AN ABSTRACT OF THE THESIS OF

Erika Snow for the degree of Honors Baccalaureate of Science in BioResource Research presented on November 28th, 2011. Title: *Mycobacterium tuberculosis* Selective Adenosine Kinase Nucleoside Polymers for Inhalation Therapy.

Abstract approved:

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John Mata

Respiratory illnesses caused by both viral and bacterial infection are serious issues for global healthcare providers. With the emergence of new, drug-resistant forms of these diseases, innovative drugs and treatment therapies are needed. *M tuberculosis* is an infection that affects a large portion of the world's population each year. Aerosol inhalation therapy has been shown to be effective in the treatment and possible prevention of diseases of the lung and nucleoside and nucleotide analogs have been shown to be therapeutically effective in the treatment of viral lung infections. This study set the pathway to develop an alternative inhalation therapy approach to treating *M. tuberculosis* using two nucleoside analog polymers 2-fluoroadenosine and 2-methyladenosine. *In vitro* experiments were designed to compare the permeability of the monomer and polymer form of 2-fluoroadenosine and 2-methyladenosine in Calu-3 lung carcinoma cells as well as establish the basic pharmacokinetic properties of the pro-drugs using a surrogate marker, 5-fluorodeoxyuridine monophosphate. An inhalation apparatus was designed, constructed and validated through flow rate and particle size analysis. The data from the permeability experiments are still under analysis and have yet to be evaluated. Drug uptake experiments did not show an uptake of 5-FdUMP into the cells in culture. The inhalation apparatus was designed to theoretically provide efficient concentrations of inhaled pro-drug to the lung through eight directed-flow nose-only delivery cones. The flow rate of the inhalation apparatus was analyzed through non-linear regression and demonstrated that the rate followed a predictable decrease as the number of open cones increased. The size of the water particles produced by the inhalation apparatus was found to have an average size of 5 μm, which falls within the ideal range for inhalation therapy. A theoretical synthesis method for a 10-subunit polymer of 2-fluoroadenosine was proposed. The results of this study demonstrate that the inhalation apparatus produces particles of appropriate size for inhalation therapy with predictable rates of administration for use in future *in vivo* testing models. The proposed synthesis scheme for a 10-subunit polymer of 2-fluoroadenosine is ready for evaluation and analysis. The results of this study thus far suggest that inhalation therapy using polymer forms of 2-methyladenosine and 2-fluoroadenosine may be a novel and efficient method for the treatment of *Mycobacterium tuberculosis.*

Keywords: inhalation therapy, *M. tuberculosis*, nucleoside analogs, respiratory illness.

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Mycobacterium tuberculosis Selective Adenosine Kinase

Nucleoside Polymers for Inhalation Therapy

By

Erika Snow

A PROJECT

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I understand my project will become part of the permanent collection of Oregon State University, University Honors College as well as the Scholars Archive collection for BioResource Research. My signature below authorizes release of my project to any reader upon request.

Erika Snow, Author

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DEDICATION

This thesis is dedicated to my family, John Mata, and Wanda Crannell. This thesis would not have been possible without your constant love, support, guidance and encouragement.

Mycobacterium tuberculosis Selective Adenosine Kinase Nucleoside Polymers for Inhalation Therapy

INTRODUCTION

The pain and suffering of humans due to respiratory illnesses caused by viral and bacterial infections is a serious issue for healthcare professionals worldwide. Such infections can have high rates of complications and mortality due to the virulent nature of such infections and the evolution of drug resistant strains of bacteria.

Influenza is an example of a common respiratory disease that affects a large portion of the world's population each year. More people died during the 1918 influenza epidemic than died in World War I. Influenza has high rates of incidence and complications, especially amongst the elderly and young children. Despite decades of intense studies concerning this illness, little progress has been made toward its significant prevention or cure.

Another example of a recent emerging respiratory illness is severe acute respiratory syndrome, otherwise known as SARS. The first case of SARS was reported in Asia in February of 2003, and within a few months it had developed into a global pandemic that spread to more than two-dozen countries worldwide.

Mycobacterium tuberculosis is a pathogen that has mutated and evolved into new drug resistant forms that now threaten global public health in many parts of the world. *M. tuberculosis* is caused by an airborne bacteria that primarily affects the lungs. One-third

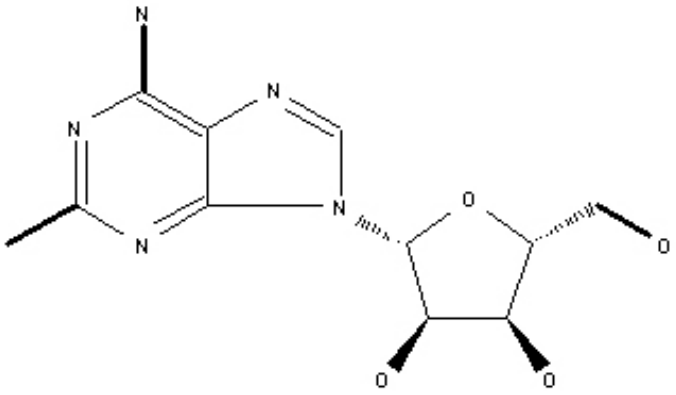
of the world's population has been infected with tuberculosis (TB) and the active rate of TB is nine million people per year. As is the case with influenza and SARS, new and more efficient drugs and drug regimens are needed to help combat this serious global medical emergency (Chien and Johnson, 2000; Laurenzi *et al.*, 2007).

Aerosol inhalation therapy has been shown to be effective in the treatment and possible prevention of certain diseases and infections of the lung. Respiratory syncytial virus (RSV) in infants has been successfully treated through inhalation therapy using aerosolized ribavirin (Rodriguez, 1987). This treatment approach requires small doses of aerosolized ribavirin to be given over long periods of time to ensure an effective dose of active drug at the site of infection (Taber, 1983). When this inhalation therapy was used to treat adults suffering from RSV, it was found to be ineffective. One strategy that has been suggested to overcome this problem is to investigate ways to increase and retain local concentrations of active drug in the lungs of adults.

Drugs that are used in the treatment of lung infections and diseases share a variety of general characteristics. First and foremost, the drugs must be able to be easily delivered to the affected area and have a long residency time at the site of infection (Scheuch, 2006; Suarez, 2001). They must be highly effective, with low toxicity, and preferably have low systemic bioavailability (Suarez, 2001; Scheuch, 2006; Zeng, 1995). The low level of absorption into systemic circulation is key to reducing the risk of toxic systemic side effects and the amount of drug required to treat the patient (Zeng, 1995). Attempts to retain high drug concentrations in the lung include: incorporating drugs in biodegradable microspheres such as liposomes, chemical structure modifications to

produce pro-drugs, the preparation of magnesium hydroxide co-precipitates, and complexes of a drug with cyclodextrins (Zeng, 1995).

Nucleotide and nucleoside analogs are compounds that have been shown to be therapeutically effective in cancer therapies, HIV antiretroviral therapies and in the treatment of viral lung infections (Balzarini, 1994; De Clercq, 2002). More recently, two nucleoside analogs, 2-methyladenosine (Fig. 1A) and 2-fluroadenosine (Fig. 1B), have demonstrated selective activity against *M. tuberculosis* (Long *et al.*, 2003).



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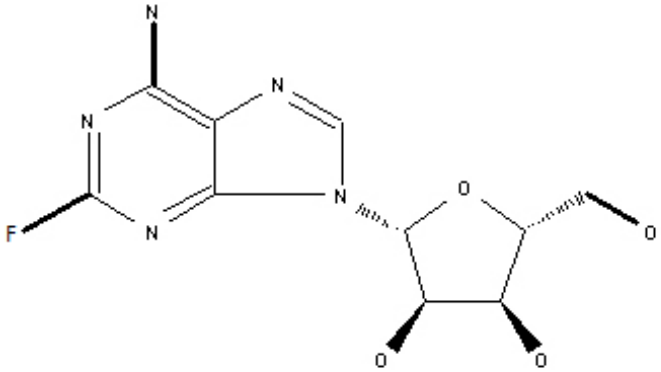


B

Fig. 1. Monomeric nucleic analogs of 2-methyladenosine (A) and 2-fluoroadenosine (B).

