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Effects of World Trade Center Particulate Matter From September 11, 2001 on Biochemical Pathways Associated with Apoptosis and Cell Viability

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Abstract

The dust that resulted from the collapse of the World Trade Center (WTC) towers on September 11th, 2001 contained organic and inorganic compounds. The survivors and first responders were breathing these compounds while fleeing and rescuing others. Some of the particles that were inhaled were small enough to affect the alveolar region of the lungs. This study looks at the effect that World Trade Center dust has on MRC-5 human lung fibroblasts. Cell viability is observed over time using an alamarBlue® assay. Previous studies have shown that MRC-5 cells that are exposed to WTC dust lose viability after 24 hours (Hernandez, Choi, & DiLorenzo, 2012). However, these studies only observed the viability of the cells at one time point. This study is different in that it shows that some viability of the cells that were exposed to WTC dust is regained at 48 hours. Western blots are performed to determine if the cells are producing proteins that would indicate apoptosis due to exposure to the dust. Western blots are performed to also observe the effect that the dust is having on the mitogen-activated protein kinases (MAPKs) c-Jun N-terminal protein kinase (JNK), p38, and extracellular signal-related kinase 1/2 (ERK1/2). The effect of the WTC dust on the MAPKs will be observed because these three proteins are involved in cell growth and apoptosis. ERK1/2 is also involved in survival and cell cycle progression. ERK1/2 is also known to have a part in cancer cell proliferation. JNK and p38 are involved also involved in inflammation. If these proteins are being expressed it could mean that survivors or first responders could be experiencing, or have experienced, one or more of these effects. Further studies should be performed to determine what immunological responses the MRC-5 human lung fibroblasts are having after being exposed to the WTC dust.

MONTCLAIR STATE UNIVERSITY

EFFECTS OF WORLD TRADE CENTER PARTICULATE MATTER FROM SEPTEMBER 11,

2001 ON BIOCHEMICAL PATHWAYS ASSOCIATED WITH APOPTOSIS AND CELL

VIABILITY

by

Eric Tobias

A Master's Thesis Submitted to the Faculty of

Montclair State University

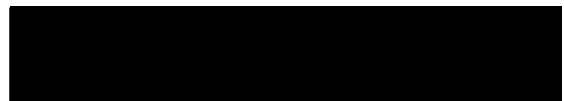In Partial Fulfillment of the Requirements

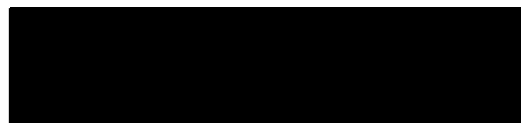For the Degree of

Master of Science

August 2018

College/School College of Science and Mathematics   Thesis Committee:

