfonyl chloride was added and an additional hour was allowed to elapse. The solution was then poured into 20 mL of ice-cold 1 M HCl, causing precipitation. The solid was filtered out and then re-dissolved into 50 mL of ethanol. Precipitation was facilitated again by pouring the solution into 50 mL of 1 M HCl. The precipitate was filtered out one last time, recrystallized in ethanol, filtered, and dried overnight before further processing.

3.3.2 *Synthesis of Cationic Glucocorticoids*

BeS, Be₂S, BuS, Bu₂S, FS, and F₂S were prepared using a reaction scheme previously described for the synthesis of DS and D₂S [23]. Briefly, the 21-mesylate form of each steroid was reacted separately into its mono- and di-substituted cationic products via a one-pot reaction involving molar excesses of both spermine and Traut's reagent (2-iminothiolane HCl) in ethanol at 40°C. Beclomethasone-21-mesylate and flumethasone-21-mesylate were synthesized as described earlier, and budesonide-21-mesylate was obtained from Steraloids (Newport, RI). Spermine and Traut's reagent were obtained from Sigma-Aldrich. The reaction was allowed to proceed for ~1 hour before being quenched with trifluoroacetic acid (Sigma-Aldrich). Ethanol was removed through evaporation under vacuum, and the reaction products were dissolved in water before the purification process.

3.3.3 Semi-preparative Purification of Reaction Products

A Shimadzu (Columbia, MD) LC-MS system was used to purify desired products from the crude reaction mixture. This system included an LC-20AB pump for solvent delivery connected to a SIL-20A autosampler, which was coupled to a SPD-20A for dual wavelength UV-Vis detection and a LCMS 2010EV mass spectrometer. A Hamilton (Reno, NV) PRP-1 column (250 x 10 mm i.d., 10- μ m particle size) was used with this LC-MS to facilitate separation. The mobile phase flow rate was 4 ml/min with a starting ratio 95% mobile phase A (water) and 5% mobile phase B (acetonitrile). For the beclomethasone- and flumethasone-derived products, the elution profile consisted of an isocratic step to 13% phase B for 35 minutes and then another isocratic step to 20% phase B for an additional 35 minutes. For separation of the budesonide products, the first step of the elution profile is the same at 13% phase B for 35 minutes, but the second step uses 25%, in lieu of 20%, phase B for the same amount of time. Solvent from collected fractions was removed via lyophilization, and final products were resuspended in nuclease-free water at concentrations between 10 and 20 mg/ml.

3.3.4 Analytical Characterization

Analytical characterization was performed with the same Shimadzu LC-MS system coupled to a Hamilton PRP-1 column (150 x 4.6 mm i.d., 5- μ m particle size). The mobile phase flow rate was 1 ml/min with a starting composition of 95% mobile phase A (water) and 5% mobile phase B (acetonitrile). The elution profile for the beclomethasone- and flumethasone-derived products consisted of an isocratic step to 13% phase B for 25 minutes and then a second step to 20% phase B for an additional 25 minutes. For the budesonide-derived products, the second step utilized 25% phase B. Results from the mass spectrometer was used to assess purity of eluent. ^1H and ^{13}C NMR analyses were performed using an AVANCE III 500 MHz

instrument (Bruker, Newark, DE) using a dual 5-mm cryoprobe. The calculated log values of the octanol/water partition coefficient, i.e. ClogP values, were predicted with ChemBioDraw Ultra software (Cambridgesoft) using fragmentation methods.

3.3.5 Cell Culture

Bovine aortic endothelial cells (BAECs) and A549 cells were cultured at 37°C and 5% CO₂ in DMEM supplemented with 10% FBS, 2% penicillin/streptomycin, and 1% L-glutamine (all materials from Gibco, Life Technologies). All experiments were conducted with cells seeded 24 hours before any treatment at 50-75% confluence in 96-well plates.

3.3.6 Nuclear Localization of the Glucocorticoid Receptor

Ability to trigger nuclear localization of the GC receptor (GR) was visualized by transfecting BAECs with pGFP-GR, a plasmid encoding for a green fluorescent protein (GFP)-GR chimeric protein. Cells were seeded as described earlier and allowed to spread overnight. Transfections were then conducted with Lipofectamine 2000 (Life Technologies), according to the manufacturer's instructions. DNA-Lipofectamine complexes were incubated with cells for two hours before the growth media was replaced with DMEM supplemented with 0.5% charcoal-treated FBS to prevent non-specific cellular responses to hormones in the serum. After an additional overnight incubation, cells were treated with cationic GCs at the specified concentrations (0.01 -1 μ M) for approximately 1 hour before being imaged using light and fluorescence microscopy (IX81; Olympus America). Total cellular fluorescence and nuclear localized fluorescence were calculated with ImageJ (NIH) for at least 10 cells per condition. These values were used to determine the average percentage of nuclear localized cellular fluorescence per condition.

3.3.7 Induction of the Glucocorticoid Response Element

Ability to induce the GC transcriptional response element (GRE) was analyzed by transfecting BAECs with pGRE-SEAP, a plasmid containing the gene for a secreted embryonic alkaline phosphatase (SEAP) under transcriptional control of the GRE. BAECs were transfected using the procedure described earlier for pGFP-GR transfection. After replacement of the media with DMEM containing 0.5% charcoal-treated FBS, cells were incubated overnight. Growth media was then refreshed with fresh media containing the specified concentrations of the synthesized cationic lipids (1 or 5 μ M). Cells were treated with the lipids for 24 hours before the media was harvested and stored at -20°C until further processing for SEAP quantification, which was conducted using the Great EscAPe™ SEAP Chemiluminescence Reporter Kit 2.0 (Clontech) according to the manufacturer's instructions. Chemiluminescence was measured with an EnVision Multilabel Plate Reader (PerkinElmer Life and Analytical Sciences, Waltham, MA).

3.3.8 Antibacterial Activity

Minimal inhibitory concentrations (MICs) and minimal bactericidal concentrations (MBCs) were determined using bacteria in their logarithmic phase of growth. Compounds were tested against the following bacteria: MRSA strain Xen30 (Caliper Life Sciences), *Escherichia coli*, and various strains of *Pseudomonas aeruginosa*, i.e. PA14, Xen5 (Caliper Life Sciences), and ten clinical strains isolated from CF sputum. Clinical strains were collected by spontaneous expectoration from patients attending the Adult Cystic Fibrosis Center, University of Pennsylvania Health System (IRB 803255). When necessary, samples were diluted with PBS and vortexed in the presence of glass beads to produce homogenous solution. Known antimicrobial agents tobramycin (TOB), cathelicidin LL-37 (Peptide 2.0), DS, and D2S, were also tested to provide reference MIC and MBC values. MIC/MBC values were determined in Luria-Burtani

(LB) media using the microdilution methods. Bacteria were incubated at 37°C for 18-24 hours before being treatment. MIC values were determined against bacterial concentrations of $\sim 5 \times 10^5$ CFU/ml, and MBC values were performed by plating each sample on appropriate solid agar mediums.

3.3.9 Preparation of Liposomes and Lipoplexes

Liposomes were formed by combining lipids with a neutral lipid, 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (Avanti Polar Lipids) in a 1:1 molar ratio, as previously described [49]. Briefly, the neutral lipid in chloroform was added to a glass tube, and the solvent was removed under vacuum to generate a lipid film. The film was rehydrated with sterile water containing appropriate amounts of the desired cationic lipids. Mixtures were then probe-sonicated for 30 seconds and briefly vortexed before use. Lipoplexes were formed by diluting the liposomes into an equal volume of Opti-MEM® I Reduced Serum Media (Life Technologies) containing plasmid DNA at the appropriate concentration. Lipoplexes were formed 15 minutes before use in all experiments.

3.3.10 Transfection and measurement of EGFP

Lipoplexes were used to transfect BAECs and A549 cells, separately, to determine transfection abilities of synthesized lipids. Plasmid DNA, pGFP-N3 (Clontech), was transfected to generate GFP as a fluorescent transgene reporter protein. Lipofectamine 2000 in a 2:1 (w/w) ratio with DNA was used as the positive control for both cell lines, and plasmid DNA without any cationic lipid was used as the negative control. Cells were transfected in quadruplicate in 96-well plates, 24 hours post-seeding. Media was removed, and cells were washed once with sterile PBS before being treated with lipoplexes. After three hours of treatment, media was aspirated and replaced with fresh DMEM without any antibiotics. After 24 hours, cells were harvested and

processed with flow cytometry to determine the percentage of cells positive for GFP. A BD Accuri™ C6 flow cytometer was used for this analysis

3.3.11 Effects on Cell Viability

A549 and BAECs cells were seeded on 96-well plates and allowed to spread overnight, as described earlier. Growth media was then replaced with media containing specified concentrations of lipids (0.01 – 50 μ M), and cells were incubated for 24 hours. A CellTiter-Glo® Luminescent Cell Viability Assay (Promega) was then used to evaluate cell viability after treatment, according to the manufacturer's instructions. Luminescence was measured with an EnVision Multilabel Plate Reader (PerkinElmer Life and Analytical Sciences, Waltham, MA).

3.3.12 Red Blood Cell Hemolysis

Hemolytic activities of synthesized cationic lipids were tested against human red blood cells (RBCs) collected from fresh blood, obtained in accordance with University of Pennsylvania's IRB. Whole blood was anti-coagulated in citrate concentrated solution (9 parts whole blood to 1 part citrate solution, SigmaAldrich), and then centrifuged for 5 minutes at 500xg. Plasma was removed, and the remaining hematocrit layer was resuspended in 150 mM NaCl to wash the RBCs. Sample was centrifuged again at 500xg for 5 minutes and then washed an additional two times, first with 150 mM NaCl and then with fresh Tyrode's solution without bovine albumin serum. After a final centrifugation cycle, a ~2% hematocrit suspension was achieved by diluting 1mL of RBCs into 49mL of fresh Tyrode's solution. Compounds were added to 100 μ L of this suspension to achieve the specified concentrations, and all samples were incubated for 1 hour at 37°C before centrifugation at 500xg for 5 minutes to pellet any intact RBCs. Supernatants were transferred into a clean 96-well plate, and relative hemoglobin (Hb)

concentration was quantified by measuring absorbance at 405 nm. To achieve 100% hemolysis, 1% Triton X-100 was as a positive control to disrupt RBC membranes.

3.4 Results

A one-pot reaction with the appropriate GC mesylate, Traut's reagent (TR), and spermine yielded the monosubstituted steroid (FS, BuS, or BeS) as the major product and the disubstituted steroid (F2S, Bu2S, or Be2S) as the minor product. The primary amines on either end of spermine reacted with TR to cause a selective ring-opening, resulting in an exposed sulfhydryl (-SH) end group. This end group then interacted with the α -keto mesylate of the modified GCs to form an α -keto thioether linkage between the steroid and polycation tail (i.e. the spermine-TR conjugate), yielding the monosubstituted cationic steroid. To form the disubstituted product, the primary amine of the monosubstituted lipid reacted with another TR molecule to eventually result in another thioether linkage with a separate GC molecule.

Desired compounds were purified from excess starting reactants and unwanted reaction intermediates with semi-preparative HPLC, and then their molecular weights and chemical structures were verified with mass spectroscopy (MS) and ^1H and ^{13}C NMR. MS results are shown in Appendix A, Figures A1-A3. ^1H and ^{13}C NMR spectra and chemical shifts are also shown in Appendix B and Appendix C, Figures B1-B6 and C1-C6, respectively. Overall yield for the entire process (synthesis to purification) was ~60-70%, i.e. ~60-70% conversion of the parent steroid mesylate to the monosubstituted cationic product and a subsequent ~6-10% conversion of the monosubstituted product to the disubstituted product in a one-pot reaction.

Hydrophobicity of compounds was also assessed through ClogP values, which are shown in Figure 3.1. Disubstituted products exhibited higher ClogP values than their monosubstituted

analogues. Budesonide-derived compounds showed the highest values, while flumethasone compounds showed the lowest.

Ability to trigger some degree of nuclear localization of the GR was observed for all cationic lipids, though some exhibited more potency than others (Figure 3.2). Only budesonide-derived compounds showed activity equivalent to that seen with the positive control dex. Flumethasone and beclomethasone compounds showed less potency than equivalent concentrations of dex at all values tested ($p < 0.01$). GC character was further evaluated by measuring ability to induce expression of the GRE. Again, budesonide-derived products are the only compounds to achieve expression equivalent to that seen with dex (Figure 3.3). Interestingly, beclomethasone products showed a comparatively limited ability to trigger GRE induction; no cellular response was observed at 1 μ M BeS or Be2S (data not shown). At 5 μ M, Be2S triggered only a modest response, while BeS triggered no effect at all. Flumethasone-derived products were able to achieve equivalently limited responses at 1 μ M for both FS and F2S.

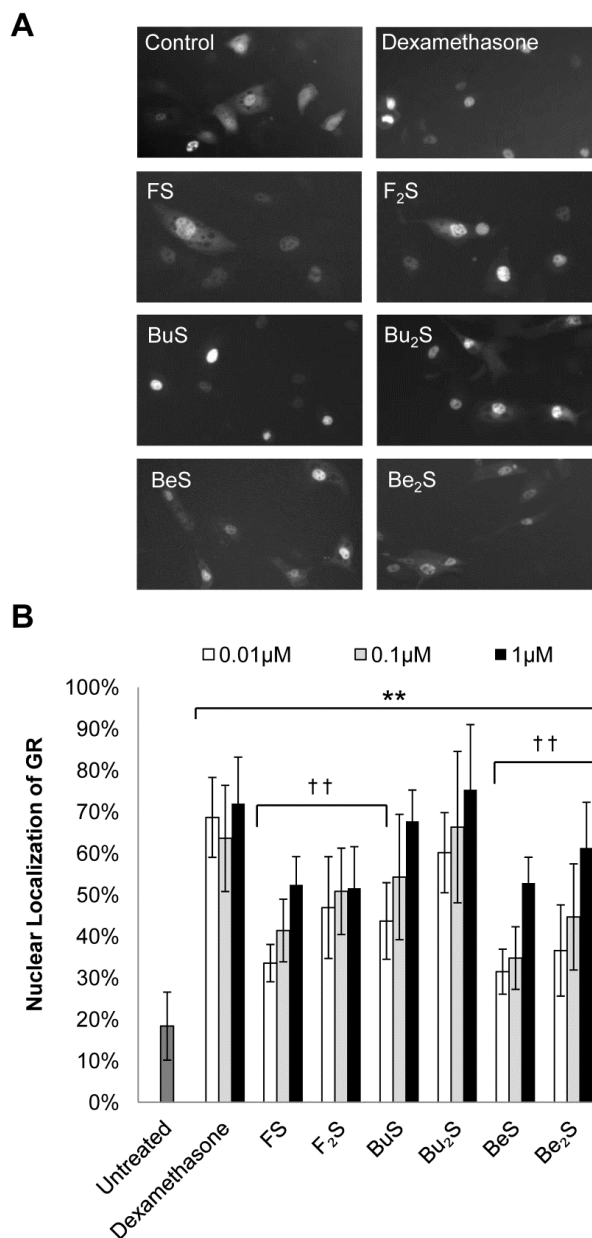


Figure 3.2: Steroid-induced nuclear localization of GFP-GR in BAECs

(A) GFP-GR translocation from cytosol to nucleoplasm. BAECs were transfected with pGFP-GR using Lipofectamine 2000 and then treated with compounds to stimulate GFP-GR nuclear localization. (B) Quantitative evaluation of cellular fluorescence localized within the nucleoplasm after treatment. Cells were treated with dexamethasone (positive control), FS, F₂S, BuS, Bu₂S, BeS, and Be₂S at various concentrations. After 4 hours of treatment, cells were imaged using fluorescence microscopy, and percentage of cellular fluorescence localized within the nucleus was assessed using ImageJ. At least 10 cells per condition were evaluated. Results are presented as means, and error bars represent the standard deviation. All experimental compounds showed some degree of GC activity greater than the untreated control at all concentrations tested (** $p < 0.01$, Student's *t*-test). Only BuS (0.1-1 μM) and Bu₂S (0.01-1 μM), however, showed activity equivalent to that seen at equivalent concentrations of the positive control dexamethasone. † † indicates statistical significance ($p < 0.01$, Student's *t*-test) when compared to dexamethasone.

