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Development of an auto-bioluminescent lung cell line for evaluation of in vitro effects of e-cigarette compounds on normal cell proliferation

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Development of an Auto-bioluminescent Lung Cell Line for Evaluation of *in vitro* Effects of E-cigarette Compounds on Normal Cell Proliferation.

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Departmental Honors Thesis

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ABSTRACT

The effects of smoking have long been studied; however, with the rise of electronic cigarettes there is a growing interest in the effects of this smoking substitute. Marketed as a safer alternative to tobacco cigarettes, the popularity of electronic cigarette (e-cigarette) use has dramatically increased. This has demanded regulatory methods to be put into effect, however, scientific study is only beginning on the new fad to determine the impacts of its use on the human body.

The present study aims to examine the biological effects of lung cell exposure to tobacco alkaloids found in electronic cigarette filling solutions. We hypothesize that e-cigarette alkaloids, namely nicotine, cotinine, myosmine, and anabasine singularly and in combination can contribute to changes in normal cell proliferation. To address the aims of this study, two directions for evaluating cell proliferation by luminescent output were pursued. One of these was a genetically engineered stable lung cell line with the pCMVlux system, whose plasmid confers an auto-bioluminescent phenotype, allowing for analysis of cell proliferation through detection of a luminescent signal. The other direction of pursuit was the Promega Cell-Titer Glo® Luminescent Viability Assay. Experimental conditions were evaluated in triplicate after 48 and 96 hours of exposure. Potential synergistic and antagonistic effects were examined by alkaloid exposure in combination. A two-tailed, 2-sample t-test ($\alpha = 0.05$) was used to evaluate statistically significant differences between experimental conditions and controls. Statistically significant differences in cell proliferation were found in exposures to all four alkaloids, but trends of their effects in combination were difficult to discriminate. Anabasine and myosmine

have potential synergistic effects in stimulating cell growth, while cotinine has the potential to act as an antagonist against their activity increasing cellular proliferation.

AKNOWLEDGEMENTS

I would like to thank Dr. Margaret Kovach, who graciously sacrificed time and effort to serve not only as my Departmental Honors project director, but also as my mentor through all my time as a student in her lab. I would like to also thank Dr. Sean Richards and Dr. Gretchen Potts for kindly serving on my committee. I would also like to thank Dr. Potts, specifically, for providing the alkaloids for this continuing study. This research was supported in part by the URaCE SEARCH Award.

INTRODUCTION

Background

Electronic cigarettes (e-cigarettes) have been rising in popularity since their introduction to the United States. It is a 2.5-billion-dollar business and as of 2014, 125 million dollars were used a year in advertising for the new tobacco alternative (FDA, 2018). It has been marketed as a safer, smarter alternative to smoking cigarettes and this has had its effect in its subsequent use. This marketing has prompted a large increase in nicotine product usage in youth in comparison to previous years. Between 2011 and 2018, there was a dramatic increase in e-cigarette usage in both middle schoolers and high schoolers. It has also been the most used tobacco product by youth since 2014. From 2017 to 2018, there was a 78% increase in electronic cigarette usage in high school students (FDA, 2018). Many reports from the Surgeon General, CDC, and FDA detail the increase in use of e-cigarettes and outline the evidence warning of the known and unknown effects caused by e-cigarette consumption. Particularly, they advise that youth and other at-risk individuals not start using electronic cigarettes if there is no prior use history due to adverse effects caused by chemicals in the filling solution (Surgeon General, 2019). These chemicals include nicotine, which is well known to be addictive as well as causing harm to developing brains.

There are many unknown impacts and consequences that e-cigarette use can have on the human body. The consumers of these products face confusion on the potential effects of their usage, therefore, scientific study and analysis of the effects should be done in every way possible. Only after thorough research can individuals make a well-informed decision on their health and future in using e-cigarettes. The purpose of this project is to add to the investigation

data on e-cigarette impact on human health by developing an auto-bioluminescent luciferase reporter system to study the effects of e-cigarette alkaloid exposure on lung cell proliferation. Briefly, this approach will entail genetically engineering lung cells to emit a light signal that can be used to measure cell activity. The advantage of this system is it does not require substrate addition or require an assay for measurement of cell proliferation, ultimately easing the process of proliferation evaluation.

Electronic Cigarettes

E-cigarettes were first introduced to the United States in 2007, however, the commonly seen e-cigarettes today were patented in 2003 by Hon Lik, a Chinese pharmacist (Grana, 2014). Also called an electronic nicotine delivery system (ENDS), the device includes a battery, a processor to control heat and light, a sensor to detect inhalation, a heater, the cartridge containing the filling solution, mouthpiece, and a light at the end that glows when inhaled (Matchless) (Figure 1). The filling solution is usually made up of propylene glycol, glycerol, nicotine, additives, and other flavorings (Grana, 2014).

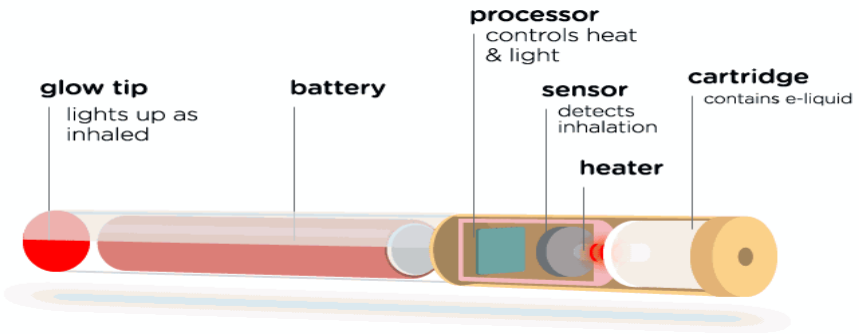


Figure 1*: Diagram of Electronic Cigarette Components*

Image obtained from Matchless

When individuals inhale through the mouth piece, the sensor detects this and activates the atomizer to start heating the filling solution, turning the liquid into an aerosol, which is often mistakenly referred to as a vapor. The filling solution is usually made up of propylene glycol, glycerol, nicotine, additives, and other flavorings (Grana, 2014). While doing this, the LED light at the end will light up and the user will inhale the aerosol through the mouthpiece and into the lungs. The resulting exhale will create a very cloud-like vapor in the air. Since e-cigarettes were introduced in the United States, many new brands, styles, and models have entered the market, but they all ultimately produce an aerosol inhaled into the mouth and to the lungs (Matchless).

Genetic Engineering of Auto-bioluminescent Human Cell Lines

Expression of autonomous bioluminescence from human cells took many years to accomplish. Early us of bioluminescence required a possibly influential chemical substrate (luciferin) to be added to the bioluminescent mammalian reporter systems (Close, 2012). However, discovery of the bacterial luciferase (lux) cassette changed this. In its original form it functioned in only one type of human cell line (HEK293), required multiple genetic constructs, and had reduced luminescence compared to the substrate dependent systems (Close, 2012). It was originally employed as a bacterial bioreporter system for the detection of specific chemical signals in environmental samples but has expanded to be used in biomedical research for toxicity screening and visualization of tumors (Close, 2012).

In the 1980s, science had advanced enough to exogenously express the full lux gene cassette, which is comprised of five genes, *luxCDABE,* in *E. coli*. The luciferase protein is a

heterodimer made up of the *luxA* and *luxB* products. *LuxC, luxD,* and *luxE* gene products are a reductase, transferase, and synthase respectively (Close, 2012). These work together to form the aldehyde substrate for the bioluminescent reaction (Close, 2012). In 2010, the lux operon was resynthesized to demonstrate self-directed bioluminescence emission in the HEK293 cell line, meaning it did not require additional substrates added for the cell line to express the auto-bioluminescent phenotype (Close, 2010). In 2014, A viral 2A-linked *lux* architecture was made that allowed the lux cassette to be expressed in a variety of human cell lines including human kidney, breast cancer, and colorectal cancer lines (Xu, 2014). This was done to improve the efficiency of the transfection procedure. The humanized *lux* cassette was redesigned to reduce its overall size and place all the required genes on a single plasmid vector under a single promoter, thus creating the pCMV*lux* plasmid (Xu, 2014).

The lux protein does not require exogenous substrate addition, it does, however, require continued access to molecular oxygen, $FMNH_2$, and aldehyde co-substrates that are produced by the cell when growing, therefore, bioluminescence can only be detected in growing cells (Xu, 2014). Its output also is directly proportional to the number of cells present. This means it can be used as a simple and non-destructive means for monitoring cell proliferation altered as a result of toxic exposure. Compared to a standard, cells exposed to different conditions can be evaluated for differences in cell growth and death without having to add substrates or change out samples, thus reducing the chance for contamination or accidental variation. The aim of this present study is to optimize the luciferase system in human lung cells in order to evaluate the effects of electronic cigarette alkaloids.

pCMVlux System of Auto-bioluminescence

Specifically, we employed the pCMVlux system for genetic engineering of a human lung cell line to an auto-bioluminescent phenotype. The pCMVlux plasmid vector (Figure 2) encodes a human expression-optimized synthetic luciferase reporter gene cassette based on the bacterial luciferase (*Photorhabdus luminescens*) operon (490 BioTek, 2019). A cassette is type of mobile genetic element that contains a gene and a recombination site. This one is under the control of a strong cytomegalovirus (CMV) promoter for auto-bioluminescent expression across a range of mammalian cell types. The plasmid contains a CMV immediate early enhancer/promoter, the synthetic luciferase cassette, neomycin resistance gene, bacterial replication origin, and ampicillin resistance gene (490 BioTek, 2019). The CMV-driven expression is responsible for the increase in auto-bioluminescent phenotype compared to previous luciferase constructs and is good for stably transfected cell lines (Xu, 2014). The stably transfected cell line maintains its phenotype without external stimulation (490 BioTek, 2019).

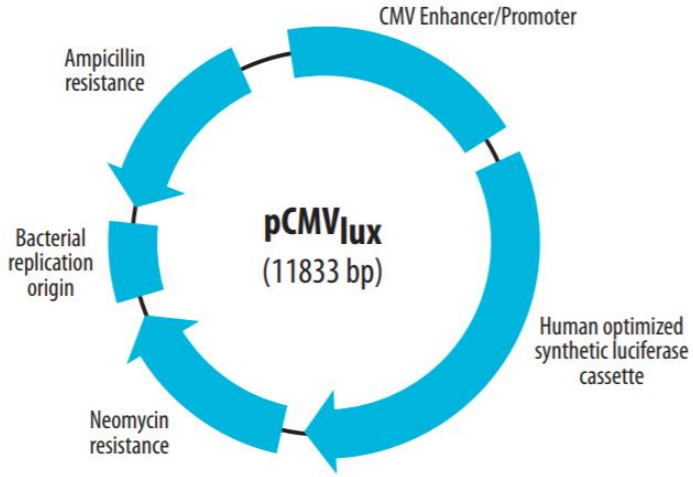


Figure 2*: Plasmid Map of pCMVlux Vector*

CMV immediate early enhancer/promoter 30 - 780 bp, Human optimized synthetic luciferase cassette 971 - 7675 bp, Neomycin resistance region 8450 - 9814 bp, Bacterial replication origin 10039 - 10720 bp, and Ampicillin resistance region 10817 - 11477 bp. Image obtained from 490 BioTek

To facilitate the process of genetic engineering, the PiggyBac Transposon system was exploited for stable transfection of lung cells with the large pCMVlux plasmid. The PiggyBac (PB) transposon is a mobile genetic element that transposes between vector and chromosome by a "cut and paste" method (Figure 3) (System Biosciences, 2019). The PB transposase recognizes transposon-specific inverted terminal repeat sequences (ITRs) located at the ends of the vector. This recognition signals the movement of the contents of the original sites to the chromosomal TTAA sites. This allows the system to integrate the genes of interest to be inserted into the target genome. First, the genes of interest are inserted into the PiggyBac base vector, forming the PiggyBac transposon vector. That vector is then co-transfected into the cells of interest with a Super PiggyBac transposase expression vector (System Biosciences, 2019).

