The effects of e-cigarette exposure on cell viability and gene expression

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The Effects of E-Cigarette Exposure on Cell Viability and Gene Expression

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Departmental Honors Thesis
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ABSTRACT

Marketed as a safer alternative to tobacco products, the popularity of e-cigarette use is on the rise. However, little is known about the potential health risks associated with their use. Many e-cigarette filling solutions are known to contain significant levels of tobacco alkaloids, including nicotine, anabasine, myosmine and cotinine. Using a panel of lung cell cultures distinguished by differences in sex and disease status, this study addresses the in vitro effects of common tobacco alkaloids found in e-cigarettes on cell proliferation and gene expression. We hypothesize that alkaloid exposure of lung cells is associated with abnormal proliferation and gene expression, and predict that cellular response to the alkaloids will present in a sex-specific manner. Alkaloid exposure on each lung cell line was evaluated at 1 µg/mL, 10 µg/mL, and 100 µg/mL concentrations throughout a 10-day time course. Cellular proliferation was measured daily using the CellTiter-Glo® Luminescent Viability Assay, and RNA isolated after 48 and 96-hour time points for gene expression analysis of 10 cancer biomarkers by qRT-PCR. Findings indicate anabasine and myosmine display a significant (p<0.05) inhibitory effect on cellular proliferation and gene expression, whereas the effect of cotinine and nicotine was minimal. Each cell line had an inherent, characteristic pattern of cellular proliferation in response to alkaloid exposure. Notably, the cancer cell line demonstrated more variability among replicates, which we attribute to the non-clonal nature of this cell line, and the female cell line displayed increased susceptibility to toxicity by the higher alkaloid concentrations. Significant differences in gene expression (p<0.05) were noted for AHR, CEACAM6, CYP1A1, MDM2, TP53, ALDH3A1 and GPX2. Correlation of abnormal patterns of cell proliferation and differential gene expression with risk for disease are discussed.
AKNOWLEDGEMENTS

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INTRODUCTION

Background

Marketed as a smarter alternative to smoking tobacco cigarettes, by 2014, the popularity of electronic cigarettes (e-cigarettes) had resulted in a 2.5 billion dollar business within the United States (Surgeon General, 2016). Though they continue to increase in popularity today, little research has been provided to validate a common assumption that e-cigarettes are a safer alternative to traditional cigarettes. This notion, regarding the perceived safety of e-cigarettes, has been examined in multiple studies including a report published in 2015 in the Journal of Behavioral Medicine. Findings from this study indicated that more people than not, believed the use of e-cigarettes resulted in fewer health effects in comparison to traditional cigarettes (Pepper, Emery, Ribisl, Rini, & Brewer, 2015). It was also found that among participants of this study, women felt more this way than men (Pepper et al., 2015).

Worse yet, two different reports found that the trend of smoking e-cigarettes extends to the youth as well, not just adults (Arrazola et al., 2015; Surgeon General, 2016). More than likely appealed to with flavorings such as bubble-gum and candy apple, in the United States alone it is estimated that in 2014 there were 2.4 million e-cigarette users that were youth and young adults (Arrazola et al., 2015). Accordingly, in 2016 the U.S. Surgeon General responded to this rise in e-cigarette use among youth and young adults with a report detailing the increasing evidence that suggests that adolescents and young adults should be warned and advised against the use of e-cigarettes due to the risk of exposure to several chemicals that could have adverse health effects on youth and young adults (Surgeon General, 2016). These chemicals included nicotine, which has been found to
cause addiction and harm to the developing brain as well as acute toxicity and/or death if larger quantities of e-cigarette filling solution containing nicotine are ingested (Surgeon General, 2016).

With so much confusion among consumers as to the impact and potential harm that e-cigarette use can have on the human body, a thorough scientific analysis of every possible health related concern should be investigated. Only with thorough scientific investigation of e-cigarette use can consumers have the tools to base a more meaningful decision that may greatly impact their present and future health. The purpose of this study is to contribute to the scientific investigation of e-cigarette use by studying tobacco alkaloids that have been found to be within e-cigarette filling solutions and their effect on cell proliferation and gene expression.

**Electronic Cigarette Design**

To better understand the effects of e-cigarette use, it is important to understand the workings of the delivery system, as well as the compounds within the filling solutions that are delivered through the e-cigarette device. Historical background into the e-cigarette and its delivery system, includes the introduction of the device to the United States in 2007, although the modern version of the electronic cigarette that is most recognized today was first developed and patented by Hon Lik, a Chinese Pharmacist, in 2003 (Grana, Benowitz, & Glantz, 2013). Simultaneously called an electronic nicotine delivery system (ENDS), the device consists of a battery, vaporizer, cartridge containing filling solution with or without nicotine, LED light at the end, heating coil, atomizer, and a mouthpiece (Figure 1).
By drawing in air through the mouthpiece, the battery of the electronic device is activated by varying methods, and which in turn initiates the atomizer, that ultimately produces aerosol from the filling solution composed of propylene glycol and/or glycerol, nicotine, additives and/or added flavorings (Cheng, 2014; Grana et al., 2013; Surgeon General, 2016). This aerosol, usually incorrectly perceived as vapor, exits through the mouthpiece and into the mouth and lungs of the user (Cheng, 2014). Since the introduction of e-cigarettes into the United States, many varying designs, makes, and models have arrived on the market, yet all still similarly produce an aerosol usually composed of nicotine, flavoring, and additives into the user’s mouth and lungs (Surgeon General, 2016).

**Tobacco Alkaloids Commonly Found in E-cigarette Filling Solutions**

There are a variety of different nicotine and non-nicotine filling solutions, and at this time, the manufacturers do not usually disclose the complete chemistry involved in e-cigarette filling solutions. As more studies show that toxic and carcinogenic compounds are present within these filler solutions, further research is warranted into how these unspecified compounds found in e-cigarettes are affecting the human body (Cheng, 2014). Besides chromatographic analysis detecting fluctuating levels of nicotine in products advertised as nicotine-free, filler solutions have also been shown to contain the chemical compounds cotinine, anabasine, and myosmine (Trehy et al., 2011). Research has shown
these common tobacco alkaloid compounds to have synergistic effects on nicotine in regards to addiction (Clemens, Caillé, Stinus, & Cador, 2009), however, data on the effects within areas of the human body other than addiction is limited, and warrants more research.

**Nicotine**

Existing as an oily liquid that is colorless to pale yellow and quickly turns brown when exposed to light or air, nicotine is a compound that can be isolated from the dried leaves of the *Nicotiana tabacum* and *N. rustica* tobacco plants (O'Neil, 2006). Within these plants it can make up between 2-8% of the plant material and combines with both malic and citric acid (O'Neil, 2006). In tobacco plants, nicotine serves as a natural defense system, effectively as an insecticide. It is known to be the most abundant alkaloid within tobacco (O'Neil, 2006). In regards to humans, the alkaloid tends to be the most addictive of the tobacco alkaloids when used recreationally (General, 1988). The addictive nature of nicotine lies in its ability to readily diffuse into brain tissue upon ingestion and entering into the bloodstream (Benowitz, 2008). Once nicotine has diffused into the brain, the compound binds to nicotinic cholinergic receptors (nAChRs), described as ligand-gated ion channels, ultimately triggering the release of neurotransmitters such as dopamine, norepinephrine, acetylcholine, serotonin, endorphins, and others that help to facilitate its physically addicting nature (Benowitz, 2008).
In regards to smoking tobacco cigarettes, nicotine is thought to be absorbed by the user at around 0.5-2 mg per cigarette on average, depending on how frequently the user inhales (puffs) on a cigarette (Benowitz, 1983; Benowitz & Jacob, 1984), though tobacco cigarettes themselves are comprised of anywhere from 10-15 mg of nicotine per cigarette on average (Kozlowski et al., 1998; Malson, Sims, Murty, & Pickworth, 2001). Within e-cigarette filling solution there is much more variance concerning the concentration of nicotine (Trehy et al., 2011). The concentration of nicotine within e-cigarette filling solution cartridges has been seen to be as low as 0 mg/cartridge and as high as 21.82 mg/cartridge, and varying levels of nicotine have even been detected among filling solutions that has been advertised as nicotine-free (Trehy et al., 2011). The amount of nicotine absorbed by the user has been determined to be concentration dependent, relying greatly on the amount of inhalations (puffs) the user takes from the device and in what time frame, since greater amounts of nicotine are seen to be absorbed with greater amounts of heat created within the e-cigarette device and these greater amounts of heat are ultimately caused by a greater amount of puffs by the user, in shorter amounts of time (Grana et al., 2013; Trehy et al., 2011).
Once inhaled, nicotine can be absorbed through not only the mucosal lining of the mouth, but also substantially in the alveoli of the lungs and the bronchial tree (Benowitz, 1983). A contributing factor to varying nicotine absorption in the mucosal lining is the pH of the smoke inhaled (Benowitz, 1983). Nicotine is a weak base with a pH of 8.0, and in its ionized state it does not readily cross membranes, such as when nicotine is within flue-cured tobacco smoke, which has an acidic pH (Benowitz, Hukkanen, & Jacob, 2009; Gori, Benowitz, & Lynch, 1986). Cigars, however, tend to have a more alkaline smoke that is inhaled by a user than that of the smoke produced by cigarettes, and because of this, more nicotine is absorbed in the user’s mucosal lining by cigars (A. Armitage & Turner, 1970).

Nicotine is also found to be readily absorbed through the skin, most notably causing an illness called, green tobacco sickness (GTS), which has been studied among individuals who handle tobacco leaves, such as tobacco workers whom are involved in the cutting, harvesting, or loading of the plants (McBride, Altman, Klein, & White, 1998). Once nicotine has been absorbed by the body, it spreads to all the bodily tissues fairly quickly, becoming metabolized primarily in the liver, and a small amount excreted by the kidneys (Benowitz, 1983). Nicotine also has an affinity for brain tissue, seen to be greater in regular tobacco smokers than non-smokers, and is known to cause both a stimulatory effect on neuronal nicotinic receptors as well as the possibility of receptor desensitization (Balfour, 1994; Benwell, Balfour, & Anderson, 1988; Breese et al., 1997; Hukkanen, Jacob, & Benowitz, 2005).

Perhaps most alarming to the health of the user of nicotine containing products, is that nicotine is also a tertiary amine, and tertiary amines are known to form stable nitrosamines with nitrosating agents, and that nicotine in particular has been implicated in
the formation of tobacco-specific nitrosamines (TSNAs) within the body (Hecht & Hoffmann, 1988; Knezevich, Muzic, Hatsukami, Hecht, & Stepanov, 2013) (Figure 3). TSNAs are regarded as a highly carcinogenic group related to tobacco and tobacco smoke (Hecht & Hoffmann, 1988; Knezevich et al., 2013). The nicotine induced TSNAs are specifically, NNK (4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone) and NNN (N’-nitrosonornicotine), both of which can contribute to tumors. NNK, in particular, demonstrates an organ localization specificity for the lung (Hecht & Hoffmann, 1988).

Once activated, the nitrosamines, NNK and NNN, also have been seen to lead to a reactive intermediate that binds to and chemically modifies DNA and proteins (Wilp, Zwickenpflug, & Richter, 2002). It has been suggested that these modifications are contributing factors to carcinogenesis (Hecht & Hoffmann, 1988).

![Figure 3: Chemical Structure of a Nitrosamine](image)

In regards to how long nicotine remains in the body, nicotine levels detected in the blood after smoking one tobacco cigarette tend to have a half-life of only 5-10 minutes, with levels varying per user due to differences among individuals in metabolism rate, smoking style, and specific nicotine product (Benowitz, 1983; Benowitz, Jacob, Jones, & Rosenberg, 1982). However, a nicotine half-life of around two hours can be detected in the
blood of individuals who are absorbing nicotine over longer periods of time or after the administration of multiple doses (Benowitz, 1983). Studies carried out to determine the arterial concentration levels of nicotine in smokers has found levels ranging in individuals from 20-60 ng/mL, and as high as 100 ng/mL (A. K. Armitage et al., 1975; Gori & Lynch, 1985; Henningfield, Stapleton, Benowitz, Grayson, & London, 1993; Hukkanen et al., 2005). Theses previous studies indicating different concentration levels at different time points among tobacco smokers, coupled with the risk of carcinogenic TSNAs being derived from nicotine, warrants further research in investigating varying ranges of nicotine exposure due to e-cigarette filling solution and possible adverse health side-effects among its users.

**Cotinine**

Described physically as a viscous oil, cotinine is a tobacco alkaloid that is a result of nicotine metabolism (Benowitz, 1983; O'Neil, 2006). It is thought that as much as 86% of nicotine is metabolized into cotinine, and that detection of cotinine can be utilized as an indicator of exposure to cigarette smoke (Benowitz, 1983; Bernert et al., 1997; Hukkanen et al., 2005). Cotinine levels are seen at their highest within the liver, and cotinine itself metabolizes even further into trans-3’-hydroxycotinine (Benowitz & Hatsukami, 1998). Cotinine has shown less variability in the time in which it is metabolized in individuals than has been seen with nicotine, and it has also been shown to have a much longer half-
life, varying from 10.9 to 37.0 hours in one study (Benowitz, 1983). This longer half-life also makes cotinine a more stable indicator in determining blood concentrations levels throughout the day of a smoker than nicotine does, and the concentration levels of cotinine in the blood of smokers tends to be much higher than nicotine (Benowitz, 1983; Hukkanen et al., 2005).