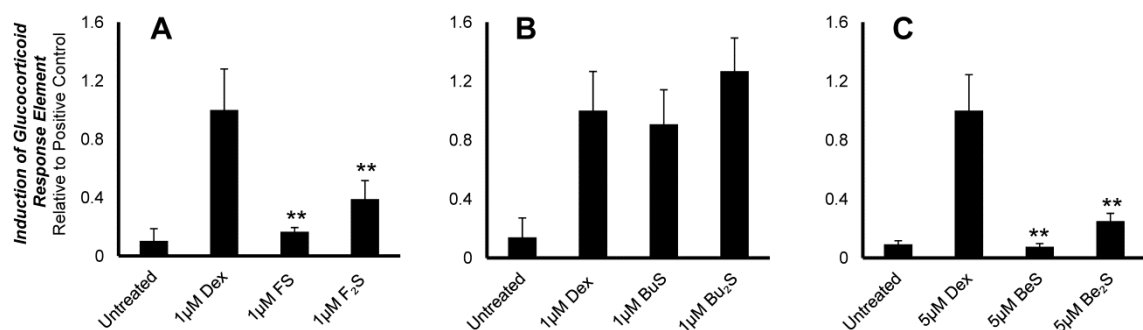


Figure 3.3: Steroid-induced activation of GRE promoter in BAECs

Cells were transfected with pGRE-SEAP using Lipofectamine 2000 and then treated with (A) 1 μ M FS and F₂S, (B) 1 μ M BuS and Bu₂S, and (C) 5 μ M BeS and Be₂S to induce activation of the GRE-SEAP gene construct. After 24 hours of treatment, media was harvested and evaluated for SEAP levels. Results were normalized relative to the positive control, dexamethasone (dex), and are presented as means. Error bars represent the standard deviation. Only BuS and Bu₂S showed GRE activation comparable to that seen with dex. Flumethasone and beclomethasone products, on the other hand, showed significantly less GRE activation (** $p < 0.01$, Student's t -test) than that seen with equivalent concentrations of dex.

Bacteria killing abilities were tested against various strains of bacteria, including several clinical isolates of *P. aeruginosa*, and the determined MICs and MBCs, as well as MBC:MIC ratios, are presented in Tables 3.1A-B. For clarity, MBCs are also presented in a cluster heat map in Figure 3.4. While generally more effective against the gram-positive bacterium tested (MRSA Xen30), many of the monosubstituted products were unable to achieve inhibitory effects at concentrations less than 128 μ g/ml with the other bacteria tested. BuS was the exception in this general trend, showing activity nearly equivalent to that seen with the more potent disubstituted products. Notably, BuS and all other disubstituted products displayed bactericidal activity greater than that observed with the positive controls, tobramycin and cathelicidin LL-37. Bu₂S showed the greatest potency of all compounds tested.

A

Bacteria Strain	MIC (µg/mL) / MBC (µg/mL)									
	TOB	LL-37	DS	D ₂ S	FS	F ₂ S	BuS	Bu ₂ S	BeS	Be ₂ S
MRSA Xen30	ND	8/16	64/64	4/8	64/128	4/4	8/8	4/8	64/64	2/4
<i>E. coli</i>	ND	16/32	256/256	8/8	256/256	16/64	8/64	1/1	256/>256	8/8
PA 14	ND	128/256	256/256	8/16	256/>256	8/8	64/64	8/8	256/>256	16/32
PA Xen5	64/128	64/128	256/256	16/32	128/128	8/16	8/32	1/1	256/>256	8/8
PA*-1	16/64	32/64	128/256	32/64	256/>256	32/32	8/16	4/4	256/>256	8/8
PA*-2	ND	ND	256/256	32/64	256/256	16/16	32/64	8/16	64/128	16/32
PA*-3	64/128	>256/>256	256/>256	32/64	256/>256	16/16	64/128	32/32	256/>256	64/64
PA*-4	32/64	>256/>256	256/256	16/32	256/256	32/64	32/32	8/16	256/>256	32/64
PA*-5	>256/>256	>256/>256	256/256	16/32	>256/>256	16/32	128/128	8/8	256/256	32/32
PA*-20	32/64	64/256	128/256	32/64	256/>256	16/32	16/32	4/8	256/>256	32/64
PA*-21	32/64	64/256	256/256	64/64	128/256	32/32	32/64	16/16	256/>256	32/64
PA*-22	>256/>256	>256/>256	128/128	16/16	128/256	16/16	64/256	8/16	128/256	16/32
PA*-63	64/256	128/256	128/128	32/32	128/128	8/8	16/64	8/16	128/256	16/16
PA*-67	>256/>256	>256/>256	>256/>256	32/32	>256/>256	16/64	128/128	8/8	>256/>256	16/32

B

Bacteria Strain	MBC/MIC									
	TOB	LL-37	DS	D ₂ S	FS	F ₂ S	BuS	Bu ₂ S	BeS	Be ₂ S
MRSA Xen30	ND	2.0	1.0	2.0	2.0	1.0	1.0	2.0	1.0	2.0
<i>E. coli</i>	ND	2.0	1.0	1.0	1.0	4.0	8.0	1.0	≥2.0	1.0
PA 14	ND	2.0	1.0	2.0	≥2.0	1.0	1.0	1.0	≥2.0	2.0
PA Xen5	2.0	2.0	1.0	2.0	1.0	2.0	4.0	1.0	≥2.0	1.0
PA*-1	4.0	2.0	2.0	2.0	≥2.0	1.0	2.0	1.0	≥2.0	1.0
PA*-2	ND	ND	1.0	2.0	1.0	1.0	2.0	2.0	2.0	2.0
PA*-3	2.0	†	≥2.0	2.0	≥2.0	1.0	2.0	1.0	≥2.0	1.0
PA*-4	2.0	†	1.0	2.0	1.0	2.0	1.0	2.0	≥2.0	2.0
PA*-5	†	†	1.0	2.0	†	2.0	1.0	1.0	1.0	1.0
PA*-20	2.0	4.0	2.0	2.0	≥2.0	2.0	2.0	2.0	≥2.0	2.0
PA*-21	2.0	4.0	1.0	1.0	2.0	1.0	2.0	1.0	≥2.0	2.0
PA*-22	†	†	1.0	1.0	2.0	1.0	4.0	2.0	2.0	2.0
PA*-63	4.0	2.0	1.0	1.0	1.0	1.0	4.0	2.0	2.0	1.0
PA*-67	†	†	†	1.0	†	4.0	1.0	1.0	†	2.0

Table 3.1: Minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of selected steroids against various pathogens.

(A) Specified pathogens were treated with FS, F₂S, BuS, Bu₂S, BeS, and Be₂S as well as known antibiotic compounds, i.e. tobramycin (TOB), cathelicidin LL-37, DS, and D₂S, to provide reference MIC and MBC values. Concentrations are in µg/ml. (B) Calculated MBC:MIC ratios. Single daggers (†) indicate ratios that could not be determined because the MBC and MIC values were both >256 µg/ml (i.e. determined without an exact value). Clinical isolates of *P. aeruginosa* (PA) in both tables are indicated by a single asterisk (*)

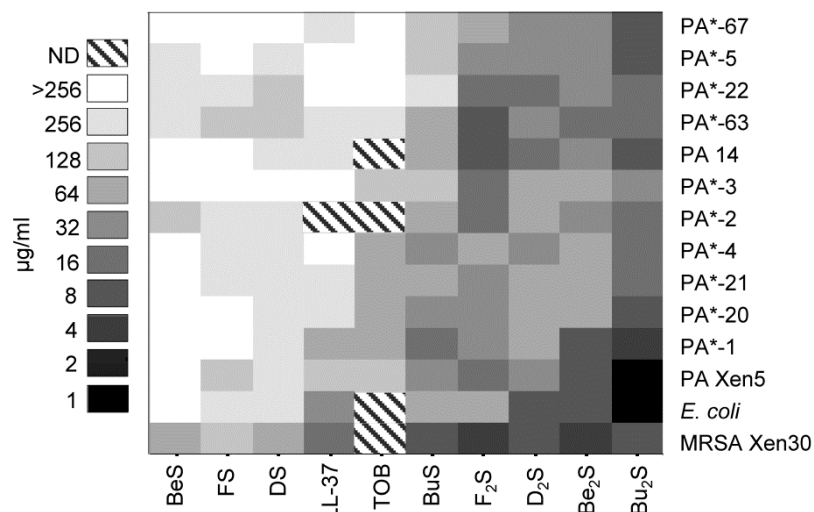


Figure 3.4: Cluster heat map of minimal bactericidal concentrations (MBCs, µg/ml) of cationic steroids against various pathogens.

Specified pathogens were treated with FS, F₂S, BuS, Bu₂S, BeS, and Be₂S as well as known antibiotic compounds, i.e. tobramycin (TOB), cathelicidin LL-37, DS, and D₂S, to provide reference MBC values. Results are presented in a cluster heat map to visually associate compounds with similar activities. Patterned boxes indicate compound/pathogen combinations that were not determined (ND). Clinical isolates of *P. aeruginosa* (PA) are indicated by a single asterisk (*).

Transfection activities of cationic lipids were assessed using mixtures of varying monosubstituted to disubstituted lipid ratios on BAECs or A549 cells. Results are shown in Figure 3.5. With either cell type, transfection mixtures containing only monosubstituted lipids showed zero to very little transfection activity. Mixtures containing only disubstituted products achieved transfection activity generally comparable to that seen with the positive control, Lipofectamine 2000. Interestingly, 60/40 and 40/60 mol% FS/F₂S mixtures showed activity equivalent to that seen with 100% F₂S with either cell type. For budesonide and beclomethasone compounds, trends in activity appeared to be cell specific. 40/60 mol% mono/disubstituted mixtures appeared more effective with BAECs, while 60/40 mol% mixtures were more effective with A549 cells.

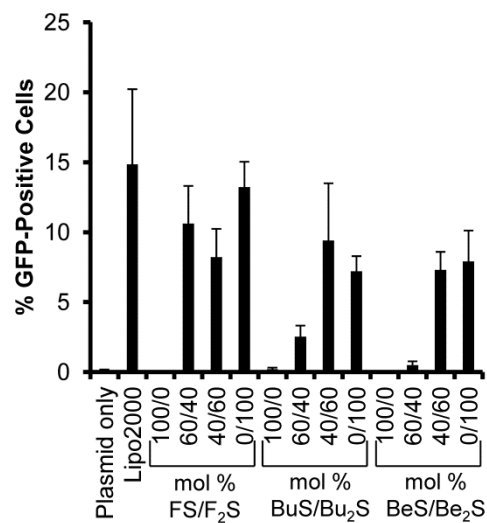
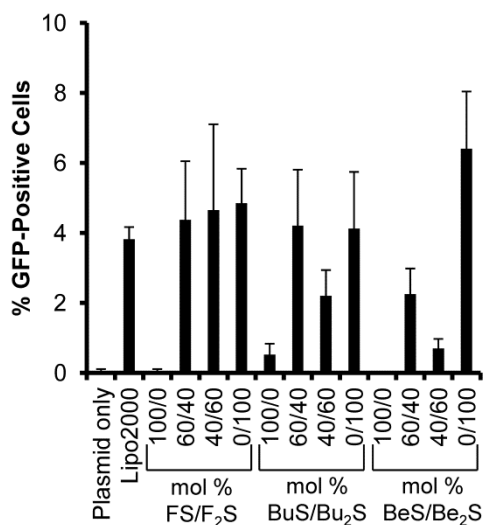
A**B**

Figure 3.5: Transfection of BAECs and A549 cells with FS, F₂S, BuS, Bu₂S, BeS, and Be₂S using GFP transgene (A) BAECs and (B) A549 were transfected with cationic lipids at a charge ratio of 6:1 or Lipofectamine 2000 (i.e. Lipo2000, positive control) at a ratio of 2:1(w/w) ratio with DNA. Cells were harvested 24 hours later and sorted for GFP-positivity using flow cytometry. Results are represented as means, and error bars show the standard deviation from four replicates of 500-1000 events per condition.

Compounds showed limited toxicity on BAECs and A549 cells at concentrations less than 50 μ M (~30 μ g/ml for monosubstituted compounds, ~60 μ g/ml for disubstituted

compounds), as shown in Figure 3.6. At these lower concentrations, loss in cell viability was never greater than ~10% for BAECs and ~20% for A549 cells. Furthermore, toxic effects on viability never exceeded that which was seen with dex at equivalent concentrations. At 50 μ M, however, much more dramatic effects were observed. Treatment with any disubstituted compound resulted in a dramatic loss in cell viability (85-100%) with either cell type. This degree of toxicity was not observed at 50 μ M dex, although this concentration proved to be more toxic than lesser concentrations of the compound. Interestingly, monosubstituted products also did not exhibit this amount of cytotoxicity at 50 μ M concentration. BuS treatment showed loss in viability comparable to that seen with dex at this concentration, while FeS and BeS barely caused a ~5-10% loss in viability.

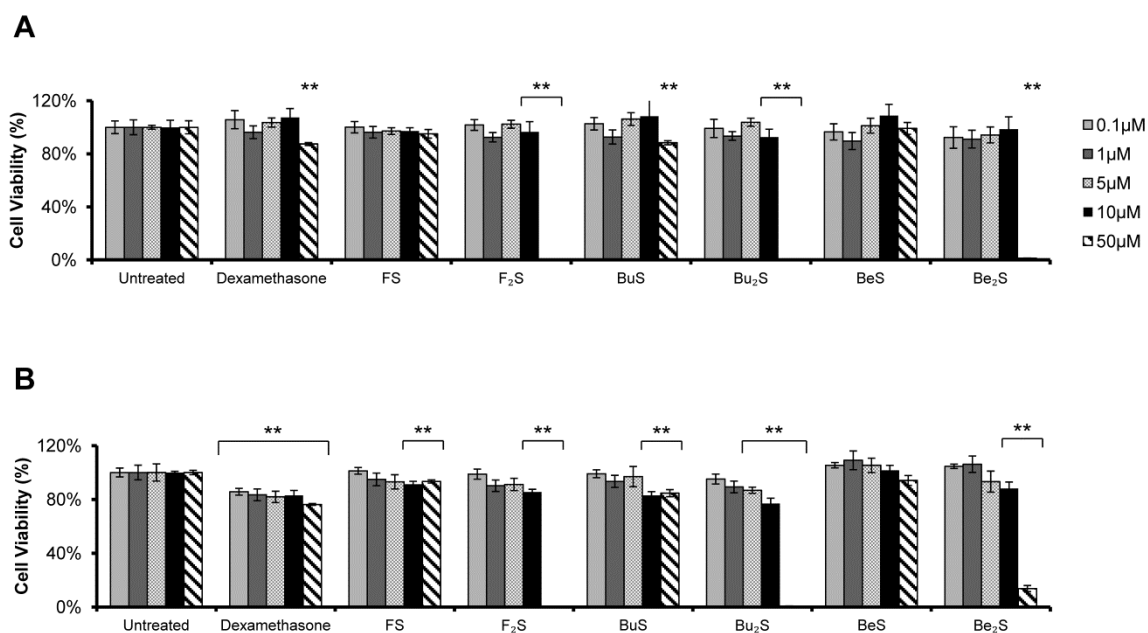


Figure 3.6 Cell viability post-treatment with FS, F₂S, BuS, Bu₂S, BeS, and Be₂S.

(A) BAECs and (B) A549 cells were treated with cationic lipids at the specified concentrations for 24 hours before cell viability was assessed. Results represent means, and error bars indicated standard deviation from four replicates at each condition. At concentrations below 50 μ M, compounds showed limited toxicity, i.e. no more than 10% or 25% loss in viability with BAECs or A549 cells, respectively. Disubstituted products showed significant toxicity at 50 μ M only, resulting in complete or near complete loss in viability with both cell types. In all other cases, toxicity was never greater than that observed with the dexamethasone control. ** indicates statistical significance ($p < 0.01$, Student's t -test) when compared to untreated control.