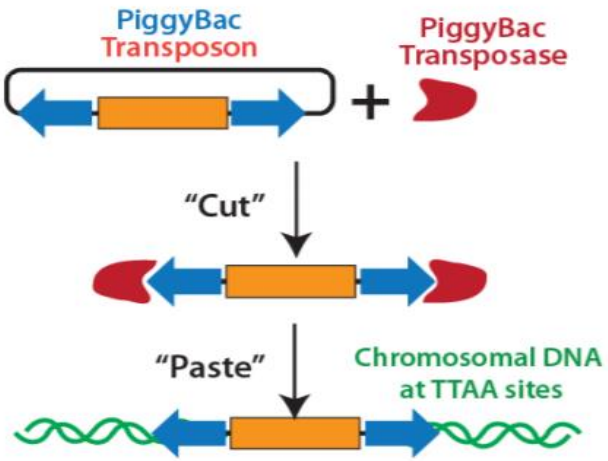


Figure 3*: PiggyBac Transposon Insertion Into DNA*

The PiggyBac (PB) transposon is a mobile genetic element that efficiently transposes between vectors and chromosomes via a "cut and paste" mechanism. Image obtained from System Biosciences.

Common Alkaloids Found in E-Cigarette Solutions

The auto-bioluminescent lung cell line will be used as an *in vitro* model to examine the effects of e-cigarette exposure on normal lung cell metabolism and proliferation. E-cigarette filling solutions can be made up of nicotine and non-nicotine solutions, and the exact chemical composition of the solution is not often disclosed by manufacturers. Research is continuing into the negative effects that these undisclosed compounds can have on human health as toxic substances are found in the filling solutions. There are some commonly found alkaloids, besides nicotine, in filling solutions including cotinine, myosmine, and anabasine. These alkaloids have been found to have synergistic effects with nicotine in relation to addiction, however, their effects on other factors of human health have not been fully explored (Shwartz, 2010). In this study, we will investigate the effect of nicotine, cotinine, myosmine and anabasine on lung cell

proliferation. The effect of these compounds will be measured individually and in combination to gain a better understanding of potential synergistic and agonistic responses.

Nicotine

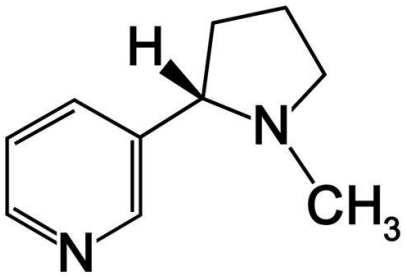


Figure 4*: Chemical Structure of Nicotine*

Nicotine is a plant alkaloid found in the tobacco plant. Its IUPAC name is 3-[(2*S*)-1-methylpyrrolidin-2-yl] pyridine and the molecular formula is $C_{10}H_{14}N_2$ (NCBI, 2020). Nicotine was first extracted from tobacco by German physicians Whilhelm Heinrich Posselt and Karl Ludwig Reimann (Mishra, 2015). It is a clear liquid with a distinctive odor that turns brown when exposed to air. It is water soluble and separates from organic solvents. Nicotine is an amine made up of pyridine and pyrrolidine rings (NCBI, 2020) Once ingested, nicotine is processed by the liver. In phase 1 metabolism, microsomal oxidation occurs, leading to many metabolites including cotinine. In phase 2 metabolism, glucuronidation of the metabolites occurs, preparing the compounds for secretion (NCBI, 2020). However, there is evidence *in vivo* that nitrosation of nicotine can occur leading to the formation of highly carcinogenic chemicals (Hecht, 1988).

Nicotine has also been linked to the formation of tobacco specific nitrosamines (TSNAs) which are viewed as highly carcinogenic. The two main nitrosamines are NNK (4-

(methylnitrosamino)-1-(3-pyridyl)-1-butanone) and NNN (N'- nitrosonornicotine), both of which can contribute to tumor formation (Hecht, 1988). Both NNK and NNN lead to a reactive intermediate that bind to DNA and proteins, contributing to carcinogenesis (Hecht, 1988).

Nicotine has three main sites of action: ganglionic transmission, nicotinic acetylcholine receptors (nAChRs) on chromaffin cells, and nAChR stimulation of the Central Nervous System (NASEM, 2019). Nicotine acts as an agonist at the nAChRs, a ligand-gated ion channel, at neuromuscular junctions, in the adrenal medulla, and the brain. Nicotine's CNS-stimulating activities may be mediated through the release of several neurotransmitters at the axon which result in the symptoms associated with nicotine intake like elevated blood pressure and tachycardia (NASEM, 2019).

It is well known that nicotine is addictive, and the Surgeon General has stated that it is as addictive as cocaine or heroin (Surgeon General, 2019). By binding the nAChRs, dopamine transmission is stimulated. Dopamine then stimulates the reward center, the nucleus accumbens, resulting in the resultant mood elevation and improved cognitive function (Surgeon General, 2019). However, if stimulation is chronic, the receptors can become desensitized, thus inducing the craving sensation of addiction. Nicotine dependence can actually be inherited on the maternal side through epigenetic effects (Schwartz, 2010).

On average, the amount of nicotine absorbed by an individual is 0.5-2 mg per cigarette (Grana, 2014). The range is due to how many times the user inhales (puffs) the cigarette. However, cigarettes contain anywhere from 10-15 mg of nicotine (Grana, 2014). When it comes to e-cigarettes, the amount of nicotine ranges much more greatly. E-cigarettes can contain 0 mg per cartridge and up to approximately 36 mg per cartridge (Mishra, 2015). There is even variance in the amount of nicotine in solution compared to what is promised on the label. This means

nicotine can be found in solutions meant to contain none and less nicotine found that what is promised in others (Mishra, 2015). The amount of nicotine an individual absorbs varies due to frequency of inhalations and the time frame of usage. More absorption of nicotine is found when the e-cigarette is heated more, meaning more puffs in a shorter amount of time (Grana, 2014). Average concentrations of nicotine in the venous blood of smokers are between 10-37 ng/mL. With first generation e-cigarette devices, blood levels of nicotine peaked at 15.75 ng/mL, and with a new generation e-cigarette the blood levels peaked at 23.47 ng/mL (Farsalinos et al, 2014). It is important to note that this present study utilized nicotine concentrations of 1 µg/mL and 10 µg/mL, which is higher than the physiological concentrations found in the blood of smokers, however, it could be more representative of the concentrations exposed to the lung epithelia prior to absorption as 15 puffs on an e-cigarette can yield anywhere from 0.5 to 15.4 mg of nicotine in the vapor inhaled (Maciej, 2012). This range will provide a baseline of data regarding potentially dose-dependent effects of the alkaloids on lung cells.

Cotinine

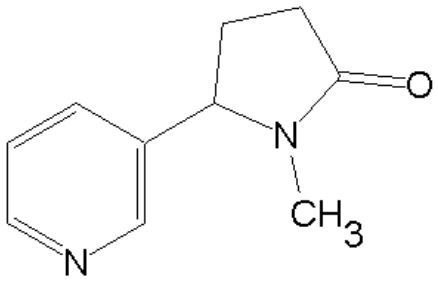


Figure 5*: Chemical Structure of Cotinine*

Cotinine is the main metabolite formed in the body after nicotine has entered the system. Approximately 80–85% of nicotine is metabolized in the liver and converted into cotinine by enzymes such as cytochrome P450 2A6 (CYP2A6) and cytochrome P4502A5 (CYP2A5) (Donato et al. 2000). Measuring cotinine is the most reliable way to measure nicotine exposure due to it persisting longer in the body than nicotine. Cotinine, despite being a metabolite of nicotine, has different pharmacological properties and passes the blood brain barrier (Donato et al., 2000). These properties make it a possible therapeutic agent for psychiatric conditions (Moran, 2012).

Cotinine concentration levels in the blood of smokers averages to approximately 250-300 ng/mL. These levels are much higher than those seen in non-smokers (N. L. Benowitz et al., 1983). Despite higher concentration levels seen within the blood of smoking individuals, research focused on any detrimental effect of the alkaloid has not found cotinine to cause cardiovascular effects on individuals. It also seems to not have any cognitive effects or noticeable withdrawal symptoms despite its ability to pass the blood brain barrier (Moran, 2012). Further investigation into the effects of cotinine is warranted in order to understand its effects in e-cigarette filling solutions.

Myosmine

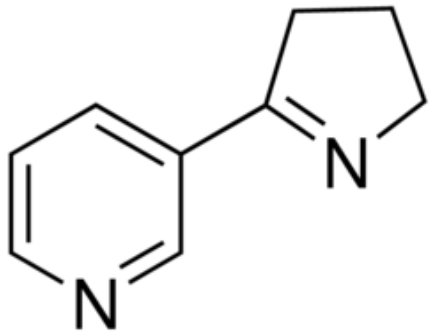


Figure 6*: Chemical Structure of Myosmine*

Mysomine, or 3-(3,4-dihydro-2*H*-pyrrol-5-yl)pyridine, is a minor tobacco plant alkaloid that is similarly structured to nicotine (NCBI, 2019). Unlike cotinine, it is not a metabolite derivative of nicotine. Its presence in the tobacco plant is believed to be caused by bacterial action or oxidation during tobacco processing (Leete, 1983). However, myosmine is also found in other foods such as nuts, cereals, cocoa, corn, etc. This presence in food results in the alkaloid being found in human toenails, saliva, and breast milk (Simeonova et al, 2012).

Mysomine metabolism is known to result in genotoxic and carginogenic effects, in which two of the five metabolites are most prevalent. These metabolites are 3-pyridyl acetic acid (3-PAA) and keto acid (Glas et al, 2007). Direct conversion of myosmine to NNN occurs under acidic conditions. NNN is implicated in the initiation of esophageal cancer in smokers. When metabolically activated, NNN can lead to agents that bind to DNA causing conversions of Guanine to Adenine resulting in mutagenesis (Vogt et al, 2006). Myosmine has also been found to give rise to another nitrosamine formed by mutagenic DNA adducts, HPB (4-hydroxy-1-(3-pyridyl)-1-butanone) (Wilp et al., 2002).