Nucleoside and nucleotide analogs are able to mimic naturally occurring DNA and RNA precursors of specific pathogens. Their mechanism of action includes absorption, phosphorylation and conversion into their active form. Once these analogs become active, they can interfere with a number of pathways of a pathogen's life cycle by inhibiting RNA and DNA polymerases, causing premature DNA chain termination, and inducing damage through the replacement of natural nucleic genomic sequences (Balzarini, 1994). While many drugs in this class are highly effective, the limited efficacy of monomeric analogs in inhalation therapy for treating lung diseases is likely due to their quick absorption into systemic circulation. Rapid absorption leads to ineffective concentrations of active compounds at the site of infection (Niven, 1995).

One strategy that has been developed to overcome the limitations of monomeric forms of nucleotide analogs is to incorporate them into polymers. Nucleic analog polymers in animal models have been shown to be an effective treatment for cancer (Liu *et al.*, 1999; Liu *et al.*, 2001; Gmeiner, 2005; Liao *et al.*, 2005; Bijnsdorp *et al.*, 2007). These polymers are polynucleotide compounds composed of sequences of pharmaceutically active nucleoside analogs separated by phosphodiester bonds that are susceptible to hydrolysis. A treatment approach using these polymer pro-drugs is attractive for pulmonary delivery therapies for multiple reasons. It allows for several active drug units to be incorporated into a single synthetic polymer molecule, theoretically leading to an increase in local concentrations of active drug at the target site. Another benefit is that these pro-drugs do not require a carrier molecule for pulmonary delivery, allowing the active drug to be delivered to the lung without leaving behind residue, reducing the risk of toxicity (Guimond *et al.*, 2008).

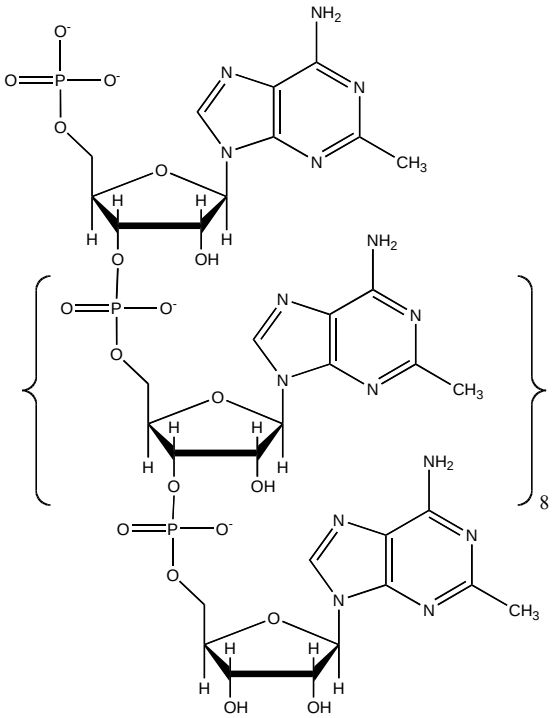
Nucleoside analogs may be an attractive treatment approach to multi-drug resistant TB because their mechanism of action is likely to be different than those of current treatment regimens. These analogs require intracellular metabolism to their active form in order to interfere with the lifecycle of *M. tuberculosis* (Long, 2003). Enzymes that catalyze this activation reaction are known as purine salvage enzymes and previous studies have documented the presence of such enzymes in strains of *Mycobacterium tuberculosis* (Long, 2003; Wheeler, 1987; Wheeler, 1987; Wheeler, 1990). Several genes that code for these particular purine salvage enzymes have been identified in the genomic sequence of *M. tuberculosis* (Cole, 1998).

2-methyladenosine (Fig. 1A) is a particular nucleoside analog that has been shown to demonstrate antitubercular activity in previous studies (Chen, 2002). The role of adenosine kinase (AK), a purine salvage enzyme that is found in humans and *M. tuberculosis* alike, in the activation of nucleoside analogs has been established in previous studies (Chen, 2002). A key step in the method of activation of 2-methyladenosine by AK is phosphorylation (Chen, 2002). Previous studies have demonstrated a difference in the rate of phosphorylation between human and *M. tuberculosis* AK's and that this difference is enough to permit selective activation of the analog, indicating that 2-methyladenosine has a selective activity against *M. tuberculosis* and has the potential to be investigated as a treatment approach to the disease (Long, 2003).

An additional nucleoside analog that has potential to treat TB is 2-fluoroadenosine (Fig.1B). Fluorinated compounds have been demonstrated in previous *in vitro* studies to act as potent synthetic agents that express activity towards a variety of

bacterial species (Wolfson, 1985). Unfortunately such fluorinated compounds have also been shown to produce adverse side effects, the most common effects involving the gastrointestinal tract, skin and central nervous system (Sarro, 2001). The attractiveness of 2-fluoroadenosine as a potential treatment approach to *M. tuberculosis* is seen in the method of damage induced by this particular analog. In its active form, 2-fluoroadenosine is able to bind to a specific site of the thymidylate kinase phosphotransferase (TMPK) enzyme found in *M. tuberculosis* (Haouz, 2003). This particular binding site is unique to the bacteria and is not found in the human TMPK enzyme, which allows for the specificity of 2-fluoroadenosine to *M. tuberculosis*, suggesting it is a viable treatment option to investigate (Haouz, 2003).

The short-term goal of this study was to investigate the efficacy of a new inhalation therapy approach to treat *M. tuberculosis* using polymer analogs of 2-methyladenosine (Fig. 2A) and 2-fluroadenosine (Fig. 2B).



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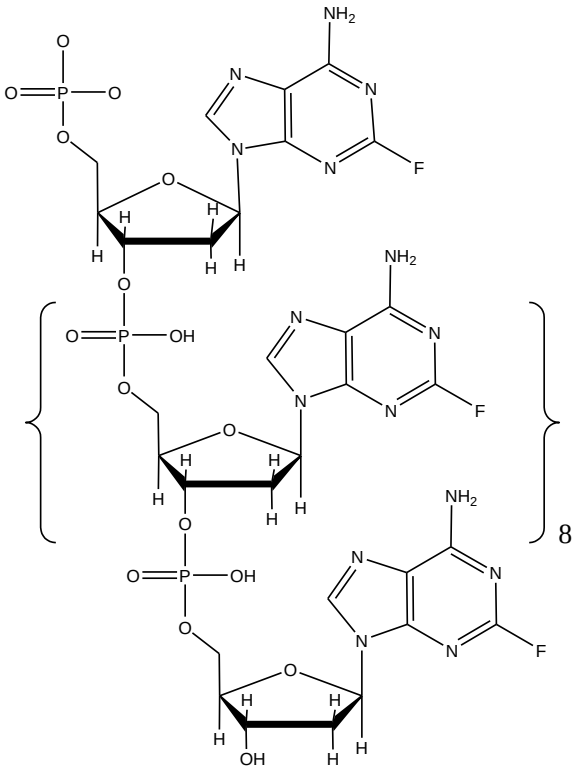


B

Fig. 2. Polymeric nucleic analogs of 2-methyladenosine (A) and 2-fluoroadenosine (B).

This was done through a series of *in vitro* cell culture experiments as well as the construction of an inhalation apparatus and a proposed synthesis scheme for the pro-drugs of interest.

In vitro experiments were designed to compare the residency time of the polymer compounds in the apical chamber of human lung carcinoma cell monolayers compared to the residency time of the monomer compound. One goal of this research was to demonstrate that the movement of the polymer from the apical to basolateral chambers in this model was reduced. Additionally, the data can later be used to determine dosages, toxicity and absorption patterns to guide future studies in animal models. These studies

used a monomer (Fig. 3) and 10-subunit polymer of 5-fluorodeoxyuridine monophosphate (5-FdUMP) as a surrogate marker for both 2-methyladenosine and 2-fluoroadenosine.