Department Biology

Dr. Ann Marie DiLorenzo
Thesis Sponsor

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Committee Member

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EFFECTS OF WORLD TRADE CENTER PARTICULATE MATTER FROM

SEPTEMBER 11, 2001 ON BIOCHEMICAL PATHWAYS ASSOCIATED WITH

APOPTOSIS AND CELL VIABILITY

A THESIS

Submitted in partial fulfillment of the requirements

For the degree of Master of Science

by

Eric Tobias

Montclair State University

Montclair, NJ

2018

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Introduction

   The collapse of the World Trade Center (WTC) towers that took place on September 11th, 2001 resulted in high concentrations of dust and particulate matter that were toxic to humans. This particulate matter and dust blanketed most of lower Manhattan, New York as seen in Figure 1. Many first responders, residents, and others were exposed to this dust. This resulted in these people being affected psychologically and physically.
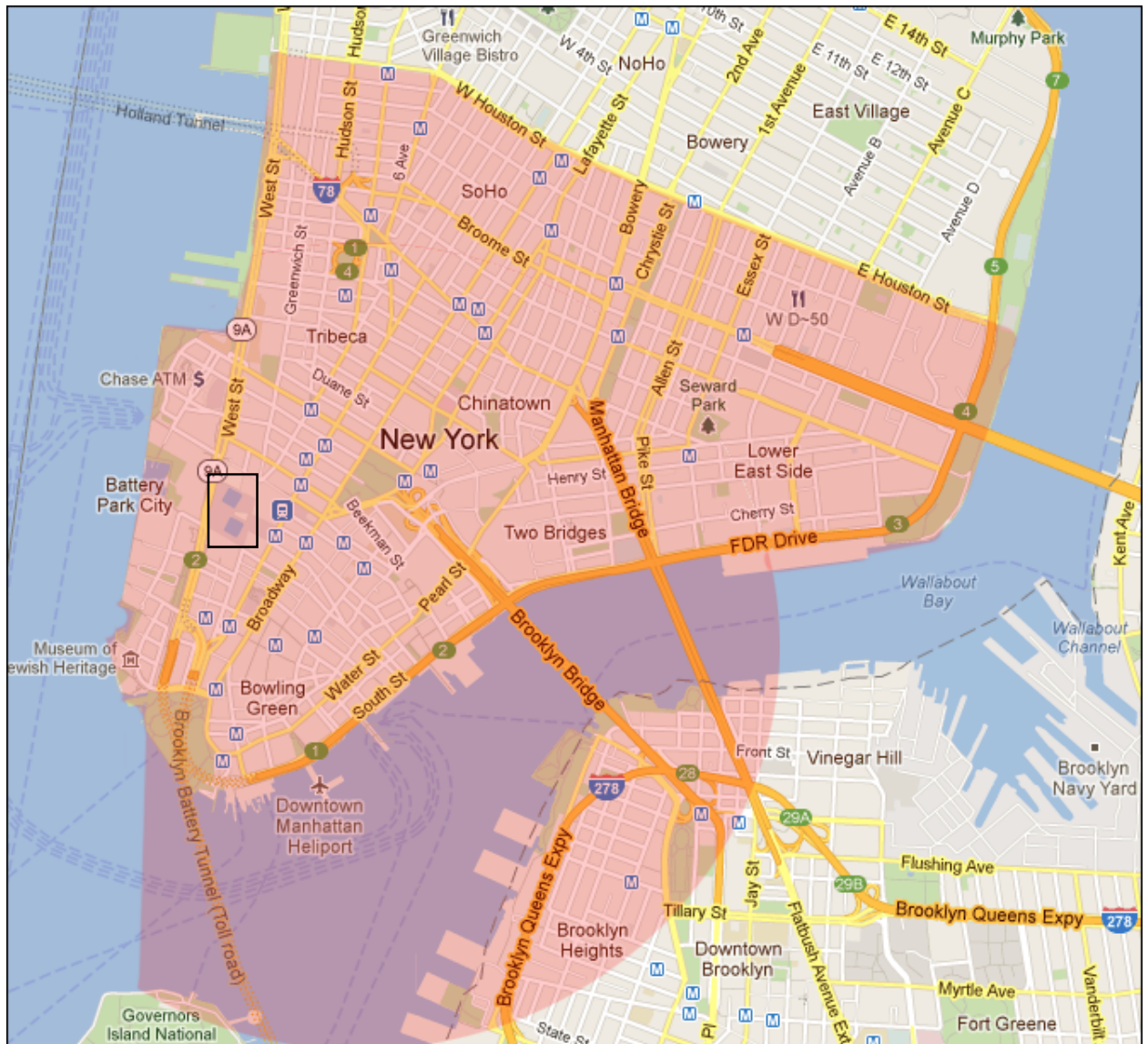
Figure 1. Area of Manhattan, New York that was affected by the WTC disaster. Ground Zero is outlined in black (from https://www.cdc.gov/wtc/define.html).

Composition Of The World Trade Center Dust

Dr. Paul Lioy and his team collected dust samples and analyzed them to determine the dust's inorganic and organic compositions. The results of the inorganic analysis showed the presence of metals, radionuclides, ionic species, asbestos, and inorganic species. The results of the organic species showed the presence of polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls, polychlorinated dibenzodioxins, polychlorinated dibenzofurans, pesticides, phthalate esters, brominated diphenyl esters, and other hydrocarbons (Lioy et al., 2002).