Anabasine

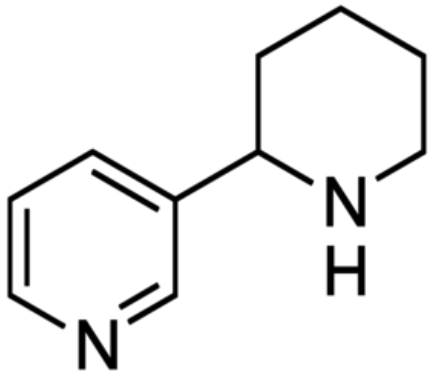


Figure 7*: Chemical Structure of Anabasine*

Anabasine, like myosmine, is a minor tobacco plant alkaloid and is not a derivative of nicotine. It is a botanical insecticide that makes up very little of the total alkaloids found in the plant (Saitoh, 1985). However, it can still be used to determine exposure to tobacco or tobacco products (Saitoh, 1985). Like nicotine, anabasine is considered a nicotinic acetylcholine receptor agonist, but is less addictive qualities in comparison (Nasirov et al, 1978). This similarity has made anabasine a possible therapy to alleviate nicotine addiction. It also has the capability of forming possible carcinogenic nitrosamines due to reacting with nitrosating agents (Hecht, 1988). This property, along with its longer half-life of metabolism, makes this an interesting alkaloid of study (Jacob et al, 2002).

Goals and Aims of Research

The effects of smoking have long been studied; however, with the rise of electronic cigarettes, there is a growing interest in the effects of the smoking substitute. The popularity of e-cigarette use has demanded regulatory methods to be put into effect, however, scientific study is only beginning on the new fad to determine the impacts of its use on the human body.

This study attempted to examine the biological effects of lung cell exposure to tobacco alkaloids found in electronic cigarette filling solutions. Previous studies have been done to elucidate possible health effects of e-cigarette alkaloids, anabasine, cotinine, myosmine, and nicotine (Trechy et al, 2011, Hale, 2017). With these previous studies in mind, it is hypothesized that these four alkaloids singularly and in combination can contribute to changes in cell proliferation and gene expression. To address this study's aim, two methods for studying cell proliferation by luminescent output were followed. A genetically engineered stable lung cell line with the pCMVlux plasmid was established and utilized. This plasmid confers an auto-bioluminescent phenotype, allowing for analysis of cell proliferation through detection of a luminescent signal. In order to confirm and compare the results of the stable cell line auto-bioluminescence, the Promega Cell-Titer Glo® Luminescent Viability Assay was used. Experimental exposures were evaluated in triplicate after 48 and 96 hours of exposure. Potential synergistic and antagonistic effects were examined by alkaloid exposure in combination. Changes in cell proliferation were measured as a function of luminescence. A two-tailed, 2-sample t-test was used to evaluate statistically significant differences between experimental conditions and untreated controls. We hypothesize that exposure of lung cell cultures to tobacco alkaloids is associated with abnormal proliferation.

MATERIALS AND METHODS

Tissue Culture Maintenance

The lung carcinoma cell line used in this study, A549 also referred to as CCL-185, was received from the American Tissue Culture Collection (ATTC). Frozen cells were seeded in

75cm^2 tissue culture flasks. CCL-185 cell line was cultured in F-12K growth medium, Kaighn's Modification of Ham's F-12 Medium, that contained 2 mM L-glutamine and 1500 mg/L sodium bicarbonate as per ATCC's recommendation. The media was also combined with 10% Fetal Bovine Serum (FBS) providing essential growth factors and 1% penicillin-streptomycin solution to prevent bacterial contamination in the solution. Cells were maintained in a CO_2 incubator at 5% CO_2 and 37˚C. Media was renewed every three days or as needed aseptically under a laminar flow hood.

When cells reached a confluency of approximately 70%, they were either sub cultured, used in transfection with the pCMVlux plasmid, or used in the cell proliferation assay. In any case, media was aseptically removed and replaced with 3mL aliquots of 0.25% trypsin solution in order to detach the cells from the surface of the flask and promote a single cell suspension. Cells were placed in the 37˚C incubator to assist in the proteolysis process. Once in a single cell suspension, F-12K medium was added to halt the trypsin activity and split 1:3 into new 75 cm^2 flasks, seeded onto 6-well plates for transfection, or seeded into 12-well plates for the CellTiter-Glo Luminescent Viability Assay.

Transfection and Stable Selection of Auto-bioluminescent Cell Line

In order to easily evaluate changes in cell proliferation and allow efficient imaging in the future aims of the e-cigarette alkaloid study, it was decided that a stable cell line that is auto-bioluminescent would ease the evaluation of alkaloid exposure effects later in the study. In order to do this, a human-optimized luciferase plasmid had to be used to insert the lux gene into lung

cells. A PureFection™ transfection system was utilized in order to insert the pCMVlux plasmid via a PiggyBac system.

PureFection™ PiggyBac Integration into Target Cells

The pCMVlux plasmid system was provided by Oak Ridge National Laboratories in two forms, one was just the pCMVlux plasmid alone and the other was in a PiggyBac delivery system that had been shown in studies (John, 2017) to be successful in stably integrating into different cell types (pPBCMVlux and SPBO). The PiggyBac system works by using System Bioscience PureFection™ protocol (System Biosciences, 2019). Plasmids underwent a large-scale plasmid preparation in order to produce and purify more plasmid for use in this experiment. 24 hours prior to transfection of cells, the CCL-185 cell line was seeded in a 6-well plate so that it would reach ~50% confluency the day of the protocol. Two hours before creating the transfection solution, media was renewed in each well of the 6-well plate. From there, for each well, 0.5μg of the pPBCMVlux plasmid, 0.2μg of the SPBO plasmid, 8μL of the PureFection™ reagent, and 50μL of serum free DMEM were combined. This solution was then vortexed and incubated at room temperature for 15 minutes. The solution was then added dropwise to each well of cultured CCL-185 cells. The transposase activity was terminated after 72 hours, at which time, antibiotic selection was applied to isolate cells that were stably transformed with the pCMVlux plasmid. Growth under geneticin exposure means those colonies became geneticin resistant due to the insertion of the geneticin resistance gene found on the pCMVlux plasmid into the genome and are successful transfectants.

Generation of Antibiotic Kill Curve

A kill curve was completed to determine the minimum concentration of geneticin antibiotic that could kill all the untrasfected cells within ten days. CCL-185 was seeded in a 6-well plate and allowed 24 hours to adhere to the well bottom. After, different concentrations of geneticin antibiotic was added to the media. The concentrations were 500 μg/mL, 600 μg/mL, 700 μg/mL, 800 μg/mL, 900 μg/mL, and 1000 μg/mL. After 10 days of evaluation of cell death under a microscope, the concentration of 800 μg/mL was decided to be the best concentration for CCL-185 antibiotic selection.

Stable Selection of Auto-bioluminescent CCL-185 Cell Line

Three days after the PureFection^{TM} PiggyBac transfection protocol, the growth media in the CCL-185 cell cultured 6-well plate was removed and replaced with F-12K media supplemented with 10% FBS and 800 μg/mL geneticin. The 1% penicillin-streptomycin solution was not added into the media, because it has a competitive inhibition interaction with geneticin, preventing full activity of the selective antibiotic. Geneticin selective media was renewed every 48 hours or as needed for 10 days until all cells except for successful transfectants were dead. Single cell colonies were allowed to grow until they were of harvestable size. Single colonies were harvested by marking their location on the plate and then adding a small amount of 0.25% trypsin solution to the area and using a pipet tip to suspend the live cells. Each successful colony was then passaged onto a 48-well plate to be grown up. Each colony was grown and maintained until they were able to be ultimately maintained in 25cm^2 flasks with the F-12K, 10% FBS, and 800 μg/mL geneticin medium.

Evaluation of Luminescent output of Successful Transfectants

Successful colonies of geneticin resistant cells were harvested using 1mL of 0.25% trypsin solution and resuspended in F-12K, 10% FBS, and 800 μg/mL geneticin medium. Cells were then counted using a hemocytometer and passaged into a white-wall 96-well plate at the lowest concentration of the colonies so each well contained the same number of cells. A blank was also included on the plate of just growth medium to compare the cells output to background luminescence. The 96-well plate was then evaluated in a BioTek Synergy plate reader. In order to evaluate which colony had the most efficient and highest output of luminescence, the wells were overloaded with cells and evaluated with an integration time of 20 seconds. The longest integration time possible is best in order to get the best luminescent results. Readings were taken every thirty minutes for two hours to see if there was an increase in luminescence. The luminescence should increase as the plate and samples cool down from the 37^{o}C incubator.

CellTiter-Glo Luminescent Viability Assay

To examine the effect of alkaloid exposure on cell proliferation, lung tissue culture samples of CCL-185 were characterized for growth and viability in 48 hour and 96-hour experiments, while exposed to 1 μg/mL and 10 μg/mL concentrations of the tobacco alkaloids: nicotine (N), cotinine (C), myosmine (M), and anabasine (A) singularly and in all combinations (Table 1). Cell viability was determined at 48 hours of exposure and 96 hours of exposure, relative to untreated controls by using the Promega CellTiter-Glo® Luminescent Viability assay. The assay relies on a thermostable luciferase reaction that detects metabolic ATP levels in the

cell (Promega Corporation, 2015). The amount of ATP present in a sample is directly proportional to the number of cells present due to the rapid degradation of ATP after cell death. The CellTiter-Glo® luminescent reagent works by lysing open the cells and releasing ATP while simultaneously inhibiting ATPases that would decompose the cellular ATP. It provides a measurable bioluminescent reaction by interaction of cellular ATP with the light-emitting compounds, luciferin, and its corresponding oxidizing enzyme, luciferase, as well as other associated reagents (Promega Corporation, 2015).

Table 1: ***Combinations of Tobacco Alkaloid Exposures***

DOUBLE COMBINATIONS	TRIPLE COMBINATIONS	TOTAL COMBINATION
Nicotine + Cotinine (NC)	Nicotine + Cotinine + Myosmine (NCM)	Nicotine + Cotinine + Myosmine + Anabasine (NCMA)
Nicotine + Myosmine (NM)	Nicotine + Cotinine + Anabasine (NCA)	
Nicotine + Anabasine (NA)	Nicotine + Myosmine + Anabasine (NMA)	
Cotinine + Myosmine (CM)	Cotinine + Myosmine + Anabasine (CMA)	
Cotinine + Anabasine (CA)		
Myosmine + Anabasine (MA)		

The above table displays the different combinations of alkaloids used in the CellTiter Glo® Luminescent Viability Assay. Combinations were separated into doubles, triples, and total combinations made up of the four tobacco alkaloids, nicotine, cotinine, myosmine, and anabasine.

Plate Set Up for Exposures

In carrying out this assay, CCL-185 cells were seeded in Falcon®12-well plates at a concentration of 20,000 cells/mL/well. This was done for both experimental and control conditions. Experimental conditions were seeded in triplicate in both concentrations (1 µg/mL and 10 µg/mL) for each combination. An untreated control was also seeded at the same density

with only the F-12K growth media added. Alkaloid dilutions were made from stocks of 1mg/mL of nicotine, cotinine, myosmine, and anabasine.