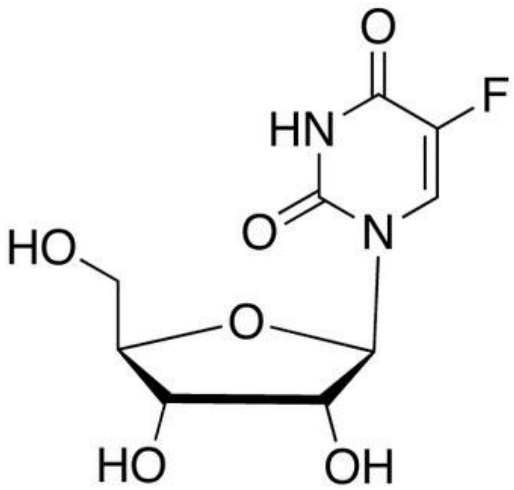


Fig. 3. 5-Fluorodeoxyruidine monophosphate (5-FdUMP).

This particular compound was chosen due to its structural resemblance to the nucleoside analogs of interest. Both compounds are similar in molecular weight and charge, however the main comparison being made is between the polymer and monomer forms. The assumption is that the monomer and polymer of 5-FdUMP will have similar pharmacokinetic properties as both the monomer and polymer of 2-methyladenosine and 2-fluoroadenosine. In addition to these experiments, an inhalation apparatus was designed and constructed for future use in rodent models of nose-only inhalation. Water vapor experiments were conducted using the inhalation apparatus to determine flow rate and consistency between individual cones. Particle size was analyzed through high speed photomicroscopic analysis to determine the approximate size of the vapor droplets.

Finally, a synthesis scheme for a 2-fluoroadenosine polymer was investigated on a theoretical basis.

It is hypothesized that active drug release through slow hydrolysis via direct inhalation therapy will lead to an increase in the local concentration of active drug in the lung, allowing for the sustained release of active drug at the site of infection. I predict that this research will establish an *in vitro* method to measure the efficacy of the polymer form of the pro-drug compounds in infected or diseased cells in culture. The following specific hypotheses were tested:

Hypothesis$_1$: The polymer form of 5-FdUMP will have a slow, sustained release from the apical side of the open-air cell culture wells, allowing the drug to remain in contact with the lung cell monolayer for a longer period of time as compared to the monomer form of 5-FdUMP.

Hypothesis$_2$: The inhalation apparatus will produce particles of appropriate size and at a flow rate that are appropriate for inhalation therapy with predictable rates of administration.

Hypothesis$_3$: Subunits of 2-fluoroadenosine can be synthesized for use in automated DNA analog polymer synthesis.

MATERIALS AND METHODS

Samples of the monomer form and of a 10-subunit polymer form of 5-FdUMP were obtained from Toronto Research Chemical Inc. (Ontario, Canada). The Calu-3 lung carcinoma cell line was obtained from the American Type Culture Collection (Manassas, VA) and maintained in a medium containing Nutrient Mixture F-12 with L-glutamate (Lonza) (Walkersville, MD), 10% fetal bovine serum (Mediatech Inc.) (Herndon, VA), 0.1 nM non-essential amino acids (Mediatech), 50 U/mL Penicillin (Mediatech), and 50 μg/mL Streptomycin (Mediatech). Hank's buffered saline solution (HBSS) 10x stock solution was obtained from Gibco (Grand Island, NY), 25 mM D-glucose from Mallinckrodt Chemicals (Phillipsburg, NJ) and 10 mM HEPES from Omnipur (Gibbstown, NJ). Phosphate buffered solution (PBS) was obtained from Mediatech and the Brdu assay was from Calbiochem (La Jolla, CA). The 96-well and 6-well Transwell^{TM} plates were obtained from Becton Dickinson Labware (Franklin Lakes, NJ). 0.5 inch plastic cones were ordered from Cole-Parmer (Vernon Hills, IL) and the mini compressor nebulizer pumps was obtained from Medquip (Bluffton, SC).

Characterization of 5-FdUMP Monomer and Polymer

H NMR spectroscopy analysis of the 5-FdUMP monomer was conducted by Toronto Research Chemicals Inc. for chemical identification. I determined the concentration of the 2-fluorouridine polymer through UV spectroscopy analysis using a Nanodrop machine using the extinction coefficients provided by the company. The

machine was calibrated with a 1 µL sample of deionized water followed by a test run using 1 µL of HBSS. A 1µL sample of the polymer was placed onto the optical pedestal and the analysis was run at a wavelength of 230 nM. Absorbance at 270 nm wavelengths was used to calculate the concentration of the 5-FdUMP polymer. A stock solution was prepared of the polymer in 10 mM aliquots frozen at -80° C. Aliquots were taken out of -80° C storage as needed and diluted for each *in* vitro experiment.

Cell Culture

Calu-3 lung carcinoma cells were cultured in a nutrient medium consisting of F-12 Nutrient Mixture and Dulbecco's modified Eagle's medium (DMEM) that contained 10% fetal bovine serum (FBS), 0.1 nM non-essential amino acids, and 50 units/mL/50 µg/mL penicillin/streptomycin liquid mixture. The cells used in all experiments fell into a passage range between 18 and 29. Using Collagen (human type IV) coated Transwell™ polycarbonate inserts of either 6-wells or 96-wells, cells were seeded into the apical surface of the wells at a density of 5×10^5 cells/cm^2. Cells were grown as "open air" cultures with media present only in the lower compartment. Experiments were conducted after day 21 for the 6-well plates once the Calu-3 cells had formed tight junctions determined by transepithelial electronic resistance (TEER) using a TEER meter with chopsticks appropriate for the Transwell™ plates. The 5-FdUMP uptake experiments were conducted 4 days after seeding 96 well tissue culture plates with 1.5×10^4 cells per well.

Permeability in Calu-3 Cells

All cell culture studies required a transport buffer consisting of HBSS with 10 mM HEPES and 25 mM D-glucose. The buffer was made with 4.5 g D-glucose and 2.38 g HEPES dissolved in 100 mL of 10x HBSS solution in a 1 L container. De-ionized water was added to the solution until a total volume of 1 L was reached. Transport studies also required stock solution preparation of both the monomer and polymer of 5-FdUMP using the prepared transport buffer. 100 mg of monomer was dissolved in 1.1 mL of 10x HBSS. 50 μL allocates of this monomer stock solution were placed in multiple vials and frozen for later use. 0.5 mL of HBSS was added to the polymer vial and 50 μL aliquots of this polymer stock solution were placed in multiple vials and frozen for later use.

Excess medium was removed from the 6-well Transwell^{TM} cell culture plates using a sterile class pasteur pipette. Working stocks of both the monomer and polymer form of 5-FdUMP were prepared using 10x HBSS. 4.8 mL HBSS was added to 46 μL of monomer stock solution while 4.8 mL HBSS was added to 4.5 μL of polymer stock solution. Because the polymer consists of 10 units of the monomer, the stocks were equivalent based on the number of monomeric units. Thus, the molarity of the polymer stock solution was 10 fold lower than that of the monomer.

All 6 wells were washed two times by adding and removing transport buffer, 2.5 mL to the top of the wells and 1.5 mL to the bottom. After washing, transport buffer (2.6 mL) was introduced to the basolateral chamber of each well and (1.6 mL) to the each apical chamber. Initial TEER measurements were taken from each well using chopstick electrodes and an EVOM voltohmmeter to determine the integrity of the monolayers. The average TEER reading for the monomer wells was 172.4 ± 68.0 Ωcm^2 and 212.0 ± 70.0

Ωcm^2 for polymer wells. These TEER readings fall within ranges found in other studies that indicate less restrictive tight junctions that are ideal for *in vitro* permeability studies (Ehrhardt, 2002; Shen, 1994; Cooney, 2004; Wan, 2000; Grainger, 2006).

Each condition had three replicates and each experiment was conducted two times. The upper chamber received 1.6 mL of prepared monomer solution and to the top of each of the remaining 3 wells, 1.6 mL of prepared polymer solution was added. 100 μL samples were taken from the AP and BP chamber of each well initially and from the BP chamber at 0.5, 1.0, 1.5, 2.0, and 2.5 hr. Equal volume was replaced using transport buffer in the BP chamber at each time point. At 3.0 hr., 100 μL samples were taken from both the AP and BP chambers. All samples were frozen and stored for further analysis.