Toxicity toward eukaryotic cell membranes was assessed by measurement of Hb release from human RBCs, and results are shown in Figure 3.7. Significant Hb release was observed only with the higher concentrations (≥ 50 μM) of the disubstituted compounds. Bu₂S caused the most Hb release at these concentrations, i.e. $\sim 17\%$ Hb release at 50 μM and $\sim 36\%$ release at 100 μM , while Be₂S resulted in the least, i.e. 0% release at 50 μM and $\sim 8\%$ release at 100 μM . All monosubstituted products failed to cause $>1\%$ Hb release at all concentrations tested.

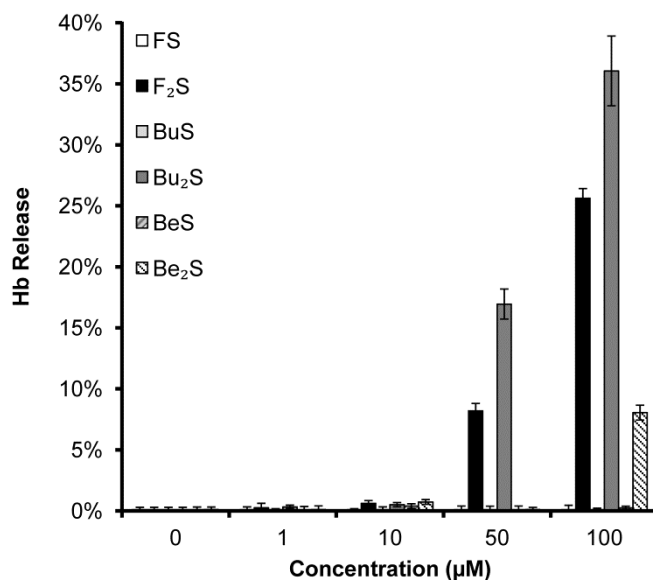


Figure 3.7: Red blood cell lysis in response to treatment with FS, F₂S, BuS, Bu₂S, BeS, and Be₂S

Freshly isolated human RBCs were treated with specified amounts of cationic lipids, and hemoglobin (Hb) release was measured after one hour of treatment at 37°C. Results are represented as means, and error bars show the standard deviation from four replicates at each condition. Monosubstituted products show very little to no RBC hemolysis at all concentrations tested. Disubstituted products show hemolysis at concentrations greater than 50 μM .

3.5 Discussion

This report presents the synthesis and in vitro characterization of six novel cationic GCs with dual antimicrobial and anti-inflammatory character. These compounds are based on previously synthesized sterol-derived cationic steroids, DS and D2S, which also displayed antimicrobial and anti-inflammatory properties. Compounds presented here exhibited a range of

anti-inflammatory and antimicrobial activities without showing dramatic toxicities at the concentrations necessary to achieve desired activities.

Disubstituted products generally demonstrated more potency than their monosubstituted analogue in many of the categories tested. This is an expected observation since the disubstituted products, as demonstrated by their CLogP values, are more lipophilic than their monosubstituted analogues. With higher hydrophobic character, these compounds are able to penetrate the hydrophobic core of a cell membrane more easily and trigger the appropriate response once inside the cell. This enhanced membrane activity is also reflected in their ability to trigger Hb release from RBCs at concentrations that triggered no such response with the monosubstituted products. Additionally, the greater anti-inflammatory activity of the disubstituted compounds can also be attributed to the presence of two steroidal side groups, rather than one. With an additional ligand to bind appropriate receptors, these compounds are more likely to bind and activate the cytosolic GR once they have bypassed the cell membrane.

Interestingly, at concentrations above 0.01 μM , one monosubstituted compound, BuS, was able to achieve levels of GC activity equivalent to that observed with dex and its disubstituted counterpart (Bu_2S) without demonstrating significant membrane disruption activity. At the concentrations tested (0-100 μM), treatment of RBCs with BuS did not cause any Hb release, indicating that the plasma membrane had not been compromised. Together, these phenomena suggest that BuS is membrane permeable without being membrane disruptive, as it must bypass the cell membrane in order to bind the cytosolic GR for GC activity. Likely, the smaller size of BuS, relative to that of the disubstituted compounds, allows it to pass through the membrane without disrupting the structural integrity.

GC activity is also known to correlate with the size and polarity of the substituent at the 16 position [50]. Hydrophobic residues at this position, like the cyclic butyraldehyde-containing acetal group of budesonide, result in higher GC potency. In fact, unmodified budesonide demonstrates GC activity that is ~10 times more potent than that of dex [50] and a GR affinity for that is ~8 times the GR affinity of dex [51]. Unmodified flumethasone and beclomethasone have GC activities that are only 2-3 times higher than GC activity of dex [50]. Consequently, the comparatively high degree of GC activity of BuS and Bu₂S is not particularly surprising. However, the lack of GC activity from flumethasone- and beclomethasone-derived compounds is. This effect suggests that addition of the cationic tails to these steroids either results in decreased membrane permeability or decreased GR affinity. The fact that BuS, a monosubstituted analogue, is able to effectively trigger GC activity suggests that this effect is not due to a loss in membrane permeability. FS and BeS are likely just as able to penetrate the cell membrane but are ultimately unable to bind the GR without sufficient affinity for activation.

The trends observed in bacteria killing ability are generally analogous to those seen with GC activity. The disubstituted compounds are more effective than their monosubstituted counterparts, and the explanations are likely the same, despite the differences in lipid membrane constituents in prokaryotic and eukaryotic cells. Disubstituted compounds are larger and more hydrophobic, enabling them to more easily associate with a lipid bilayer membrane and cause disruptions in the structural integrity when they do so. Notably, however, BuS once again shows unexpectedly high activity. Despite being less effective than its disubstituted counterpart, it was much more effective than any of the other monosubstituted compounds tested. In fact, the antimicrobial activity of BuS was more comparable to that of the disubstituted compounds than that of the monosubstituted ones. BuS is thus a particularly promising candidate for a future

therapeutic because it exhibits high potencies in GC and antimicrobial activity but little in cytotoxic and hemolytic activity.

Results from the bacteria killing assay also demonstrated two other key findings. First, BuS and all disubstituted compounds tested were generally more effective at killing the tested pathogens than antimicrobial compounds cathelicidin LL-37 and tobramycin were, and the latter is frequently used to treat bacterial infections during CF airway exacerbations. Second, all compounds of this study, both mono- and disubstituted, showed particularly high efficacies against the Gram-positive bacterium MRSA Xen30. Gram-positive bacteria are generally more susceptible to cationic lipids [52], but they may show especial susceptibility with cationic antibiotics because of their peptidoglycan outer layer. Anionic polymers, i.e. teichoic acids, are exposed on the surface of Gram-positive bacteria and thread through their peptidoglycan outer layer. They impart to it a negative charge that facilitates easy association with cationic antibiotics. Gram-negative bacteria, on the other hand, have an outer membrane layer that contains LPS molecules, which reduce the fluidity, and thus the permeability, of the membrane. LPS typically allows resistance against hydrophobic antibiotics for this reason, but the antibiotics presented here are also cationic in nature. This characteristic likely enables them to weaken the LPS structure by displacing the divalent cations (Ca^{2+} and Mg^{2+}) that normally stabilize it, thereby allowing permeabilization of the outer membrane that render the bacteria unviable.

Transfection activity of the cationic steroids was also assessed, and while no compound was more efficacious than the commercially available Lipofectamine 2000, many of the formulations tested showed comparable activity. Those formulations could therefore be used to facilitate equivalent gene transfer while also effecting anti-inflammatory and antimicrobial changes as well. The value of these compounds is not in enhancing any single particular effect but in achieving simultaneous actions with a single formulation.

The compounds presented here offer a unique advantage in treating infections associated with elevated tissue inflammation. As novel antibiotics with dual anti-inflammatory and bactericidal activities, they are able to treat the infection itself as well as the inflammatory environment that promotes the development of hypermutable bacteria strains and antibiotic resistance. The abilities of these compounds to facilitate gene transfer provide additional value with treatments that target the relevant diseases at their genetic source. More notably, however, the results presented here additionally demonstrate that these compounds can be synthesized with different parent glucocorticoids to produce cationic steroids that retain their dual functionalities but achieve potencies that correlate with that of the starting steroid. Synthesis of these lipids can thus be modified accordingly to tailor potential treatments for a variety of applications and potencies.

4.0 Coordinated cationic glucocorticoid treatment with adeno-associated virus gene transfer during acute airway infection

4.1 Abstract

Airway bacterial infections, whether currently on-going or recently resolved, are known to reduce efficacy of airway gene transfer by certain viral vectors. This effect is particularly detrimental for the development of genetic therapies for cystic fibrosis airway disease, which is characterized by chronic infections with opportunistic bacteria and associated inflammation. In this study, negative effects of infection were partially reversed by coordinating gene transfer with pre-treatments involving anti-inflammatory and antimicrobial cationic glucocorticoids. These cationic glucocorticoids, budesonide spermine and disubstituted budesonide spermine, were formulated into liposomes with a neutral lipid and administered to *Pseudomonas aeruginosa* PAO1-infected mice 24 hours before treatment with an adeno-associated virus vector. All mice treated with cationic liposomes showed higher expression levels of the reporter gene, and one mouse even showed expression higher than that seen with healthy, uninfected mice. These results confirm that cationic glucocorticoid treatment can be coordinated with gene therapies for CF airway diseases to regain gene transfer efficiency that was lost due the presence of infection.

4.2 Introduction

As discussed earlier, infections negatively impact gene transfer in the airway, regardless of whether they are currently on-going or recently resolved at the time of gene administration, and the mechanism behind this observed effect was linked to the immune response occurring at the time of gene delivery [53]. These observed phenomenon have strong implications for potential gene therapies of CF airway disease, a condition characterized by on-going infection and concomitant inflammation. Without first addressing this hostile environment for gene transfer, successful gene transfer for treating the underlying genetic disease is likely unachievable. The multifunctional cationic glucocorticoids discussed in chapter 2 have particular potential for such applications, as they possess anti-inflammatory and antimicrobial activities in addition to their abilities to facilitate gene transfer.

In this study, treatment with two cationic glucocorticoids, BuS and Bu₂S, was coordinated with AAV-mediated gene transfer in SP-A KO mice transiently infected with *P. aeruginosa* PAO1. BuS and Bu₂S were the most likely candidates to perform well *in vivo* due to their encouraging potencies observed *in vitro*. These cationic glucocorticoids were formulated into liposomes with a neutral lipid, DOPE, and then intranasally delivered to mice 24 hours before AAV administration. Loss in gene expression in pre-treated groups was only a fraction of that observed in infected, untreated mice. These results confirm that cationic glucocorticoids can be coordinated with gene therapies of CF airway disease to produce beneficial results.

4.3 Materials and Methods

4.3.1 Mice

C57BL/6 male mice were purchased from Charles River Laboratories (Wilmington, MA), while SP-D and SP-A knockout mice [40] were maintained in-house at the University of Pennsylvania. For all experiments, a group size of at least 3 mice was used for each experimental cohort. All animals were maintained at the Animal Facility of the Translation Research Laboratories at the University of Pennsylvania under protocols reviewed and approved by the University of Pennsylvania's Institutional Animal Care and Use Committee. Prior to all intranasal administrations, mice were anesthetized by an intraperitoneal injection of ketamine/xylazine (70/7 mg/kg).

4.3.2 Preparation of Cationic Liposomes

Separately, BuS and Bu₂S were synthesized and combined with neutral lipid DOPE (Avanti Polar Lipids) in a 1:1 molar ratio to form liposomes, as described earlier. Briefly, the neutral lipid in chloroform was added to a glass tube, and the solvent was removed under vacuum to generate a lipid film. The film was rehydrated with sterile water containing appropriate amounts of the desired cationic lipids. Mixtures were then probe-sonicated for 30 seconds and briefly vortexed. Liposomes were then diluted 10x into PBS to achieve the appropriate concentration. Lipids were formulated 30 minutes before use and kept on ice until administration to animals.

4.3.3 AAV Preparation

The AAV9 vectors flanked with AAV2 inverted terminal repeats (ITRs) contained a firefly luciferase (*ffluc*) reporter gene fused to a nucleus localization sequence at the N-terminus

under the transcriptional control of the cytomegalovirus (CMV)-enhanced chicken- β -actin promoter. Vectors were produced by the Penn Vector core as previously described [39].

4.3.4 Preparation of PAO1 Inoculum

P. aeruginosa PAO1 was cultured at 37°C in Miller's Lysogeny Broth (LB) (Mediatech, Herndon, VA) or on ceftrimide agar from Sigma Aldrich. Bacteria were grown overnight in LB at 37°C with shaking at 250 rpm. Approximately 16-18 hours later, the bacteria were diluted into fresh broth to an optical density (OD) ($\lambda=600\text{nm}$) reading of 0.1 and were allowed to grow until mid-logarithmic phase ($\text{OD}_{600} \approx 0.4$). At that point, the bacteria were harvested and washed once in phosphate-buffered saline (PBS) before being diluted into fresh PBS at the desired concentration (controlled by evaluation of optical density at 600 nm).

4.3.5 Airway Infection with PAO1 and Treatment with Cationic Liposomes and AAV

Before challenge, mice were anesthetized intraperitoneally as described earlier. Mice were then intranasally (i.n.) challenged with a 20- μL bolus (delivered as two 10 μL aliquots, one into each nostril) with *P. aeruginosa* PAO1 to achieve approximately 10^6 CFU/mouse. At 24 hours post-infection, mice received 20 μL of cationic liposomes at specified concentrations. After an additional 24 hours, mice were i.n. administered 5×10^{10} genome copies (GC) of AAV vector in 20 μL (10 μL into each nostril).

To evaluate *ffluc* expression, mice (~20 grams) were anesthetized and suspended before 20 μL of D-luciferin (15 mg/mL) was i.n. administered as two 10 μL aliquots, one into each nostril. After 5 minutes, mice were imaged for 60 seconds with the IVIS Xenogen imaging system. Quantification of signal was calculated with the Living Image 2.5.1 software.

4.3.6 Evaluation of Bacterial Load

Samples of nasal lavage fluid (NLF) were taken from each randomly selected mouse (n=2-3 per group) at the time of AAV administration to evaluate bacterial load. Mice were decapitated and a cannula attached to a 1-mL syringe containing 300 μ L of sterile PBS was placed into the tracheal remnant. PBS was then flushed through the nasal passages and collected through the nares into a 1.5-mL tube. This recovered fluid was used to flush the nasal cavity another two times, for a total of three flushes. All samples were kept on ice until further processing. To determine the CFU counts in each sample, 50-microliter aliquots of each sample were plated on solid selection agar for *P. aeruginosa* and incubated at 37°C for 16 hours. The number of colonies was then counted, and the total CFU count of each sample was back calculated from that number.

4.4 Results

SP-A KO mice were infected with $\sim 10^6$ CFU PAO1 per mouse, treated with cationic liposomes (50 μ M BuS/DOPE or 25 μ M Bu₂S/DOPE) 24 hours later, and then finally given AAV9.*ffluc* 48 hours after initial infection with PAO1. A randomly selected group of mice (n=2-3) were tested for bacterial load at the time of AAV administration (Figure 4.1A). Interestingly, while the BuS/DOPE treated group showed a lower average bacterial load compared to the untreated group, the difference was not significant. Additionally, the Bu₂S/DOPE treated group showed no difference compared to untreated mice.

Significant differences, however, were observed in reporter gene expression, when measured with live whole-animal luminescent imaging one week later (n=3, Figure 4.1B). Although differences were not significant for the BuS/DOPE treated group, mice, on average and individually, showed higher levels of *ffluc* expression than that seen in infected, untreated mice.

Two of these mice showed ~1.5-fold higher expression than infected, untreated mice, and the last mouse showed ~6-fold higher expression. This high expression value was even two-fold higher than that seen in the healthy mice. Recovery in gene expression was much more uniform in the Bu₂S/DOPE treated group, and average expression was significantly higher (~1.7-fold) than that observed in the infected, untreated group ($p < 0.01$, Student's t -test).