![Figure 4: Chemical Structure of Cotinine](image)

Cotinine blood concentration levels in smokers average about 250 to 300 ng/mL, and can get up to 900 ng/mL, which is much higher than the concentration levels of nicotine seen in smokers (N. L. Benowitz et al., 1983). Even with these higher concentration levels seen within the blood of individuals, research carried out to discover any detrimental effect of the alkaloid has found that cotinine does not seem to cause cardiovascular effects on individuals, nor does it seem to have any cognitive effects such as an effect on performance or noticeable withdrawal symptoms (Benowitz, Kuyt, Jacob, Jones, & Osman, 1983; Hatsukami, Grillo, Pentel, Oncken, & Bliss, 1997; Zevin, Jacob Iii, Geppetti, & Benowitz, 2000). By studying this alkaloid at different concentrations levels in regards to cell exposure; evidence promoting or contradicting previous research to its relatively mild effects will prove to be beneficial in evaluating the effect this alkaloid has within e-cigarette filling solution, on the e-cigarette user.
Myosmine is a naturally occurring minor alkaloid within tobacco plants, and though it is structurally like nicotine, it is not produced through the metabolism of nicotine such as with cotinine (Fejér-Kossey, 1972; Simeonova, Vitcheva, Gorneva, & Mitcheva, 2012). However, myosmine is similar to nicotine in that, along with studies showing that nicotine, under conditions of nitrosation, gives rise to NNK (4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone) and NNN (N’-nitrosonornicotine) nitrosamines, myosmine has also been shown upon nitrosation to give rise to the NNN nitrosamine, a known esophageal carcinogen, as well as another nitrosamine, HPB (4-hydroxy-1-(3-pyridyl)-1-butanone) (Hecht & Hoffmann, 1988; Wilp et al., 2002). HPB is different from NNN, in that it is the product of the formation of DNA adducts (sections of DNA covalently bound to a carcinogen(s)), and can even be used as an indicator of exposure to TSNAs from tobacco (Wilp et al., 2002).

In some studies, myosmine was seen to be readily nitrosated in the stomach and lower esophagus under the conditions of GERD/GORD (gastro-esophageal reflux disease (Vogt, Fuchs, & Richter, 2006; Wilp et al., 2002), posing another possible risk factor for smokers who also have been diagnosed with GERD/GORD and the possibility of carcinogenic nitrosamines forming more readily. Along with its ability to readily undergo nitrosation, myosmine has also been shown to have genotoxic effects on human

![Figure 5: Chemical Structure of Myosmine](image)

Aside from the concern of this study, myosmine also naturally occurs among other organisms besides the tobacco plant, and research has found that human exposure can occur with ingestion of not only tobacco products, but also foods such as peanuts, hazelnuts, rice, milk, carrots, and many other items (Tyroller, Zwickenpflug, & Richter, 2002; Zwickenpflug, Meger, & Richter, 1998). As a result, foods such as peanuts and hazelnuts have also been found to be a possible risk to developing human cancer due to the nitrosation of the myosmine found within them (Wilp et al., 2002).

Regarding detectable concentration levels of the alkaloid within tobacco cigarettes, in a plain, unblended tobacco cigarette, myosmine concentration levels have been analyzed within studies to be between 13-33µg/cigarette in mainstream smoke and 73-224µg/cigarette in side stream smoke (Sakuma, Kusama, Yamaguchi, Matsuki, & Sugawara, 1984). Myosmine has also been found to be in detectable levels within toenails, saliva, and plasma (Schütte-Borkovec, Heppel, Heling, & Richter, 2009), but in a study involving IV
administration of myosmine to Long-Evans rats, myosmine detection in plasma found it to have a short half-life of around an hour (Glas et al., 2007; Schutte-Borkovec, Heppel, Heling, & Richter, 2009). The same study involving the IV administration of myosmine in Long-Evans rats, also found seemingly high levels of myosmine within the esophagus and accessory genital gland (Glas et al., 2007). Due to this detection of myosmine in various tissue throughout the body, its indication of nitrosation into carcinogenic nitrosamines, and its genotoxic and mutagenic effects on human lymphocytes, more research is warranted into how this alkaloid can affect lung tissue cells of a user when concentrated within e-cigarette filling solution.

**Anabasine**

Anabasine is another common tobacco alkaloid that, like myosmine, is not the result of the metabolism of nicotine, but yet is found within the stems and leaves of the tobacco plant itself (Saitoh, Noma, & Kawashima, 1985). Commonly described as a piperidine botanical insecticide that exists as a colorless liquid and becomes dark upon air exposure, anabasine makes up ~0.3% of all of the alkaloids found within the tobacco plant and although it is considered to be a minor tobacco alkaloid, it can still be used to determine whether or not someone has had exposure to tobacco or tobacco products (Armstrong, Wang, Lee, & Liu, 1999; Saitoh et al., 1985). Interestingly, like nicotine, anabasine is also
considered a nicotinic acetylcholine receptor agonist, and though it has reportedly less naturally addictive qualities than nicotine, it has been studied as a potentially useful therapy in relieving nicotine addiction (Nasirov, Ryabchenko, Khalikova, Khazbievich, & Kashkova, 1978). Anabasine, due to being a secondary amine, also has the capability of reacting with nitrosating agents to form a more stable, possibly carcinogenic nitrosamine, similar to nicotine (Hecht & Hoffmann, 1988).

![Figure 6: Chemical Structure of Anabasine](image)

In regards to safety hazards when dealing with the compound, anabasine is easily absorbed through the skin and mucous membranes, and is potentially fatal for humans at a dose speculated to be less than 5mg/kg (Benowitz, 1983; Neal L. Benowitz et al., 1983; Gosselin, Smith, & Hodge, 1984; McBride et al., 1998; National Center for Biotechnology Information). Metabolism of the compound, when based on its half-life determined by rate of urinary excretion, has been determined to be around 16 hours (Jacob et al., 2002). Due to evidence of this long half-life in comparison to nicotine metabolism, as well as its potential to become carcinogenic through interaction with nitrosating agents, makes investigation of anabasine within e-cigarette filling solution an interesting inquiry within this study.
Candidate Genes Associated with Tobacco-Induced Disease Pathology

The potential cellular and health effects of tobacco use in individuals has been investigated by researchers throughout the years by varying methods, including, but not limited to, investigation of differential gene expression among genes with evidence as being potential cancer and disease biomarkers (U.S. Department of Health and Human Services, 2010). Genes such as ALDH3A1, which is associated with higher levels of expression when in the beginning stages of malignant transformation associated with lung cancer (Patel et al., 2008), CYP1A1, which is associated with xenobiotic (drug) metabolism (Androutsopoulos, Tsatsakis, & Spandidos, 2009), GPX2, which is associated with changes seen in oxidative stress-induced apoptosis (Yan & Chen, 2006), and SLIT1, a tumor suppressor gene (Dickinson et al., 2004), are just a few of the genes that have been utilized in previous studies throughout the years, as biomarkers, to investigate the potential health hazards of tobacco use.

The investigation of differential gene expression among known cancer and disease biomarkers concerning tobacco use also holds potential as a promising method to be utilized in studies investigating potential cellular and health effects related to tobacco alkaloids found within e-cigarette filling solution. Within this study, a panel of ten candidate genes linked to modified gene expression patterns associated with tobacco smoke exposure, as well as two reference genes, are utilized for gene expression analysis under varying experimental conditions to investigate any potential hazards that these tobacco alkaloids may pose to human health (Table 1).
The gene TP53 is a known tumor suppressor gene in humans that is located on the human chromosome 17p13.1 (National Center for Biotechnology Information), and in 2013, there were at least 12 known isoforms of its protein, p53, often referred to as the “guardian of the genome,” due to its ability to protect the cell during times of stress (Sigal & Rotter, 2000; Surget, Khoury, & Bourdon, 2014). The TP53 gene has many other homologous forms in other organisms as well, and once the p53 protein is activated, it can be used to induce various cancer halting mechanisms (Hartl, 2012; Hollstein, Sidransky, Vogelstein, & Harris, 1991; Surget et al., 2014). Certain types of cellular stress can trigger the p53 protein to be activated by posttranslational modifications, such as acetylation and phosphorylation, and these stressors can include a lack of oxygen within the cell, sensed DNA damage, or a shortage in nucleoside triphosphates (Hartl, 2012). Once the protein has been activated, it carries out the transcriptional activation and repression of other genes, in order to achieve effects within the cell such as: apoptosis (also known as the death of cells), cell cycle arrest, DNA repair, inhibition of angiogenesis and metastasis, as well as other functions known to be associated with tumor suppression (Hartl, 2012; Ko & Prives, 1996).

**Table 1: Candidate Genes for Evaluation of Tobacco Alkaloid Induced Carcinogenesis**

<table>
<thead>
<tr>
<th>Candidate Gene SYMBOL</th>
<th>Gene Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AHR</td>
<td>Oncogene</td>
</tr>
<tr>
<td>CEACAM6</td>
<td>Oncogene</td>
</tr>
<tr>
<td>PIR**</td>
<td>Oncogene</td>
</tr>
<tr>
<td>CYP1A1*</td>
<td>Oncogene</td>
</tr>
<tr>
<td>MDM2</td>
<td>Proto-Oncogene</td>
</tr>
<tr>
<td>SLIT1**</td>
<td>Tumor Suppressor</td>
</tr>
<tr>
<td>TP53*</td>
<td>Tumor Suppressor</td>
</tr>
<tr>
<td>CX3CL1**</td>
<td>Immune Response</td>
</tr>
<tr>
<td>ALDH3A1</td>
<td>Antioxidant, Cancer-related Enzyme</td>
</tr>
<tr>
<td>GPX2</td>
<td>Antioxidant, Cancer-related Enzyme</td>
</tr>
</tbody>
</table>

*Possible differences in gene expression between sexes.
** Unable to investigate expression, at this time, due to unsatisfactory binding of designed primers and/or low expression levels.
Believed to be inactivated in nearly 50% of all human cancers (Levine, Momand, & Finlay, 1991; Surget et al., 2014), TP53 plays an important role in tumorigenesis of lung epithelial tissue, and mutations of the gene have become some of the most studied concerning gene mutations involved with lung cancer (Toyooka, Tsuda, & Gazdar, 2003). While there can be germline mutations within TP53 which can directly cause Li-Fraumeni syndrome, and germline mutations can pre-dispose one to early cancer onset, it is the somatic mutations that have been studied extensively among lung cancer research, especially when lung cancer has been potentially caused by tobacco smoke, a well-known mutagen (Olivier, Hollstein, & Hainaut, 2010; Toyooka et al., 2003).

In correlation with this study, we hypothesize that TP53 expression is beneficial in determining if tobacco alkaloids in e-cigarette filling solution can induce a mutated state within the TP53 gene that can lead to lung cancer. Furthermore, we propose to examine whether gender has an effect on differential genetic expression of the TP53 gene. This question has been specifically raised due to a previous study, where it was seen that women may be more susceptible to lung cancer in the setting of TP53 mutations, due to the finding of more G:C and T:A transversions within the TP53 gene in female cigarette smokers than in female non-smokers (Toyooka et al., 2003).

MDM2

An important gene involved in the regulation of the protein produced in humans by TP53 is the MDM2 gene, which is a known proto-oncogene and is also involved in protein coding (National Center for Biotechnology Information). The MDM2 gene produces a nuclear-localized E3 ubiquitin ligase, which mediates the ubiquitination of p53 from TP53,
which leads to the p53 protein’s subsequent degradation by the proteasome (Harris & Levine, 2005; National Center for Biotecnology Information). The p53/MDM2 interaction though is one of an auto-regulatory feedback loop, meaning that p53 induces the expression of \textit{MDM2} as a positive regulator, and then the protein of \textit{MDM2} in turn becomes a negative regulator of p53, limiting its abundance within the cell when under normal cellular conditions (Harris & Levine, 2005; Hernandez-Monge, Rousset-Roman, Medina-Medina, & Olivares-Illana, 2016; Shmueli & Oren, 2007). The protein MDM2 is also the binding substrate for ATM kinase, and in times of genotoxic stress within the cell, ATM phosphorylates MDM2, exposing a site on the protein that promotes translation of \textit{TP53} mRNA (Candeias et al., 2008; Gajjar et al., 2012; Hernandez-Monge et al., 2016).

An overexpression of \textit{MDM2} has been seen in many different cancers, speculatively from its repressive abilities over p53 (Chauhan, Ramakrishnan, Kollareddy, & Martinez, 2016; National Center for Biotecnology Information). It has also been found, however, that in addition to factors facilitating overexpression and amplification of the \textit{MDM2} gene directly involving degradation of p53, there is also evidence that overexpression of \textit{MDM2} has a carcinogenic effect on the cell that is completely independent from any activity involving p53 (Jones, Hancock, Vogel, Donehower, & Bradley, 1998). Furthermore, in a study published in 2006, it was seen that genetic polymorphisms that arise within the promoter region of \textit{MDM2} have a great impact on the development of lung cancer within an individual, and that tobacco smoking was also seen to have an interactive effect with regulation of gene expression of the \textit{MDM2} promoter (Zhang et al., 2006). One particular polymorphism, MDM2-GG, was significantly
overrepresented in patients with lung cancer (26%) when compared to the control population (20%) (Zhang et al., 2006).