In vitro Drug Uptake

5-FdUMP monomer and polymer were serially diluted to final concentrations of 10, 5.0, 2.5, 1.25, 0.625, 0.3125, 0.15625 and 0.78125 μM. A bromodeoxyuridine (Brdu) assay was used to detect the presence of incorporated 5-FdUMP into the genome of the cultured cells. Previous studies have demonstrated that there is indeed cross-reactivity between anti-Brdu monoclonal antibodies and 5-FdUMP (Boisvert, 2000). The assay was conducted in 96-well plates and the columns were designated as either control, blank, monomer, or polymer. The blank column received 20 μL of Brdu solution/well and the control column received 20 μL of media/well. The columns directly adjacent to these two columns were left empty. 4 of the remaining wells were designated as monomer wells and each column received 20 μL/well of serially diluted monomer solution. The remaining 4 wells were designated as polymer wells and each column underwent a serial

dilution with the polymer solution with 20 μL of solution per well. The Brdu assay was then carried out as specified by the directions of the assay kit with one exception.

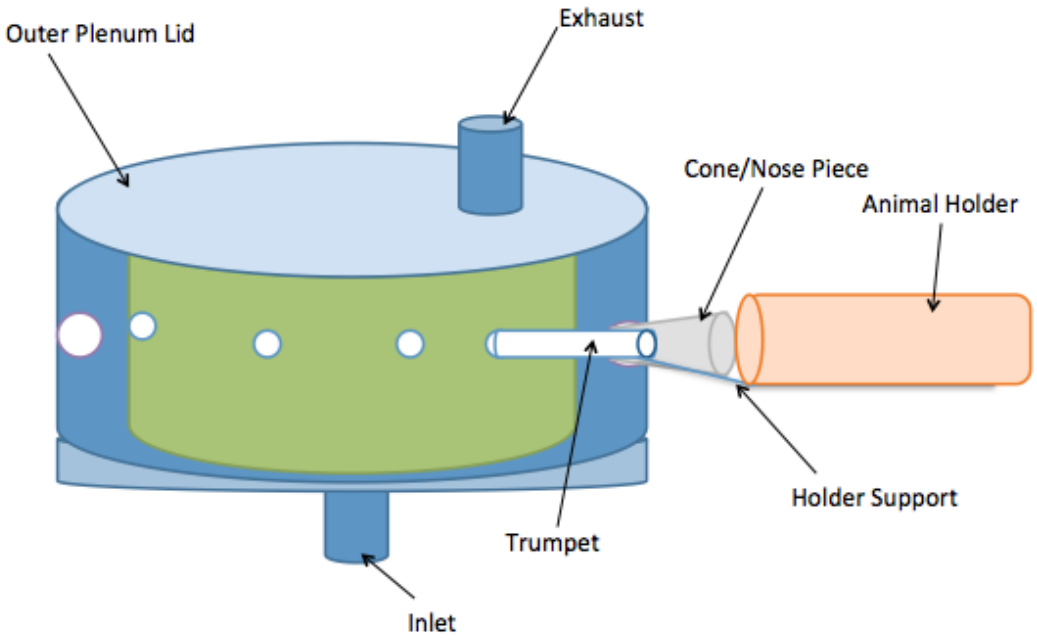
The exception to the standard Brdu assay protocol was the incubation of the cells with Brdu. A normal Brdu assay involves incorporation of Brdu into the genome and RNA of cultured cells and the addition of antibodies that bind to the incorporated Brdu. These antibodies are used to quantify the uptake of Brdu into the cells following incubation. In my experiment the incubation and incorporation of Brdu was replaced with the incubation and incorporation of 5-FdUMP. The addition of antibodies was used to indicate the incorporation of 5-FdUMP into the genome of the cultured cells following drug uptake during the incubation period.

In order to enable binding of the antibodies to the incorporated 5-FdUMP, the cultured cells needed to be fixed, permeabilized, and the DNA/RNA denatured. This was done through the treatment of the cultures with Fixative/Denaturing solution as specified in the assay protocol. Detector anti-Brdu monoclonal antibody was added to the wells and allowed to incubate as indicated in the assay protocol. This allowed the antibody to bind to the incorporated 5-FdUMP. Any unbound antibody was washed away and horseradish peroxidase-conjugated goat anti-mouse antibody was added to each well and was allowed to bind to the detector antibody. This allowed for a color change to occur within each well based on the amount of horseradish peroxidase-conjugated secondary antibody present. The initially colorless solution turned blue upon the addition of the horseradish peroxidase-conjugated goat anti-mouse. Following the addition of stopping reagent, the blue color was converted to a shade of yellow, the intensity of which was proportional to the amount of incorporated 5-FdUMP in the DNA/RNA of the cultured cells. This

colored reaction product was quantified using a spectrophotometer to measure absorbance at dual wavelengths of 450-540 nm.

Inhalation Apparatus Design/Construction

An inhalation apparatus was designed and constructed to ensure directed-flow, nose-only chambers that would theoretically deliver efficient concentrations of inhaled pro-drug to the lungs. The apparatus design was based on a modular nose-only inhalation exposure system (CH technologies) (Minnetonka, MN). This tier system is made of an inner and outer plenum that are connected though rectangular trumpets (tubes). Testing vapor is delivered to each test subject from the inner plenum, through the trumpet, and is exhaled into the space between the inner and outer plenum. The design (Fig. 4A) and flow (Fig. 4B) of the CH technology exposure system is shown in Figure 4.



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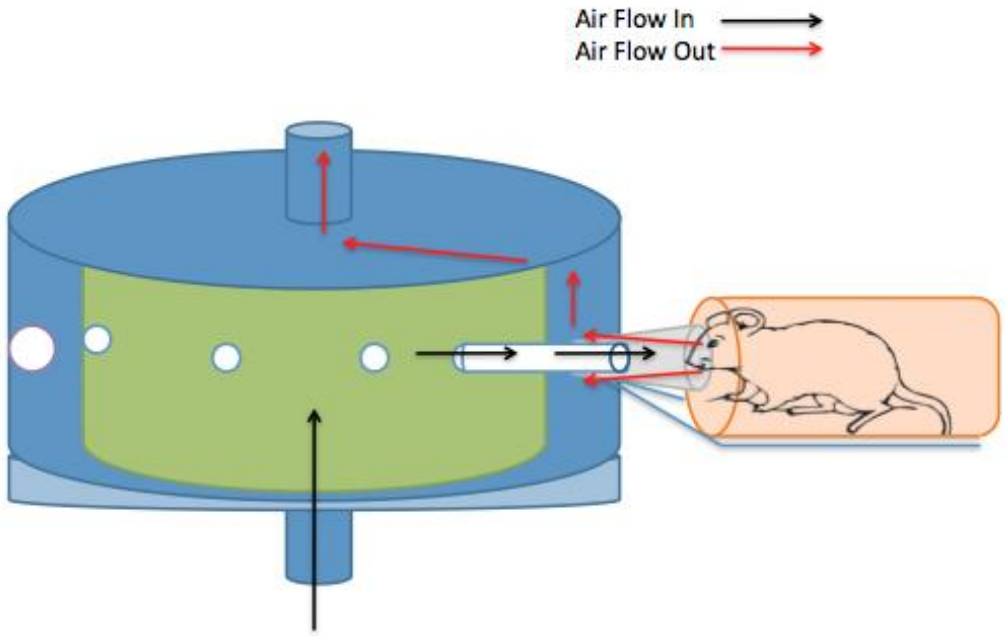