Element	ng/g
Hg	0
Tl	1,290
Cs	1,327
Bi	1,466
Ag	2,247
As	2,613
Be	3,754
U	4,213
Cd	7,459
Co	10,460
Rb	21,710
Li	29,520
Ga	34,060
V	42,610
Ni	47,290
Cr	171,500
Mg	179,000
Pb	289,200
Cu	325,600
Ba	406,500
Sr	720,800
Mn	828,100
Al	908,700
Ti	1,797,000
Zn	2,992,000
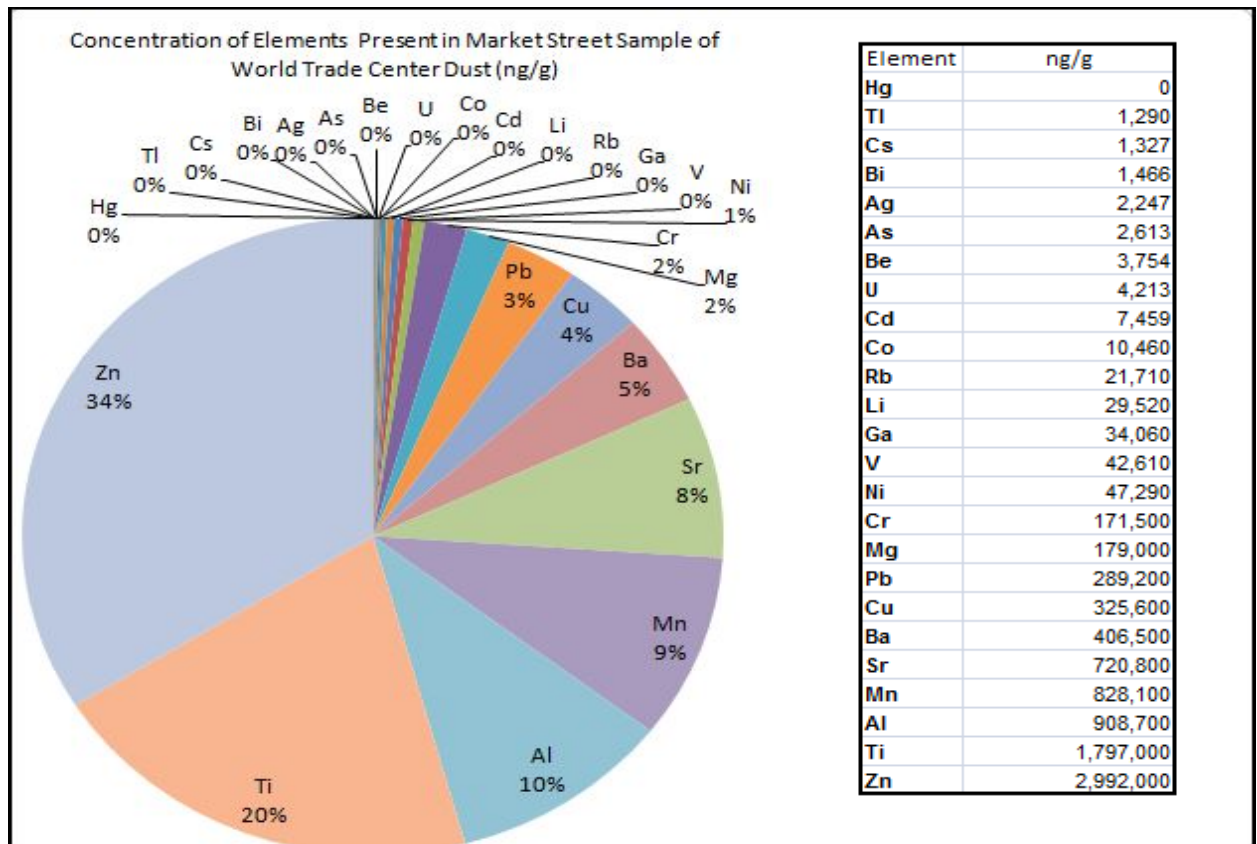
Figure 2. Relative concentrations in the Market Street Sample of World Trade Center dust. (adapted from Lioy et al., 2002)

Effects Of The WTC Dust On The Respiratory System

Three regions make up the respiratory system. The nasopharygeal region is composed of the nose, mouth, larynx, and pharynx. The tracheobronchial region is composed of the trachea, bronchi, and bronchioles. The alveolar region is composed of the alveoli which is where the exchange of oxygen and carbon dioxide takes place ("Anatomy of the Human Respiratory System", n.d.).

Particulate matter (PM) is composed of solid or liquid airborne particles that are micometers in diameter ("Particulate Matter (PM) Basics", 2016). WTC dust particles that were 3-10 μm in diameter affected the nasopharyngeal region. Particles that were 5-10 μm in diameter affected the nose and throat region, while particles that were 3-5 μm in diameter affected the trachea. WTC dust particles that were 1-3 μm in diameter affected the tracheobronchial region. Particles that were 2-3 μm in diameter affected the bronchi, while particles that were 1-2 μm in diameter affected the bronchioles. WTC dust particles that were less than or equal to 1 μm affected the alveolar region. Particles that were 0.1-1 μm in diameter affected the alveoli. (Oberdörster et al., 2005).
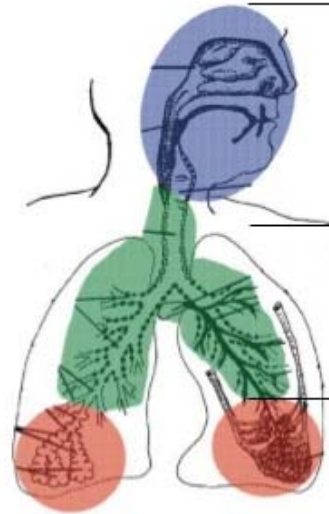
Figure 3. The size of WTC PM affected different regions of the respiratory system based on the particle's diameter. (adapted from Oberdörster et al., 2005)

Project Description

The goal of this project is to observe how WTC dust affects cell viability and/or apoptosis, expression of proteins associated with intrinsic apoptosis, and expression of proteins associated with inflammation. To determine whether or not the WTC dust was having an effect on cell death or cell viability an alamarBlue® assay will be performed. The proteins associated with intrinsic apoptosis that will be measured will be p53, Bax, cleaved caspase-3, and cleaved caspase-6. The proteins associated with inflammation that will be measured will be ERK1/2, p38, and JNK.. The expression of these proteins kinases will be measured by western blot. The ERK1/2, p38, and JNK are activated upon phosphorylation. The western blots will look for phospho-ERK1/2, phospho-p38, and phospho-JNK to see the respective activity of these kinases.