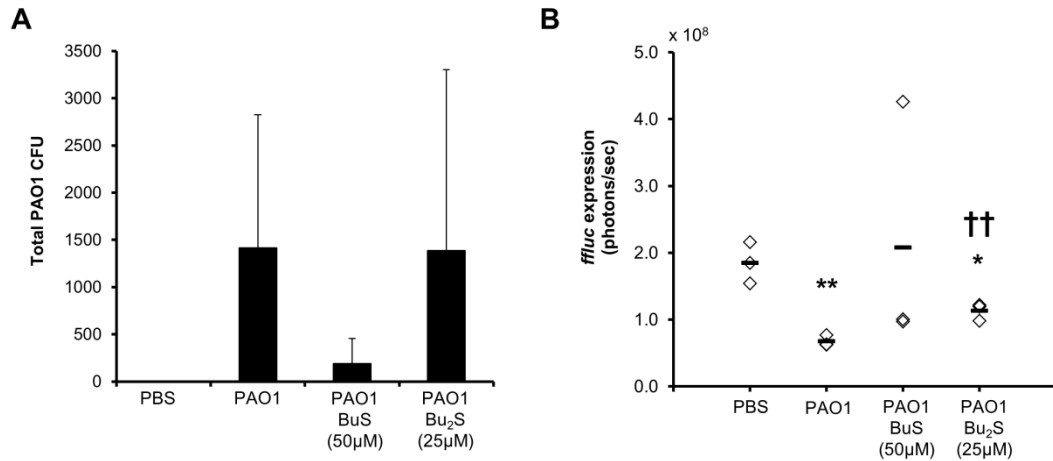


Figure 4.1 Effects of cationic glucocorticoid pre-treatment on AAV9-mediated transduction during acute *Pseudomonas aeruginosa* PAO1 respiratory infection in SP-A KO mice

(A) PAO1 colonization in SP-A KO mouse airway with or without cationic liposome pre-treatment. Approximately 10^6 CFU of bacteria were i.n. delivered to mice, and ~24 hours later, mice were treated with 20 µL of BuS/DOPE or Bu₂S/DOPE cationic liposomes at the specified concentrations. After an additional 24 hours, i.e. 48 hours after initial infection, bacterial load was measured through harvest of NLF from randomly selected mice. Samples from the BuS/DOPE-treated group showed the lowest average bacterial load, but differences were not determined to be significant. Results are shown as means, and error bars represent the standard deviations for $n = 2-3$ mice in each group ($n = 3$ for the PBS-treated group and the BuS/DOPE-treated group; $n = 2$ for the PAO1-only-treated group and the Bu₂S/DOPE-treated group). **(B)** AAV9-mediated transduction in PAO1-infected SP-A KO mice with or without cationic liposome pre-treatment. Two days after PAO1 infection (24 hours after cationic liposome treatment) mice were i.n. dosed with AAV9-*ffluc*. One week later, mice were imaged for gene expression. Plots show quantification of luminescence for mouse nasal cavity of each mouse imaged ($n=3$ for all groups). The PAO1-only-treated group and Bu₂S/DOPE-treated group showed significantly lower signal than the uninfected, PBS-treated mice did (** $p < 0.01$, $p < 0.05$, Student's t -test). Importantly, however, expression in the Bu₂S/DOPE group, while lower than that of the uninfected mice, was significantly higher than that seen in the untreated, infected group (i.e. PAO1-only-treated group, †† $p < 0.01$, Student's t -test). The BuS/DOPE group showed less uniform increases over the PAO1-treated group, and differences between that or the PBS-treated group were determined to be insignificant.

4.5 Discussion

Coordination of gene therapies for CF airway disease with dual functioning anti-inflammatory, antimicrobial cationic lipids could be an effective means for creating viable genetic therapies of this monogenic disease. These lipids could potentially treat not only the local

respiratory infection often seen with many CF patients, but they could also potentially address the concomitant hyperinflammation that so characteristically defines these patients as well. The preliminary results described here in this study validate the potential of using sterol-based cationic lipids to enhance potential gene therapies of CF airway disease.

Pre-treatment with BuS/DOPE or Bu₂S/DOPE liposomes at 24 hours before AAV administration during an active PAO1 infection resulted in partial restoration of reporter gene expression to normal levels seen in uninfected mice. While these differences were not significant in the BuS/DOPE group, none of the mice in this group showed lower expression than any of the mice in the untreated, infected group (n = 3 for both groups). Statistical significance could not be attained due to high variability in expression due to a single high expressing mouse. Notably, this high expresser displayed reporter gene expression that was two-times greater than that observed in the healthy group. Thus, while differences were not significant, trends indicate that BuS/DOPE treatment was beneficial for restoring or even enhancing gene expression to normal levels. Repeat studies with greater n-values are required to determine true variability of treatment and potential reasons why such dramatic variability exists.

Interestingly, the Bu₂S/DOPE-treated mice did show significantly higher gene expression than the infected, untreated group. Preliminary analysis of bacterial load, however, showed limited efficacy of the Bu₂S/DOPE liposome to reduce bacterial load at the time of AAV administration at the concentration tested, i.e. 25 μ M (n=2). This was half the concentration used for the BuS/DOPE formulations, which showed more obvious reduction in bacterial load at 50 μ M (n=3). Higher concentrations of Bu₂S were not tested due to toxicity observed *in vitro* 50 μ M. Regardless, the observed results suggest that gene expression can be restored to normal levels without reducing overall bacterial load. However, our current experimental protocol prevents us from assessing both bacterial load and gene expression in the same mouse, and with such

variability in effects, we cannot determine with certainty whether this conclusion is valid. Mice selected for analysis of gene expression may have had lower bacterial loads than those taken for bacterial load analysis, thereby skewing the true results. Additional experiments are required to further investigate these effects with larger n-number.

The results presented here are preliminary in nature, but they show potential in validating the use of cationic glucocorticoids to enhance gene therapies of CF airway disease. Additional studies with larger cohorts to determine true significance of events. Importantly, however, all of the infected mice treated with cationic liposomes showed higher expression than untreated, infected mice. Future studies should address the mechanism behind this observed effect by more complete analysis of effects on bacterial load as well as characterization of effects on immune response at the time of AAV administration.

5.0 Conclusions and Future Work

5.1 Conclusions

A viable genetic therapy for cystic fibrosis airway disease does not yet exist, despite the more than 20 years that have elapsed since the discovery of the single gene that causes the disease. The lack of a convenient animal model to test potential therapies has hindered progress to a successful approach. The current work addresses this issue by establishing three different mouse models of airway infection, one chronic and two acute, to study the effects of infection on potential therapeutic methods. Individually, these models capture different aspects of clinical manifestations of CF: chronic infection, exaggerated inflammatory responses, and/or less effective clearance of bacteria from the airway. In each model, efficiency of AAV9-mediated transduction was decreased by ~2.5- to 3-fold when AAV was administered on day 2 of infection. Postponing administration of the vector to a later time point gave different results in the chronic and acute infection models. During the chronic infection, postponement resulted in an even greater loss in transduction efficiency (~8-fold), while during the acute infection, the result was the opposite, i.e. partial regain of efficiency was observed. In both cases, loss in efficiency was linked to immune responses occurring at the time of AAV administration. Notably, the more dramatic loss in gene expression at day 14 was associated CD4 T cells and IL-17 secretion, which is associated with stimulation of airway mucus production.

This work is the first to show the negative effects of active infection on AAV-mediated gene transfer, and the implications are serious. Optimal therapeutic efficacy of gene therapies for CF airway disease cannot be achieved unless these effects are addressed as part of the treatment. Previous work in this lab demonstrated that sterol-based cationic lipids could be useful as adjunctive treatment to potential CF gene therapies. These lipids not only facilitated gene transfer but also demonstrated both anti-inflammatory and antimicrobial characteristics as well. In this

study, six additional cationic glucocorticoids were developed and characterized to further understanding of this family of compounds. Results revealed that lipids exhibited potencies that correlated to that of the parent glucocorticoid. Additionally, the two lipids that showed the highest potencies *in vitro*, BuS and Bu₂S, were particularly ideal for applications in treating airway disease due to the low ratio of systemic to local activity of the parent glucocorticoid, budesonide.

Logically, this work concludes with the evaluation of these cationic glucocorticoids on reversing the negative effects of infection on AAV-mediated gene transfer. Preliminary results were encouraging; all mice that received BuS or Bu₂S pre-treatment 24 hours before AAV administration displayed higher gene expression than that seen with infected mice untreated with lipid. Interestingly, one mouse treated with BuS showed enhanced gene expression over that seen with healthy mice. Generally, however, restoration of gene expression was only partial. Lipid treatment was able to reduce the loss in expression by ~25-47% in all other treated mice.

5.2 Future Work

BuS and Bu₂S, thus, show strong potential for enhancing AAV-mediated gene transfer in an infected environment. Additional studies, however, are required to more accurately characterize these effects and the mechanisms behind them. Experiments need to be repeated with larger n-value to determine true significance of events. Studies should also include characterization of the immune response at the time of AAV administration to evaluate any effects on inflammation. Methods to further enhance the therapeutic value of these coordinated gene therapies can also be investigated. Such methods could include varying the dosages and dosing schedule of the administered lipids. Dosages should differ by total concentration as well as by composition of mono- to disubstituted compounds. Eventually, lipids can also be tested in the chronic infection models to determine if therapeutic effects are still observed with the changes in immune response that eventually occur during a sustained infection. Effects of long-term usage

should also be considered if these compounds will be used to as an ongoing treatment in cases of chronic infection.

While the present work has focused the application of these cationic glucocorticoids for enhancing gene therapies of CF, the therapeutic potential of these compounds be extended to other applications as well. In particular, the dual anti-inflammatory and antimicrobial functionality are particularly suited for treating infections associated with elevated tissue damage. Examples include bacterial keratitis (bacterial infections of the cornea) and otitis externa (inflammation or infection of the ear canal). Such conditions benefit from combined antibiotic/steroid treatment over treatment that only utilized one or the other [54,55]. The cationic glucocorticoids presented here could provide a useful alternative to current methods by eliminating the need for a dual-compound therapy for these conditions.

Appendix A: Mass spectrometry data for FS, F₂S, BuS, Bu₂S, BeS, and Be₂S

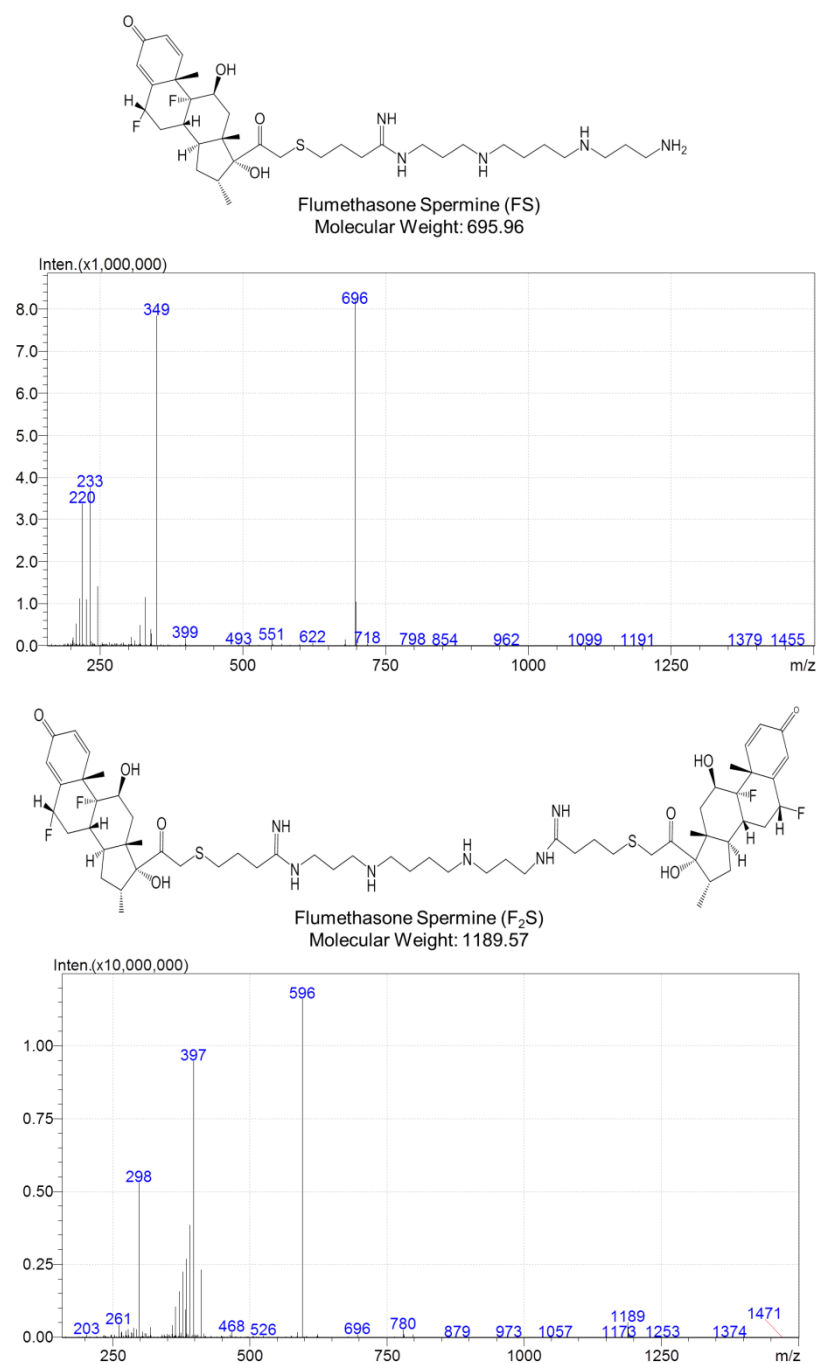


Figure A1: Structures of FS and F₂S with mass spectrometry data showing multiple ionizations.

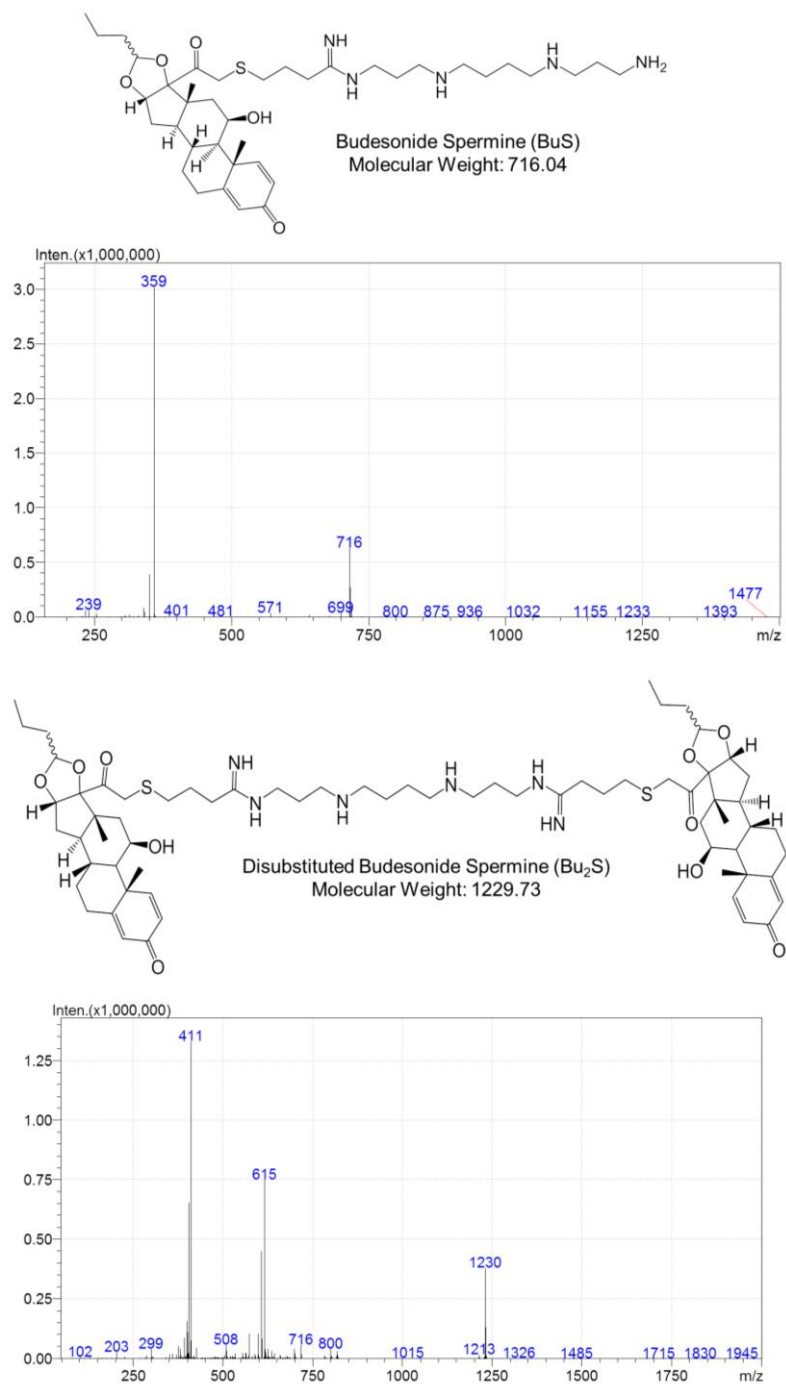


Figure A2: Structures of BuS and Bu₂S with mass spectrometry data showing multiple ionizations.