**SLIT1**

The human *SLIT1* gene is one of a family of three known genes that contribute to SLIT proteins within humans (Dallol et al., 2002). Within this family, *SLIT1* is primarily considered to be expressed in neuronal tissue, with *SLIT2* and *SLIT3* being expressed in greater amounts in various tissues throughout the body in comparison (Dallol et al., 2002). Though *SLIT1* expression levels within human tissues other than neuronal has been thought to be low (Dickinson et al., 2004), studies have shown it to play a role in prostate cancer, where expression levels have been shown to be elevated (Latil et al., 2003), as well studies regarding differential gene expression within lung tissue (Huang, Zheng, VanBuren, & Zhao, 2010; Spira et al., 2004). In regards to lung cancer found within both tobacco smokers and non-smokers, *SLIT1* has been shown to be downregulated among smokers with lung cancer (Huang et al., 2010; Spira et al., 2004). This downregulating effect seen within lung cancer suggests *SLIT1* to be a putative tumor suppressor within lung tissue, and possibly a good biomarker for disease within lung tissue exposed to tobacco alkaloids (Huang et al., 2010; Spira et al., 2004).

**CX3CL1**

In studying any diseased state that could occur within the body due to exposure of e-cigarettes and their filling solution, it would be beneficial to not only study the expression of genes linked to cancer, but also genes known for their role in immune system response,
making them key indicators of any resultant inflammation caused by/in response to the potential hazardous substance being studied. *CX3CL1* is one such gene, and encodes for a cytokine protein that is also known by the names: neurotactin or fractalkine (FKN) (Imaizumi, Yoshida, & Satoh, 2004). The importance of the CX3CL1 protein is that it functions as a transmembrane protein that can be expressed/found on stressed/inflamed endothelium, dendritic cells, neurons, epithelial cells, and plays a role in adhering and guiding leukocytes with its important chemoattractant signaling as well as its adhesion properties (Ancuta et al., 2003; Harrison et al., 1998; Imai et al., 1997; Lucas et al., 2001; Papadopoulos et al., 1999).

CX3CL1 and its stimulating effects/cooperativeness with immune system response, in regards to apoptotic cells, has made it useful as an inflammatory marker for diseased states such as COPD (chronic obstructive pulmonary disease)(Ning et al., 2004). Expression of CX3CL1 is upregulated in cigarette-smoke induced emphysema, as well as in response to prolonged tobacco smoke exposure (McComb et al., 2008). When studied with its ligand receptor, CX3CR1, which has also been seen to be upregulated in expression, in response to prolonged tobacco smoke exposure, a CX3CL1-CX3CR1 pathway is thought to possibly be a front-runner for recruiting the immune system within lung tissue when damage has occurred due to cigarette smoke (McComb et al., 2008). We hypothesize that the study of *CX3CL1* expression could also potentially prove it to possibly be a beneficial marker for lung tissue damage in the setting of e-cigarette exposure as well, and could shed light into any possibly increased immune response due to tobacco alkaloid exposure.
**GPX2**

One of the ways a diseased state in the lung can be identified is by observing an increase in the protective enzymes being translated by host machinery to help protect the cell from damage. One such protective enzyme is a protein produced by the *GPX2* gene (Brigelius-Flohe & Kipp, 2012; Singh et al., 2006). The GPX2 protein is part of a family of glutathione peroxidases, which are antioxidant enzymes that reduce hydrogen peroxide and organic hydroperoxides by way of glutathione, and by doing so, help to protect the cell from acquired oxidative stress (National Center for Biotechnology Information; Singh et al., 2006). In studies carried out concerning airway gene expression differentiation when exposed to tobacco smoke in cigarettes, *GPX2* showed an irreversible differentiation in expression (Banerjee et al.; Beane et al., 2007). In another study, *GPX2* was seen to be consistently upregulated in current and former smokers with adenocarcinomas, and could possibly serve as a biomarker in its upregulated state for lung cancer risk (Woenckhaus et al., 2006).

**ALDH3A1**

Along with *GPX2*, another useful biomarker for visualizing oxidative stress within the tissues of the lung is upregulation of the *ALDH3A1* gene (Gebel et al., 2004; Woenckhaus et al., 2006). The protein produced by *ALDH3A1* belongs to the ALDH superfamily of proteins, a family of proteins which are known to oxidize aldehydes that can originate both endogenously and exogenously within the human body, and that can potentially become hazardous to the human body due to their electrophilic state and tendency to react with amino and thiol groups, sometimes leading to carcinogenic effects.
Some endogenously formed aldehydes can occur through the metabolism of lipids, vitamins, and steroids within the body, whereas some exogenously formed aldehydes may occur through exposure to environmental toxins and ingested recreational drugs such as tobacco smoke (Vasiliou & Nebert, 2005).

In context as a useful biomarker within this study for determining cytotoxic damage from tobacco alkaloids, studies have previously found *ALDH3A1* to be upregulated within the respiratory tissue of rats upon exposure to tobacco cigarettes (Gebel et al., 2004). *ALDH3A1* has also been observed as being inducible, if not the most inducible of the *ALDH* family, to tobacco cigarette smoke exposure among human airway epithelial cells (Patel et al., 2008). In its upregulated state among non-small cell lung cancer cells, however, its role in carcinogenesis is still un-determined, and there is even some evidence linking its overexpression of its encoded aldehyde dehydrogenase protein to the reduction and possibly protection of cytotoxicity and DNA damage within the cell due to tobacco cigarette smoke exposure (Jang et al., 2014; Patel et al., 2008).

**PIR**

Another gene that is similar to *GPX2*, in that it has been also shown in studies as being irreversible in its consequential differential gene expression in response to tobacco smoke exposure, is the gene **PIR** (Banerjee et al.). The **PIR** gene encodes a member of the cupin protein superfamily, a family of proteins distinguished by their β-barrel fold structure and from being composed of proteins that are enzymatic as well as proteins that are non-enzymatic (Dunwell, Culham, Carter, Sosa-Aguirre, & Goodenough, 2001). Present in nearly all mammalian tissue cells, **PIR** is thought to be involved in DNA transcription and
regulation through its interaction with nuclear factor I/CCAAT box transcription factor (NFI/CTF1) as well as its studied interaction with B-cell lymphoma protein, BCL-3, a regulator of NF-κB/Rel activity (Licciulli et al., 2011; Wendler, Kremmer, Forster, & Winnacker, 1997). In a study carried out that evaluated PIR expression in melanocytes, PIR was shown to be a negative regulator of cellular senescence, and so once upregulated, appears to contribute an oncogenic state (Licciulli et al., 2011). By studying the PIR gene in response to tobacco alkaloids, we can evaluate whether tobacco alkaloids can induce an oncogenic state within lung tissue, but within the spectrum of also studying the effects of e-cigarette use.

**CYP1A1**

In expanding our investigation into the possible disease initiating/modifying potential of the tobacco alkaloids found in e-cigarette filling solution, differential response between gender is also of concern. To study these differences, we investigate expression within **TP53**, as mentioned previously, and also **CYP1A1**, which is also known as aryl hydrocarbon hydroxylase and a member of the cytochrome P450 superfamily of enzymes (National Center for Biotechnology Information). The proteins produced by this known superfamily are responsible in catalyzing reactions involved in drug metabolism as well as the synthesis of steroids, lipids, and cholesterol (McLemore et al., 1990).

One of the modes of action for CYP1A1 that is of concern, involves the metabolism of xenobiotics such as polycyclic aromatic hydrocarbons (PAHs) found in tobacco smoke into potentially harmful, carcinogenic intermediates, especially with certain polymorphisms of the gene (McLemore et al., 1990; Nakachi, Imai, Hayashi, & Kawajiri,
PAHs are formed by the combustion of fossil fuels and can be found not only in tobacco cigarettes, but throughout our environment in various forms such as our soil and even grilled meat (Lee, 2000). Some PAHs are known to have individual carcinogenic properties, and some PAHs can be bioactivated to a carcinogenic state by enzymes such as CYP1A1 (Iwano, Asanuma, Nukaya, Saito, & Kamataki, 2005; Lee, 2000).

Not only has CYP1A1 been shown to metabolize PAHs into harmful, carcinogenic intermediates, but studies between non-tobacco cigarette smokers and tobacco cigarette smokers have also shown that PAHs stimulate the production of CYP1A1, indicated by higher levels of CYP1A1 expression within smokers as opposed to non-smokers (McLemore et al., 1990). This links CYP1A1 and its upregulation in translation due to PAHs, to an increase in lung cancer risk, also giving CYP1A1 the potential to be used as a lung cancer biomarker (McLemore et al., 1990). Interestingly, studies have also shown an upregulation in CYP1A1 with exposure to the tobacco alkaloid, nicotine, which makes it a potentially valuable biomarker in this study as well, regarding potential differential gene expression from e-cigarette use due to the tobacco alkaloids found within the e-cigarette filling solutions (Michael M. Iba & Fung, 1999; M. M. Iba, Scholl, Fung, Thomas, & Alam, 1998).

In analyzing differences in gene expression among gender, investigation of CYP1A1 is of benefit due to previous research linking abnormal upregulation of the gene within female smokers, in comparison to male smokers, as well as an increase in PAH adduct levels, which have been studied as a contributing factor of an increased susceptibility to lung cancer (Mollerup et al., 2006; Mollerup, Ryberg, Hewer, Phillips, &
By studying differences in gene expression among TP53 and CYP1A1 among the male lung tissue cell lines and the female lung tissue cell line within this study, a correlation, or lack thereof, may help shed light as to whether tobacco alkaloids found within e-cigarette filling solutions can cause different physical effects between genders.

**AHR**

In direct relation with CYP1A1, AHR is another gene of interest. As a protein coding gene, AHR encodes for a transcription factor known to carry out a receptor-ligand activated response to PAHs (Lin, Hu, & Chang, 2003; National Center for Biotechnology Information). After activation of the receptor by the ligand, AHR travels from the cytoplasm of the cell to the nucleus, heterodimerizes with AhR nuclear translocator (Arnt), and in turn stimulates the transcription of xenobiotic metabolizing enzymes such as CYP1A1, which was stated previously as a metabolizer of xenobiotics such as PAHs found in tobacco smoke (Lin et al., 2003; National Center for Biotechnology Information).

With regard to the effects of tobacco alkaloids found in e-cigarettes, investigating genetic expression of AHR is of interest due to previous evidence that the AHR receptor is not only activated by PAH ligands, but seems to also be activated by nicotine, due to an increase in AHR levels being seen when lung fibroblast cells in culture were treated with the alkaloid (Sohn et al., 2008). This AHR activating CYP1A1 pathway, therefore, would seem to not only recommend the investigation into the expression levels of both genes when investigating the potential of an increased carcinogenic state due to the exposure of PAHs, but also when there has been exposure to the tobacco alkaloid, nicotine.
Among the genes that have been previously studied as putative oncogenes in a setting of tobacco smoke exposure, CEACAM6 has been shown, along with MDM2, PIR, and CYPIA1, as a top candidate for use as a biomarker in determining lung cancer susceptibility due to its upregulated state in exposure to cigarette smoke (Beane et al., 2007; Blumenthal, Hansen, & Goldenberg, 2005; Gonzales et al., 2015; Huang et al., 2010; Spira et al., 2004). Importantly, this upregulated state has also been shown to be irreversible (Beane et al., 2007). The protein encoded for by CEACAM6 is a glycosyl phosphatidyl inositol (GPI) anchored cell surface glycoprotein that belongs to the carcinoembryonic antigen (CEA) family of proteins (Blumenthal, Leon, Hansen, & Goldenberg, 2007; Gonzales et al., 2015). The CEACAM6 protein can be found within various epithelium tissue and has a role in cell differentiation, as well as innate immune response, but its adhesion and anti-apoptotic properties are of concern due to the speculative role in facilitating tumor growth and migration within the tissues (such as the lung, gastrointestinal tract, pancreas, and breast) (Blumenthal et al., 2005; Blumenthal et al., 2007; Gonzales et al., 2015; Ilantzis, DeMarte, Screaton, & Stanners, 2002). It is also of interest due to its upregulatory expression seen within the lung tissue of smokers, making it a potential biomarker for tumorigenesis, and possibly a very beneficial candidate gene to investigate in regards to the tobacco alkaloids found within e-cigarettes (Huang et al., 2010; Spira et al., 2004).
Goals/Aims of Research

In this study, we aim to investigate the effects of tobacco alkaloids found within e-cigarette filling solution on lung cell proliferation and gene expression. With electronic cigarette use on the rise among consumers looking for a safer alternative or drawn in by its trendy appeal, data concerning the effects of e-cigarette use and the effects of the chemical compounds that make up its filling solution is still greatly lacking (Pepper et al., 2015; Surgeon General, 2016). To shed more light on possible health outcomes that could become associated with e-cigarette use, we have conducted a study focusing on the tobacco alkaloids: anabasine, cotinine, myosmine, and nicotine, previously found within e-cigarette filling solution (Trehy et al., 2011). We hypothesize, based on prior research indicating tobacco alkaloids as contributing factors to diseased states of the human body (Hecht & Hoffmann, 1988; McBride et al., 1998; Wilp et al., 2002), that these four tobacco alkaloids have the capability of contributing to differential cell proliferation and abnormal gene expression with e-cigarette use. In addition to studying the effects of these tobacco alkaloids on the human body in a broad sense, we aim to also expand our investigation to include a study on any differences seen among gender and disease status by testing the alkaloids within this study against a panel of lung tissue cell lines varying in gender (male/female) and disease status (normal/cancer).