<u>Cell Viability</u>

The first goal of this project is to determine how the WTC dust affected cell viability. This will be done by performing an alamarBlue® assay (Thermo Fisher Scientific, Waltham, MA). The active compound for this assay is resazurin (Munshi, Twining, & Dahl, 2014). This molecule is permeable across the cell membrane, non-fluorescent, and blue in color. Once inside the cell, resazurin is then reduced to resorufin, a molecule that is fluorescent and red in color. One way that resazurin can be reduced to resorufin is by NADH dehydrogenase (O'Brien, Wilson, Orton, & Pognan, 2000).

NADH dehydrogenase is Complex I of the electron transport chain. It is located in the inner membrane of the mitochondria. The way NADH dehydrogenase normally functions is that it oxidizes NADH to NAD^+ while transferring two electrons from the N-

2 Fe-S center to ubiquinone which reduces it to ubiquinol. While this electron transfer is taking place, four protons are being pumped from inside the mitochondrial matrix into the intermembrane space (Treberg & Brand, 2011). Cells that are viable will be able to constantly perform the oxidation-reduction reaction that reduces resazurin to resorufin resulting in the emission of a fluorescent wavelength that can be measured. The more viable cells that are present, the more fluorescent emission wavelength will be produced.

Apoptosis

It is not known if the cells will undergo apoptosis, but if loss of cell viability is observed the possibility of apoptosis must be examined. Apoptosis is programmed cell death. It is a normal process that takes place in organisms that helps to control cellular populations within an organism. Apoptosis can take place to defend an organism in the event of cellular stress and to prevent cancer from developing. There are two main pathways by which apoptosis occurs: the intrinsic pathway and the extrinsic pathway. This research will be focused on the intrinsic pathway.

The intrinsic pathway of apoptosis occurs when intracellular signals are made and received within a cell. Some examples of pro-apoptotic signals that can cause a cell to undergo apoptosis through the intrinsic pathway are: toxins, free radicals, viral infections, radiation, hypoxia, and hyperthermia. Certain signals can inhibit apoptosis from proceeding. Some examples of these anti-apoptotic signals are cytokines, hormones, and growth factors. When the signals that inhibit apoptosis are not present, apoptosis can take place (Elmore, 2007).

When pro-apoptotic signals are present, or anti-apoptotic signals are not present, changes can result in modifications to the inner mitochondrial membrane which can lead to the mitochondrial permeability transition pore becoming more open, a reduction in the mitochondrial transmembrane protein potential, and the expulsion of pro-apoptotic proteins into the cytosol from the mitochondrial intermembrane space (Elmore, 2007).

One of the main pro-apoptotic proteins that is released from the mitochondria into the cytosol is cytochrome *c*. Cytochrome *c* is a water-soluble, hemeprotein. It is a part of the electron transport chain. It is able to carry one electron from Complex III to Complex IV. Cytochrome *c* is held in the mitochondrial inner membrane by binding to cardiolipin, forming a cardiolipin-cytochrome *c* complex. This attraction is electrostatic because of the positive charge that cytochrome *c* has, and is also hydrophobic due to a hydrophobic tail of cardiolipin interacting with the hydrophobic portion of cytochrome *c*. When apoptosis starts, mitochondrial reactive oxygen species are produced. This causes the cardiolipin to become oxidized which stops the binding of the cardiolipin-cytochrome *c* complex and releases cytochrome *c* into the cytosol through pores in the outer mitochondrial membrane (Orrhenius & Zhivotovsky, 2005).

Once cytochrome *c* is released into the cytosol it can then bind to monomers of apoptotic protease activating factor 1 (APAF-1). This binding promotes the extension of APAF-1. These monomers then arrange themselves to so that they are in a heptameric complex. This complex will then bind caspase-9 on each of the APAF-1 monomers which will activate the caspase-9 catalytic domain (Suen, Norris, & Youle, 2008). This activated caspase-9 can now activate caspase-3 by cleaving caspase-3 dimers. Activated

caspase-3 can then regulate downstream effects that cause parts of the cell to disassemble leading the cell to die (Ow, Green, Hao, & Mak, 2008).

Another pro-apoptotic protein that is released from the mitochondria into the cytoplasm is Caspase-Activated DNAse (CAD). CAD is found complexed to inhibitor of Caspase-Activated DNAse (ICAD) when cells are proliferating. During apoptosis activated caspase-3 releases CAD from the CAD-ICAD complex by cleaving ICAD at its caspase recognition sites which are located at Asp^{117} and Asp^{224} (Yuste et al., 2005). Once released from the CAD-ICAD complex, CAD then translocates to the nucleus to and proceeds to degrade chromosomal DNA and cause chromatin condensation. When the cell's DNA is cleaved it can no longer form proteins to carry out its various functions and will die.

Other pro-apoptotic proteins that are released from the mitochondria during apoptosis are apoptosis inducing factor (AIF) and endonuclease G. AIF translocates from the cytoplasm into the nucleus and will cause DNA fragmentation and chromatin condensation. Like AIF, endonuclease G will also translocate from the cytoplasm into the nucleus. When endonuclease G is in the nucleus it degrades chromatin. Both AIF and endonuclease G are able to carry out their functions without caspase activation (Elmore, 2007).