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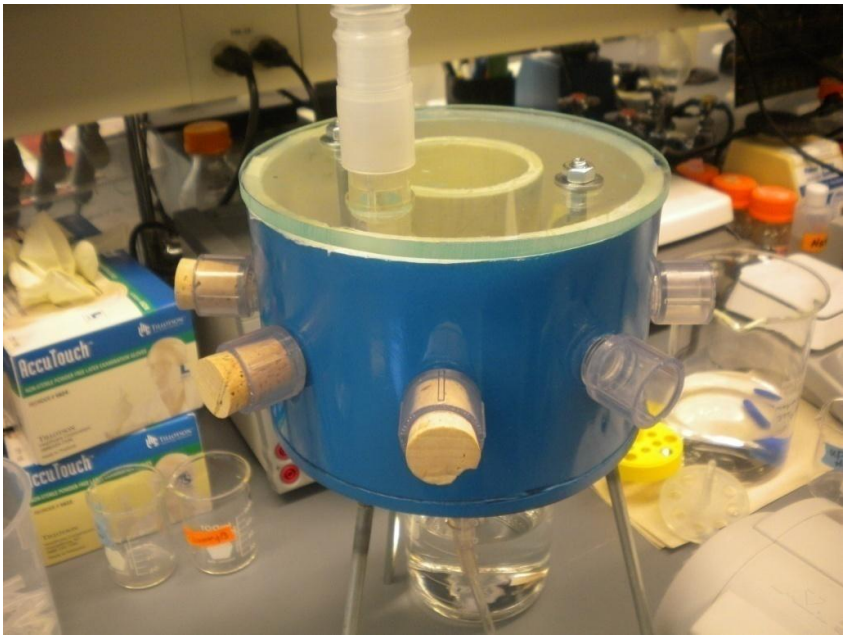
Fig. 4. Figure A shows the general design pattern followed in the construction of the inhalation apparatus and the various parts of the apparatus are labeled. Figure B shows the airflow pathway of vaporized particles through the inhalation apparatus in mouse *in vivo* experiments.

Using this design schematic as reference, I designed a directed-flow, nose-only inhalation apparatus with 8 ports available for drug delivery. My machine included eight delivery cones, an inner plenum made of scrap PVC pipe for entrance of aerosolized pro-drug connected to plastic tubing (trumpets) to connect the flow of vaporized particles from the inner plenum to each cone. The apparatus also consisted an outer PVC plenum that housed each cone and created a space inside the apparatus for exhaled vapor. Finally, the machine was equipped with an exhaust system made of scrape tubing to dispose of exhaled test vapor. The inhalation apparatus was equipped with a nebulizer pump to

aerosolize the testing solution placed in the apparatus. Figure 5 shows the completed inhalation apparatus.



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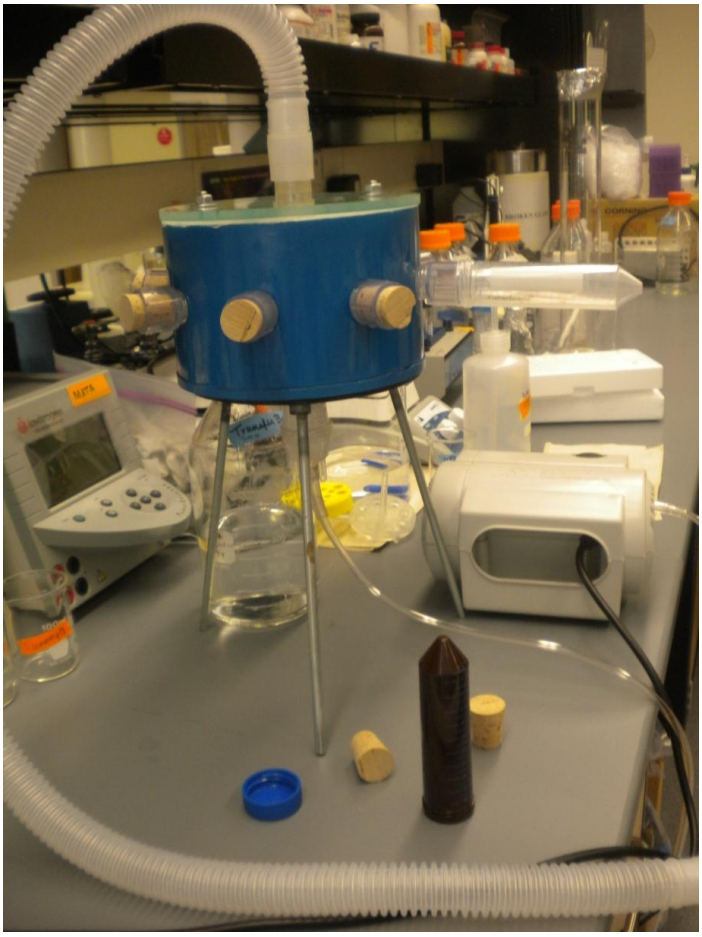


B

Fig. 5. Both A and B in this figure show the constructed apparatus and from these figures the theoretical path of aerosolized pro-drug can be seen. Figure B shows the pump connected to the inner plenum of the apparatus. The flow of aerosolized pro-drug goes from the pump through the connecting tube into the inner plenum where it is dispersed to each cone. Once the pro-drug has been inhaled, the exhaled air is forced back into the outer plenum where it flows up and out through the exhaust tube shown connected to the top of the apparatus.

Flow Rate Analysis

The flow rate of the inhalation apparatus was determined using deionized water. 10 mL of water was added to the nebulizer pump and the machine was allowed to run for nine minutes. For the first trial, only one cone was open and the other cones were closed using cork stoppers. The number of open cones was then increased and data was collected for 1-8 open cones. At the end of nine minutes, the water remaining in the nebulizer was measured and the flow per cone was estimated using the following calculations.

(1)

(2)

The second set of experiments was conducted measuring the water exiting each cone using a cotton ball to trap the water vapor leaving the cones. The purpose of these experiments was to determine the consistency between cones to ensure that each animal receives similar drug exposure. The mass of each cotton ball was measured both before and after a trial period of four minutes and all eight cones remained open. The change in the mass of the cotton balls was used to ensure that each cone was receiving the same amount of water vapor. All experiments were conducted in triplicate.

Particle Size Analysis

This analysis was run by the Microproducts Breakthrough Institute using high-speed photomicroscopic analysis to provide data on particle size.

Theoretical Synthesis of 2-fluoroadenosine

To propose a synthesis scheme for a polymer of 2-fluoroadenosine, two general ideas were taken into consideration. Oligonucleotide synthesis is a well-established approach to synthesizing short fragments of nucleic acids with a specific chemical structure and was considered as an ideal approach to synthesizing the desired 2-fluoroadenosine polymer compound (Reese, 2005). The other important detail that I kept in mind when formulating the synthesis scheme was the fluorination reaction required for the introduction of fluorine in the purine 2-position. A reaction scheme using adenosine as a starting reactant has been shown to be effective in creating a final product of 2-fluoroadenosine (Fig. 6) (Braendvang, 2006).

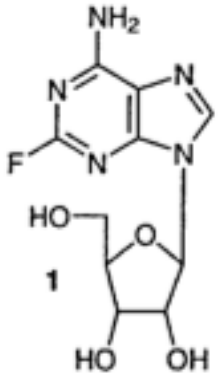


Fig. 6. 2-fluoroadenosine.

Based in these two established methods, my approach to proposing a synthesis scheme for a 2-fluoroadenosine polymer was to combine these two synthesis methods

into a single method that would theoretically result in the desired final product, a 10-subunit polymer of 2-fluoroadenosine.

RESULTS

Pro-drug Analysis

The absorption analysis of the wells that underwent Brdu assay analysis did not show any difference in the intensity of yellow coloration. The wavelengths emitted by all columns containing either monomer or polymer 5-FdUMP solutions were equal. The expected results of this assay were to see a difference in the intensity of yellow coloring and absorption patterns of the wells as you move down each serially diluted column. Ideally, the intensity of yellow coloring would decrease as the amount of 5-FdUMP available for incorporation into cells is reduced. Generally, with a standard Brdu assay, these results would indicate that no 5-FdUMP molecules were present in the DNA of the cultured cells. However, previous studies have shown that 5-FdUMP's method of action in cells is indeed incorporation into the cellular genome (Haouz, 2003). Therefore, the results that were obtained from the Brdu assay indicate a problem with the experimental design and/or protocol rather than a lack of incorporation of pro-drug into the cellular DNA. There are two possible explanations why I obtained results different than what was expected.