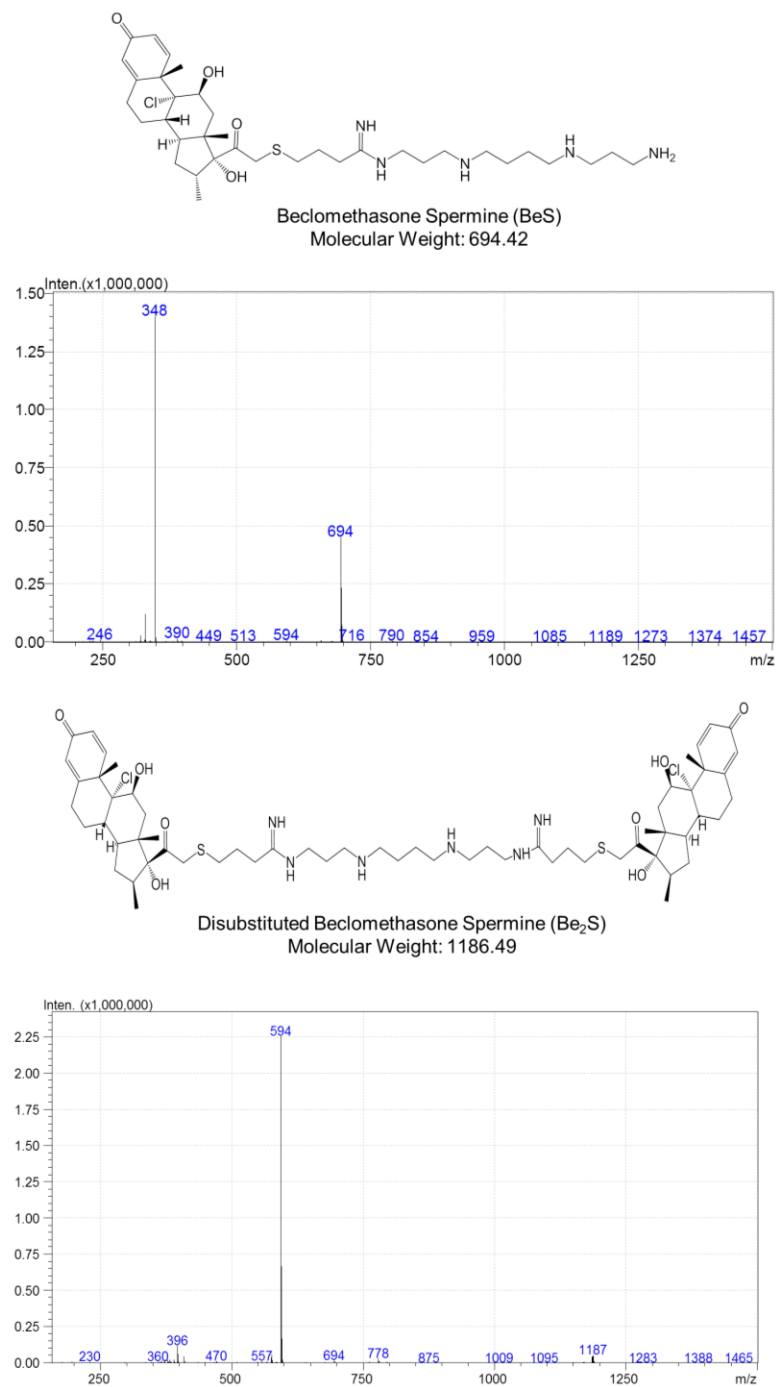


Figure A3: Structures of BeS and Be₂S with mass spectrometry data showing multiple ionizations.

Appendix B: ^1H NMR Data for FS, F_2S , BuS, Bu_2S , BeS, and Be_2S

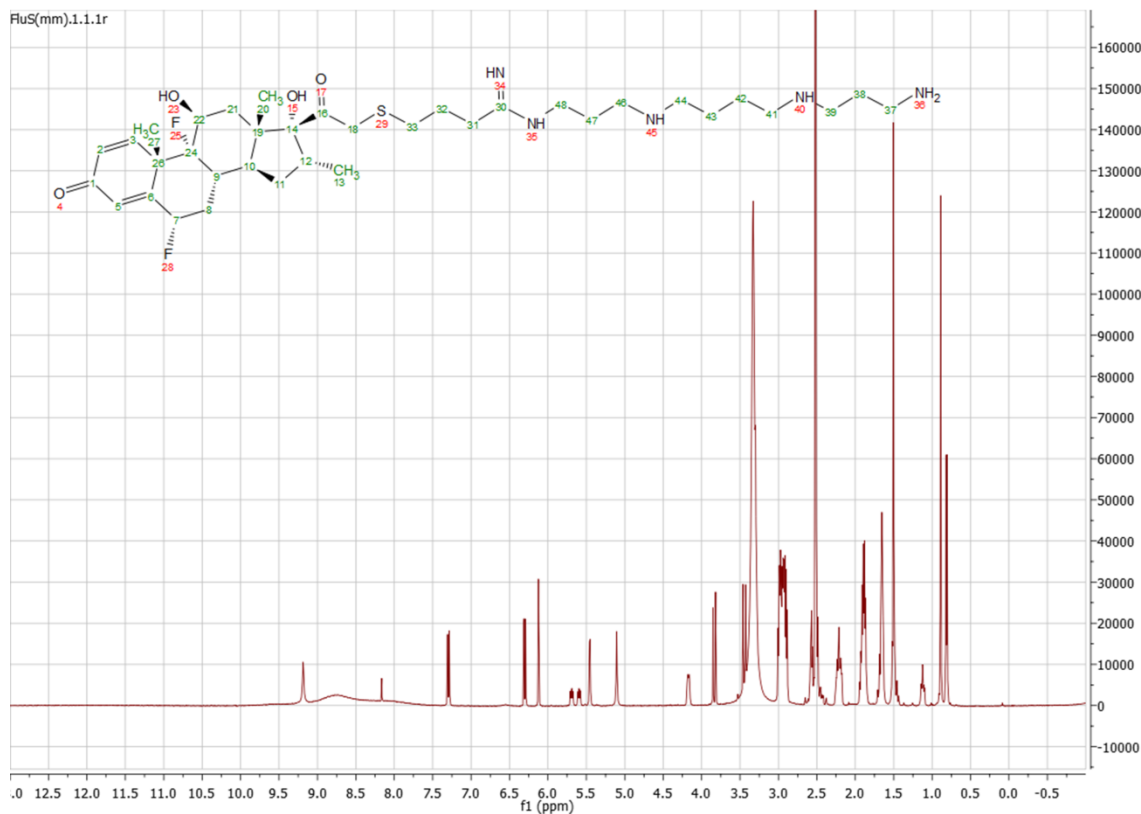


Figure B1: ^1H NMR spectrum of FS

Flumethasone Spermine, **FS**, N-(3-((4-((3-aminopropyl)amino)butyl)amino)propyl)-4-((2-((6S,8S,9R,10S,11S,13S,14S,16R,17R)-6,9-difluoro-11,17-dihydroxy-10,13,16-trimethyl-3-oxo-6,7,8,9,10,11,12,13,14,15,16,17-dodecahydro-3H-cyclopenta[a]phenanthren-17-yl)-2-oxoethyl)thio)butanimidamide ^1H NMR (500 MHz, $\text{DMSO}-d_6$ with TMS) δ 9.19 (s, 1H), 8.16 (s, 0H), 7.30 (dd, $J = 10.2, 1.5$ Hz, 1H), 6.30 (dd, $J = 10.1, 1.9$ Hz, 1H), 6.12 (s, 1H), 5.69 (ddd, $J = 11.4, 6.6, 2.0$ Hz, 1H), 5.59 (ddd, $J = 11.5, 6.7, 2.0$ Hz, 1H), 5.46 (dd, $J = 4.7, 1.6$ Hz, 1H), 5.11 (s, 1H), 4.17 (td, $J = 6.4, 3.1$ Hz, 1H), 3.83 (d, $J = 16.6$ Hz, 1H), 3.44 (d, $J = 16.6$ Hz, 1H), 3.28 (s, 1H), 3.03 – 2.87 (m, 10H), 2.57 (td, $J = 7.1, 2.4$ Hz, 2H), 2.48 (dd, $J = 19.8, 6.3$ Hz, 2H), 2.21 (tt, $J = 11.2, 4.3$ Hz, 3H), 1.90 (dp, $J = 14.9, 7.6$ Hz, 6H), 1.73 – 1.62 (m, 5H), 1.55 – 1.47 (m, 1H), 1.50 (s, 3H), 1.51 – 1.41 (m, 1H), 1.12 (ddd, $J = 12.2, 8.1, 4.2$ Hz, 1H), 0.89 (s, 3H), 0.81 (d, $J = 7.3$ Hz, 3H).

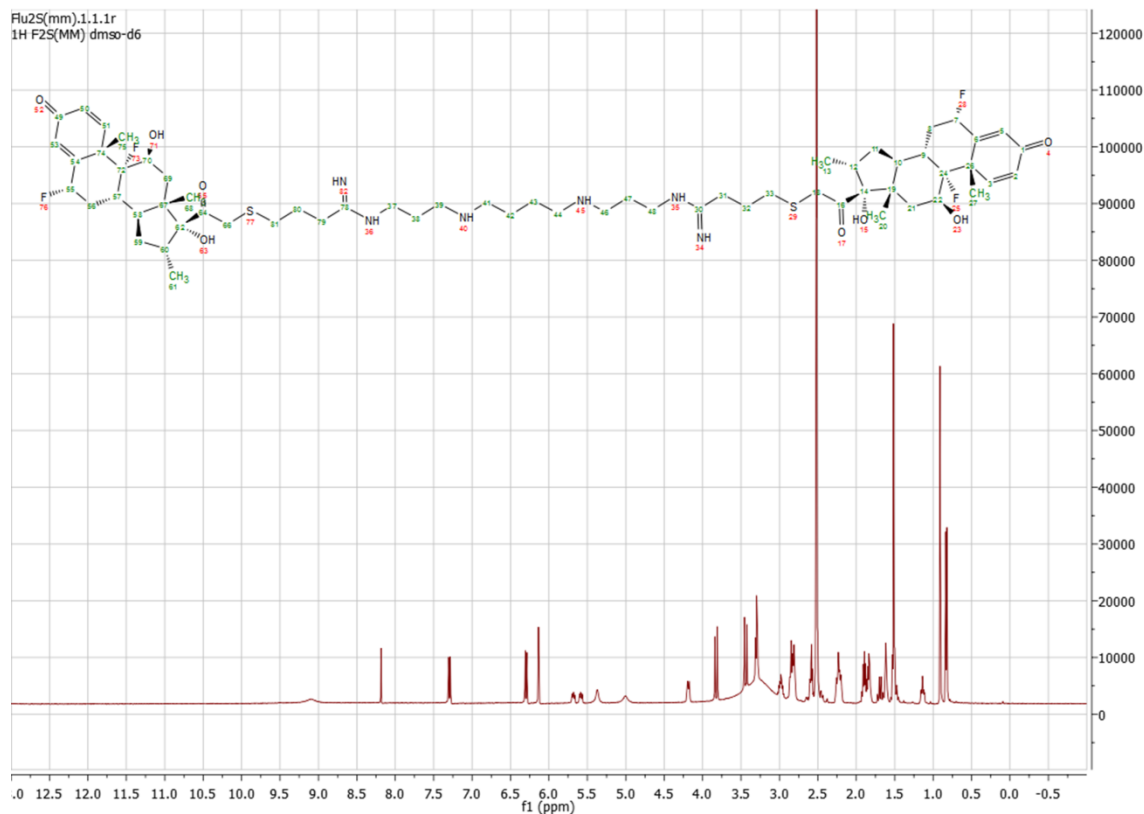


Figure B2: ^1H NMR spectrum of F_2S

Disubstituted flumethasone spermine, F_2S , 4-((2-((6R,8R,9S,10R,11R,13R,14R,16S,17S)-6,9-difluoro-11,17-dihydroxy-10,13,16-trimethyl-3-oxo-6,7,8,9,10,11,12,13,14,15,16,17-dodecahydro-3H-cyclopenta[a]phenanthren-17-yl)-2-oxoethyl)thio)-N-(1-((6S,8S,9R,10S,11S,13S,14S,16R,17R)-6,9-difluoro-11,17-dihydroxy-10,13,16-trimethyl-3-oxo-6,7,8,9,10,11,12,13,14,15,16,17-dodecahydro-3H-cyclopenta[a]phenanthren-17-yl)-7-imino-1-oxo-3-thia-8,12,17-triazaicosan-20-yl)butanimidamide ^1H NMR (500 MHz, $\text{DMSO}-d_6$ with TMS) δ 9.10 (s, 1H), 8.19 (s, 0H), 7.29 (dd, J = 10.2, 1.5 Hz, 1H), 6.30 (dd, J = 10.2, 1.9 Hz, 1H), 5.63 (dddd, J = 48.7, 11.4, 6.7, 1.9 Hz, 1H), 5.37 (s, 1H), 5.01 (s, 1H), 4.22 – 4.15 (m, 1H), 3.82 (d, J = 16.5 Hz, 1H), 3.44 (d, J = 16.5 Hz, 1H), 3.30 (t, J = 7.0 Hz, 1H), 2.84 (dd, J = 16.5, 9.0 Hz, 2H), 2.59 (td, J = 7.1, 2.9 Hz, 1H), 2.58 – 2.41 (m, 1H), 2.23 (ddt, J = 13.8, 7.8, 5.6 Hz, 2H), 1.87 (dp, J = 28.8, 7.4 Hz, 2H), 1.69 (q, J = 12.5, 11.8 Hz, 1H), 1.65 – 1.58 (m, 1H), 1.52 (s, 2H), 1.55 – 1.45 (m, 1H), 1.14 (ddd, J = 12.1, 8.1, 4.1 Hz, 1H), 0.91 (s, 2H), 0.83 (d, J = 7.2 Hz, 2H).