To carry out this investigation into potential differential cell proliferation among the cell lines, varying alkaloid concentrations are exposed to a panel of cell lines in a 10-day time-course, with viability determined through the Promega CellTiter-Glo® Luminescent Viability Assay. Investigation of gene expression is carried out with the isolation of RNA from within the alkaloid treated and un-treated cell lines after 48 and 96-
hour time-points, followed by creation of cDNA samples from those RNA transcripts. The cDNA samples are then utilized to proficiently quantitate targeted gene expression by way of quantitative reverse transcription polymerase chain reaction technology (qRT-PCR), carried out in real time. This process is meant to measure the amount of mRNA transcript being made by the cell, which is a direct reflection of what proteins are going to be produced due to specific gene expression. The cDNA synthesized from RNA of control and treated samples and quantified by qRT-PCR technology, is analyzed for the expression of 10 candidate genes, chosen due to their potential as a useful biomarker for disease and/or sex-specific expression. Differential cell proliferation and potential gene expression abnormalities, due to exposure to the alkaloids mentioned previously, are then evaluated using statistical methods.

MATERIALS AND METHODS

Description of Cell Lines

Evaluation of cell growth and viability was investigated by treating human lung cells in culture with varying concentrations of nicotine, cotinine, myosmine, and anabasine. Cell cultures exposed to the tobacco alkaloids were then compared to non-exposed cell cultures for analysis of cell growth and viability, as well as differential gene expression. To examine any differences related to sex specific gene expression in exposure to electronic cigarettes, as well as any differences related to disease state, a panel of three different cell lines was utilized, varying in gender (male/female) and disease status (normal/cancer). The three cell lines used in the panel were: CCL-185, CCL-171, and CCL-186, and were purchased from the American Tissue Culture Collection (ATCC) (Manassas,
VA) (Table 2). CCL-171 and CCL-186 are normal cell lines capable of senescence, and CCL-185 is considered a transformed cell line due to its cancerous state, and therefore considered immortal (Table 2). CCL-171 was documented by the ATCC of being capable of 42-46 population doublings before the onset of senescence, and CCL-186 was documented as having 58. CCL-171 was received from ATCC in anticipation of this study at passage number 16, whereas CCL-186 was received from ATCC at passage number 10.

### Table 2: Human Lung Tissue Cell Lines Exposed to Tobacco Alkaloids

<table>
<thead>
<tr>
<th>ATCC No.</th>
<th>Alternate Name</th>
<th>Tissue Type</th>
<th>Organism</th>
<th>Sex</th>
<th>Disease Status</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCL-185</td>
<td>A549</td>
<td>Lung</td>
<td>Homo sapiens</td>
<td>Male</td>
<td>Carcinoma</td>
</tr>
<tr>
<td>CCL-171</td>
<td>MRC-5</td>
<td>Lung</td>
<td>Homo sapiens</td>
<td>Male</td>
<td>Normal</td>
</tr>
<tr>
<td>CCL-186</td>
<td>IMR-90</td>
<td>Lung</td>
<td>Homo sapiens</td>
<td>Female</td>
<td>Normal</td>
</tr>
</tbody>
</table>

### Tissue Culture Growth and Maintenance

The three lung tissue cell lines that were examined in this study were seeded from frozen into vented, Falcon® 75 cm² tissue culture flasks. Cell lines, CCL-171 and CCL-186 were cultured in Eagle’s Minimum Essential Growth Medium supplemented with 1.5 g/L sodium bicarbonate, non-essential amino acids, L-glutamine, and sodium pyruvate, and adjusted to contain an added 10% fetal bovine serum (FBS), used to provide essential and rich growth factors, as well as 1% penicillin-streptomycin solution, utilized for prevention of bacterial contamination. Cell line CCL-185 was cultured in HyClone™ DMEM/High Glucose growth medium containing 4.00 mM L-glutamine, 4500 mg/L glucose, sodium pyruvate, 10% FBS and 1% penicillin-streptomycin. All cells were maintained within a
CO₂ incubator set at 37°C and 5% CO₂, with media renewal every three days underneath a laminar flow hood, using aseptic practices.

To prevent contact inhibition and to promote a healthy environment, when the lung tissue cells in culture reached a confluency of ~70%, the cells were either sub-cultured, to allow for continued growth, or utilized in the seeding of a 10-day experimental time course. In either case, media was aseptically removed, and 3 mL of 0.25% trypsin solution was added into each flask to detach the cells from the tissue culture flask and to promote a single cell suspension. Cells were incubated within the trypsin solution for no more than 5 minutes until a single cell suspension was reached. Once cells reached a single cell suspension, they were either split 1:2, 1:3, or 1:5 (depending upon confluency) and seeded into fresh 75 cm² flasks containing fresh growth media, or utilized in seeding tissue culture plates that were to be used for the 10-day experimental time course.

**CellTiter-Glo® Luminescent Viability Assay**

To examine the effect of alkaloid exposure on cell proliferation, lung tissue culture samples of CCL-185, CCL-186, and CCL-171 were characterized for growth and viability in 10-day time course experiments, while exposed to 1 µg/mL, 10 µg/mL, and 100 µg/mL concentrations of the tobacco alkaloids: anabasine, cotinine, myosmine, and nicotine, individually. Cell viability was determined daily, relative to untreated controls by using the Promega CellTiter-Glo® Luminescent Viability assay which relies on a thermostable luciferase reaction that detects metabolic ATP levels in the cell (Promega Corporation, 2015). The amount of ATP present 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, releasing ATP while simultaneously inhibiting ATPases that would decompose the cellular ATP, and providing 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).

**Plate Set-up for Time Course**

In carrying out this assay, lung tissue cells were seeded at a concentration of 20,000 cells/mL/well in 12-well Falcon® tissue culture plates under experimental and control conditions. Experimental exposure conditions, performed in technical triplicate, included 1 µg/mL, 10 µg/mL, and 100 µg/mL of either anabasine, cotinine, myosmine, or nicotine, individually, as well an untreated control of 20,000 cells/mL within cell growth media alone. Alkaloid dilutions were made from 1 mg/mL stock solutions of anabasine, cotinine, myosmine, and nicotine. Due to differences within molecular weight of each compound, the molar concentration of each exposure condition varied slightly across the panel of alkaloids (Table 3).

<table>
<thead>
<tr>
<th>Alkaloid</th>
<th>Stock Solution Concentration</th>
<th>Molarity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anabasine</td>
<td>1.00 µg/mL</td>
<td>6.16 µM</td>
</tr>
<tr>
<td>Cotinine</td>
<td>1.00 µg/mL</td>
<td>5.68 µM</td>
</tr>
<tr>
<td>Myosmine</td>
<td>1.00 µg/mL</td>
<td>6.84 µM</td>
</tr>
<tr>
<td>Nicotine</td>
<td>1.00 µg/mL</td>
<td>6.16 µM</td>
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</table>
A plate containing experimental conditions of the four alkaloids, along with a separate plate of controls, was used for each day, totaling 20 plates for the 10-day time course (Figure 7). Throughout the 10-day time course, the experimental plates were maintained in the CO\(_2\) incubator at 37˚C and 5% CO\(_2\).

![Figure 7: 12-Well Tissue Culture Plate-Setup of a Cell Line Under Experimental Conditions.](image)

Each exposure treatment is evaluated in a technical triplicate. For each 10-day time course, 10 plates were independently seeded on day 1 and sequentially analyzed by proliferation assay throughout the time course.

**Measurement of Daily Cell Proliferation**

Cell viability and proliferation was measured daily throughout the time course, using the Promega CellTiter-Glo® Luminescent Viability Assay, per manufacturer’s instructions. Briefly, 350µL of cell growth media, without FBS or antibiotics, was added to each well of the 12-well plates containing the cultured cells. Next, 350µL of CellTiter-Glo® reagent was applied to the same wells, immediately producing a visible color change within the media/cell solution. The experimental plates were then incubated on an orbital shaker for 10 minutes. Once incubation was complete, each well containing either
Experimental conditions or control was assayed for metabolic ATP by loading each sample, in triplicate, for each well of the 12-well plate they originated, totaling 9 wells per experimental condition, onto a 96-well white-walled, reflective plate (Figure 8).

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<td>A</td>
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<td>C</td>
<td>Myosmine</td>
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<td>Nicotine</td>
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<td>G</td>
<td>5 x 10^{-11} moles ATP</td>
<td>5 x 10^{-11} moles ATP</td>
<td>5 x 10^{-11} moles ATP</td>
<td>1 x 10^{-12} moles ATP</td>
<td>1 x 10^{-12} moles ATP</td>
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<tr>
<td>H</td>
<td>2 x 10^{-10} moles ATP</td>
<td>2 x 10^{-10} moles ATP</td>
<td>2 x 10^{-10} moles ATP</td>
<td>1 x 10^{-11} moles ATP</td>
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Figure 8: 96-well Plate Set-up 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 (in alkaloid concentrations of 1 µg/mL, 10 µg/mL of 100 µg/mL), as well as the moles of ATP for the ATP standard curve that was included on each plate. A plate control of only media (no cells or substrate) was also included on each plate.

A no template control, to monitor background luminescence, was also loaded in triplicate, as well as a standard curve of known ATP concentrations (2 x 10^{-10}, 5 x 10^{-11}, 1 x 10^{-11}, 5 x 10^{-12} and 1 x 10^{-12} moles) to extrapolate metabolic ATP levels in samples (Figure 9). Subsequent reading of the 96-well plate was carried out by a Biotek® Synergy plate-reader for luminescence detection, and results of cell proliferation from the Promega CellTiter-Glo® Luminescent Viability Assay were then statistically analyzed in Microsoft Excel™.
**Statistical Analysis of Alkaloid Exposure on Cellular Proliferation**

A two-tailed, 2-sample equal variance student t-test was applied to the cell proliferation results, to evaluate statistical differences between the mean value of each experimental condition, against the mean value of the untreated control wells. P-values obtained from the student t-test highlighted the difference in values obtained between groups (control and experimental) compared to the difference in value obtained within groups. A p-value cutoff of ≤ 0.05, between experiment and controls, was considered statistically significant. A p-value obtained that was ≤ 0.05 was accepted as being against 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
conditions and the control was due to random chance, and thus, the null hypothesis was accepted.

To account for inherent differences in cell proliferation across cell lines, all experimental values were normalized to the untreated control using the following equation:

\[
\% \text{ Change} = \left( \frac{\text{Experimental} - \text{Control}}{\text{Control}} \right) \times 100
\]

*Equation 1: Equation of Percent Change for Normalization of Data to Control*

**Evaluation of Differential Expression of Candidate Genes by qRT-PCR**

Differential gene expression patterns were analyzed by qRT-PCR for each cell line under untreated and treated conditions, to evaluate the effect of alkaloid exposure on the expression of a panel of candidate genes speculated to be important in pathology of tobacco-associated disease (Table 1). The experimental method of qRT-PCR relies on fluorescent dye technology to indirectly quantify the amount of mRNA transcript in a sample through specific PCR amplification of target genes that, in the presence of intercalating dyes, fluoresce when bound to double-stranded DNA product, SYBR green. Visualization, in real time, of the amplified product, gives the ability to evaluate by quantitative analysis, a necessary component in comparing our experimental RNA transcripts against the control, RNA transcripts. There are four basic steps to qRT-PCR: RNA isolation, cDNA synthesis by reverse transcription, SYBR green, and real-time PCR and quantitative analysis relative to a standard curve of known concentration values.

Differential gene expression patterns associated for tobacco alkaloid exposure were evaluated for each cell line at 48 and 96 hours, post treatment. Towards this goal, the three
different lung cell lines, CCL-185, CCL-186, and CCL-171, were seeded at a density of 20,000 cells/mL into 75cm² Falcon® tissue culture flasks for a total volume of 19 mL (cells + media), and exposed to the tobacco alkaloids in concentrations of either 1µg/mL, 10 µg/mL, or 100 µg/mL. A control consisting of a cell suspension of each cell line that had not been treated by alkaloids was also seeded simultaneously.