One explanation is that the concentrations of testing solutions of the monomer and polymer of 5-FdUMP were too high. If the concentrations were not dilute enough, the addition of polymer or monomer 5-FdUMP to the wells may have proved toxic to the cultured cells and produced the results that were observed. Lysed cells would not allow for the incorporation of 5-FdUMP into the DNA and therefore there would be no antibody binding occurring during the assay. Another possible explanation is that the antibodies simply were not able to bind to the 5-FdUMP that had been incorporated into the genome of the cultured cells.

Apparatus Analysis

Non-linear regression analysis of water vapor demonstrated that the flow rate followed a predictable decrease as the number of open cones varied. Analysis was done using the GraphPad™ computer program. The X variable was defined as the number of open cones and the Y variable was defined as the calculated flow rate. Nonlinear regressing analysis with one-phase decay was used to analyze the data, which was fit to the following equation.

(1)

The data points were extrapolated X=0 using equation 1 and plotted on an XY graph based on defining Y as a function of X (Fig. 7).

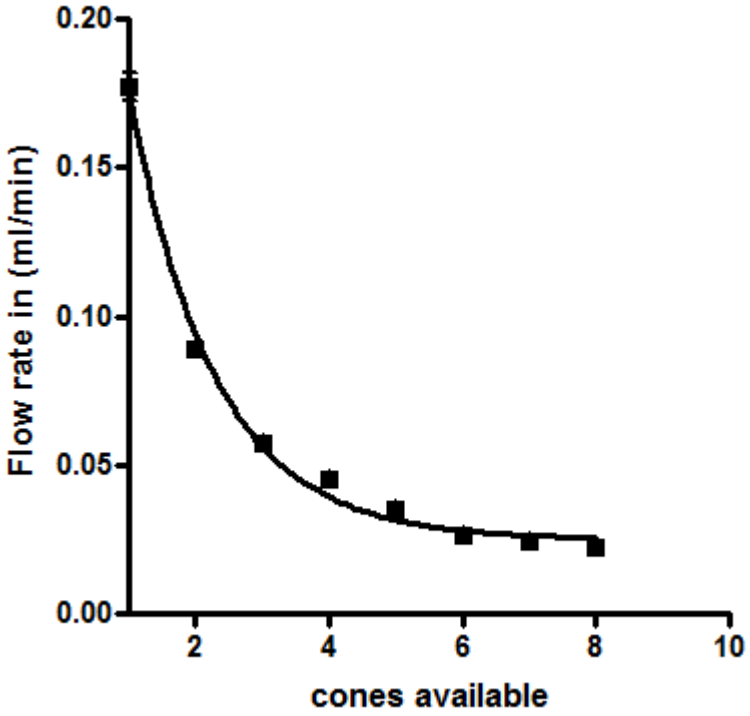


Fig. 7. Graph of the flow rate in mL/min of water vapor as a dependent variable of the number of open cones. Graph indicates a predictable inverse relationship between the number of open cones relative to the flow rate as a 1st order process.

The flow rate changed as a 1st order process dependent on the number of open cones (Fig. 7). For example, the average flow rate when two cones were open was 0.08886 mL/min (Fig. 7). This is approximately half of the average flow rate, 0.17749 mL/min (Fig. 7), that was observed when one cone was open. The curve of the data was shown to fit very closely to the standardized 1st order process curve. The approximation as a 1st order process is also demonstrated by the calculated R^2 value of 0.994 (Fig.7) suggesting that the apparatus is behaving in a predictable manner.

To determine if each cone was receiving the same amount of vapor, the mass of water leaving each cone was collected via a cotton ball and the results are summarized in Figure 8.

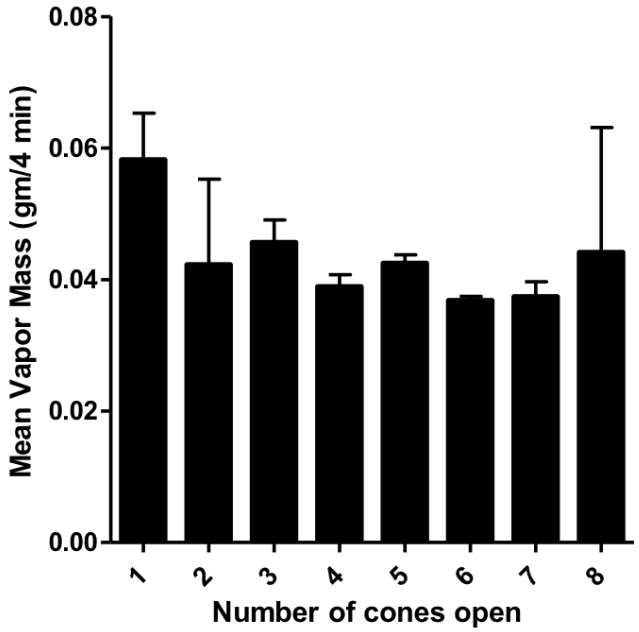
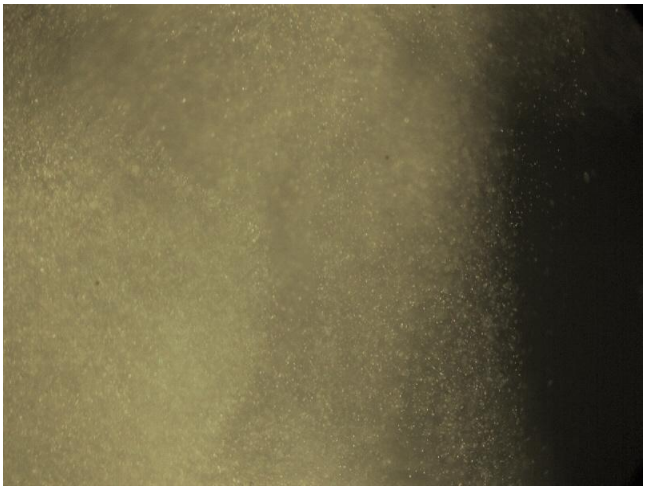


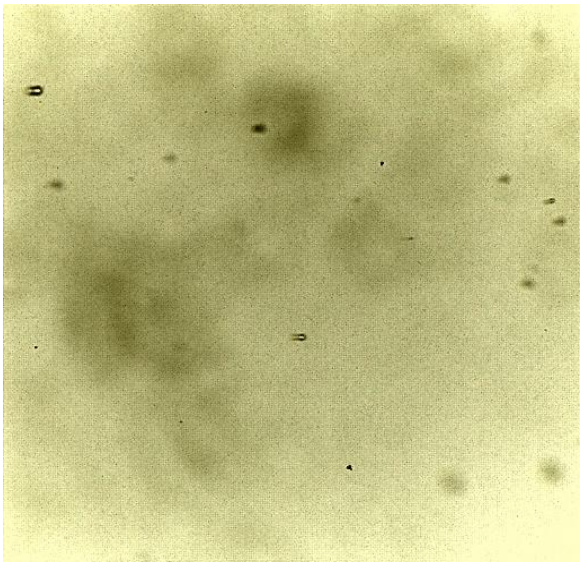
Fig. 8. Bar chart that indicates the mean vapor mass for each individual cone 1-8. Vapor mass is measure in gm/4 min and has minimal variability from cone to cone with negligible error.

The average mean vapor mass for each cone ranged from 0.03680 g/4min to 0.05823 g/4min, with a total average of 0.04322. g/4min (Fig. 8). The standard deviation for each cone ranged from 0.00064 g/4min to 0.019 g/4min, with an average standard deviation of 0.00607 g/4min. Thus, the variability of flow to each cone was small.

The average particle size of water vapor droplets was determined to be approximately 5 μm. Images of the particles were captured using a high-speed camera and are shown in Figure 9.



A



B

C

Fig. 9. A-C are high-speed captured photographs of water vapor leaving the cones of the inhalation apparatus. Figure A shows a spray of various water vapor droplets as they leave a cone. Figure B displays individual water vapor droplets. Figure C is a picture of the flow of the vapor as it is expelled from the apparatus cones.