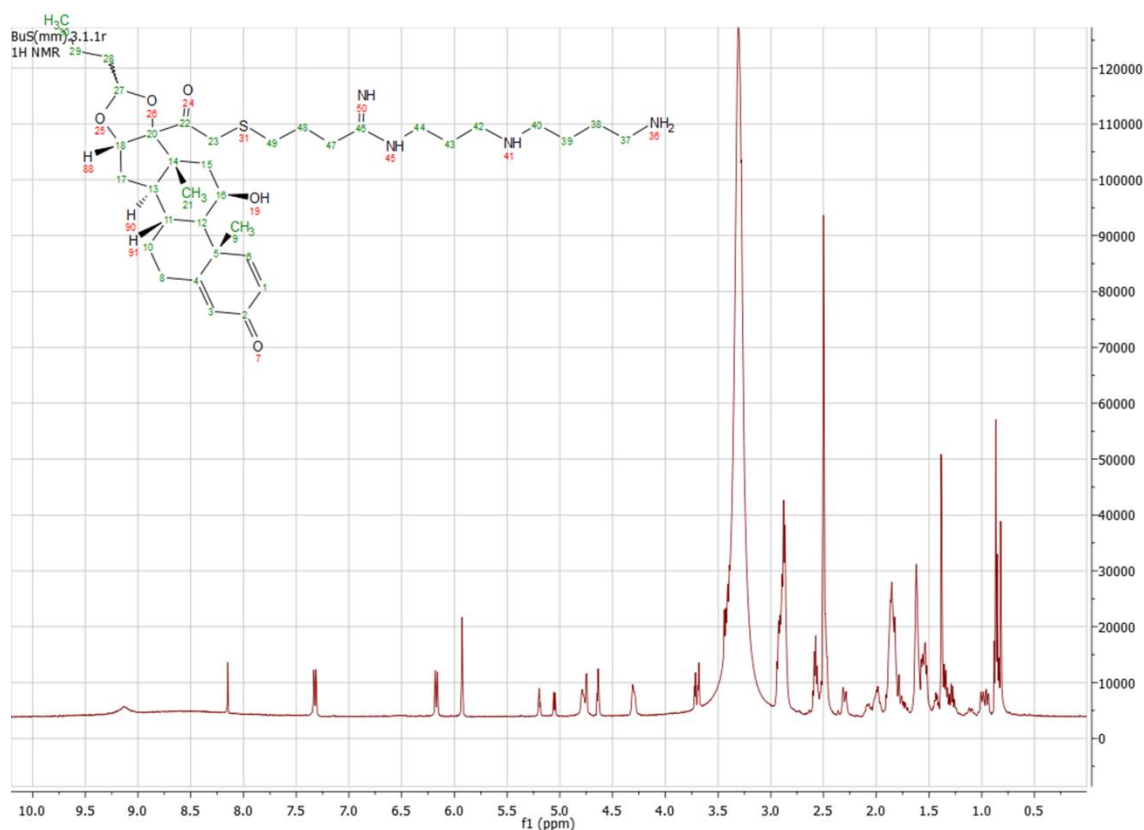


Figure B3: ^1H NMR spectrum of BuS

Budesonide Spermine, **BuS** N-(3-((4-((3-aminopropyl)amino)butyl)amino)propyl)-4-((2-((6aS,6bR,7R,8aR,8bR,10(R,S),11aS,12aR,12bR) -7-hydroxy-6a,8a-dimethyl-4-oxo-10-propyl-1,2,4,6a,6b,7,8,8a,11a,12,12a,12b-dodecahydro-8bH-naphtho[2',1':4,5]indeno[1,2-d][1,3]dioxol-8b-yl)-2-oxoethyl)thio)butanimidamide ^1H NMR (500 MHz, $\text{DMSO}-d_6$ with TMS) δ 9.13 (s, 1H), 8.55 (s, 4H), 7.32 (dd, J = 10.1, 3.1 Hz, 1H), 6.17 (d, J = 10.0 Hz, 1H), 5.93 (s, 1H), 4.81 – 4.72 (m, 1H), 4.30 (d, J = 9.7 Hz, 1H), 3.70 (dd, J = 16.1, 4.6 Hz, 1H), 3.42 (dd, J = 16.1, 8.1 Hz, 1H), 2.90 (dt, J = 24.2, 7.7 Hz, 6H), 2.57 (t, J = 6.7 Hz, 1H), 2.30 (d, J = 13.4 Hz, 1H), 2.00 (dt, J = 19.1, 8.9 Hz, 1H), 1.83 (ddt, J = 32.5, 24.6, 10.5 Hz, 5H), 1.65 – 1.52 (m, 4H), 1.53 (d, J = 7.7 Hz, 1H), 1.38 (s, 2H), 1.32 (ddd, J = 32.3, 16.0, 8.5 Hz, 2H), 1.21 (s, 0H), 1.11 (qd, J = 12.4, 4.6 Hz, 0H), 1.03 (s, 0H), 1.03 – 0.91 (m, 1H), 0.85 (dt, J = 15.7, 7.9 Hz, 4H).

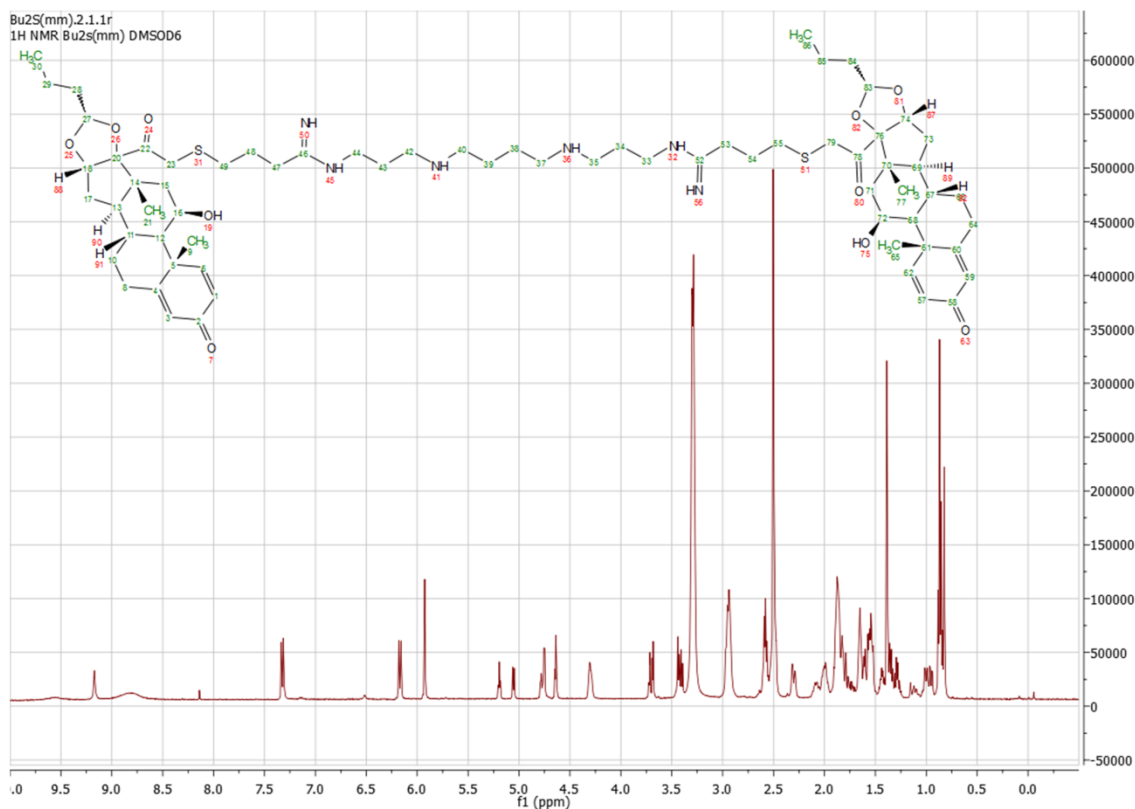


Figure B4: ^1H NMR spectrum of Bu₂S

Disubstituted Budesonide Spermine, **Bu₂S** 4-((2-((6aR,6bS,7S,8aS,8bS,10(R,S),11aR,12aS,12bS)-7-hydroxy-6a,8a-dimethyl-4-oxo-10-propyl-1,2,4,6a,6b,7,8,8a,11a,12,12a,12b-dodecahydro-8bH-naphtho[2',1':4,5]indeno[1,2-d][1,3]dioxol-8b-yl)-2-oxoethyl)thio)-N-(1-((6aS,6bR,7R,8aR,8bR,10(R,S),11aS,12aR,12bR)-7-hydroxy-6a,8a-dimethyl-4-oxo-10-propyl-1,2,4,6a,6b,7,8,8a,11a,12,12a,12b-dodecahydro-8bH-naphtho[2',1':4,5]indeno[1,2-d][1,3]dioxol-8b-yl)-7-imino-1-oxo-3-thia-8,12,17-triazaicosan-20-yl)butanimidamide ^1H NMR (500 MHz, DMSO- d_6 with TMS) δ 9.17 (s, 0H), 8.82 (s, 1H), 8.14 (s, 0H), 7.32 (dd, J = 10.1, 3.0 Hz, 1H), 6.17 (dd, J = 10.0, 2.3 Hz, 1H), 5.93 (s, 1H), 5.19 (t, J = 4.9 Hz, 0H), 5.05 (d, J = 7.4 Hz, 0H), 4.81 – 4.73 (m, 1H), 4.64 (t, J = 4.4 Hz, 0H), 4.30 (dp, J = 9.7, 3.3 Hz, 1H), 3.70 (dd, J = 16.1, 4.9 Hz, 1H), 3.42 (dd, J = 16.1, 7.7 Hz, 1H), 3.29 (t, J = 6.8 Hz, 7H), 2.94 (p, J = 6.6, 5.5 Hz, 2H), 2.59 (q, J = 6.8 Hz, 1H), 2.51 – 2.45 (m, 1H), 2.34 – 2.27 (m, 1H), 2.11 – 1.95 (m, 1H), 1.87 (td, J = 16.7, 16.1, 10.1 Hz, 3H), 1.84 – 1.59 (m, 1H), 1.65 (s, 1H), 1.62 – 1.53 (m, 1H), 1.56 – 1.50 (m, 1H), 1.46 – 1.23 (m, 3H), 1.20 – 1.02 (m, 0H), 0.98 (ddt, J = 25.2, 11.1, 5.4 Hz, 1H), 0.85 (dt, J = 15.5, 7.6 Hz, 3H).

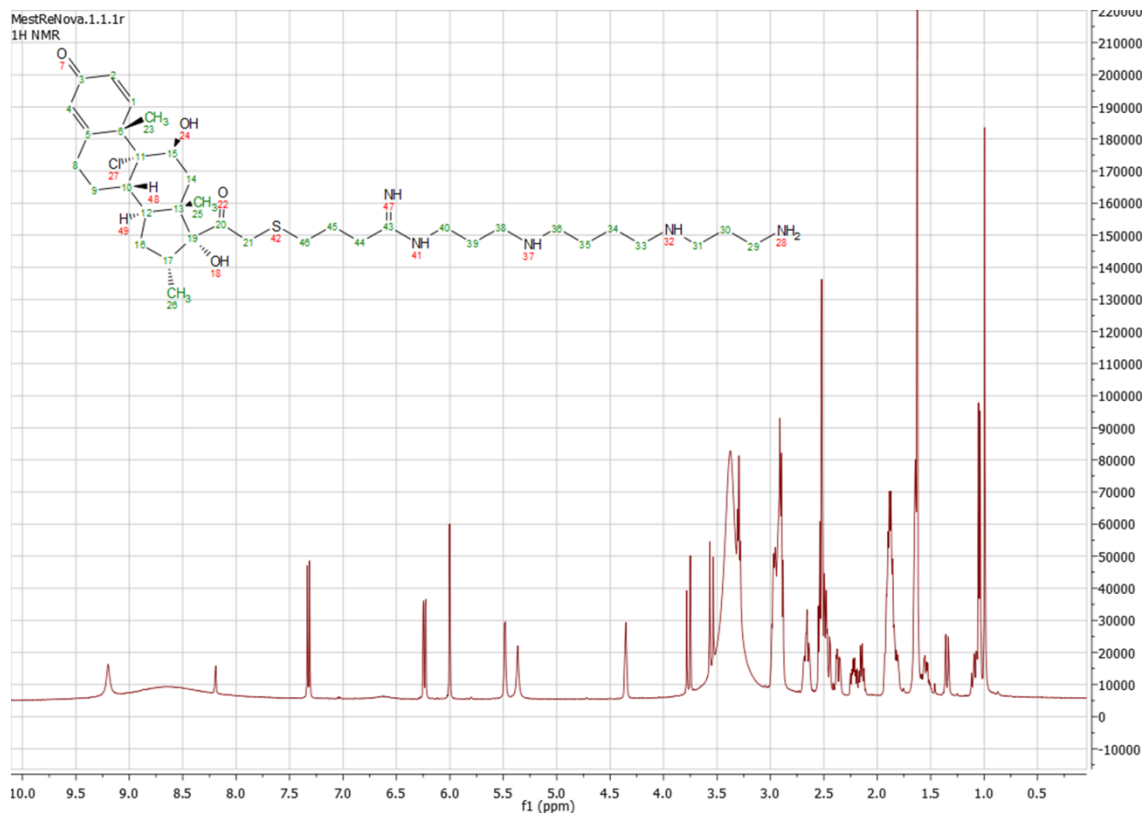


Figure B5: ^1H NMR spectrum of BeS

Beclomethasone Spermine, **BeS** N-(3-((4-((3-aminopropyl)amino)butyl)amino)propyl)-4-((2-((8S,9R,10S,11S,13S,14S,16S,17R)-9-chloro-11,17-dihydroxy-10,13,16-trimethyl-3-oxo-6,7,8,9,10,11,12,13,14,15,16,17-dodecahydro-3H-cyclopenta[a]phenanthren-17-yl)-2-oxoethyl)thio)butanimidamide ^1H NMR (500 MHz, $\text{DMSO}-d_6$ with TMS) δ 9.20 (s, 1H), 8.64 (s, 6H), 7.32 (d, $J = 10.1$ Hz, 1H), 6.24 (dd, $J = 10.1, 1.9$ Hz, 1H), 6.00 (d, $J = 1.9$ Hz, 1H), 5.48 (d, $J = 4.6$ Hz, 1H), 5.36 (s, 1H), 4.35 (q, $J = 3.4$ Hz, 1H), 3.77 (d, $J = 16.5$ Hz, 1H), 3.55 (d, $J = 16.5$ Hz, 1H), 3.29 (t, $J = 6.9$ Hz, 1H), 2.97 (ddd, $J = 10.6, 6.9, 2.7$ Hz, 2H), 2.93 (s, 3H), 2.93 – 2.86 (m, 2H), 2.66 (td, $J = 11.6, 11.1, 4.2$ Hz, 1H), 2.54 (d, $J = 7.2$ Hz, 1H), 2.52 – 2.42 (m, 2H), 2.36 (dd, $J = 13.8, 4.9$ Hz, 1H), 2.27 – 2.11 (m, 1H), 1.95 – 1.78 (m, 5H), 1.64 (d, $J = 9.2$ Hz, 4H), 1.60 – 1.48 (m, 1H), 1.35 (dd, $J = 13.9, 2.4$ Hz, 1H), 1.14 – 1.02 (m, 2H), 1.00 (s, 2H).