The Bcl-2 family, as presented in Figure 4, is a group of proteins that regulate the release of these apoptotic proteins from the mitochondria into the cytosol. The Bcl-2 protein family accomplishes this function by controlling how permeable the mitochondrial membrane is, thereby controlling the release of cytochrome c. The Bcl-2 family is made up of proteins that are both anti-apoptotic and pro-apoptotic. These

proteins contain common sequence homology in α-helical regions that are conserved among them. These conserved regions are known as Bcl-2 homology (BH) domains. Each of the anti-apoptotic proteins is a multi-domain protein, meaning they have more than one BH domain in common. There are pro-apoptotic proteins that are multi-domain, and there is a subset of the pro-apoptotic proteins known as BH3-only proteins, and these proteins only have the BH3 domain in common.

Some of the Bcl-2 family proteins that have an anti-apoptotic function include Bcl-2, Bcl-x, Bcl-XL, Bcl-XS, Bcl-w, BAG, MCL-1, Bcl-b, and BFL-1/A1. The Bcl-2 family proteins that have a pro-apoptotic function and are multi-domain are Bax and Bak. The Bcl-3 family proteins that have a pro-apoptotic function and are BH3-only include Bcl-10, Bid, Bad, Bim, Bik, Blk, Puma, Noxa, Bmf, and HRK (Aubrey, Kelly, Janic, Herold, & Strasser, 2017).
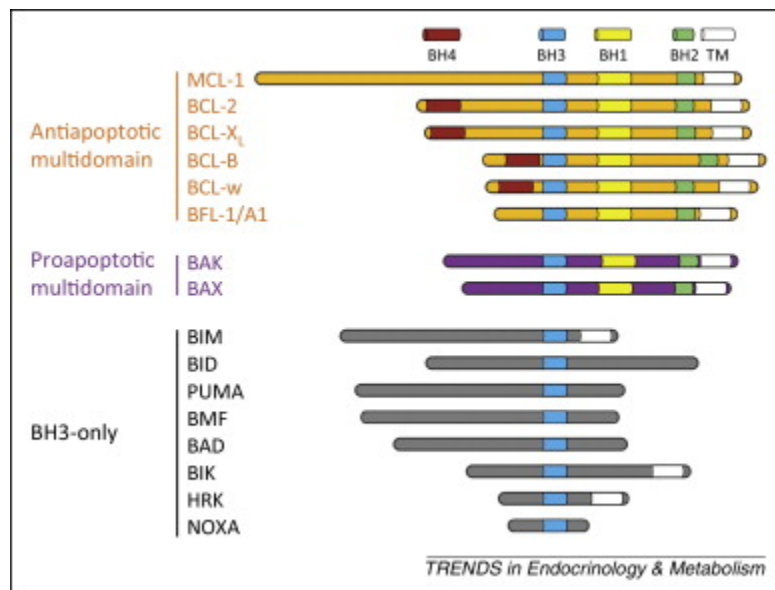
Figure 4. Some of the Bcl-2 family proteins. The proteins are organized as to whether or not they are multi-domain and anti-apoptotic or pro-apoptotic. The TM is an abbreviation for transmembrane domain (from Giménez-Cassina and Danial, 2015).

There are two types of BH3-only proteins. Bad and Noxa are known as sensitizers and inhibit the anti-apoptotic Bcl-2 proteins by binding to them. Bid, Bim, and Puma are known as activators and promote the oligomerization of Bax/Bak (Giménez-Cassina and Danial, 2015). When Bax/Bak have oligomerized the mitochondrial outer membrane become permeabilized and harmful proteins such as cytochrome c are released in the cytosol and apoptosis can proceed (Vaseva & Moll, 2009).

When a stress signal is present p53 that is in the cytoplasm will translocate to the mitochondria. The p53 protein will then interact with the pro-apoptotic and anti-apoptotic multi-domain members of the Bcl-2 family proteins (Danial & Korsmeyer, 2004). These interactions can either activate or inhibit the Bcl-2 family proteins. Mitochondrial p53 that is stress-induced antagonizes the mitochondrial outer membrane stabilizing activity of the Bcl-2, Bcl-XL, and Mcl-1 anti-apoptotic proteins. Under unstressed conditions Bak can be found in the mitochondrial outer membrane as part of an inhibitory complex with Mcl-1. Under stress conditions mitochondrial p53 will release Bak from the inhibitory complex it is part of and promote the oligomerization of Bak. Once Bak oligomerizes it can form pores in the mitochondrial outer membrane which will allow for cytochrome c to be released into the cytosol (Wolff, Erster, Palacios, & Moll, 2008).