Proposed Chemical Synthesis Scheme for 2-fluoroadenosine Polymer

Chemical synthesis begins with a basic phosphoramidite building block (Compound A) (Fig. 10).

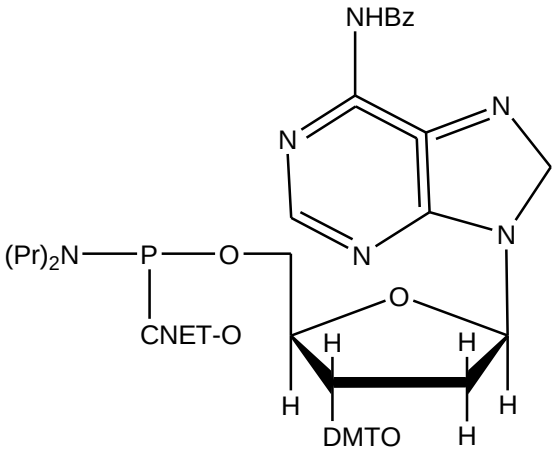


Fig. 10. Compound A. Phosphoramidite building block used in proposed oligonucleotide synthesis scheme.

Step one is to react Compound A (Fig. 10) with BzCl and pyridine at 65°C for four hours followed by treatment with TBAN, TFFA and CH_2Cl_2 at 0°C for 14 hours. This reaction will produce a protected, nitrated intermediate (Compound B) (Fig. 11).

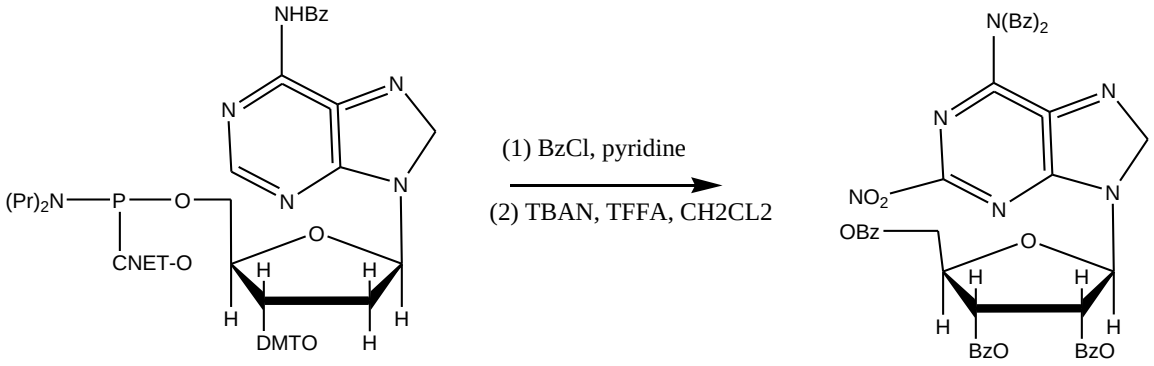


Fig. 11. Protection/nitration reaction of Compound A with a final product of Compound B.

Step two is a substitution reaction. React Compound B (Fig. 11) with TBAF, THF and DMF at room temperature for 1 hour, yielding a fluorinated monomer compound (Compound C) (Fig. 12).

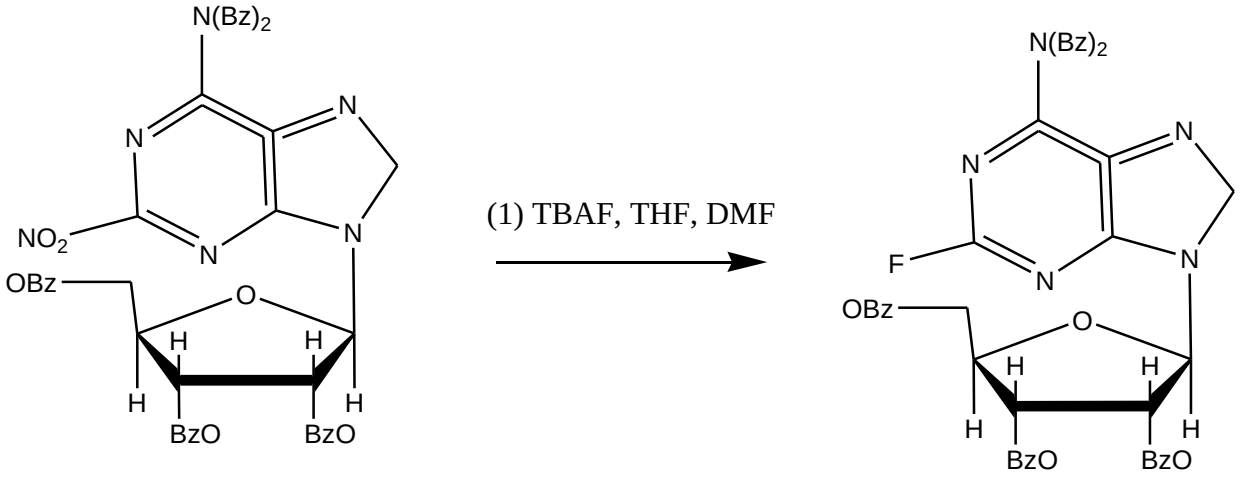


Fig. 12 Fluorination reaction scheme. Compound B reacted with TBAF, THF and DMF to produce the final fluorinated Compound C.

Step three is to bind Compound C (Fig. 12) to a universal oligonucleotide synthesis support with CPG followed by a cleavage and 3'-dephosphorylation using

NH_3/MeOH for 30 minutes at room temperature. This process will yield a protected polymer of 2-fluoroadenosine (Compound D) and can be repeated to extend the chain to the desired length using an automated DNA synthesizer (Fig. 13).

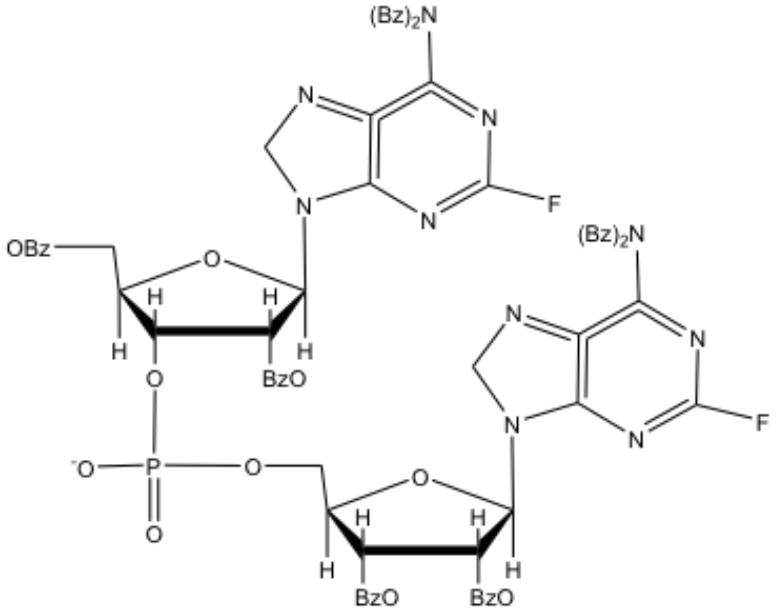


Fig. 13. Protected polymer compound following oligonucleotide synthesis.

Step four is to react Compound D polymer with NH_3 and MeOH at room temperature for 21 hours, which will yield the final desired product, a 2-fluoroadenosine polymer (Fig. 14).