Each plate held three wells of experimental combinations or single exposures and three wells of control. Plates were separated by concentration of exposure and a total of 10 plates were used (Figure 8). Experimental plates were maintained in the 37^{o}C incubator at 5% CO_2.

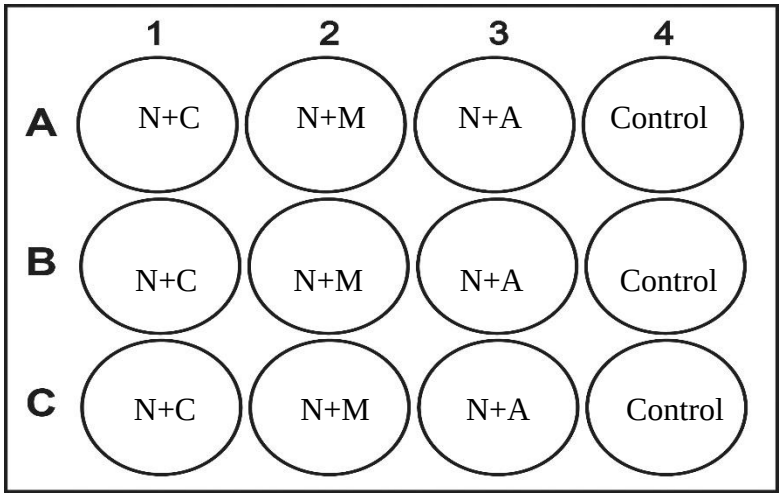


Figure 8*: Example of 12-Well Plate Layout for Alkaloid Exposures for CCL-185*

Each exposure treatment is evaluated in a technical triplicate. Plates were independently seeded on day one and the above plate layout example was made for both 1 µg/mL and 10 µg/mL concentrations.

Measurement of Cell Proliferation

Cell viability and proliferation was measured every 48 and 96 hours for every experimental combination using the Promega CellTiter-Glo® Luminescent Viability Assay, per

manufacturer's instructions. The day of measurement, media was removed and replaced with 350 µL of F-12K growth media without the added FBS or penicillin-streptomycin solution. Next to the 12-well plate of cultured cells, 350 µL of CellTiter Glo® Reagent was added to each well, producing a color change. The plates were then incubated at room temperature for 10 minutes on an orbital shaker. After the incubation, each well containing an experimental condition or control were plated in triplicate into a 96-well plate (Figure 9). Thus, there were nine samples per experimental condition on the 96-well plate (the single exposures were done in duplicate due to it being a confirmation of past research). The 96-well white walled, reflective plate was then assayed for metabolic ATP.

	1	2	3	4	5	6	7	8	9	10	11	12
A	NC	NC	NC	NA	NA	NA	CA	CA	CA	MA	MA	MA
B	NC	NC	NC	NA	NA	NA	CA	CA	CA	MA	MA	MA
C	NC	NC	NC	NA	NA	NA	CA	CA	CA	MA	MA	MA
D	NM	NM	NM	CM	CM	CM	CONTROL	CONTROL	CONTROL	CONTROL	CONTROL	CONTROL
E	NM	NM	NM	CM	CM	CM	CONTROL	CONTROL	CONTROL	BLANK	BLANK	BLANK
F	NM	NM	NM	CM	CM	CM	5.00E-12	5.00E-12	5.00E-12	1.00E-12	1.00E-12	1.00E-12
	2.00E-10	2.00E-10	2.00E-10	1.00E-10	1.00E-10	1.00E-10	5.00E-11	5.00E-11	5.00E-11	1.00E-11	1.00E-11	1.00E-11

	1	2	3	4	5	6	7	8	9	10	11	12
A	NCM	NCM	NCM	NMA	NMA	NMA	NCMA	NCMA	NCMA	Control	Control	Control
B	NCM	NCM	NCM	NMA	NMA	NMA	NCMA	NCMA	NCMA	Control	Control	Control
C	NCM	NCM	NCM	NMA	NMA	NMA	NCMA	NCMA	NCMA	Control	Control	Control
D	NCA	NCA	NCA	CMA	CMA	CMA						
E	NCA	NCA	NCA	CMA	CMA	CMA				BLANK	BLANK	BLANK
F	NCA	NCA	NCA	CMA	CMA	CMA	5.00E-12	5.00E-12	5.00E-12	1.00E-12	1.00E-12	1.00E-12
	2.00E-10	2.00E-10	2.00E-10	1.00E-10	1.00E-10	1.00E-10	5.00E-11	5.00E-11	5.00E-11	1.00E-11	1.00E-11	1.00E-11

	1	2	3	4	5	6	7	8	9	10	11	12
A	N 1	N 1	N 1	M 1	M 1	M 1	N 10	N 10	N 10	M 10	M 10	M 10
B	N 1	N 1	N 1	M 1	M 1	M 1	N 10	N 10	N 10	M 10	M 10	M 10
C	C 1	C 1	C 1	A 1	A 1	A 1	C 10	C 10	C 10	A 10	A10	A10
D	C 1	C 1	C 1	A 1	A 1	A 1	C 10	C 10	C 10	A 10	A10	A10
E	Control	Control	Control	Control	Control	Control				BLANK	BLANK	BLANK
F	Control	Control	Control	Control	Control	Control	5.00E-12	5.00E-12	5.00E-12	1.00E-12	1.00E-12	1.00E-12
	2.00E-10	2.00E-10	2.00E-10	1.00E-10	1.00E-10	1.00E-10	5.00E-11	5.00E-11	5.00E-11	1.00E-11	1.00E-11	1.00E-11

Figure 9*: 96-well Layout for CellTiter Glo® Luminescent Viability Assay*

Cells were sampled from each well of the 12-well plate used for seeding with experimental alkaloid exposure, into triplicates, totaling nine wells per experimental condition. The wells in the figure are labelled by the experimental

condition exposure of the cells as well as the moles of ATP for the ATP standard curve that was included on each plate. A plate control, labeled as blank, of only media (no cells or substrate) was also included on each plate.

A no template control (blank) was added to the plate to monitor background luminescence was also added in triplicate (100 µL) along with a standard curve of know ATP concentrations ($2x10^{-10}$, $1x10^{-10}$, $5x10^{-11}$, $1x10^{-11}$, $5x10^{-12}$, and $1x10^{-12}$). These standards were used to extrapolate the ATP levels of the unknown samples (Figure 10). Equal volumes of the CellTiter Glo® Reagent was added to the blank and standard curve samples. Luminescent reading of the 96 well plate was subsequently carried out by a BioTek® Synergy plate reader. Cell proliferation results from the Promega CellTiter-Glo® Luminescent Viability Assay were then statistically analyzed in Microsoft Excel™.

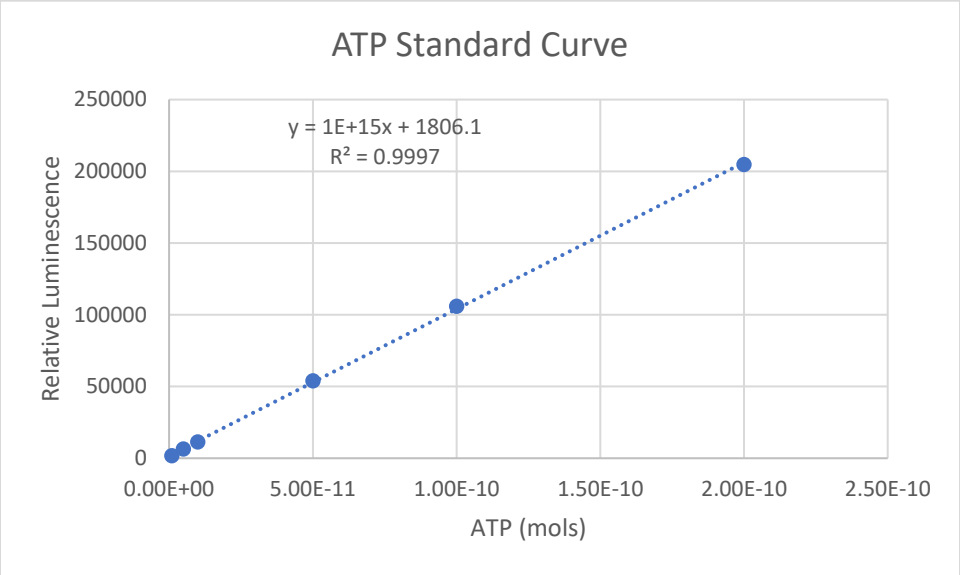


Figure 10*: ATP Standard Curve Generation in Moles of ATP*

In order to extrapolate the metabolic ATP levels in the samples, a standard curve of known concentrations of ATP was made and read for luminescence ($2x10^{-10}$, $1x10^{-10}$, $5x10^{-11}$, $1x10^{-11}$, $5x10^{-12}$, and $1x10^{-12}$). The standards were loaded in triplicate.

Statistical Analysis of Alkaloid Exposure on Cellular Proliferation

A two-tailed, 2 sample equal variance student t-test ($\alpha = 0.05$) was applied in order to determine if the differences between the condition samples and the controls were statistically significant. The mean value of each sample was evaluated against the mean value of the control wells. P-values obtained from the student t-test displayed the differences between groups compared to the differences in values within groups. A p-value of ≤ 0.05 between experiment and controls was deemed statistically significant. A p-value obtained that was ≤ 0.05 was accepted as being against rejecting the null hypothesis that any differentiation seen between the experimental value and the control was due to random chance. Any p-value > 0.05, for the experimental conditions, was accepted as probable evidence that any differentiation between the experimental value and the control was due to random chance.

To compare the experimental values to the controls, they were normalized to the untreated control using the following equation:

$$\% \text{ Change} = (\text{Experimental} - \text{Control} / \text{Control}) \times 100$$

By converting the values into percent difference, different experimental combinations can be compared.

RESULTS

The aim of this study was to examine the biological effects of tobacco alkaloids found in electronic cigarette filling solutions on human lung cells in culture. This was to be done by establishing an auto-bioluminescent stable lung cell line by use of the pCMVlux system to

evaluate cell proliferation as a measurement of an endogenous luminescent signal. In addition, a commercially available luminescent cell viability assay was employed to evaluate the biological effects of tobacco alkaloid exposure in the event that we were unsuccessful in genetically engineering an auto-bioluminescent lung cell line. Another aim was to expand prior studies in the laboratory to examine potential synergistic and antagonistic effects of these alkaloids in combination. These aims were to be carried out by evaluating the differences in cell proliferation of the CCL-185 lung cell line when exposed to different combinations of the e-cigarette alkaloids: nicotine, cotinine, myosmine, and anabasine in concentrations of 1 µg/mL and 10 µg/mL. We hypothesized, based on prior research indicating tobacco alkaloids contributions to human diseased states (Hecht, 1988, Hale, 2017), that exposure of lung cell cultures to tobacco alkaloids is associated with changes in normal cellular proliferation. These changes in cell growth could offer understanding of potential factors for lung disease associated with exposure to e-cigarette compounds.