The p53 gene is a tumor suppressor gene that codes for a tumor suppressor protein. The structure of the protein includes two N-terminal transactivation domains, a proline-rich domain, DNA binding domain, nuclear localization signal, tetramerization

domain, and a C-terminal regulatory domain which includes a nuclear export sequence. The p53 protein has different anticancer functions. Some of these anticancer functions include: activation of DNA repair proteins when DNA has been damaged; regulating the cell cycle at the G1/S checkpoint; and start apoptosis (Harris, 1996). The p53 protein can be regulated by post-translational modifications (Childs, Baker, Kirkland, Campisi, & van Deursen, 2014). Two of the post-translational modifications that regulate that regulate p53 are ubiquitination and phosphorylation (Chao, 2015).
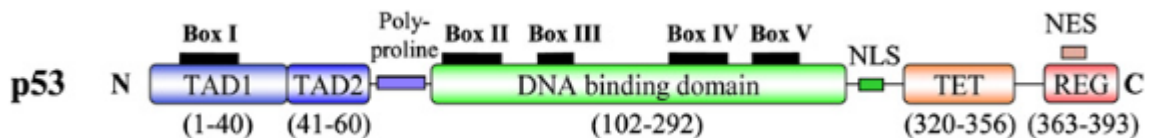
Figure 5. The domains of the p53 protein. TAD1 and TAD2 are the transcriptional activation domains. NLS is the nuclear localization sequence. TET is the tetramerization domain. REG is the C-terminal regulatory domain. NES is the nuclear export sequence (from Meek, 2015). Boxes I-V are regions of p53 that are conserved between different species (from Soussi & May, 1990).

When a cell is not under stress p53 is inactivated by the protein Mdm2 (Lukashchuk & Vousden, 2007). The p53 protein is able to activate Mdm2 expression transcriptionally through negative feedback (Wu, Bayle, Olson, & Levine, 1993). Under homeostatic conditions Mdm2 will bind to the TAD1 portion of the N-terminal domain of p53 and form a complex that can inhibit p53-dependent transactivation (Kulikov et al., 2010). Once both proteins have complexed with each other Mdm2 can act as an E3 ligase and ubiquitinate the C-terminal REG domain (Jenkins, Durell, Mazur, & Appella, 2012).

The p53 protein is then degraded by the 26S proteasome after it has been ubiquitinated (Tsvetkov, Reuven, & Shaul, 2010).

Another post-translational modification of p53 is phosphorylation. Upon phosphorylation p53 becomes activated and can inhibit gene expression for genes involved in cell survival. Many different proteins can phosphorylate p53. The mitogen-activated protein kinases (MAPKs) are among these proteins (Wu, 2004). The proteins that make up the MAPKs include c-Jun N-terminal protein kinase (JNK), p38, and extracellular signal-related kinase (ERK).

Mitogen-activated protein kinases (MAPKs)

Mitogen-activated protein kinases (MAPKs) are a group of protein kinases whose targets are the amino acids serine and threonine (Xu et al., 2016). These proteins phosphorylate the hydroxyl group of serine or threonine. These proteins help with the regulation of different cellular functions that include apoptosis, differentiation, stress response, motility, survival, and proliferation (Plotnikov, Zehorai, Procaccia, & Seger, 2010). The MAPKs of interest include c-Jun N-terminal protein kinase (JNK), p38, and extracellular signal-related kinase 1/2 (ERK1/2). Some of the stimuli that MAPKs respond to include proinflammatory cytokines, heat shock, osmotic stress, and mitogens (Kim & Choi, 2010).

The MAPKs function by cascade pathways as presented in Figure 6. These MAPKs have three main kinases associated with them: MAP kinase kinase kinase (MAP3K), MAP kinase kinase (MAP2K), and MAP kinase (MAPK) (Dhillon, Hagan, Rath, & Kolch, 2007). The downstream targets of each kinase is known as mitogen-

activated protein kinase-activated protein kinase (MAPKAPK). Signals are transmitted from one kinase to the next kinase by phosphorylation (Sabio & Davis, 2014).

The function of the JNK cascade as presented in Figure 6 is to mediate intracellular and extracellular stresses. JNK is able to phosphorylate substrates that are involved with apoptosis, insulin signaling, neuronal activity, and immunological effects. One of the functions of JNK is that it is able to phosphorylate the protein c-Jun. c-Jun is part of the transcription factor activator protein 1 (AP-1) (Norbury & Hickson, 2001). Some of the processes that AP-1 is involved with include apoptosis, differentiation, and cell growth.

The p38 cascade also presented in Figure 6 is another pathway that respond to extra-cellular stresses. The p38 protein is able to activate substrates that are involved with apoptosis, inflammation, and cell differentiation (Zarubin, 2005). One of the functions of p38 is to phosphorylate p53 after a cell has been exposed to UV radiation (Bulavin et al., 1999). The phosphorylation of p53 occurs at Ser^{33} and Ser^{46} (Boehme & Blattner, 2009).