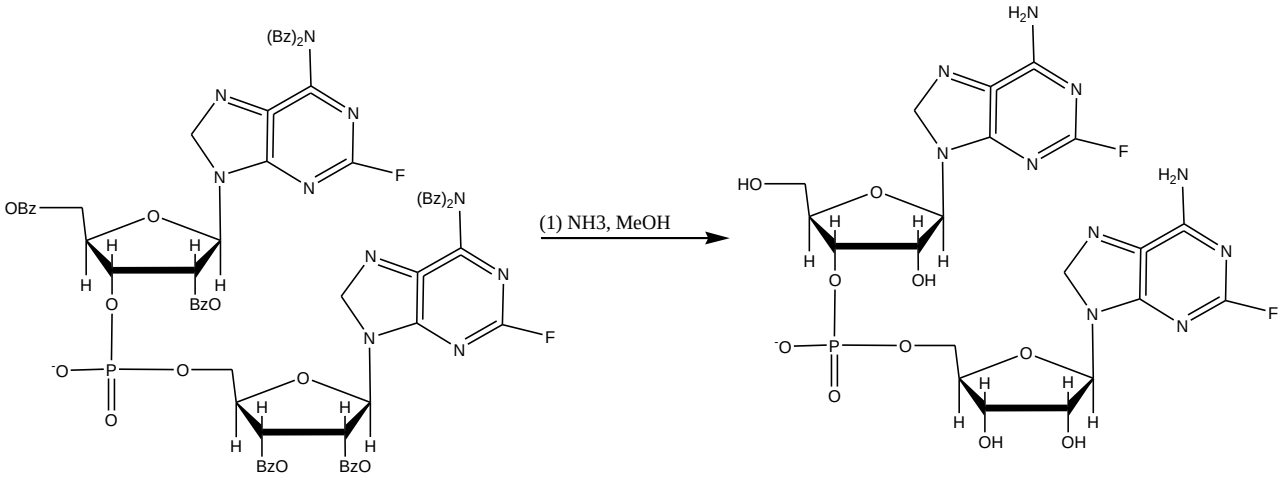


Fig. 14. Deprotecting reaction scheme. Protected polymer chain of Compound C reacted with NH_3 and MeOH to produce the final, deprotected polymer chain.

DISCUSSION

Although the HPLC analysis of cell permeability is still underway, the results of the analysis are expected to show that 5-FdUMP will efflux across the epithelial cell monolayer in the basolateral chamber while the 5-FdUMP polymer will have significantly reduced apparent permeability (Pe). From the HPLC analysis, we will determine the apparent permeability (Pe) of the compounds in the Calu-3 lung cells using the following equation.

— (1)

The results are expected to demonstrate that the active monomer form of 5-FdUMP transports across the cell layer faster, therefore showing a higher Pe while the

polymer form is found to have a slow, sustained release from the apical side and therefore a lower Pe. This slow, sustained release allows the active drug to remain in contact with the lung cells for a longer period of time. The expectation is that this will produce higher efficacy by providing higher concentrations of active drug in the lung for longer periods of time following administration. In the treatment of patients suffering from lung infections, the expectation is that smaller doses of drugs in polymer form at less frequent intervals could be used, resulting in an improvement in the efficacy of treatment. Another possible advantage would be increased patient compliance and satisfaction. Smaller doses of inhaled pro-drug will also help to reduce the possible risk of toxic side effects by reducing systemic peak concentrations.

The Brdu assay used to analysis the *in-vitro* drug uptake experiments gave no results. There are two possible explanations for this. One is that the antibodies did not bind to the 5-FdUMP and therefore did not produce any change in absorption patterns. The other possibility is that the solutions of monomer and polymer 5-FdUMP were too highly concentrated and toxic to the cultured cells. Because previous studies indicated cross-reactivity between 5-FdUMP and the antibodies used in the Brdu assay, I believe the most likely explanation of the unexpected results that I observed was due to using toxic concentrations of monomer and polymer 5-FdUMP. It will be necessary to repeat the experiment using more diluted solutions of 5-FdUMP monomer and polymer and use the same Brdu assay and absorption analysis methods. If the results of this second experiment give similar results as were already observed from the first trial, it might indicate that the issue with the experiment is a lack of binding between the antibodies of the assay and the incorporated 5-FdUMP. This is unlikely because detection of 5-FdUMP

has been reported in the literature (Boisvert, 2000). If this is the case, it will be necessary to explore other possible assay options to be used for data analysis.

The flow rate analysis of the inhalation apparatus showed an inverse relationship between the number of open cones and the flow rate. As the number of open cones increased, the flow rate per cone decreased in a predictable fashion. The variability in mean vapor mass per cone was low demonstrating that each cone received approximately the same amount of vapor. However, more work will have to be done to validate this apparatus with the test articles in phosphate buffered saline. Our results demonstrate that the mass of the liquid delivered to each cone will be consistent. Because the flow rate analysis results follow a 1^{st} order process and the variability of dosage between cones was small, the results can be used to calculate expected dosage deliveries in future *in vivo* testing models. However, before those dosages can be calculated, more research will be necessary to determine the expected dose in mice based on flow volumes and time of exposure. Addition work may also be necessary to determine if the small variability that was observed in the water vapor experiments is significant.

Another important aspect of inhalation therapy is particle size. Previous studies have shown that dispersal of aerosolized particles in the lung is directly dependent on particle size (Mitchel, 1987). The optimal size for aerosolized particles in the treatment of tuberculosis is 1-10 μm (Pillay, 2006; Barrow, 1998). Particles that are too large tend to disperse along the upper airways and therefore never reach the treatment target site while particles that are too small become deposited by sedimentation and therefore are exhaled in large amounts rather than remaining in the lung (Mitchel, 1987). This means that in order to be an effective treatment therapy, the inhalation apparatus needs to produce

aerosolized particles that fall within the ideal size range. The water vapor particles produced by the inhalation apparatus were found to have an average size of 5 μm, which falls within the ideal range.

The synthesis scheme to produce the polymer compound of 5-fluoroadenosine proposes a final product through a series of smaller steps. The initial step is to produce a protected, fluorinated form of the active monomer compound. Using these subunits as building blocks, the next step is to follow a standard protocol (Glen Research, Sterling, VA) method of oligonucleotide synthesis to create the desired 10-mer chain of pro-drug. Once this chain as been created, the final step is to deprotect the polymer compound, which in theory will produce the desired 5-fluoroadenosine compound. However, in discussion with scientists from Glen Research, we found that a 5-fluoroadenosine subunit had been attempted in the past but it was found to be unstable under the conditions it was subjected to during polymerization, deprotection, and cleavage from the column. While the synthesis of 2-fluoroadenosine monomers has been shown to be effective in previous studies, these compounds tend to be relatively unstable (Braendvang, 2006). Using these subunits as building blocks for oligonucleotide synthesis may not produce the desired results due to the harsh conditions and chemicals used during this process.

As a possible solution to the potential complications with the original synthesis scheme, it has been proposed that the oligonucleotide synthesis occur as the first step in the process. This synthesis will use the same phosphoramidite building block as the original synthesis scheme but the building blocks will not be fluorinated, only protected. Once the 10-mer chain has been synthesized it will undergo the same fluorination reaction, adjusting the amounts of reactants to account for the difference in the number of

reactant sites. Following the fluorination reaction, the compound will be deprotected using the same reaction (Fig. 14), which will produce the final desired product of 5-fluoroadenosine polymer. Theoretically once the synthesis scheme for this polymer has been shown to be successful the same process can be used to produce the other desired polymer pro-drug, 5-methyladenosine.

The remaining steps in this research project are to complete the HPLC analysis of the cell permeability experiments as well as resolve the issues that arose with the Brdu analysis of the drug uptake experiments. Once this data has been analyzed, it will be possible to determine the toxicity, dosages and absorption patterns of 5-fluoroadenosine and 5-methyladenosine for use in future animal models. The new proposed synthesis scheme will need to be demonstrated and the two polymer compounds originally proposed synthesized. Once this has been done the platform for *in vitro* efficacy testing and *in vivo* trials will be laid. The efficacy of both polymers, 2-fluoroadenosine and 2-methyladenosine, can be evaluated in multiple strains of *M. tuberculosis* to determine the activity of the compounds as well as in the macrophage test system. *In vivo* trials will allow for further efficacy testing of the constructed apparatus as well as provide a testing model to compare the efficacy of the proposed inhalation treatment to intravenous therapy using the polymer pro-drug compounds.

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APPENDICES

APPENDIX A

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