Establishment of Stable, Auto-bioluminescent CCL-185 Cell Line

After optimizing conditions for stable transfection of CCL-185 cell line with the pCMVlux system, six colonies originating from separate transformation events remained after antibiotic selection. Each were grown up to be analyzed for highest output of luminescence. This means those colonies became geneticin resistant due to the insertion of the geneticin resistance gene found on the pCMVlux plasmid into the genome. Measurement of the luminescent output was measured in comparison to a control in order to determine if insertion of the luciferase cassette was successful as well as putting out readable luminescent signal. Four readings were

taken and put in order of increasing luminescent output to determine if there was a sample that was continuously higher than the others (Table 2).

Table 2*: Luminescent Readings of Transfected CCL-185 Colonies Compared to Control*

	Colony 1	Colony 2	Colony 3	Colony 4	Colony 5	Colony 6	Control
Read 1	977	922	948	928	927	890	841
Read 2	929	959	942	975	1026	987	967
Read 3	1166	1137	1137	1158	1167	1116	1147
Read 4	1259	1290	1258	1311	1266	1254	1256

Cells were placed in technical duplicate in a 96well plate and averaged into the above values for each reading. Colonies were labeled 1-6 at formation and maintained as that number through growth and evaluation. The control sample was made up of cells that were not transfected at the same concentration as the colony samples.

The luminescent readings of all six colonies and control all increased as they cooled. This was expected as luminescence increases when temperature decreases. However, the grown-up colonies did not show a pattern of continual, high output of luminescence. Even the control of nontransfected cells and media at times read as higher in luminescent output. After these results, the developer of the pCMVlux system, Dr. TingTing Xu (Oak Ridge National Laboratories) was contacted to validate that transfection of the plasmid was unsuccessful. She reported back that our transfection efficiency was low and that it seemed as though we were unsuccessful in establishing a stable line with high enough expression of the luciferase gene to show increase luminescent signal (T. Xu, personal communication, February 11, 2020). With this in mind, more transfections were started to see if new successful colonies could be grown.

The growth of colonies but nondifferential luminescent output could be due to insertion of the plasmid being only integrated partially into the genome of the CCL-185 lung cell. It could also have been inserted into a poor region of expression in the genome. Or, it could be due to only partial transfection of the plasmid into the cell due to its large size. Antibiotic resistance to

geneticin could have also been due to a genetic mutation that occurred randomly in the lung cell genome, allowing colonies to grow.

Evaluation of Tobacco Alkaloid Exposure on Lung Cell Proliferation

The effect of e-cigarette alkaloid exposure of cellular proliferation and viability was examined for the CCL-185 cell line. The lung tissue cell line displayed a growth trend over the time points evaluated in this study (Figure 11).

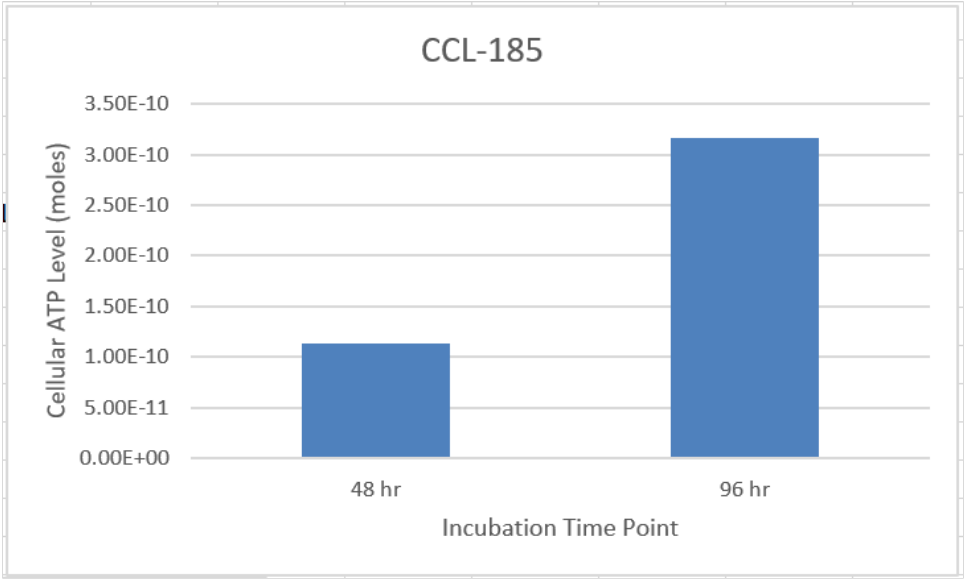


Figure 11*: Cell Growth Pattern of CCL-185 Lung Cell Line*

The CCL-185 lung tissue cell line's growth is characterized in the above graph at the time points used in this study. For both the 48 hour and 96 hour time points, three wells of 12 well plate were seeded, without any exposure to alkaloids. Cells were analyzed by the Cell-Titer Glo® Luminescent Viability Assay and the means were obtained for each 96 well plate analyzed. The above figure displays the growth rate of the CCL-185 cell line given by moles of ATP.

This cell line was exposed to e-cigarette tobacco alkaloids: nicotine, cotinine, myosmine, and anabasine, singularly and in combination in 1 µg/mL and 10 µg/mL concentrations, over a

time period of 96 hours. The effects of cellular proliferation were then evaluated by use of Promega CellTiter Glo® Luminescent Viability Assay at time points, 48 hours and 96 hours, over the course of the story. An equation of percent difference to the control was applied to the resultant ATP level data from the proliferation assays to normalize the data to the control. Results obtained with a percent difference of 15% or greater and a p-value of ≤ 0.05, obtained from the two-tailed student t-test, were considered the most significant findings and are included in this section and marked by an asterisk in the figures. In this section, the results of the single exposures are compared to a study done by Christine Hale, who began the overarching project to study the effects of e-cigarette alkaloids on lung cells in 2016. This comparison of the results is to validate the findings of the experiment, as her projects included performing multiple rounds of single exposures over a ten-day time course.

Single Exposures of Alkaloids

Overall, the effect of alkaloid exposure on lung cell proliferation differed between alkaloids. Nicotine showed a general stimulatory proliferation response. Cotinine showed initial inhibition followed by some stimulation. Myosmine showed an inhibitory response at 48 hours followed by a larger jump in proliferation stimulation at 96 hours. Lastly, anabasine showed an increasingly stimulatory pattern for both concentrations of exposures (Figure 12).

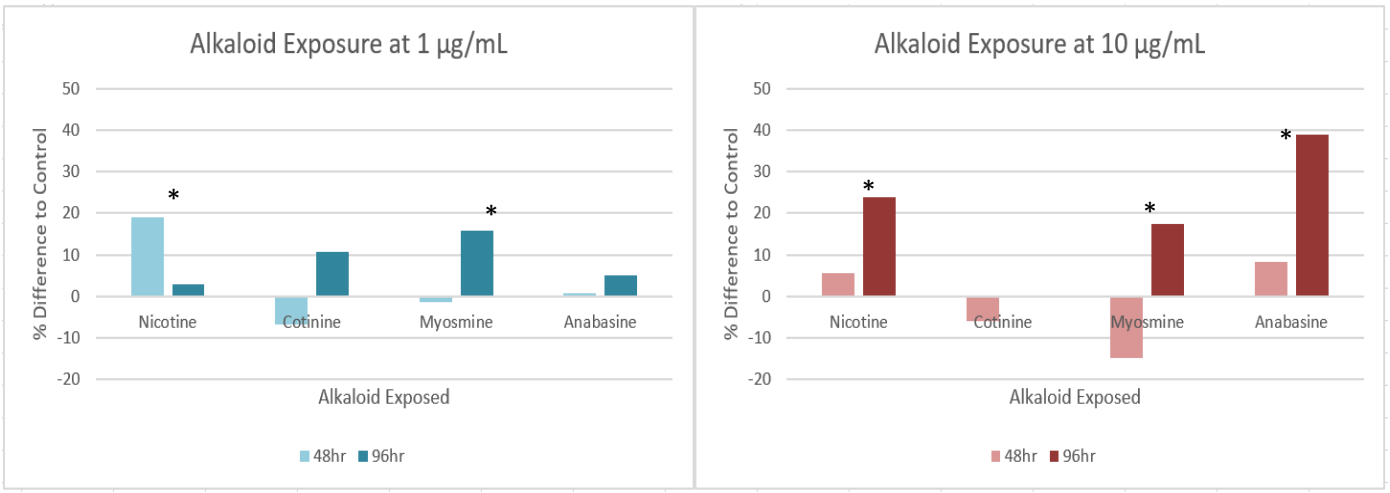
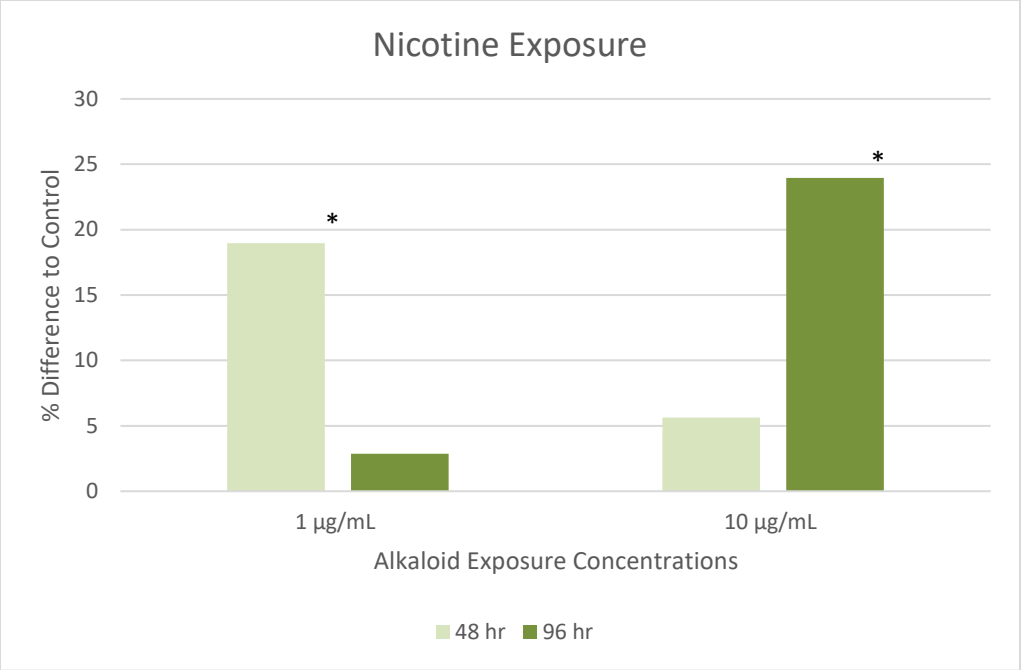


Figure 12*: Cell Growth of CCL-185 under Single Alkaloid Exposures, Normalized to Control*

Data obtained every 48 hours under exposure to e-cigarette alkaloids singularly at 1 µg/mL and 10 µg/mL concentrations by the CellTiter Glo ® Luminescent Viability Assay. Normalized to control by applying equation of percent difference for each experimental condition. Most significant values within the study are marked by an asterisk (*).