*Isolation of RNA from Cell Lines*

The QIAGEN® RNeasy RNA Isolation kit was used to isolate total RNA from all experimental and control samples per manufacturer’s instructions. Briefly, cells were detached from tissue culture flasks by trypsination, followed by application of cell suspension to the supplied QIA shredder column, which results in homogenization and lysis of cells. Genomic DNA was then removed with use of a gDNA eliminator spin column, provided by the manufacturer. Ethanol was added to the total RNA, and the suspension was then passed through the manufacturer provided RNeasy spin column, that binds the RNA to the filter. The column was then washed twice before the total RNA was eluted from the column with 30 µL of nuclease-free water.

RNA isolated was quantified using the Thermo Scientific™ Nanodrop™ and analyzed for integrity through native agarose gel electrophoresis (Figure 10) that was prepared with RNase free reagents and consisted of RNase free agarose at a 1.2% concentration. The native agarose gel was then electrophoresed in RNase free 1X TBE buffer, and ran at ~ 5-6 Volts/cm, until the bromophenol blue that was added to the RNA samples had migrated to approximately a third of the way down the gel. The gel was then visualized under a UV transilluminator for the presence/absence of contaminating genomic
DNA, as well as visualization of the clarity and sharpness of the 28S and 18S eukaryotic rRNA bands within the total RNA samples (Figure 10).

For analysis of differential gene expression by qRT-PCR, isolated experimental RNA was converted to complementary DNA (cDNA) with the Bio-Rad iScript™ Reverse Transcription Supremix for qRT-PCR. cDNA synthesis was carried out by following the manufacturer’s protocol for a 40 µL cDNA reaction (Table 4), and programmed for the following thermal cycler settings: priming of the template at 25°C for 5 minutes, reverse
transcription at 42°C for 30 minutes, and inactivation of reverse transcriptase at 85°C for 5 minutes.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bio-Rad iScript™ Reverse Transcription Supermix *</td>
<td>8 µL</td>
</tr>
<tr>
<td>Experimental total RNA sample</td>
<td>2 µg</td>
</tr>
<tr>
<td>Nuclease-free water</td>
<td>varying**</td>
</tr>
</tbody>
</table>

Total Volume = 40 µL

*Bio-Rad iScript™ Reverse Transcription Supermix includes: reverse transcriptase, RNase inhibitor, dNTPs, oligo(dT), random primers, buffer, MgCl₂, and stabilizer.

**Nuclease-free water added to bring the reaction up to 40 µL/reaction.

**SYBR Green Real-Time PCR**

**Description of Candidate Genes and Primer Design**

Analysis of gene expression by qRT-PCR was carried out by using markers targeting specific candidate genes speculated to be important in pathology of tobacco-associated disease. Table 1 summarizes the ten candidate genes selected and their focused function for gene expression analysis. Two reference genes (also known as housekeeping genes) were also utilized within this study as a reference to normal cell function and metabolism that should be seen, regardless of the different experimental conditions imposed.

To isolate the candidate genes within experimental cDNA template as well as to create gene standards for a standard curve, primers to amplify their target sequence in a polymerase chain reaction were designed through idtDNA (Integrated DNA Technologies, 2017) and purchased through the companies Eurofins Genomics and Invitrogen. The parameters for design of forward and reverse primers were set to amplify across exon: exon
junctions, generate a product size in the range of 75-150 bps, be matched for % G/C and annealing temperatures between 55 - 65°C, and have minimal primer: dimer and self-annealing properties. Each validated primer pair used yielded a single peak of dissociation upon melting curve analysis. Primer sequences designed are listed in Table 5.

Table 5: Candidate Genes to Be Evaluated for Differential Gene Expression and their Designed Primers

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Primer Sequence</th>
<th>Mature Amplicon Length</th>
<th>Genomic Amplicon Length</th>
</tr>
</thead>
<tbody>
<tr>
<td>AHR</td>
<td>5'-TCATTGTAATCAGAGACCCTAAC-3' 5'-ATTTATGGGAGAAAGGGGTG-3'</td>
<td>137 bp</td>
<td>3,336 bp</td>
</tr>
<tr>
<td>ALDH3A1*</td>
<td>5'-CCTTAAATACGTCCCCCTTGG-3' 5'-TCGCTGATCTGTGCTTGGG-3'</td>
<td>92 bp</td>
<td>3,216 bp</td>
</tr>
<tr>
<td>PIR*</td>
<td>5'-AGTAAAGGATGTTGACAGTGG-3' 5'-AGGATAGGTTGGGAATGGT-3'</td>
<td>135 bp</td>
<td>29,992 bp</td>
</tr>
<tr>
<td>CEACAM6*</td>
<td>5'-CTCAAAAGAGGTGGACAGAAG-3' 5'-AAATGAGCTCCTTGCCAGG-3'</td>
<td>149 bp</td>
<td>996 bp</td>
</tr>
<tr>
<td>CX3CL1*</td>
<td>5'-CACCTCTGCCCATCTGAATG-3' 5'-TGCTCCTGTGAGATGGT-3'</td>
<td>130 bp</td>
<td>7,125 bp</td>
</tr>
<tr>
<td>CYP1A1*</td>
<td>5'-CCTACACCTTTCCTGAATG-3' 5'-TTCTCCTGACAGTGAACCT-3'</td>
<td>146 bp</td>
<td>701 bp</td>
</tr>
<tr>
<td>MDM2</td>
<td>5'-TGGCAAGCTCTGTGGAAG-3' 5'-TCTTTGTATTCACTCCCAAC-3'</td>
<td>148 bp</td>
<td>7,639 bp</td>
</tr>
<tr>
<td>SLIT1*</td>
<td>5'-GACTGCGTACAAGGAACCG-3' 5'-TGGAAAAAGAGGACAGAC-3'</td>
<td>149 bp</td>
<td>2,291 bp</td>
</tr>
<tr>
<td>TP53</td>
<td>5'-CCTACAGATCTTCGTGGATG-3' 5'-ACATGTAGTTGTGATAGGGTTG-3'</td>
<td>143 bp</td>
<td>710 bp</td>
</tr>
<tr>
<td>GPX2*</td>
<td>5'-GCTTTCCCTTGGACAATTTG-3' 5'-TTCTCGCCATTACACCTC-3'</td>
<td>139 bp</td>
<td>2,805 bp</td>
</tr>
<tr>
<td>ACTB*</td>
<td>5'-GCGCGCGGTGTAACAAACAGTG-3' 5'-CCCGCGCCCTCAGCCTCTG-3'</td>
<td>225 bp</td>
<td>319 bp</td>
</tr>
<tr>
<td>GAPDH*</td>
<td>5'-CCTCAACGACCACCTTGT-3' 5'-TGTCAGGGGTCTTACT-3'</td>
<td>116 bp</td>
<td>220 bp</td>
</tr>
</tbody>
</table>

* Primers previously designed by Max Marlowe and/or provided by Dr. Margaret Kovach

Determination of Reference Gene Expression Stability

The two reference genes chosen for this study were selected by testing untreated and treated experimental samples of the three cell lines: CCL-185, CCL-186, and CCL-171, for 14 candidate reference/housekeeping genes by qRT-PCR, using the Bio-Rad Reference Genes H96 predesigned 96-well panel for use with SYBR® green technology.
Using the Bio-Rad CFX Connect software, the candidate reference genes tested were ranked individually for their associated M-value, with an M-value < 0.5 being indicative of greater gene stability among both the treated and untreated samples and favored as opposed to those reference genes tested simultaneously with a resultant higher M-value (Hellemans, Mortier, De Paepe, Speleman, & Vandesompele, 2007). The housekeeping genes, *GAPDH* and *ACTB*, ranked as the reference genes with two of the lowest M-values and were subsequently both chosen to be reference genes utilized for normalization of gene expression throughout this study.

**Preparation of a Standard Curve**

Each of the candidate genes listed in Table 5 were amplified by polymerase chain reaction to synthesize PCR product of known concentration for use in generating a standard curve for qRT-PCR analysis of gene expression within the experimental samples. The reactions for the synthesis of the gene standards included the reagents and volumes outlined by New England BioLabs® for their Hot Start *Taq* 2X Master Mix protocol for routine PCR (Table 6), amplified to create a 5x reaction, and synthesized by polymerase chain reaction in a thermal cycler set to the following: 1 cycle of initial denaturation at 95°C for 5 minutes, followed by 40 cycles at 95°C for 30 seconds (denaturation), 58°C for 30 seconds (annealing), and 72°C for 1 minute (extension), and then final extension was carried out with 1 cycle at 72°C for 7 minutes.
Once gene standards were amplified, the synthesized product was loaded onto a 2% native agarose gel for electrophoresis with 1X TBE buffer. The agarose gel was then visualized under a UV transilluminator and PCR products of size appropriate to the target gene mature amplicon length (Table 5), were extracted, and purified using the QIAGEN Gel Extraction Kit, followed by quantification by Nanodrop™. A solution with $2 \times 10^{10}$ copies of product per microliter was prepared. This solution was serially diluted tenfold to prepare a standard curve of DNA with concentrations ranging from $2 \times 10^{10}$ to $2 \times 10^2$ copies of product per microliter. If each reaction performed near 100% efficiency (DNA doubling in count per cycle), then the linear relationship derived from the standard curve can be used to predict the copy number in samples of unknown copy number.

Table 6: New England BioLabs® Hot Start Taq 2X Master Mix PCR Reaction Protocol

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hot Start Taq 2x Master Mix*</td>
<td>12.5 µL</td>
</tr>
<tr>
<td>Template DNA</td>
<td>&lt; 1,000 ng</td>
</tr>
<tr>
<td>Candidate Gene Forward Primer</td>
<td>0.2 µM</td>
</tr>
<tr>
<td>Candidate Gene Reverse Primer</td>
<td>0.2 µM</td>
</tr>
<tr>
<td>Nuclease-free Water</td>
<td>varying**</td>
</tr>
<tr>
<td><strong>Total Volume = 25 µL</strong></td>
<td></td>
</tr>
</tbody>
</table>

*Hot Start Taq 2x Master Mix includes: Hot Start Taq DNA Polymerase, dNTPs, Standard Taq Reaction Buffer, and stabilizers

**Nuclease-free water added to bring the reaction up to 25 µL/reaction.

qRT-PCR

In order to carry out quantification by qRT-PCR, cDNA derived from corresponding RNA isolated from control and treated samples, served as template for
amplification of the candidate genes listed in Tables 1 and 5, as well as the reference genes, *ACTB* and *GAPDH*. Each reaction was carried out in triplicate for each experimental condition/per cell line with the reagents/volumes listed in Table 7.

**Table 7: Bio-Rad 2x iQ™ SYBR Green Supermix Reaction Protocol**

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>2X iQ™ SYBR® Green Supermix *</td>
<td>12.5 µL</td>
</tr>
<tr>
<td>Template DNA</td>
<td>1 µg</td>
</tr>
<tr>
<td>Candidate Gene Forward Primer</td>
<td>10 pmol (of a 20 pmol/µL stock working solution)</td>
</tr>
<tr>
<td>Candidate Gene Reverse Primer</td>
<td>10 pmol (of a 20 pmol/µL stock working solution)</td>
</tr>
<tr>
<td>Nuclease-free Water</td>
<td>varying**</td>
</tr>
</tbody>
</table>

Total Volume = 25 µL

*2X iQ™ SYBR® Green Supermix is brought to a final concentration of 1X by addition of reagents listed.

**Nuclease-free water added to bring the reaction up to 25 µL/reaction.

Each 25 µL reaction was then loaded onto a 96-well plate (each experimental condition, for each cell line, in triplicate) along with a no template control (NTP), a no primer control (NPC), and an interplate control (IPC), each loaded in duplicate. The 96-well plate also contained standard dilutions of the gene being analyzed for that specific plate to test for efficiency of the candidate gene primers. The standards ranged in copy numbers of 1 x 10⁸ to 1 x 10², with each standard being loaded onto the plate in duplicate. The layout of the 96-well plate is illustrated in Figure 11. The 96-well plate, once loaded, was then subject to real time qRT-PCR by the Bio-Rad CFX Connect Real-Time PCR detection system.
Statistical Analysis of Quantitative Reverse-Transcription PCR Data

In evaluating gene expression by qRT-PCR, the target sequence of the gene can be analyzed with fluorescent technology in real-time, by observing the pace at which the fluorescent signal of the target sequence reaches a detection threshold, which is then charted out onto a graph for analysis (Figure 12). The threshold cycle number (Ct) is the number at which the signal of the targeted sequence reaches the threshold. The threshold cycle number can then be used to calculate the quantity of the initial sample copies since the Ct is inversely related to the amount of starting material. When the sample reaches the threshold, the cycle number recorded is used to calculate the difference between starting material, by factoring in that the material approximately doubles after every cycle. So, a sample passing the threshold at cycle number 10, in comparison to a sample passing the threshold at cycle number 20, would correlate to a $2^{10}$ difference between the two samples since the cycles reached their respected thresholds ten cycles apart from each other.
Differential gene expression among the experimentally treated cell lines was evaluated by utilizing the gene expression analysis profile tool in the Bio-Rad CFX Connect Real-Time PCR Detection software program. The two reference genes, ACTB and GAPDH, were also analyzed for every cell line tested under the varying experimental conditions, and the data from these qRT-PCR plates for reference gene expression was then used to normalize the experimental expression values that were obtained for the other candidate genes listed by way of the gene expression analysis tool. The efficiency of the gene standard curve, also loaded onto the experimental plate, was used as well to determine efficiency within calculations of the gene expression analysis.