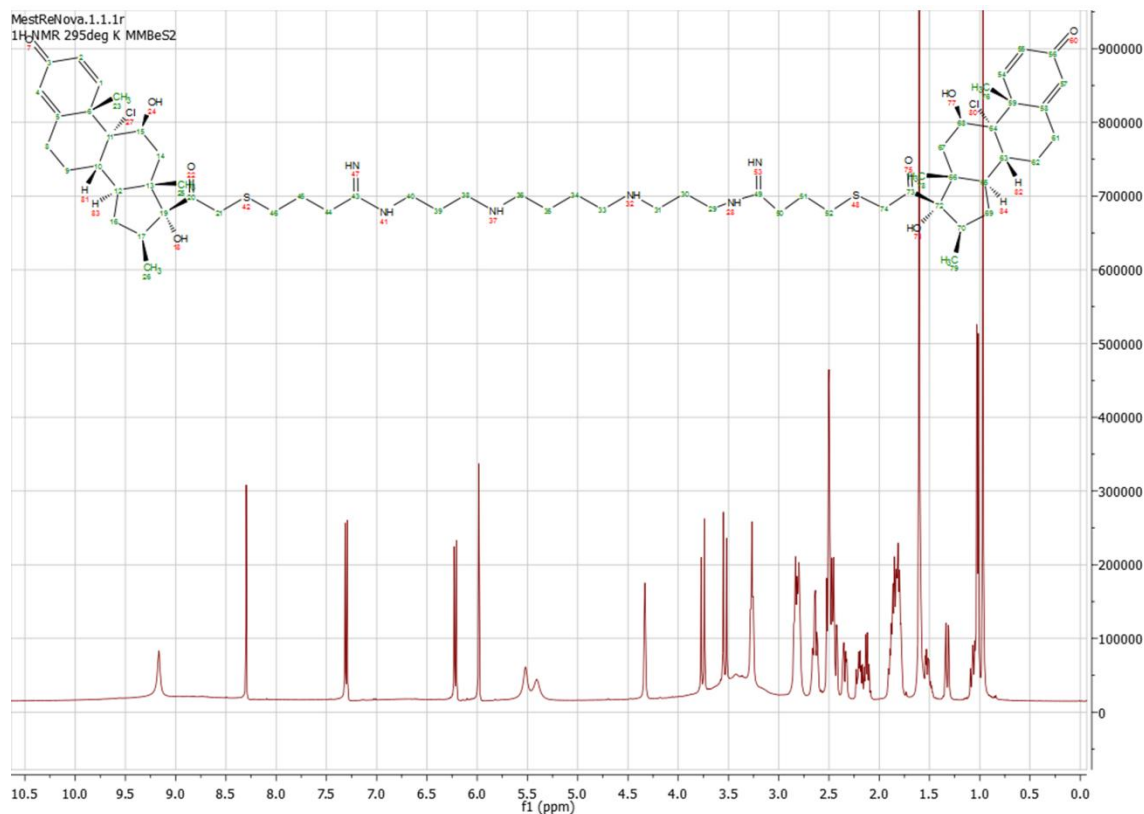


Figure B6: ^1H NMR spectrum of Be_2S

Disubstituted Beclomethasone Spermine, Be_2S 4-((2-((8R,9S,11R,13R,14R,16R,17S)-9-chloro-11,17-dihydroxy-10,13,16-trimethyl-3-oxo-6,7,8,9,10,11,12,13,14,15,16,17-dodecahydro-3H-cyclopenta[a]phenanthren-17-yl)-2-oxoethyl)thio)-N-(1-((8S,9R,10S,11S,13S,14S,16S,17R)-9-chloro-11,17-dihydroxy-10,13,16-trimethyl-3-oxo-6,7,8,9,10,11,12,13,14,15,16,17-dodecahydro-3H-cyclopenta[a]phenanthren-17-yl)-7-imino-1-oxo-3-thia-8,12,17-triazaicosan-20-yl)butanimidamide ^1H NMR (500 MHz, $\text{DMSO}-d_6$ with TMS) δ 9.17 (s, 1H), 8.30 (s, 0H), 7.30 (d, $J = 10.2$ Hz, 1H), 6.22 (dd, $J = 10.1, 1.9$ Hz, 1H), 5.98 (d, $J = 1.8$ Hz, 1H), 5.52 (s, 1H), 5.41 (s, 1H), 4.33 (d, $J = 3.1$ Hz, 1H), 3.76 (d, $J = 16.5$ Hz, 1H), 3.54 (d, $J = 16.5$ Hz, 1H), 3.27 (t, $J = 6.9$ Hz, 1H), 2.82 (dt, $J = 14.9, 6.8$ Hz, 3H), 2.64 (td, $J = 11.2, 4.1$ Hz, 1H), 2.56 – 2.40 (m, 2H), 2.38 – 2.30 (m, 1H), 2.25 – 2.08 (m, 1H), 1.85 (dddq, $J = 25.5, 19.6, 11.2, 5.5, 4.8$ Hz, 4H), 1.60 (s, 3H), 1.51 (td, $J = 12.9, 12.5, 5.2$ Hz, 1H), 1.32 (dd, $J = 13.8, 2.5$ Hz, 1H), 1.11 – 0.99 (m, 2H), 0.97 (s, 2H).

Appendix C: ^{13}C NMR Data for FS, F₂S, BuS, Bu₂S, BeS, and Be₂S

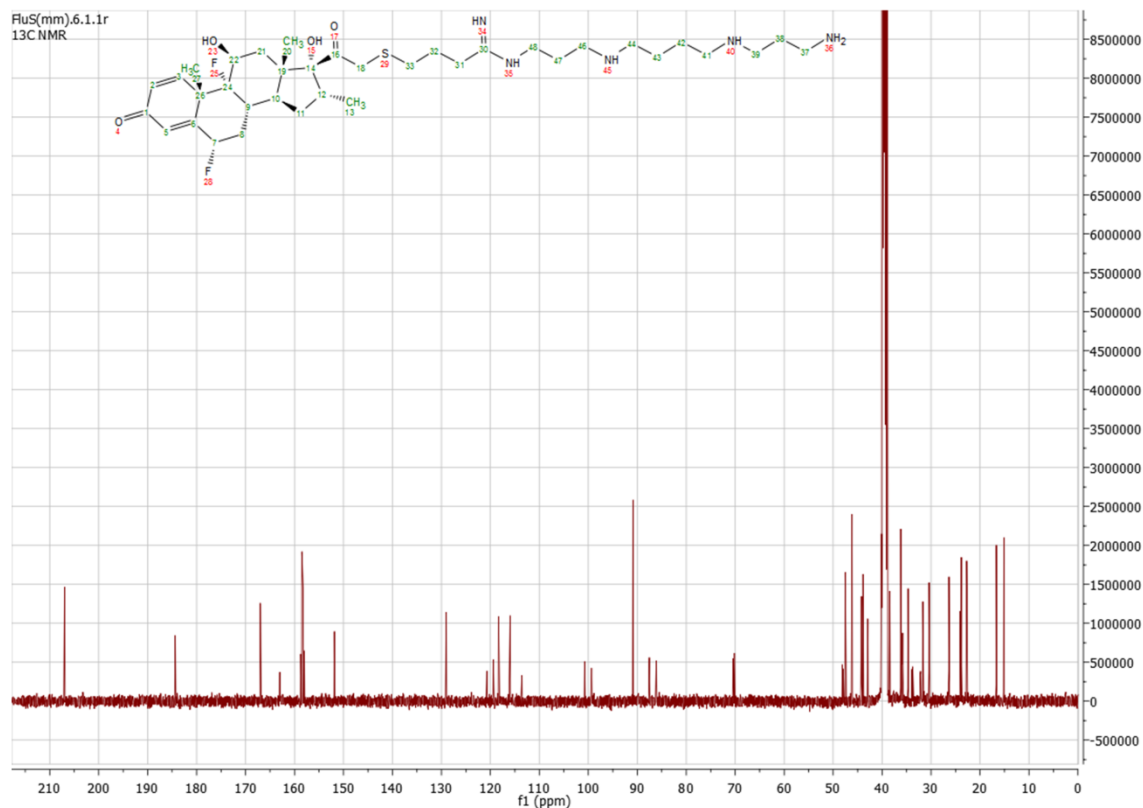


Figure C1: ^{13}C NMR spectrum of FS

Flumethasone Spermine, FS, N-(3-((4-((3-aminopropyl)amino)butyl)amino)propyl)-4-((2-((6S,8S,9R,10S,11S,13S,14S,16R,17R)-6,9-difluoro-11,17-dihydroxy-10,13,16-trimethyl-3-oxo-6,7,8,9,10,11,12,13,14,15,16,17-dodecahydro-3H-cyclopenta[a]phenanthren-17-yl)-2-oxoethyl)thio)butanimidamide
 ^{13}C NMR (126 MHz, DMSO- d_6 with TMS) δ 206.96 (16), 184.38 (1), 166.98 (30), 163.02 (6), 162.91 (6), 151.86 (3), 129.02 (2), 119.42 (5), 119.32(5), 100.75 (24), 99.35 (24), 90.87 (14), 87.53 (7), 86.10 (7), 70.4 4(22), 70.16 (22), 48.14 (26), 48.12 (26), 47.97 (26), 47.94 (26), 47.52 (19), 46.15 (41, 44), 44.19 (46), 43.90 (39), 42.92 (10), 39.05 (12), 38.43 (18), 36.19 (37), 35.81 (31), 34.69 (11), 33.89 (21), 33.75 (21), 32.23 (8), 32.14(8), 32.08 (8), 31.99 (8), 31.69 (9), 31.61 (9), 30.41 (33), 26.34 (47), 24.02 (38), 23.79 (32), 22.76 (27), 22.73 (42, 43), 22.65 (27), 16.67 (20), 15.12 (13).

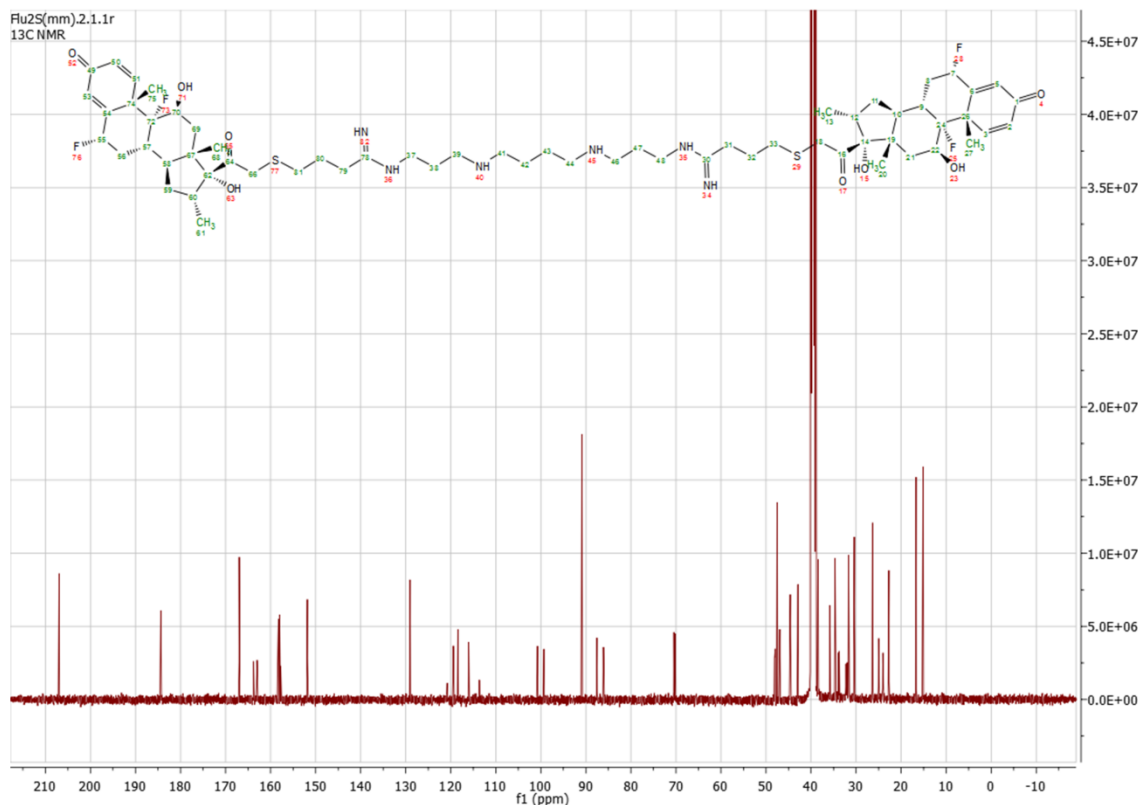


Figure C2: ^{13}C NMR spectrum of F_2S

Disubstituted-flumethasone spermine, F_2S , 4-((2-((6R,8R,9S,10R,11R,13R,14R,16S,17S)-6,9-difluoro-11,17-dihydroxy-10,13,16-trimethyl-3-oxo-6,7,8,9,10,11,12,13,14,15,16,17-dodecahydro-3H-cyclopenta[a]phenanthren-17-yl)-2-oxoethyl)thio)-N-(1-((6S,8S,9R,10S,11S,13S,14S,16R,17R)-6,9-difluoro-11,17-dihydroxy-10,13,16-trimethyl-3-oxo-6,7,8,9,10,11,12,13,14,15,16,17-dodecahydro-3H-cyclopenta[a]phenanthren-17-yl)-7-imino-1-oxo-3-thia-8,12,17-triazaicosan-20-yl)butanimidamide ^{13}C NMR (126 MHz, DMSO-d_6 with TMS) δ 206.94 (16, 64), 184.37 (1, 49), 166.96 (30, 78), 163.79 (6,54), 163.00 (6,54), 162.89 (6,54), 151.83 (3, 51), 129.03 (2, 50), 119.42 (5,53), 119.32 (5,53), 100.75 (24,72), 99.34 (24,72), 90.87 (14, 62), 87.52 (7,55), 86.09 (7,55), 70.45 (22,70), 70.16 (22,70), 48.14 (26,74), 48.11 (26,74), 47.96 (26,74), 47.93 (26,74), 47.53 (41,44), 46.95 (19,67), 44.61 (39, 46), 42.92 (10, 58), 39.60 (37, 48), 39.43 (12,60), 38.42 (18, 66), 35.82 (31, 79), 34.69 (11, 59), 33.89 (21,69), 33.74 (21,69), 32.23 (8,56), 32.14 (8,56), 32.08 (8,56), 31.99 (8,56), 31.68 (9,57), 31.61 (9,57), 30.39 (33, 81), 26.35 (38, 47), 24.98 (32, 80), 24.05 (42, 43), 22.76 (27,75), 22.71 (27,75), 16.67 (20, 68), 15.12 (13, 61).

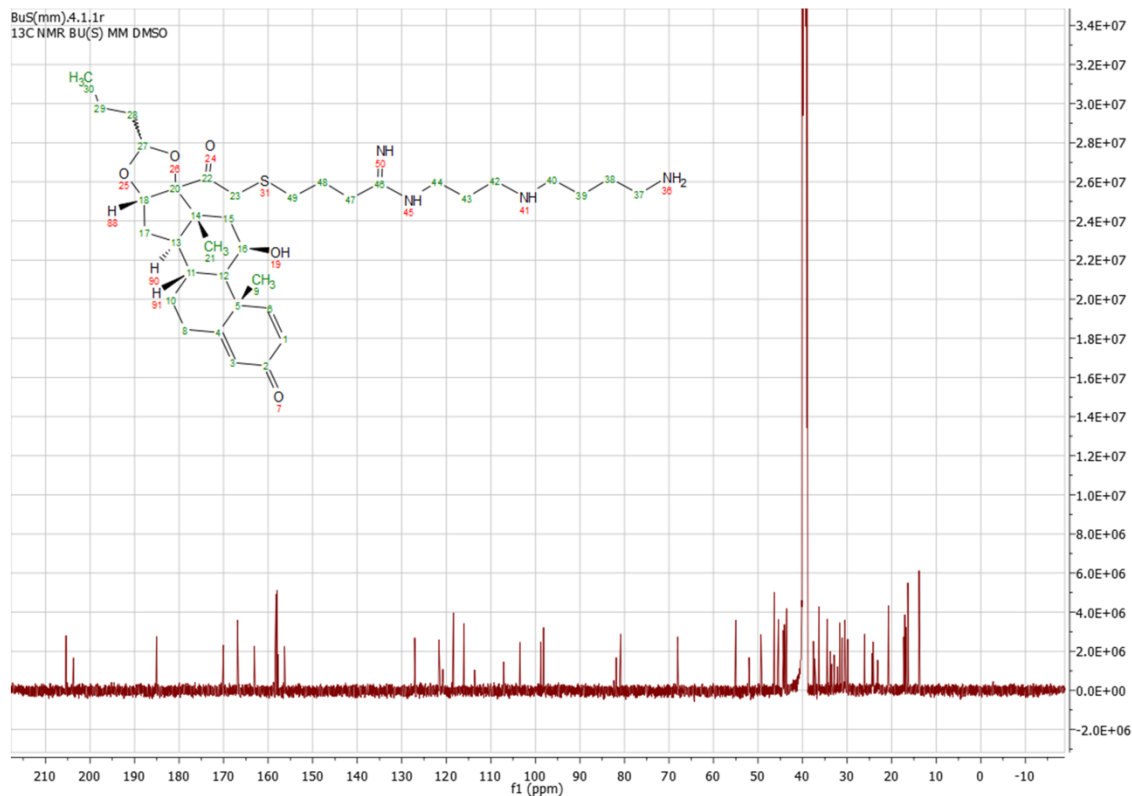


Figure C3: ^{13}C NMR spectrum of BuS

Budesonide Spermine, **BuS** N-(3-((4-((3-aminopropyl)amino)butyl)amino)propyl)-4-((2-((6aS,6bR,7R,8aR,8bR,10(R,S),11aS,12aR,12bR)-7-hydroxy-6a,8a-dimethyl-4-oxo-10-propyl-1,2,4,6a,6b,7,8,8a,11a,12,12a,12b-dodecahydro-8bH-naphtho[2',1':4,5]indeno[1,2-d][1,3]dioxol-8b-yl)-2-oxoethyl)thio)butanimidamide ^{13}C NMR (126 MHz, $\text{DMSO}-d_6$ with TMS) δ 205.38 (22), 203.73 (22), 185.05 (2), 170.13 (4), 170.04 (4), 166.89 (46), 163.10 (46), 156.33 (6), 127.09 (1), 127.06 (1), 121.67 (3), 121.61 (3), 107.10 (27), 103.46 (27), 98.75 (20), 98.17 (20), 81.86 (18), 80.84 (18), 68.07 (16), 68.02 (16), 55.00 (12), 52.04 (13), 49.35 (13), 46.36 (14), 45.40 (14), 44.35 (37, 40), 44.11 (5), 43.60 (42), 40.18 (15), 39.93 (44), 39.59 (23), 37.57 (47), 37.29 (37.29), 36.41 (28), 36.34 (28), 34.49 (28), 33.81 (10), 33.44 (10), 32.92 (17), 32.19 (17), 31.63 (8), 31.11 (8), 30.54 (49), 30.49 (49), 29.89 (11), 26.16 (43), 26.11 (43), 24.37 (48), 24.14 (48), 23.14 (38, 39), 20.72 (9), 20.68 (9), 17.30 (21), 17.06 (21), 16.78 (29), 16.37 (29), 13.84 (30), 13.77 (30).