Nicotine

The effect of nicotine exposure on cell proliferation showed a non-specific trend within the CCL-185 cell line. For both concentrations, it caused an increase in cell proliferation in comparison to the control, but at 1 µg/mL, the percent difference decreased from time point 1 to 2, and at 10 µg/mL, the percent difference increased. Ultimately, a stimulatory response was seen for both concentrations. Significant stimulatory response for nicotine exposure at 1 µg/mL was observed at 48 hours at 18.97% difference from the control (3.64E-7 p-value) (Figure 13). This was followed by a still stimulatory response, but a decrease in percent difference for the 96 hour time point. For the 10 µg/mL concentration, the second time point was significant in its percent difference at 23.95% (0.002 p-value) increase in proliferation from control (Figure 13).



	48 hr 1 µg/mL	96 hr 1 µg/mL	48 hr 10 µg/mL	96 hr 10 µg/mL
% Difference from Control	18.97	5.64	2.86	23.95
P-value	3.64E-7	0.959	0.267	0.002

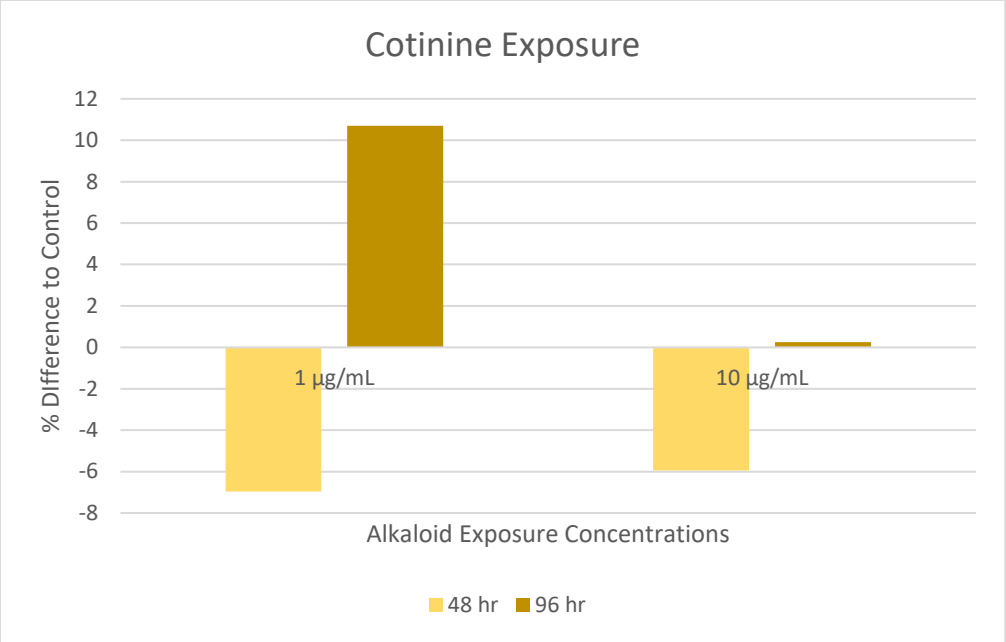
Figure 13*: Cell Growth of CCL-185 with Exposure to Nicotine, Normalized to Control*

Data obtained every 48 hours under exposure to e-cigarette alkaloids under the exposure of nicotine at 1 µg/mL and 10 µg/mL concentrations by the CellTiter Glo ® Luminescent Viability Assay. Normalized to control by applying equation of percent difference for each experimental condition. Most significant values within the study are marked by an asterisk (*). The table included in the figure shows the percent difference from control each value was and the p-value obtained by the student t-test.

These findings are consistent with results from a previous study done by Christine Hale, in that the 10 µg/mL concentration showed an overall stimulatory effect on cell growth. The percent difference from control decreased from 48 hours of exposure to 96 hours as well (Hale, 2017). However, her studies showed a much larger percent difference at both time points.

Cotinine

In contrast to the stimulatory response shown by nicotine, cotinine exposure caused an inhibitory response at 48 hours for both concentrations. However, by 96 hours this inhibitory effect was lost. In fact, at the lower concentration of cotinine there appeared to be a transition to a stimulatory effect (although not statistically significant). Likewise, at the higher concentration, the inhibitory effect seen at 48 hours was inapparent at 96 hours and cell proliferation returned to untreated control levels. This observation may imply that in culture, cotinine has a short-term, transient effect that could be due to innate cellular recovery responses or chemical instability. In summary, none of the cotinine exposures resulted in a significant percent difference within the bounds of this study, but the biggest difference was at 1 µg/mL at 96 hours, when there was an increase in cellular proliferation by 10.7% (0.113 p-value) (Figure 14).



	48 hr 1 µg/mL	96 hr 1 µg/mL	48 hr 10 µg/mL	96 hr 10 µg/mL
% Difference from Control	-6.96	10.7	-5.95	0.26
P-value	0.0007	0.113	0.0003	0.701

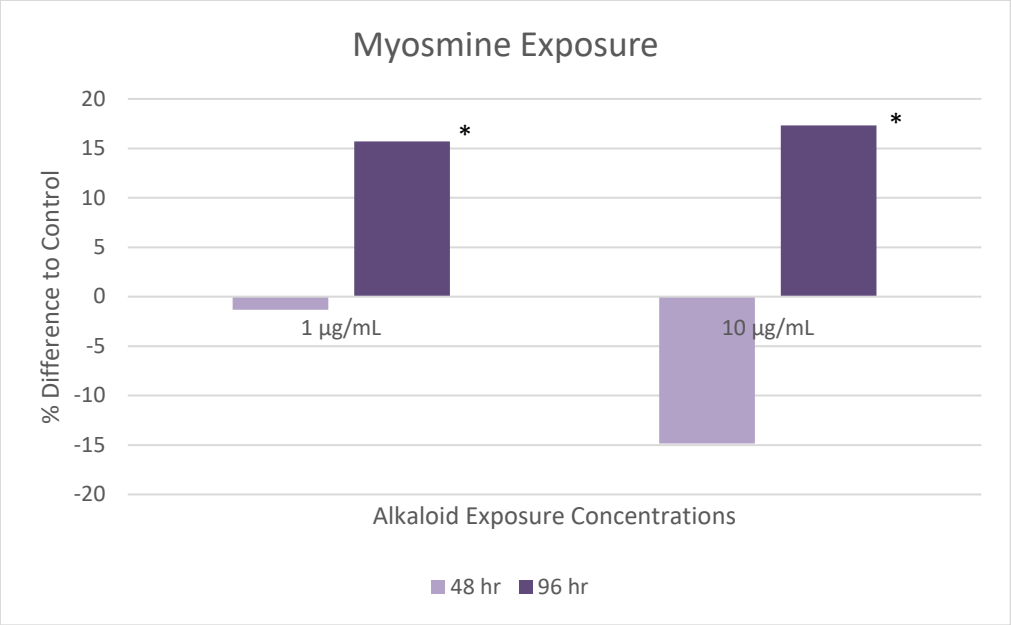
Figure 14*: Cell Growth of CCL-185 with Exposure to Cotinine, Normalized to Control*

Data obtained every 48 hours under exposure to e-cigarette alkaloids under the exposure of cotinine at 1 µg/mL and 10 µg/mL concentrations by the CellTiter Glo ® Luminescent Viability Assay. Normalized to control by applying equation of percent difference for each experimental condition. The table included in the figure shows the percent difference from control each value was and the p-value obtained by the student t-test.

In the study completed by Hale, at 10 µg/mL, cotinine showed only an inhibitory effect on cellular proliferation, at 48 hours, it was nonsignificant, but at the 96 hour time point, the decrease in cell growth was statistically significant (Hale, 2017). However, at 96 hours in this study, the cell proliferation change was non-significant with a p-value of 0.701 (Figure 14). This difference could have been due to differences in cell density or experimental error within this study.

Myosmine

Similar to cotinine, myosmine exposure began with an initial inhibitory cellular proliferation response followed by a stimulatory one. However, myosmine exposure caused a more significant response in proliferation. Both concentrations followed a similar trend with a reduced cell growth relative to control at 48 hours and followed by increase in cellular proliferation at 96 hours. The most significant percent differences were both seen on day 4 (96 hours) when at 1 µg/mL, myosmine had a 15.7% increase in growth (0.025 p-value) and at 10 µg/mL, a 17.33% increase was seen (0.0309 p-value). Day 2 for the 10 µg/mL concentration showed a -14.86% decrease (1.99E-6 p-value) in cell proliferation compared to the control (Figure 15).



	48 hr 1 µg/mL	96 hr 1 µg/mL	48 hr 10 µg/mL	96 hr 10 µg/mL
% Difference from Control	-1.31	15.7	-14.86	17.33
P-value	0.659	0.025	1.99E-6	0.0309

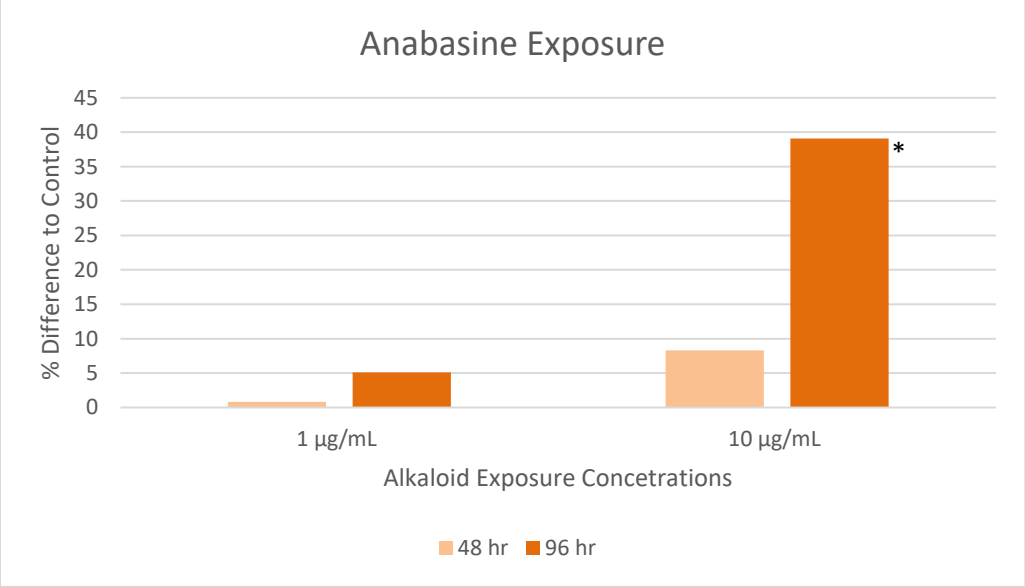
Figure 15*: Cell Growth of CCL-185 with Exposure to Myosmine, Normalized to Control*

Data obtained every 48 hours under exposure to e-cigarette alkaloids under the exposure of myosmine at 1 µg/mL and 10 µg/mL concentrations by the CellTiter Glo ® Luminescent Viability Assay. Normalized to control by applying equation of percent difference for each experimental condition. Most significant values within the study are marked by an asterisk (*). The table included in the figure shows the percent difference from control each value was and the p-value obtained by the student t-test.