Figure 12: qRT-PCR Quantification of Gene Expression for Analyzation of Isolated RNA Under Varying Conditions.
The Y-axis of the graph represents the relative fluorescence, and the X-axis represents the cycle number associated with that amount of fluorescence. The green horizontal line going directly across the graph represents the threshold for detecting fluorescence. The Ct values, indicated by arrows, for the represented samples A, B, and C are 14, 19 and 23, respectively. Thus, in this example there is a $2^5$ (32-fold) difference in expression between samples A and B, and a $2^9$ (512-fold) difference in expression between samples A and C.
RESULTS

Evaluation of Tobacco Alkaloid Exposure on Lung Cell Proliferation

The effect of tobacco alkaloid exposure on lung cellular proliferation and viability was examined across cell lines that differed with respect to gender and disease status of tissue. Three lung tissue cell lines were included in this study: CCL-171, a male, normal lung tissue cell line, CCL-186, a female, normal lung tissue cell line, and CCL-185, a male, transformed lung tissue cell line (Table 2). Each lung tissue cell line in the panel displayed a characteristic growth curve unique to that cell line and differing between the other cell lines within the panel (Figure 13).

Figure 13: Cell Growth Patterns of Experimental Cell Lines. The lung tissue cell lines used in this study are characterized in the above graph by their cell growth curve, when untreated for experimental conditions, for comparison between cell lines. Three wells of a 12-well tissue culture plate were seeded, without exposure to any alkaloids, for each time point of a ten-day time course, and for each individual cell line. The cells were analyzed by the CellTiter-Glo® Luminescent Viability Assay, and the mean was obtained. The above figure is a graph that depicts the average growth rate of these cells, per day, given by mols of ATP detected, for the 10-day time course.
These cell lines were then exposed individually to the tobacco alkaloids: anabasine, cotinine, myosmine, and nicotine, found to be within e-cigarette filling solutions, in 1 µg/mL, 10 µg/mL, and 100 µg/mL concentrations, over a period of 10 days, and effects on cell proliferation were then evaluated by utilizing the Promega CellTiter-Glo® Luminescent Viability Assay every 24 hours, over the course of the 10-day study. An equation of percent difference to control (Equation 1) was applied to the resultant experimental data from the cell proliferation assays, to normalize the data to the control. Results obtained that were greater than or equal to a 25% change from control, and with a p-value ≤ 0.05, obtained through the two-tailed student t-test, were considered significant findings and are highlighted in this section.

**Anabasine**

Overall, the effect of anabasine exposure on lung cell proliferation demonstrated an inhibitory dose-response, associated with increasing concentration of the alkaloid. Anabasine exposure to the panel of lung tissue cell lines, in the concentration of 1 µg/mL, showed minimal change in cellular proliferation to control throughout the 10-day time courses, with a percent difference to the control (Equation 1) being greater than or equal to 25% only occurring at two time points within the study: an increase in cellular proliferation of 27.2% on day 1 for the normal, male cell line, and a decrease in cellular proliferation of 26.3% on day 5 for the cancerous cell line.

However, with exposure to higher concentrations of anabasine, there was a more pronounced effect on proliferation profile across all cell lines, particularly at the highest exposure concentration (100 µg/mL). The effect varied slightly between stimulatory and
inhibitory, but ultimately the predominant effect displayed was that of inhibition of cellular growth. Exceptions to this inhibitory effect of anabasine was observed only in the normal male cell line, in which an increase of 48.2% was observed on day 10 of the study, upon exposure to 10 µg/mL anabasine (Figure 14).

![Figure 14: Cell Growth with Exposure to 10 µg/mL Anabasine, Normalized to Control.](image)

Data obtained daily on the three experimental cell lines, under the exposure of anabasine at the 10 µg/mL concentration, by the CellTiter-Glo® luminescent viability assay, was normalized to the control by applying an equation of percent difference for each time-point within the 10-day time course. Most significant values within the cell lines are marked by an asterisk (*). The legend indicates the associated colors for the three varying cell lines: normal male = CCL-171, normal female = CCL-186, and cancerous male = CCL-185.

The other time points studied for the individual cell lines, at the 10 µg/mL and 100 µg/mL concentrations, showed a predominately inhibitory effect, with the greatest percent difference to control, regarding inhibition being displayed, being seen within the normal, female cell line, as well as the cancer cell line. The normal, female cell line was observed to display the greatest decrease in cell growth, when compared to control, of 57.6%, when exposed to 100 µg/mL anabasine, for day 6 (Figure 15). The cancer cell line was observed
to display its greatest decrease in cellular growth, when exposed to 10 µg/mL anabasine, on day 6, where there was a decrease in cellular growth, when compared to control, of 52.6% (Figure 14).

Cotinine

Similar to the findings observed for the effect of anabasine exposure on lung cell proliferation, cotinine exposure demonstrated minimal effect at the lowest concentration. Only two time-points, with a percent difference to the control (Equation 1) being greater than or equal to 25%, occurred within the study: an increase in cellular proliferation of 25.1% on day 1 for the normal male cell line, and an increase in cellular proliferation of 36.7% on day 7 for the cancer cell line.

*Figure 15: Cell Growth with Exposure to 100 µg/mL Anabasine, Normalized to Control.*

Data obtained daily on the three experimental cell lines, under the exposure of anabasine at the 100 µg/mL concentration, by the CellTiter-Glo® luminescent viability assay, was normalized to the control by applying an equation of percent difference for each time-point within the 10-day time course. Most significant values within the cell lines are marked by an asterisk (*). The legend indicates the associated colors for the three varying cell lines: normal male = CCL-171, normal female = CCL-186, and cancerous male = CCL-185.
The greatest effect of cotinine was observed at the 10 µg/mL concentration, and interestingly, this effect was inhibitory and specific to the cancer cell line. The greatest percent difference to control for the cancer cell line was observed on day 7, with a decrease of cell growth by 69.0% (Figure 16). In contrast, cotinine exposure of the normal lung cell lines, both CCL-186 and CCL-171, showed a stimulatory effect on cellular growth, where the greatest percent difference to control for the two cell lines was portrayed by the normal, male cell line, on day 10, with an increase in cell growth of 52.2% (Figure 16).

Upon exposure to 100 µg/mL cotinine, the effect on proliferation was less pronounced and only significant after long exposure periods. The normal, male cell line displayed the greatest increase in cell growth, with an increase of 37.6%, on day 10 of the study (Figure 17). The female cell line showed just a slight decrease in cell growth, with...
the greatest percent difference to control being a 28.4% decrease, observed on day 9 (Figure 17). The cancer cell line displayed both inhibitory and stimulatory results on cell growth with 100 µg/mL cotinine. The greatest percent difference to control was a 30.4% increase in cell growth on day 8 (Figure 17).

![Cotinine Exposure at 100 µg/mL](image)

**Figure 17: Cell Growth with Exposure to 100 µg/mL Cotinine, Normalized to Control.** Data obtained daily on the three experimental cell lines, under the exposure of cotinine at the 100 µg/mL concentration, by the CellTiter-Glo® luminescent viability assay, was normalized to the control by applying an equation of percent difference for each time-point within the 10-day time course. Most significant values within the cell lines are marked by an asterisk (*). The legend indicates the associated colors for the three varying cell lines: normal male = CCL-171, normal female = CCL-186, and cancerous male = CCL-185.

**Myosmine**

Of all the alkaloids tested in this study, myosmine exposure resulted in the most negative and detrimental effect on lung cell proliferation. In fact, exposure was tolerated only at the 1 µg/mL and 10 µg/mL concentrations. Exposure at the highest concentration level (100 µg/mL) demonstrated complete cytotoxicity. Specifically, at the lowest exposure level, minimal changes in cell proliferation were seen, except after prolonged exposure of
the cancer cell line. On the days resulting in the greatest percent difference to control, the cancer cell line increased in cellular proliferation values by 36.8% on day 9, and 56.1% on day 10.

Exposure to 10 µg/mL myosmine demonstrated strikingly different effects on cellular proliferation, characteristic of each cell line. The female cell line was characterized by a decrease in cellular proliferation, when compared to control, of more than 25% throughout the length of the 10-day time course, with the greatest percent difference to control observed on day 7, when a 44.2% decrease was recorded (Figure 18).

In contrast, the normal, male cell line, displayed a biphasic growth curve, characterized by an inhibitory effect during the first phase, with the greatest percent difference to control being obtained at 30.7%, on day 4, followed by a stimulatory effect on cellular proliferation for the remaining five days of the 10-day study, with the greatest percent difference to control on day 10, at 46.9% (Figure 18).

Lastly, cancer cell line, showed a strong stimulatory effect on cellular growth during the first five days of exposure, with the maximum increase of 138% observed on day 1, but returned to relatively normal growth for the remaining length of the 10-day study (Figure 18).
At 100 µg/mL, the toxicity of myosmine completely inhibited all cellular growth, for all three lung cell lines, by day 3 of the 10-day study. Associated p-values for the three cell lines correlated with this extreme decrease in cell viability, with the p-values for each time point, and for every cell line, being greatly under the 0.05 threshold of significance (Figure 19).
Nicotine

Surprisingly, the most well-known of the tobacco alkaloids, nicotine, exhibited the most varied response upon exposure to the panel of lung tissue cell lines, with no real trends observed in stimulation or inhibition of cell growth. Similar to anabasine and cotinine, the lowest concentration showed minimal change in cellular proliferation to control throughout the 10-day time courses, with no significant values in cellular growth obtained. Exposure to the higher concentrations (10 µg/mL and 100 µg/mL), however, displayed a variant effect on the lung cell lines.

Within the normal, female cell line, this variance was observed upon exposure to 10 µg/mL nicotine as a biphasic effect on cell growth, with a decrease observed during the first five days of the 10-day time course, and then an increase in cell growth towards the last five days. The greatest percent difference to control for the female cell line, regarding

![Figure 19: Cell Growth with Exposure to 100 µg/mL Myosmine, Normalized to Control.](image)

Data obtained daily on the three experimental cell lines, under the exposure of myosmine at the 100 µg/mL concentration, by the CellTiter-Glo® luminescent viability assay, was normalized to the control by applying an equation of percent difference for each time-point within the 10-day time course. There was a complete loss of cell viability among all three cell lines by day 3. The legend indicates the associated colors for the three varying cell lines: normal male = CCL-171, normal female = CCL-186, and cancerous male = CCL-185.
a decrease in cell viability, was recorded on day 2, with a decrease of 26.7%, and the greatest increase in cell growth was recorded on day 9, with an increase of 30.7% (Figure 20).

In contrast, the normal, male cell line displayed fewer significant changes upon exposure to 10 µg/mL nicotine, where only a slight stimulatory effect was noted for the last six days of the 10-day time course (Figure 20). The greatest percent difference to control within the 10-day time course for the male cell line was recorded on day 10, where there was an increase in cell growth of 27.8% (Figure 20). The cancer cell line, however, showed a more significant stimulatory response upon exposure to 10 µg/mL nicotine, with the greatest percent difference to control observed on day 1 of the 10-day time-course, with an increase in cellular growth at a remarkable 166% (Figure 20).

![Figure 20: Cell Growth with Exposure to 10 µg/mL Nicotine, Normalized to Control.](image)

Data obtained daily on the three experimental cell lines, under the exposure of nicotine at the 10 µg/mL concentration, by the CellTiter-Glo® luminescent viability assay, was normalized to the control by applying an equation of percent difference for each time-point within the 10-day time course. Most significant values within the cell lines are marked by an asterisk (*). The legend indicates the associated colors for the three varying cell lines: normal male = CCL-171, normal female = CCL-186, and cancerous male = CCL-185.
Finally, at the 100 µg/mL concentration of nicotine exposure, the normal, female cell line displayed variance once more, by showing little to no effect within cellular growth. The cancer cell line, also continued this trend of variance by portraying a decrease in cellular growth upon exposure to the highest concentration. The most profound decrease within the cancer cell line occurred on day 1 of the study, where a decrease in percent difference when compared to control was recorded at 56.7% (Figure 21). In contrast, the normal, male cell line, did not display variance but continued to display a slight stimulatory effect in cell growth from exposure to nicotine at the 100 µg/mL concentration. On day 10, the normal male cell line exhibited the greatest increase in cell growth, recorded at 46% (Figure 21).