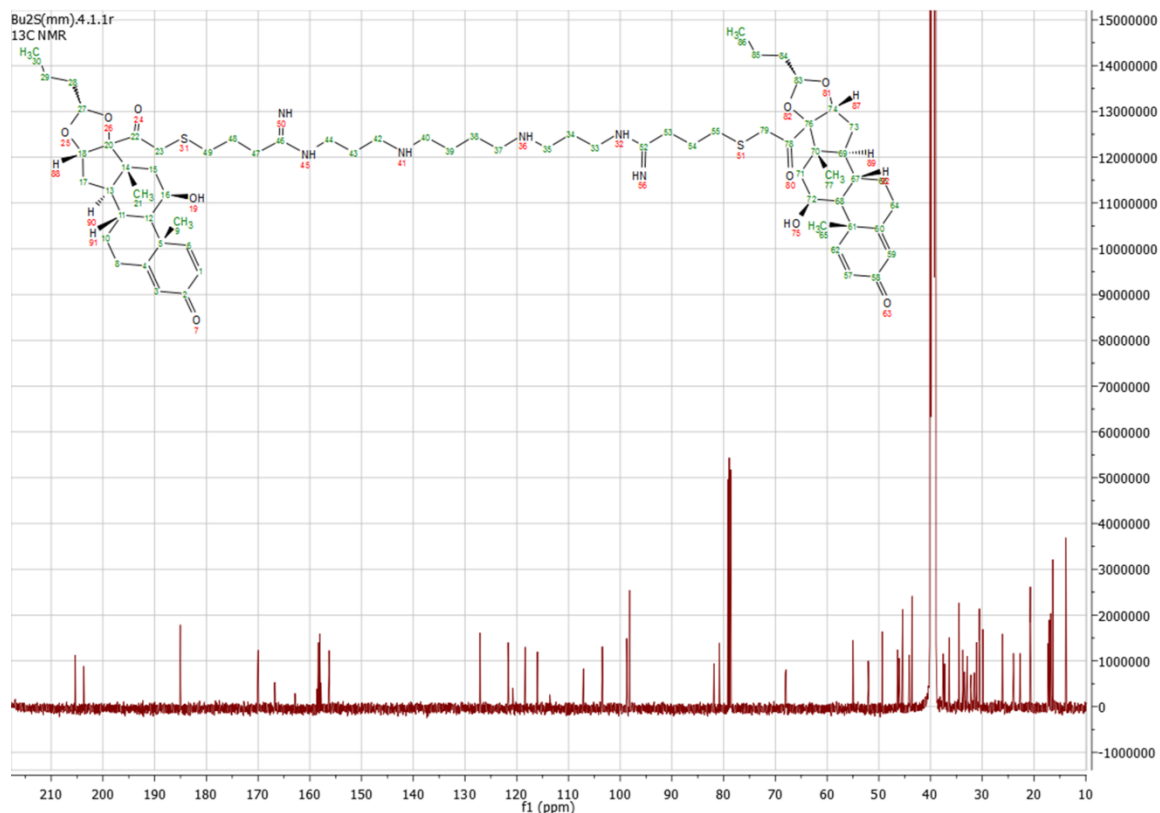


Figure C4: ^{13}C NMR spectrum of Bu_2S

Disubstituted Budesonide Spermine, **Bu₂S** 4-((2-((6aR,6bS,7S,8aS,8bS,10(R,S),11aR,12aS,12bS)-7-hydroxy-6a,8a-dimethyl-4-oxo-10-propyl-1,2,4,6a,6b,7,8,8a,11a,12,12a,12b-dodecahydro-8bH-naphtho[2',1':4,5]indeno[1,2-d][1,3]dioxol-8b-yl)-2-oxoethyl)thio)-N-(1-((6aS,6bR,7R,8aR,8bR,10(R,S),11aS,12aR,12bR)-7-hydroxy-6a,8a-dimethyl-4-oxo-10-propyl-1,2,4,6a,6b,7,8,8a,11a,12,12a,12b-dodecahydro-8bH-naphtho[2',1':4,5]indeno[1,2-d][1,3]dioxol-8b-yl)-7-imino-1-oxo-3-thia-8,12,17-triazaicosan-20-yl)butanimidamide ^{13}C NMR (126 MHz, $\text{DMSO}-d_6$ with TMS) δ 205.35 (22,78), 203.70 (22,78), 185.02 (2,58), 170.07 (4,60), 169.98 (4,60), 166.83 (46,52), 166.76 (46,52), 162.87 (46,52), 156.32 (6,62), 156.28 (6,62), 127.09 (1,57), 127.05 (1,57), 121.67 (3,59), 121.61 (3,59), 107.10 (27,83), 103.46 (27,83), 98.75 (20,76), 98.17 (20,76), 81.86 (18,74), 80.83 (18,74), 68.08 (16,72), 68.02 (16,72), 67.96 (16,72), 67.90 (16,72), 54.99 (12,68), 54.96 (12,68), 52.05 (13,69), 49.35 (13,69), 46.36 (14,70), 46.08 (14,70), 45.39 (37,40), 44.12 (5, 61), 43.59 (35,42), 40.10 (15,71), 39.93 (33,44), 39.59 (23,79), 37.55 (47,53), 37.28 (47,53), 36.42 (28,84), 34.50 (28,84), 33.80 (10,66), 33.45 (10,66), 32.91 (17,73), 32.19 (17,73), 31.57 (8,64), 31.12 (8,64), 30.55 (49,55), 30.49 (49,55), 29.90 (11,67), 26.14 (34,43), 26.08 (34,43), 23.97 (48,54), 22.70 (38,39), 20.71 (9,65), 20.68 (9,65), 17.29 (21,77), 17.05 (21,77), 16.78 (29,85), 16.38 (29,85), 13.83 (30,86), 13.76 (30,86).

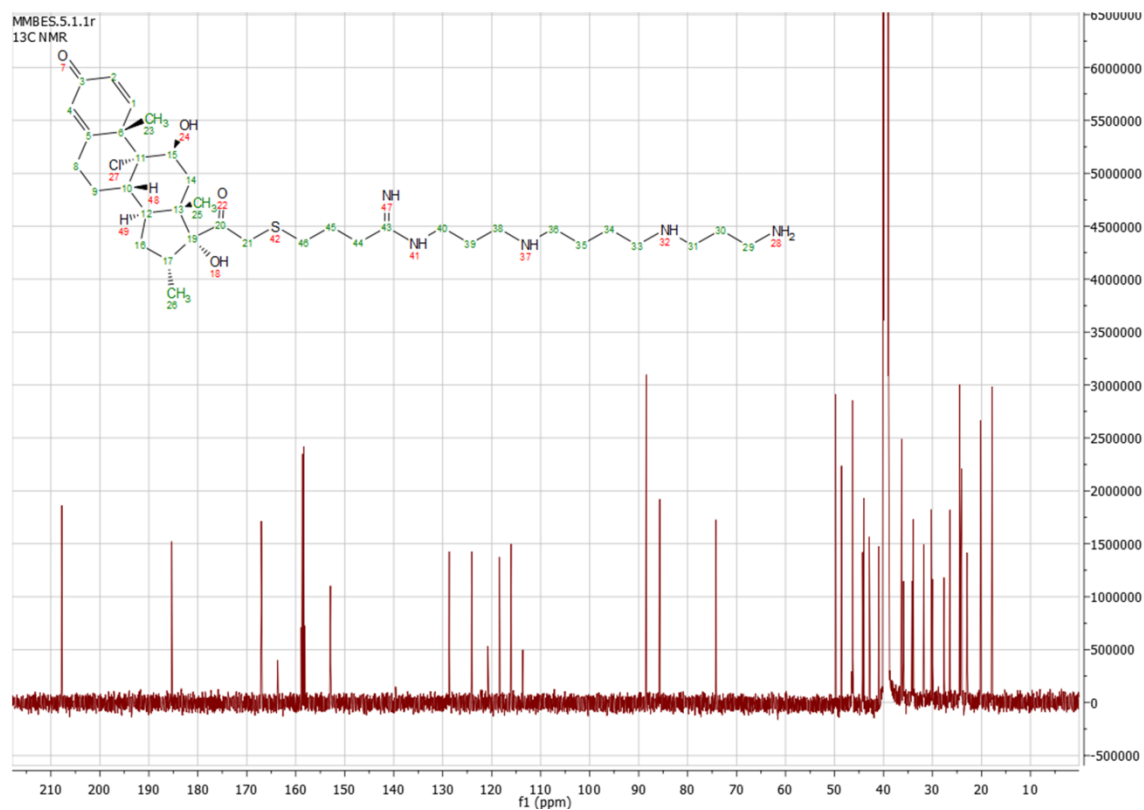


Figure C5: ^{13}C NMR spectrum of BeS

Beclomethasone Spermine, **BeS** N-(3-((4-((3-aminopropyl)amino)butyl)amino)propyl)-4-((2-((8S,9R,10S,11S,13S,14S,16S,17R)-9-chloro-11,17-dihydroxy-10,13,16-trimethyl-3-oxo 6,7,8,9,10,11,12,13,14,15,16,17-dodecahydro-3H-cyclopenta[a]phenanthren-17-yl)-2-oxoethyl)thio)butanimidamide ^{13}C NMR (126 MHz, $\text{DMSO}-d_6$ with TMS) δ 207.78 (20), 185.34 (3), 167.04 (5), 166.90 (5), 163.70 (43), 152.91 (1), 128.65 (2), 124.08 (4), 88.44 (19), 85.70 (11), 74.20 (15), 49.77 (6), 48.59 (13), 46.46 (33, 36), 46.31 (12), 44.30 (31), 44.03 (38), 42.91 (17), 40.96 (40), 40.11, 39.78 (44), 36.30 (14), 35.94 (29), 34.18 (10), 33.96 (16), 31.77 (46), 30.25 (21), 29.98 (8), 27.63 (9), 26.46 (39), 24.44 (23), 24.24 (45), 24.03 (30), 23.00 (34,35), 22.93 (34,35), 20.17 (25), 17.81 (26).

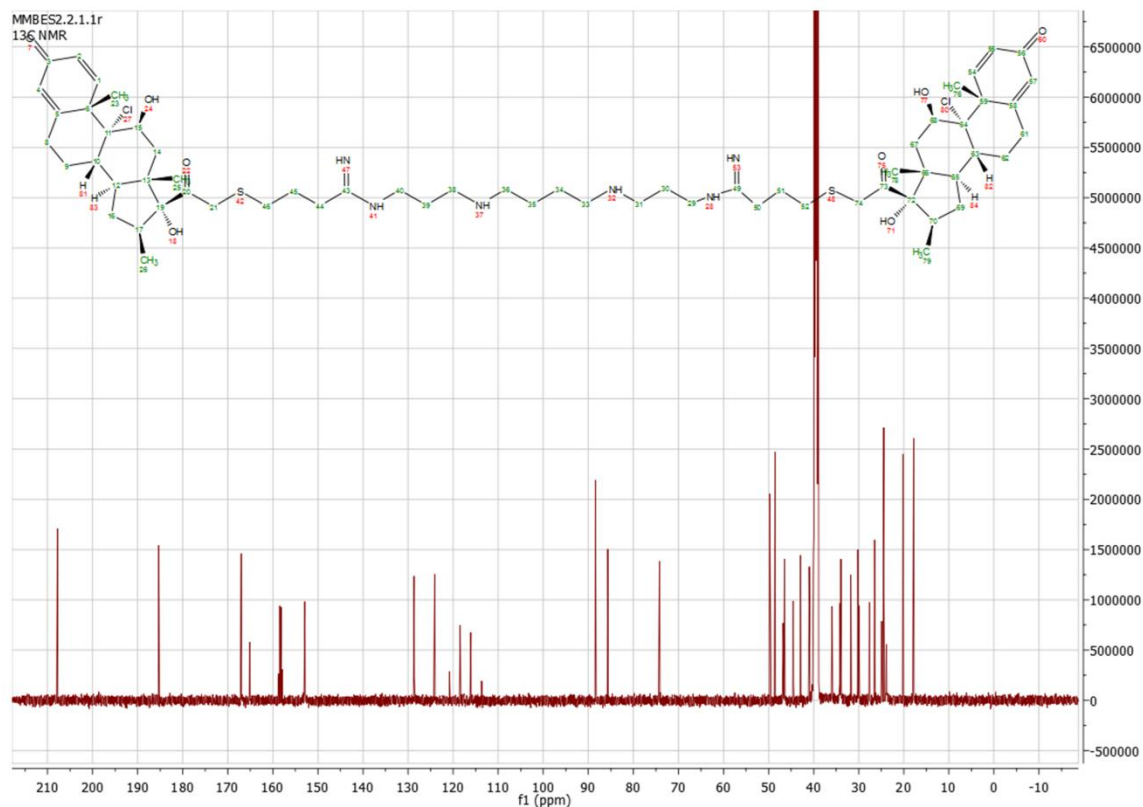


Figure C6: ¹³C NMR spectrum of Be₂S

Disubstituted Beclomethasone Spermine, **Be₂S** 4-((2-((8R,9S,11R,13R,14R,16R,17S)-9-chloro-11,17-dihydroxy-10,13,16-trimethyl-3-oxo-6,7,8,9,10,11,12,13,14,15,16,17-dodecahydro-3H-cyclopenta[a]phenanthren-17-yl)-2-oxoethyl)thio)-N-(1-((8S,9R,10S,11S,13S,14S,16S,17R)-9-chloro-11,17-dihydroxy-10,13,16-trimethyl-3-oxo-6,7,8,9,10,11,12,13,14,15,16,17-dodecahydro-3H-cyclopenta[a]phenanthren-17-yl)-7-imino-1-oxo-3-thia-8,12,17-triazaicosan-20-yl)butanimidamide ¹³C NMR (126 MHz, DMSO- *d*₆ with TMS) δ 207.79 (20, 73), 185.33 (3, 56), 167.02 (5,58), 166.88 (5,58), 165.14 (43, 49), 152.91 (1, 54), 128.65 (2,55), 124.08 (4, 57), 88.42 (19, 72), 85.69 (11,64), 74.20 (15, 68), 49.77 (6, 59), 48.57 (13,66), 46.79 (33, 36), 46.47 (12, 65), 44.53 (31, 38), 42.91 (17, 70), 40.97 (29, 40), 39.78 (44, 50), 35.93 (14, 67), 34.18 (16, 69), 33.96 (10, 63), 31.75 (46, 52), 30.22 (21, 74), 29.98 (8, 61), 27.63 (9, 62), 26.48 (30, 39), 24.89 (45,51), 24.44 (23, 76), 23.87 (34, 35), 20.17 (25, 78), 17.81 (26, 79).

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