The last item of interest in Figure 6 presents the ERK cascade which also responds to extra-cellular stresses (Wortzel & Seger, 2011). The ERK1/2 proteins are able to activate substrates involved with apoptosis, cell survival, cell proliferation, and cell determination (Shaul, Gibor, Plotnikov, & Seger, 2009). This cascade helps regulate cell survival through phosphorylation of Bcl-2 (McCubrey et al., 2007). Bcl-2 is an anti-apoptotic protein. However, the mechanism that helps promote cell survival is not currently known (Ruvolo, Deng, & May, 2001).
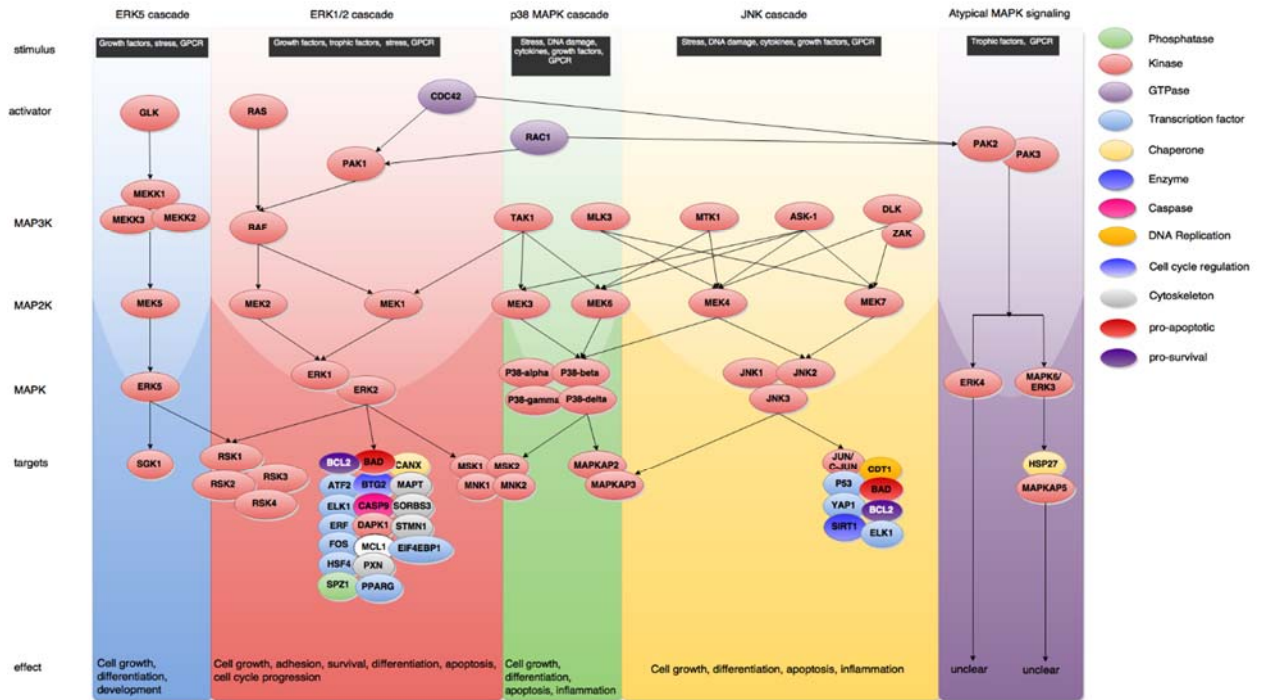
Figure 6. The MAPK signaling cascades. The different protein kinases of the different MAPK cascades are shown as well as their targets and what cellular function those targets are involved with. (from https://www.antibodies-online.com/mapk-signaling-pathway-3/)

The work presented in this thesis project will demonstrate the role of the WTC dust as a factor in cell viability and changes in production of proteins identified in the apoptosis and mitogen-activated protein kinase cascades. The data presented should clarify which specific proteins in these biochemical pathways of interest are affected by the WTC dust.

Materials & Methods

Dust Sample

The World Trade Center dust sample was received from Dr. Paul Lioy at Rutgers University. The WTC PM$_{2.5}$ was chosen because the size of the dust particles that penetrated the lungs was 2.5 µm. The WTC dust contains many components including ceiling tiles, concrete, glass fibers, wallboard, cement aggregate, etc. (Lioy et al., 2002).

Figure 7. An image of the WTC dust sample obtained from Dr. Paul Lioy.

Dust Sample Preparation

One gram of non-heat treated WTC dust was weighed out in a fume hood. The dust was then dissolved in sufficient media to create a 100 mL solution of "stock" WTC dust media. The stock media was subdivided adjusted to the 10% Fetal Bovine Serum (FBS) level and was then diluted to each of the experimental concentrations (25 and 125 ppm).