![Figure 21: Cell Growth with Exposure to 100 µg/mL Nicotine, Normalized to Control.](image)

Data obtained daily on the three experimental cell lines, under the exposure of nicotine at the 100 µg/mL concentration, by the CellTiter-Glo® luminescent viability assay, was normalized to the control by applying an equation of percent difference for each time-point within the 10-day time course. Most significant values within the cell lines are marked by an asterisk (*). The legend indicates the associated colors for the three varying cell lines: normal male = CCL-171, normal female = CCL-186, and cancerous male = CCL-185.
Expression of Candidate Genes by qRT-PCR

To evaluate candidate gene expression patterns among the alkaloid treated and untreated cell cultures, control and alkaloid-treated, experimental samples of the lung tissue cell lines were analyzed by qRT-PCR, for seven candidate genes listed within Table 1. Due to loss of cell viability, experimental RNA samples of the cell lines treated with myosmine, at the 100 µg/mL concentration, were unattainable, and therefore gene expression analysis was not carried out for experimental samples treated with myosmine, at any of the alkaloid concentrations or time points. Also, due to time constraints and funding, as well as little variance being observed on cell proliferation and viability, the experimental samples that were exposed to only a 1 µg/mL concentration of the four alkaloids, were excluded from gene expression analysis. Of the experimental samples that could be analyzed, expression levels that displayed a 4-fold increase or decrease, along with a correlating p-value of ≤ 0.01, were evaluated as statistically significant. Three candidate genes: PIR, CX3CLI, and SLIT1, listed in Table 1, were unable to be analyzed due to either unsatisfactory binding of designed primers and/or low expression levels, as noted in Table 1.

Anabasine

The most significant effect seen on differential expression within candidate genes from exposure to the alkaloids, was seen from exposure to anabasine. Upon exposure to the anabasine, at different concentrations, an upregulation of gene expression was observed for many of the candidate genes, which were chosen due to their usefulness as biomarkers for cytotoxic damage and carcinogenesis. Notably, this upregulatory effect was also seen across all cell lines.
Upon exposure to 10 µg/mL anabasine, the normal, male cell line, showed no significant change in expression levels of the seven candidate genes analyzed (Table 1.5), however, the cancer cell line displayed an upregulatory response in gene expression for the candidate genes: AHR, ALDH3A1, CEACAM6, CYP1A1, and GPX2, at the 96-hour time point. The most significant increase in expression seen within the cancer cell line was in ALDH3A1, where a 19.3-fold increase was observed, when normalized to the control. The normal, female cell line also displayed this upregulatory response in expression as well, for the candidate genes: ALDH3A1, CEACAM6, GPX2, and TP53, at the 48-hour time point. Within the normal, female cell line, the highest increase in expression was also observed in ALDH3A1, where there was a 6.81-fold increase in expression when normalized to the control.

Figure 22 presents a volcano plot summarizing the significant findings from the 10 µg/mL exposure on the cell lines. Though differentially expressed genes, CEACAM6 and GPX2, common to both the normal, female cell line and the cancer cell lines are highlighted within the plot together, AHR, also highlighted within the plot, was only differentially expressed within the cancer cell line. The volcano plot can quickly be interpreted for gene expression relative to control by visualizing any plot to the right of the vertical, green dotted line as at least a + 4.0-fold change in expression for that gene, and any plot to the left of the vertical, red dotted line as at least a – 4.0-fold change in expression for that gene.
Following the normal, female cell line and cancer cell line, at 100 µg/mL anabasine exposure, the normal male cell line also started to display significant upregulation within the candidate genes serving as disease biomarkers. An upregulatory response in expression of \textit{ALDH3A1}, \textit{CEACAM6}, \textit{CYP1A1}, \textit{GPX2}, \textit{MDM2}, and \textit{TP53}, was seen in the normal male cell line at the 48-hour time point (Figure 23), with \textit{GPX2} evaluated as having the highest increase in expression, displaying a 15.8-fold increase, when normalized to the control. The normal female cell line, when exposed to 100 µg/mL anabasine, showed a surprising downregulatory response in expression of \textit{MDM2} at the 48-hour time point, with a -4.70-fold decrease in expression recorded, when normalized to the control.
Lastly, the cancer cell line showed insignificant changes in expression within the seven candidate genes (Table 1) upon exposure to 100 µg/mL anabasine. Significant findings in differential gene expression, after exposure to 100 µg/mL anabasine, are featured within the volcano plot, Figure 23, listed below, where there was no common upregulation or downregulation seen within the either the normal, female or normal, male cell lines.

![Volcano Plot](image)

**Figure 23: Relative Normalized Gene Expression with Exposure to Anabasine at 100µg/mL.**

Data obtained at 48 hours and 96 hours, on the three experimental cell lines, under the exposure of anabasine at the 100 µg/mL concentration, was analyzed by qRT-PCR for differential gene expression of seven candidate genes. Only cell lines displaying significant differential expression of candidate genes are shown. All values obtained for CCL-171 and CCL-186 were obtained at the 48 hr time-point. Legend: Blue horizontal line= p-value threshold of 0.01; Red vertical dotted line= -4.0-fold change in expression and green vertical dotted line= +4.0-fold change in expression, any gene expression data lying in between the two values was evaluated as insignificant; Blue transparent oval= indicative of data for CCL-171; Red transparent oval= indicative of data for CCL-186.

**Cotinine**

In contrast to anabasine exposure, lung cells exposed to cotinine at varying concentrations showed limited findings, with the only significant upregulation in gene
expression seen within the cancer cell line. Upon exposure to 10 µg/mL cotinine, the normal, male cell line, and the normal, female cell line, displayed no significant changes in the seven candidate genes analyzed (Table 1). The cancer cell line displayed an upregulatory response, at the 48-hour time-point, in AHR, ALDH3A1, CYP1A1, GPX2, MDM2, and TP53. The greatest increase in expression was seen in ALDH3A1, displaying a 15.1-fold increase in expression, when normalized to the control. Figure 24 depicts these significant values for differential gene expression within the cancer cell line, below.

![Differential Gene Expression When Exposed to Cotinine at 10 µg/mL](image)

**Figure 24: Relative Normalized Gene Expression with Exposure to Cotinine at 10µg/mL.**

Data obtained at 48 hours and 96 hours, on the three experimental cell lines, under the exposure of cotinine at the 10 µg/mL concentration, was analyzed by qRT-PCR for differential gene expression of seven candidate genes. Only cell lines displaying significant differential expression of candidate genes are shown. All values obtained for CCL-185 were obtained at the 48 hr time-point. Legend: Blue horizontal line= p-value threshold of 0.01; Red vertical dotted line= -4.0-fold change in expression and green vertical dotted line= +4.0-fold change in expression, any gene expression data lying in between the two values was evaluated as insignificant; Green transparent oval= indicative of data for CCL-185.

At the 100 µg/mL concentration of cotinine, both the normal, male cell line, and the cancer cell line, showed no significant changes in expression for the seven candidate
genes analyzed (Table 1). Oddly, the normal, female cell line, when exposed to the alkaloid at the 100 µg/mL concentration, portrayed a decrease in expression, at the 48-hour time-point, in AHR, ALDH3A1, CEACAM6, CYP1A1, GPX2, MDM2, and TP53. The most prominent decrease in expression seen within the normal, female cell line was observed in GPX2, where there was a decrease in fold change of -9.04 when normalized to the control. Figure 25 features a volcano plot pertaining to this decrease in gene expression within the normal, female cell line.

Figure 25: Relative Normalized Gene Expression with Exposure to Cotinine at 100µg/mL.
Data obtained at 48 hours and 96 hours, on the three experimental cell lines, under the exposure of cotinine at the 100 µg/mL concentration, was analyzed by qRT-PCR for differential gene expression of seven candidate genes. Only cell lines displaying significant differential expression of candidate genes are shown. All values obtained for CCL-186 were obtained at the 48 hr time-point. Legend: Blue horizontal line= p-value threshold of 0.01; Red vertical dotted line= -4.0-fold change in expression and green vertical dotted line= +4.0-fold change in expression, any gene expression data lying in between the two values was evaluated as insignificant; Red transparent oval= indicative of data for CCL-186.
**Nicotine**

Perhaps the most surprising results found within the gene analysis studies were the limited, significant differences in gene expression examined within the cells, upon exposure to the well-known tobacco alkaloid, nicotine. Upon exposure to 10 µg/mL nicotine, the normal, female cell line, and the cancer cell line, showed no significant changes in expression in the seven candidate genes analyzed (Table 1), when normalized to the control. The normal, male cell line, when exposed to the alkaloid at the 10 µg/mL concentration, only showed an increase in expression in three candidate genes: *ALDH3A1*, *GPX2*, and *TP53*, at the 48-hour time-point (Figure 26). Of these genes, the greatest increase in expression was recorded in *GPX2*, where there was a 5.73-fold increase in expression when normalized to the control. These significant findings within the normal, male cell line, are featured in the volcano plot, Figure 26, below.

![Figure 26: Relative Normalized Gene Expression with Exposure to Nicotine at 10µg/mL.](image)

Data obtained at 48 hours and 96 hours, on the three experimental cell lines, under the exposure of nicotine at the 10 µg/mL concentration, was analyzed by qRT-PCR for differential gene expression of seven candidate genes. Only cell lines displaying significant differential expression of candidate genes are shown. All values obtained for CCL-171 were obtained at the 48 hr time-point. Legend: Blue horizontal line= p-value threshold of 0.01; Red vertical dotted line= -4.0-fold change in expression and green vertical dotted line= +4.0-fold change in expression, any gene expression data lying in between the two values was evaluated as insignificant; Blue transparent oval= indicative of data for CCL-171.
At the highest exposure of nicotine, 100 µg/mL, findings were surprisingly similar to that of cotinine at the highest concentration, where the normal, female cell line displayed a primarily downregulatory response. In contrast, the normal, male also displayed downregulation in at least one gene and continued a response of upregulation in another. The normal, female cell line, when exposed to nicotine at the 100 µg/mL concentration, showed a decrease in expression of AHR, ALDH3A1, CEACAM6, GPX2, MDM2, and TP53, at the 48-hour time point. The greatest decrease was evaluated to be in ALDH3A1, where there was a -6.69-fold change decrease, when normalized to the control.

The normal, male cell line, when exposed to nicotine at the 100 µg/mL concentration, also showed a -5.08-fold-change decrease in expression for ALDH3A1, when normalized to the control, at the 96-hour time-point. The male cell line also showed a 4.06-fold-change increase, at the 48-hour time-point, for GPX2, when normalized to the control. The volcano plot displayed in Figure 27, highlights these significant findings of differential gene expression within the normal, female and normal, male cell lines. Common genes that displayed a downregulatory response, between the two cell lines, include ALDH3A1, where the -6.7-fold value is indicative of the normal, female lung cell line, and the -5.1-fold value is indicative of the normal, male lung cell line.
The aim of this study was to evaluate and analyze potential adverse effects of electronic cigarette filling solutions on the growth and viability of human lung cells in culture. This was to be carried out by investigating differences in cell proliferation and gene expression among a panel of lung tissue cell lines that were exposed to the tobacco alkaloids: anabasine, cotinine, myosmine, and nicotine, which have been recognized in previous studies to be within e-cigarette filling solution (Trehy et al., 2011). We hypothesized, based on prior research indicating tobacco alkaloids as contributing factors to diseased states of the human body upon their exposure (Hecht & Hoffmann, 1988; McBride et al., 1998; Wilp et al., 2002) that these four tobacco alkaloids would

**DISCUSSION**

![Figure 27: Relative Normalized Gene Expression with Exposure to Nicotine at 100µg/mL.](image)

Data obtained at 48 hours and 96 hours, on the three experimental cell lines, under the exposure of nicotine at the 100 µg/mL concentration, was analyzed by qRT-PCR for differential gene expression of seven candidate genes. Only cell lines displaying significant differential expression of candidate genes are shown. All values obtained for CCL-171 were obtained at the 48 and 96 hr time-points. Values shown for CCL-186 were obtained at the 48 hr time point. Legend: Blue horizontal line= p-value threshold of 0.01; Red vertical dotted line= -4.0-fold change in expression and green vertical dotted line= +4.0-fold change in expression, any gene expression data lying in between the two values was evaluated as insignificant; Blue transparent oval= indicative of data for CCL-171. Red transparent rectangle= indicative of data for CCL-186.
demonstrate an effect on the normal proliferation and viability of lung cells and modify normal gene expression patterns in a manner that is both sex-specific and/or disease specific. We further hypothesized that these changes in cellular growth and gene expression patterns could offer insight to potential risk factors for lung disease associated with exposure to e-cigarette compounds.

**Effect of Alkaloid Exposure on Normal Cell Proliferation**

Of the four alkaloids tested within this study, exposure to anabasine and myosmine displayed the most notable significance in differential cellular proliferation and viability within the panel of lung cell lines examined. Though brief, significant, increases in cellular proliferation were observed after individual exposure to the two individual alkaloids, anabasine and myosmine, ultimately, exposure to each alkaloid resulted in a pattern of decreased cellular proliferation and viability within the lung cell lines. Myosmine, in particular, presented the most significant results of this inhibition on cellular growth, where surprisingly, exposure to this alkaloid at the highest concentration studied, resulted in a complete loss of cellular viability within all three of the lung cell lines (Figure 19).

In contrast to the results seen with anabasine and myosmine, exposure to the well-known tobacco alkaloid, nicotine, and its metabolite, cotinine, resulted in a more varied response among the panel of lung cell lines, where few trends were able to be observed. For example, both nicotine and cotinine were seen to individually demonstrate both stimulatory and inhibitory effects on the normal, female cell line, and the cancer cell line, and these effects differed not only between the different lung cell lines, but also between different concentrations, and time-points. The only trend that was somewhat observed,
however, was that of a slight, stimulatory effect seen on the normal, male cell line, by both nicotine and cotinine, individually, and more of an inhibitory effect on the cancer cell line, by cotinine.