These results differed greatly to those found in Hale's study. Myosmine at 10 µg/mL showed an extreme stimulatory effect on cellular proliferation for the cancer cell line. At day 1, an increase of 138% was found (Hale, 2017). However, these levels returned to much lower levels later in the study. Myosmine at higher concentrations was also found to induce complete cytotoxicity in the study (Hale, 2017).

Anabasine

Anabasine exposure, like nicotine, resulted in a stimulatory response of cellular proliferation. Both at 1 µg/mL and 10 µg/mL, there was an increase in percent difference of cell growth from 48 hours to 96 hours of exposure. The largest percent difference from all single exposures was seen in anabasine at 10 µg/mL at hour 96 with a value of 39.07% (0.0009 p-value) (Figure 16).



	48 hr 1 µg/mL	96 hr 1 µg/mL	48 hr 10 µg/mL	96 hr 10 µg/mL
% Difference from Control	0.84	5.09	8.32	39.07
P-value	0.781	0.613	2.73E-5	0.0009

Figure 16*: Cell Growth of CCL-185 with Exposure to Anabasine, Normalized to Control*

Data obtained every 48 hours under exposure to e-cigarette alkaloids under the exposure of anabasine at 1 µg/mL and 10 µg/mL concentrations by the CellTiter Glo ® Luminescent Viability Assay. Normalized to control by applying equation of percent difference for each experimental condition. Most significant values within the study are marked by an asterisk (*). The table included in the figure shows the percent difference from control each value was and the p-value obtained by the student t-test.

Like myosmine, anabasine exposure in this study resulted in a different trend from the previous study. The previous study showed an inhibitory response caused by anabasine at the 10 µg/mL concentration. However, on day 3 of that study, an increase in cell growth was observed, so it is possible there is cell growth in the time range (Hale, 2017).

Alkaloid Exposures in Combination

Exposures of e-cigarette alkaloids in combination lead to varying effects of cellular proliferation. The effects of the double exposures: nicotine + cotinine (NC), nicotine + myosmine (NM), nicotine + anabasine (NA), cotinine + myosmine (CM), cotinine + anabasine (CA), and myosmine + anabasine (MA) all resulted in similar trends in cell growth. Each of these exposures at the 1 µg/mL and 10 µg/mL concentrations showed an inhibitory response of cell proliferation. At 10 µg/mL, each exposure resulted in an average of -36.95% difference in cell growth compared to the control at hour 48 and an average of -80.54% difference at hour 96 (Figure 17). However, the 1 µg/mL concentration exposures showed more variety in their differences to the control. NC, NM, CM, and MA, all showed an increase in their difference from control over the course of the study. NA and CA showed a decrease in their differences from control, and at 96 hours, showed no significant difference from the control. The largest difference from control at 1 µg/mL was at 96 hours of exposure of CM and showed a -26.45% difference (Figure 17). The largest difference from control for the 10 µg/mL concentration exposure was CA at -85.06% difference (Figure 17).

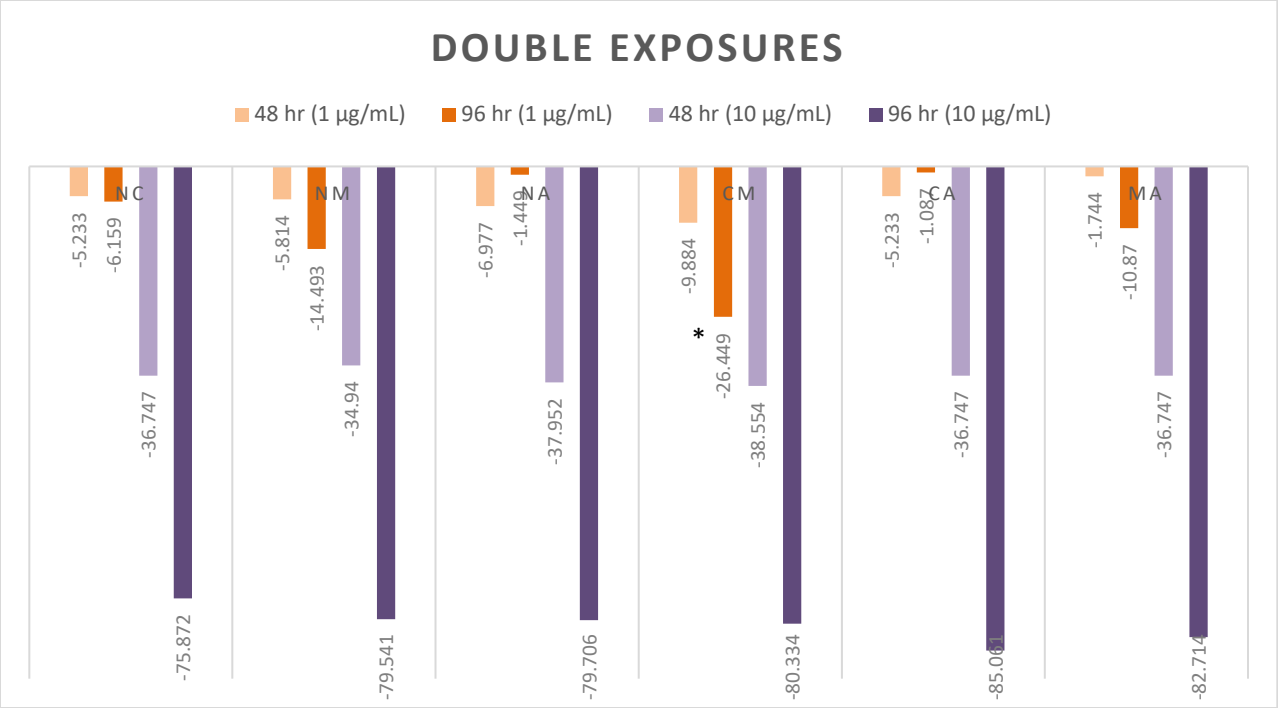


Figure 17*: Cell Growth of CCL-185 with Exposure to Double Combinations, Normalized to Control*

Data obtained every 48 hours under exposure to e-cigarette alkaloids under the exposure of double combinations (NC, NM, NA, CM, CA, and MA) at 1 µg/mL and 10 µg/mL concentrations by the CellTiter Glo ® Luminescent Viability Assay. Normalized to control by applying equation of percent difference for each experimental condition. Most significant values within the study are marked by an asterisk (*). All values for the 10 µg/mL concentration exposures are considered most significant as well.

Within the double exposures, combinations including cotinine showed the largest percent differences from control. Cotinine, when singularly exposed, showed an initial inhibitory response, that, when in the presence of other alkaloids, may be compounded and causes a larger inhibitory effect. Both combinations at 1 µg/mL that showed a decrease in percent difference from control contained anabasine (NA and CA), which had a stimulatory effect of CCL-185 cell proliferation when exposed in singularity. This could be what is causing the cells to have a closer to normal cell growth compared to the control.

Exposures of three alkaloids in combination and all the alkaloids together had much more varying results in cell proliferation changes. The combinations for these exposures were: nicotine + cotinine + myosmine (NCM), nicotine + cotinine + anabasine (NCA), nicotine + myosmine + anabasine (NMA), cotinine + myosmine + anabasine (CMA), and nicotine + cotinine + myosmine + anabasine (NCMA). In contrast to the double exposures that all caused an inhibitory response, the triple and total exposures resulted in mostly increases in cell growth. The only inhibitory effects seen were statistically nonsignificant by the two-tail student t-test.

The largest percent difference from control seen in this group of combined exposures was seen in the exposure with all four e-cigarette alkaloids. At 1 µg/mL, there was an increase in cell growth of 33.97% difference from control (Figure 18). The total combination exposure of all four alkaloids showed a trend of increased cell proliferation that decreased in percent difference from 48 hours to 96 hours of exposure. This same trend of initial increase and then smaller percent difference is seen in other experimental conditions like CMA exposures, NMA and NCM 10 µg/mL exposures, and NCA 1 µg/mL exposure (Figure 18).

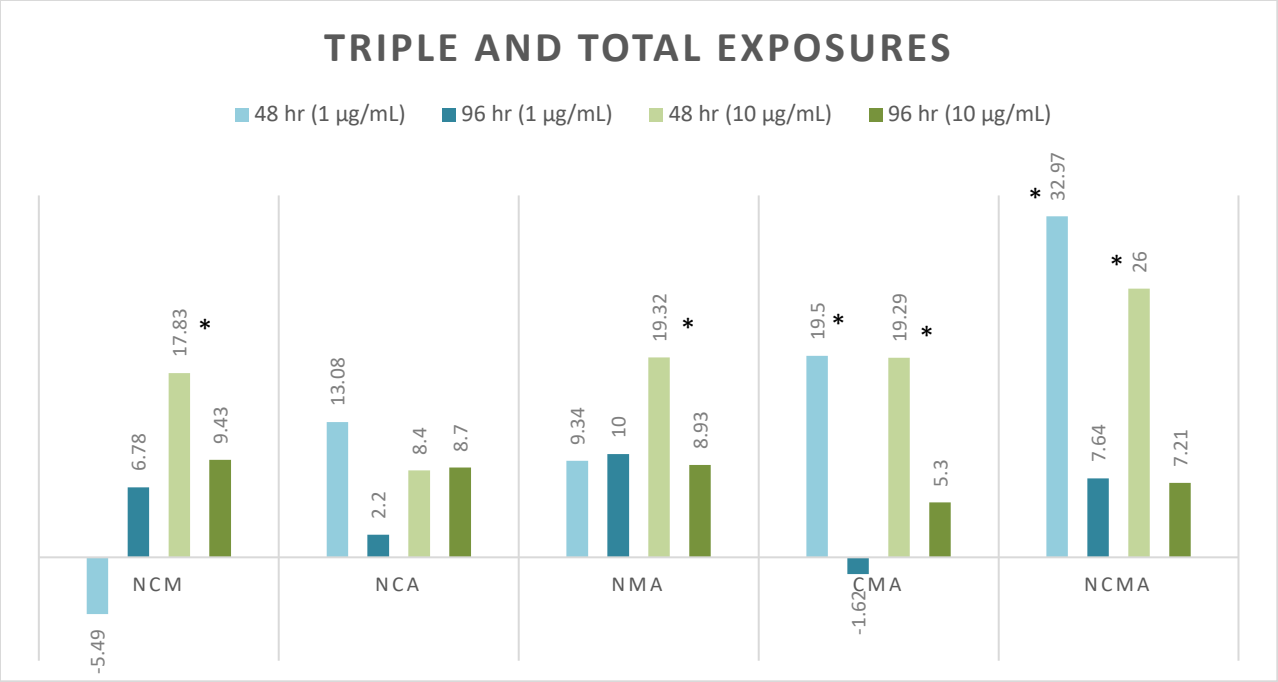


Figure 18*: Cell Growth of CCL-185 with Exposure to Triple and Total Combinations, Normalized to Control*

Data obtained every 48 hours under exposure to e-cigarette alkaloids under the exposure of triple and total combinations (NCM, NCA, NMA, CMA, and NCMA) at 1 µg/mL and 10 µg/mL concentrations by the CellTiter Glo ® Luminescent Viability Assay. Normalized to control by applying equation of percent difference for each experimental condition. Most significant values within the study are marked by an asterisk (*).