Examination of Differential Gene Expression Patterns Associated with Alkaloid Exposure

Seven candidate genes were investigated within this study to serve as biomarkers for potential disease risks that may be associated with exposure to the tobacco alkaloids, anabasine, cotinine, and nicotine, within the panel of lung cell lines studied. Table 1 within this study outlines the potential biomarker function for each of the seven genes analyzed, and the results of those that were differentially expressed are visualized in Table 8 below.

Table 8: Differential Expression of Candidate Genes by Lung Tissue Cell Lines

<table>
<thead>
<tr>
<th>Gene</th>
<th>10 µg/mL Concentration</th>
<th>100 µg/mL Concentration</th>
</tr>
</thead>
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<tr>
<td></td>
<td>CCL-185</td>
<td>CCL-171 ♀</td>
</tr>
<tr>
<td></td>
<td>Anabasine</td>
<td>Cotinine</td>
</tr>
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<td>AHR</td>
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<td>Green</td>
</tr>
<tr>
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<td>Green</td>
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<tr>
<td>CYP1A1</td>
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<td>Green</td>
</tr>
<tr>
<td>MDM2</td>
<td>Green</td>
<td>Green</td>
</tr>
<tr>
<td>TP53</td>
<td>Green</td>
<td>Green</td>
</tr>
<tr>
<td>ALDH3A1</td>
<td>Green</td>
<td>Green</td>
</tr>
<tr>
<td>GPX2</td>
<td>Green</td>
<td>Green</td>
</tr>
</tbody>
</table>

Red=indicative of downregulation; Green=indicative of upregulation; Grey=indicative of no differential expression observed; All time points (48 hr and 96 hr) are included within the table.
Of the tobacco alkaloids studied by exposure on the lung cell lines, anabasine seemed to induce the most significant features of abnormal, differential gene expression, by displaying upregulation in practically all seven candidate genes, chosen for their use as biomarkers for cytotoxic damage and oxidative stress (GPX2, ALDH3A1, TP53), as well as potential carcinogenesis (CYP1A1, CEACAM6, MDM2, TP53), among all three of the lung cell lines, at varying concentrations and time-points within the study. This trend was followed by cotinine, which also displayed significant upregulation in gene expression, within all seven of the candidate genes, except for the tumor suppressor, TP53. This trend was specifically observed, however, within the cancer cell line, at exposure to the lower concentration of cotinine. Interestingly, as a contrast in response, significant downregulation of the exact same candidate genes was also observed within the normal, female cell line upon exposure to cotinine at the highest concentrations.

Unexpectedly, of all the alkaloids examined, nicotine was perhaps the least influential on differential expression of the seven candidate genes studied. The only real significance in upregulatory response was seen within three genes: ALDH3A1, GPX2, and TP53, and only within the normal, male cell line at exposure to 10 µg/mL nicotine. Interestingly still, significant downregulation within the majority of the candidate genes, with the exclusion of TP53, was once again seen in the normal, female cell line, at exposure to the highest concentration of nicotine, which was similar to the effect observed within the normal, female cell line from exposure to the highest concentration of cotinine.
Potential Risk of Disease Pathology Associated with Alkaloid Exposure

**Anabasine and Myosmine**

Resultant cellular proliferation and viability observed within the lung cell lines exposed to anabasine and myosmine, individually, displayed a downward trend in cell growth and viability. This trend could be suggestive of an environment within the lung tissue that is similar to diseased states such as COPD and other respiratory illnesses, where an increased apoptotic cellular response is hallmark (Henson, Vandivier, & Douglas, 2006; U.S. Department of Health and Human Services, 2010). The brief increase that was also observed in cellular proliferation after exposure to the two individual alkaloids could also be suggestive that anabasine/myosmine could possibly support a state of carcinogenesis within lung tissue by suppressing apoptosis. Interestingly, this possibility is supported by prior research indicating both anabasine and myosmine of reacting with nitrosating agents to form carcinogenic compounds (Hecht & Hoffmann, 1988; Wilp et al., 2002), and also by gene expression analysis studies carried out for anabasine exposure on the lung cell lines.

Though myosmine was unable to be studied in gene expression analysis due to the loss of cell viability when the lung cell lines were treated with higher concentrations of the alkaloid, anabasine was able to be analyzed, and the resultant differential gene expression data expectantly followed a similar trend as the resultant cell proliferation data obtained by the cell lines exposed to anabasine. Upregulation that was seen in the xenobiotic metabolizing protein producing genes: \textit{ALDH3A1}, \textit{CYP1A1}, and \textit{AHR}, as well as cytotoxic biomarkers such as \textit{GPX2}, could possibly be suggestive of oxidative stress and cellular damage within the cell (Patel et al., 2008; Yan & Chen, 2006). Also, the upregulation that
was observed in at least one or all of the oncogenes and proto-oncogenes, *CEACAM6*, *CYP1A1*, *AHR*, and *MDM2*, upon exposure at varying concentrations and time points, as well as upregulation of the tumor suppressor gene, *TP53*, could also be possibly suggestive of a state which is supportive of carcinogenesis within the cell (Blumenthal et al., 2007; Chauhan et al., 2016; Hartl, 2012; Jones et al., 1998; McLemore et al., 1990; Nakachi et al., 1993).

**Nicotine and Cotinine**

In contrast to anabasine and myosmine, exposure of the lung cell lines to nicotine, and its metabolite, cotinine (individually), resulted in very diverse cell proliferation and viability data, as well as gene expression analysis. The only real trend observed that could be suggestive of an increased risk of disease, when taking both cellular proliferation and gene expression analysis data into consideration, was the effect seen on the cancer cell line, by exposure to cotinine. Upregulation of almost all seven candidate genes, upon exposure to cotinine, was observed within the cell line, coupled with a significant downward trend seen in cell proliferation and viability. These findings could be suggestive of non-cancerous diseased states within lung tissue, which could be supported by the upregulation observed in the cytotoxic and oxidative stress biomarkers, *GPX2* and *ALDH3A1* (Gebel et al., 2004; Patel et al., 2008; Yan & Chen, 2006). The findings could also, however, be suggestive of possibly the beginnings to a more carcinogenic state within the lung cells, which could be supported by the observance of significant upregulation within the proto-oncogenes/oncogenes, *CYP1A1*, *CEACAM6*, *MDM2*, and *AHR* (Blumenthal et al., 2005;
In regards to nicotine, the normal, male cell line did display slightly differential cell proliferation as well as differential expression of candidate genes, which could be indications of disease risk, however, the results for the gene expression analysis studies were still very close to control, and any upregulation that was displayed was only observed in a few genes. These few genes the upregulation was observed in were biomarkers that are mostly suggestive of slight cytotoxic damage and/or oxidative stress.

**Differences Seen Across Gender and Disease Status Upon Alkaloid Exposure**

In regards to differences in cellular proliferation and expression of candidate genes, across gender, when exposed to the four alkaloids observed within this study, the most significant differences were seen within the normal, female cell line, when exposed to the highest concentration of nicotine, and for the highest concentration of cotinine. When exposed to either 100 µg/mL nicotine or 100 µg/mL cotinine, a downregulation in expression of almost all candidate genes, excluding TP53, was observed within the normal, female cell line. These findings could possibly be suggestive of a more cytotoxic response of the cell line, in comparison to the normal, male cell line, upon exposure to higher concentrations of nicotine and/or cotinine.

In regards to differences observed in cellular proliferation and expression of candidate genes across disease status, when exposed to the four alkaloids, the most significant differences were seen upon exposure of cotinine to the cancer cell line. At
exposure to 10 µg/mL cotinine, the cancer cell line was observed to display upregulation in all but one of the seven candidate genes analyzed. Neither the normal, male cell line, nor the normal, female cell line displayed this upregulatory response in expression within the candidate genes, upon exposure to the alkaloid, at any concentration.

CONCLUSION

In conclusion, the tobacco alkaloids, anabasine and myosmine found within e-cigarette filling solution, have been shown within this study to cause abnormal gene expression among lung tissue cells, within candidate genes serving as biomarkers for tobacco-smoke induced diseases of the lung, as well as carcinogenesis of the lung, and/or to cause differential cellular proliferation and viability among the lung tissue cell lines studied. Surprisingly, the most well-known of the tobacco alkaloids, nicotine, as well its lesser known metabolite, cotinine, were shown to have minimal effect on the lung tissue cells. Of the two, only the alkaloid, cotinine, presented the possibility of an increased risk of disease and carcinogenesis, but only among the already diseased, cancer cell line that was utilized within this study.

In regards to differential cellular response to the alkaloids, presenting in a sex-specific manner, little support was seen within the cellular proliferation and viability results that could firmly be attributed to differences due to gender alone, and differential expression for the candidate genes, TP53 and CYP1A1, utilized for analyzation of any gender differences, was also not observed within the study. However, the normal, female cell line did, in comparison to the normal, male cell line, show a downregulation in most of the analyzed candidate genes, upon exposure to the higher concentrations of nicotine.
and cotinine. The normal, female cell line also displayed an earlier response in differential cellular proliferation and abnormal gene expression, from exposure to anabasine. These results could possibly indicate an increased susceptibility in women to toxicity from exposure to the three alkaloids, nicotine, cotinine, and anabasine, at lower concentrations than what would cause the same effect in males. Further research supporting these conclusions would be necessary, however, due to the limited findings.

LIMITATIONS/FUTURE AIMS

In this pilot study examining the effects of tobacco alkaloid exposure on lung cell proliferation, variable concentrations of each alkaloid were tested on a panel of lung cell lines that differed with respect to gender and disease status. Moving forward, taking in consideration the findings from this report, we recommend repeating the study with focus on the 10 μg/mL exposure concentration, which was the dose that resulted in a measurable effect on cell proliferation, but did not cause cell death. At this concentration, we can then begin to address any synergistic or agonistic effects of exposure of the alkaloids in combination. Ideally, however, 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 more readily mimics in vivo exposure to the e-cigarette filling solution, once it becomes an aerosol that is inhaled by the e-cigarette user.

Another concern within this study was the growing pattern of the normal, female lung tissue cell line, which seemed to be lower in its normal cellular proliferation growth
curve than that of the other two lung tissue cell lines used within the study. The normal, female lung tissue cell line was also the only lung tissue cell line used within this study, that showed downregulation of candidate genes. In order to ensure that the observation of differences seen between the normal, female lung tissue cell line and that of the male, was due to gender differences alone, the incorporation of another normal, female cell line should be used, in order to support or oppose results seen within this study concerning the results obtained for the normal, female cell line, CCL-186.
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doi:10.1016/j.lungcan.2007.08.033


### Appendix 1: Two-Tailed Student T-Test Results

#### Statistical Evaluation of the Effect of Tobacco Alkaloid Exposure on Cell Proliferation

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<th>Day</th>
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<th>100 µg</th>
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<tr>
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<td></td>
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#### Tobacco Alkaloids

- **Anabasine**
- **Cotinine**
- **Myosmine**
- **Nicotine**

### Test Results

- Statistical Evaluation of the Effect of Tobacco Alkaloid Exposure on Cell Proliferation

### APPENDICES
Appendix 2: Cell Growth with Exposure to 1 µg/mL Anabasine, Normalized to Control.

Data obtained daily on the three experimental cell lines, under the exposure of anabasine at the 1 µg/mL concentration, by the CellTiter-Glo® luminescent viability assay, was normalized to the control by applying an equation of percent difference for each time-point within the 10-day time course. The legend indicates the associated colors for the three varying cell lines: normal male = CCL-171, normal female = CCL-186, and cancerous male = CCL-185.
Appendix 3: Cell Growth with Exposure to 1 μg/mL Cotinine, Normalized to Control

Data obtained daily on the three experimental cell lines, under the exposure of cotinine at the 1 μg/mL concentration, by the CellTiter-Glo® luminescent viability assay, was normalized to the control by applying an equation of percent difference for each time-point within the 10-day time course. The legend indicates the associated colors for the three varying cell lines: normal male = CCL-171, normal female = CCL-186, and cancerous male = CCL-185.
Appendix 4: Cell Growth with Exposure to 1 µg/mL Myosmine, Normalized to Control

Data obtained daily on the three experimental cell lines, under the exposure of myosmine at the 1 µg/mL concentration, by the CellTiter-Glo® luminescent viability assay, was normalized to the control by applying an equation of percent difference for each time-point within the 10-day time course. The legend indicates the associated colors for the three varying cell lines: normal male = CCL-171, normal female = CCL-186, and cancerous male = CCL-185.
Appendix 5: Cell Growth with Exposure to 1 µg/mL Nicotine, Normalized to Control

Data obtained daily on the three experimental cell lines, under the exposure of nicotine at the 1 µg/mL concentration, by the CellTiter-Glo® luminescent viability assay, was normalized to the control by applying an equation of percent difference for each time-point within the 10-day time course. The legend indicates the associated colors for the three varying cell lines: normal male = CCL-171, normal female = CCL-186, and cancerous male = CCL-185.