The presence of anabasine and myosmine seems to help in increasing cellular proliferation under exposure conditions, for combinations including the two saw a higher 48 hour increase in cell growth than others. Samples including cotinine had the more varied results with lower/nonsignificant changes in cellular proliferation.

DISCUSSION

The aim of this study was to examine the biological effects of tobacco alkaloids found in electronic cigarette filling solutions on the growth and viability of human lung cells in culture. It was also to evaluate potential synergistic and agonistic effects of these alkaloids in combination. These aims were to be carried out by evaluating the differences in cell proliferation of the CCL-185 lung cell line when exposed to different combinations of the e-cigarette alkaloids: nicotine, cotinine, myosmine, and anabasine. We hypothesized, based on prior research indicating tobacco alkaloids contributions to human diseased states such as lung disease and cancer (Hecht, 1988, Hale, 2017), that exposure of lung cell cultures to tobacco alkaloids is associated with abnormal cellular proliferation. These changes in cell growth could offer understanding of potential factors for lung disease associated with exposure to e-cigarette compounds.

Stable Auto-bioluminescent CCL-185 Cell Line

The establishment of the stable auto-bioluminescent lung cell line was unsuccessful within this study, however, there was movement towards the successful creation of this cell line. The kill curve generated for geneticin antibiotic selection allowed optimization of the concentration dose used for killing untransfected cells while allowing successful transformants to grow within a 10 day time frame. The generation of six successful transformation events shows promise in the methodology for generating the stable cell line, however, more trials should be done in order to obtain a successful transfectant that not only grows under geneticin selection, but also has an auto-bioluminescent signal output differentiable from the control. This may take

a larger number of cells undergoing the PureFection™ protocol to increase transformation efficiency.

Effect of Alkaloid Exposures on Normal Cell Proliferation

The exposures to single alkaloids was to establish a base line for the study to then understand the synergistic or agonistic effects of the alkaloids in combination. Within this study, anabasine showed the most stable trend of a stimulatory effect on cellular proliferation. Both of the 48 hour exposures of anabasine showed no statistical significance from the control, showing an increase in cell growth after the 48 hour time point. Myosmine also showed an increase in cell growth after an initial inhibitory effect at the 48 hour time point. Cotinine had initial inhibition for both concentrations. Nicotine was the most varied in its results, but ultimately had a stimulatory effect of cell proliferation.

The exposures of two alkaloids in combination had similar results across the board. This run of the assay was done separately from the single, triple, and total exposures. This could explain why the results are so different between the double exposures and the others. It is possible experimental error occurred in the process of one of the assays, most likely in the double exposures due to the immense similarity in the results. The only large differences between the different alkaloid combination exposures was at the 1 µg/mL concentration. Two of the three combinations including anabasine (NA and CA), which showed a stimulatory effect in singular exposure, showed difference from the trend. Instead of increasingly getting a larger percent difference as time continued, it actually approached a more normal amount of cell growth. Both 96 hour values for the 1 µg/mL concentration exposure of NA and CA were not

statistically significant from the control, meaning they had normal amounts of cell proliferation at 96 hours. As other samples' cell proliferation at this concentration continued to be inhibited, the interaction between anabasine, nicotine, and nicotine's metabolite, cotinine, prevented inhibition of cell growth.

The results of the triple and total exposures of e-cigarette alkaloids to lung cells in culture were much more varied than the doubles and ultimately had a different effect of cell proliferation. As the doubles caused immense amounts of inhibition, the triples and total exposure usually caused an increase in cell growth, causing a stimulatory effect of cell proliferation. Again, anabasine, which showed stimulatory effects in singular exposure, seems to have an effect on increasing cellular proliferation. The exposures including anabasine had more growth that the one that did not. Myosmine could also be causing a stimulatory effect, and when combined with anabasine, has the largest percent differences from control. Samples with cotinine in their exposure, were more varied and had less significant changes in cellular proliferation from the control. NMA was the sample containing every alkaloid but cotinine and saw general levels of increased percent difference from control. The other samples all saw a larger drop in percent difference and approached more normal levels of cellular proliferation. Cotinine, in its singular exposure, showed an initial decrease in cell proliferation and when compared to the other single alkaloid exposures, had the most inhibitory effect on the CCL-185 cell line. This inhibitory effect could be counteracting the stimulatory effects of other alkaloids within the exposure combinations.

From the data obtained from the different combinations of alkaloids, anabasine and cotinine seemed to have the largest effects on cell proliferation. Anabasine in the case of the double alkaloid exposures at the 1 µg/mL concentration seemed to counteract the inhibitory

effects the other alkaloids were having on it. In the triple exposures, anabasine along with myosmine showed the greatest differences from the control in terms of cell growth, acting in a synergistic relationship. Together, they had the largest stimulatory effect upon initial exposure. Cotinine on the other hand seems to have an inhibitory effect and agonistic relationship with the other alkaloids. It seemed to reduce the effect the others had on cell proliferation.

Potential Disease Pathology Risk Associated with Alkaloid Exposure

Anabasine showed an increase in positive percent difference from control and myosmine showed an initial inhibition of cell growth followed by stimulation of cell proliferation. The increase in cell proliferation seen in anabasine and myosmine could be suggestive that anabasine and/or myosmine could possibly support a state of carcinogenesis within lung tissue by suppressing apoptosis. This possibility is actually supported by prior research indicating both anabasine and myosmine of reacting with nitrosating agents to form carcinogenic compounds (Wilp et al., 2002). This is further expanded upon in that combinations of the two e-cigarette alkaloids together resulted in stimulation of growth (double exposures excluded). A study done by Mateva in 2019 found that myosmine actually inhibited growth of blood and liver cancer cell lines. However, their study was focusing on the consumption of myosmine through food (Mateva, 2019). The chemical processes it would go through in the body's environment would not occur between the e-cigarette aerosol and epithelial lung cells. Cell proliferation and viability studies done on these individual studies are just beginning to be published in the rush for scientific data on e-cigarette effects. Anabasine had no studies done on it individually and myosmine only included other cell lines under different exposure routes.

Conversely, cotinine displayed a similar trend as myosmine in its singular exposure with less growth afterwards. However, in its exposures with other alkaloids, it provides an inhibitory factor, counteracting the stimulatory effects. This perhaps suggests that cotinine plays a role in creating an environment of inhibited cell growth and viability. This is seen in diseased states such as COPD and other respiratory illnesses, where an increased apoptotic cellular response is hallmark (U.S. Department of Health and Human Services, 2010). However, cotinine exposure cell proliferation response was varied, and more study would need to be done to draw this conclusion. Other studies on cotinine cytotoxity found that MRC-5 cells continued to proliferate after 48 hours of cotinine exposure (Vlasceanu, 2018). MRC-5 is a normal human lung cell line, meaning it is not in a continuous diseased state and is more similar to physiological lung cell within the human body. The study also showed that cotinine, despite still growing did showcase weak proliferative potential compared to the control at a 2mM concentration (Vlasceanu, 2018). The differences seen in the results of the Vlasceanu study and this one could be due to the cell line differences and the much higher concentration of cotinine used in exposure for their study. Continuous and normal cell lines will have different cell proliferation due to a cancerous and non-cancerous state, leading to potential differences seen between studies. Nicotine showed varied results throughout the cell proliferation assays in this project, but individually showed an increase in cell growth compared to the control. It has been demonstrated that nicotine induces cell proliferation in mouse lung cancer and enhances the growth of tumors induced by tobacco carcinogens (Davis, 2009). However, at high concentrations (2mM), nicotine was found to reduce viability of the normal lung cell line in the Vlasceanu study by 42% (Vlasceanu, 2018). Nicotine exposure causes a does dependent response on lung cells as seen by this study and the others.

CONCLUSION

In conclusion, the tobacco alkaloids, nicotine, cotinine, myosmine, and anabasine, found within e-cigarette filling solutions, have been shown within this study to cause differential cell proliferation and viability among lung tissue cells. Anabasine and myosmine showed increases in cell proliferation when in triple combinations and in the presence of each other, possibly showing a risk of an increased state of carcinogenesis. Nicotine showed a general increase in cell proliferation compared to the control in a single exposure but did not have a noticeable trend in combination exposures. Cotinine acted opposite of anabasine and myosmine in the triple exposures. Combinations including cotinine resulted in a smaller percent difference from control. Ultimately, all four alkaloids resulted in changes in normal cell proliferation, but trends of their effects in combination were difficult to discriminate.

LIMITATIONS AND FUTURE AIMS

There were many limitations in this study due to time and funding. There was not enough CellTiter Glo® Luminescent Viability Assay reagent to do multiple runs of the assay in order to test for reproducibility or account for error. Funding was used up early in trying to establish the stable CCL-185 pCMVlux cell line. The CellTiter Glo® assay was started later in the project when stable colonies were not showing differential luminescent output. Moving forward, additional runs of the assay should be done for all combinations again to test if the results are similar. Not being able to do the experiment again means we do no know if the results are true or

by mistake in practice. Additionally, more time points for analysis would allow for a trend to be found easier. Ideally, the best exposure level would be one that is physiologically relevant and reflects exposure encountered from normal e-cigarette inhalation. A major limitation to the experimental design of this study is the mechanism and exposure route of tobacco alkaloid administration in a liquid tissue culture system. Future studies should investigate an exposure system that mimics *in vivo* exposure to the e-cigarette filling solution, once it becomes an aerosol that is inhaled by the e-cigarette user. Future studies could also investigate establishing a way to structure the cells into a more lifelike form. Despite the ideal of *in vivo* studies, this study still maintains relevance within the field. The exposure of these alkaloids to lung epithelial cells is as physiologically relevant this *in vitro* study can get. In normal e-cigarette inhalation, these alkaloids do get to the epithelial cells of a human lung without any interaction in other parts of the body. This means, of all cell types to study in e-cigarette alkaloid exposure, this is the best for real life application. This current system of study is a valid way of evaluating the effects of e-cigarette alkaloids on lung cell proliferation despite the other directions the study could take to make it more *in vivo*.

Another future direction for this research could be the analysis of changes in gene expression when the cells are exposure to e-cigarette alkaloids in combination. A previous study (Hale, 2017), studied the effect of gene expression of disease biomarkers of the e-cigarette alkaloids in singular exposure. Further research should be done studying the synergistic and agonistic effects they have of gene expression. Lastly, the auto-bioluminescent CCL-185 stable cell line could still be pursued. It would allow easy and efficient analysis over multiple time points and not be reliant on ATP levels as a study for cellular proliferation. Other transfection

methods could be explored to successfully integrate the plasmid into the genome of the lung cells.

RESOURCES

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