Media Preparation

Eagle's Minimal Essential Media (MEM), 1% of Penicillin Streptomycin (PS), Glutamax (G), Kanamycin (K) and 10% (FBS) were used to prepare WTC stock dust solutions to make a 100 mg mL^{-1} stock solution. Exposure to UV radiation to the WTC dust sample was necessary for sterilization.
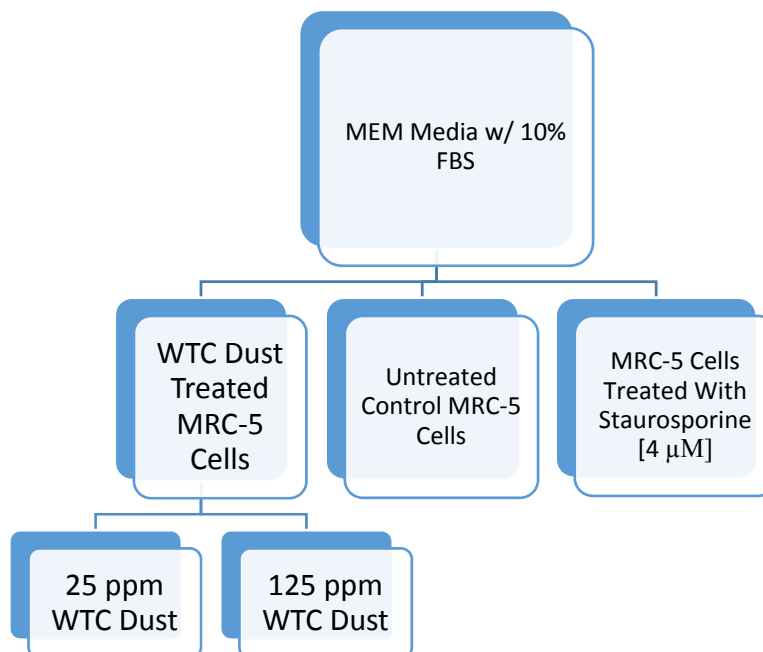
Figure 8. Various WTC dust concentrations were made using MEM media with 10% FBS. Staurosporine was used as a positive control for cell viability and western blot studies.

Cell Culture Maintenance

MRC-5 (ATCC, Manassas, VA), fibroblast cells found in human lung tissue, were cultured in Eagle's Minimal Essential Media and 10% FBS that has 1% PSGK. Cells were then plated, sub-cultured and incubated at 37°C for 24 hours. Media was then

removed and replaced with media containing different concentrations of the WTC dust. Cells were then incubated for 24 hours.

<u>alamarBlue® Cell Viability Assay Protocol</u>

This assay was used to determine if MRC-5 human lung fibroblast cells were viable after exposure to either 25 ppm or 125 ppm of WTC dust. MRC-5 cells were plated in 96-well plates and were grown in media, media containing a WTC dust concentration, or media containing Staurosporine at a concentration of 4 μM. The cells were incubated in time courses of 0.5-72 hours for the 25 ppm WTC dust exposed cells and 1-48 hours for the 125 ppm WTC dust exposed cells.

MRC-5 cells were grown to about 80% confluency in a T-75 flask. Once the desired confluency was reached the cells were then plated into a 96-well plate, leaving the wells on the perimeter of the plate empty. The volume of cells and media were plated at 100 μL. The cells were left for 24 hours to settle and adhere to the bottom of the wells. After 24 hours the cells to be poisoned with WTC dust concentrations or Staurosporine had their media removed and the appropriate poisoned media was added. The MRC-5 cells were left to incubate for the desired amount of time before the alamarBlue® (Thermo Fisher Scientific, Waltham, MA) reagent was added. When the desired time came to check the viability of the MRC-5 cells, 10 μL of the alamarBlue® reagent was added to each well that contained cells and media.

The cells were then incubated for 1 hour at 37°C in the incubator. Once the incubation was complete cell viability observed by measuring fluorescence of the alamarBlue® reagent. The 96-well plates were each placed into a BioTek® Synergy™ H1 Hybrid Multi-Mode Reader microplate reader. The software that was used to interpret

the readings was Gen5™. The fluorescence excitation wavelength was 540 nm and the fluorescence emission wavelength was 585 nm.

<u>Western Blot Protocol</u>

MRC-5 cells were grown to about 80% confluency in T-75 flasks. Cells were then poisoned with 125 ppm WTC dust or 4 µM Staurosporine. Once the cells were poisoned they were allowed to incubate with the cells for 1 hour, 2 hours, or 8 hours before they were pelleted to be used for the western blot.

The cells poisoned with media that contained 125 ppm WTC dust were the experimental cells. The cells poisoned with media that contained 4 µM Staurosporine were the positive control cells. The negative control cells did not have their media changed.

Cells were lysed using M-PER™ Mammalian Protein Extraction Reagent (Thermo Fisher Scientific, Waltham, MA) and Protease inhibitor. MRC-5 cells were centrifuged for 10 minutes at $21{,}000 \times$ g. The Loading Dye consisted of NuPAGE® LDS Sample Buffer (4X) (Thermo Fisher Scientific, Waltham, MA) and 500 mM DTT.

The proteins were loaded onto a NuPAGE™ 4-12% Bis-Tris Protein Gel, 1.0 mm, 10-well and run with NuPAGE™ MOPS SDS Running Buffer 20X (69.3 mM SDS, 20.5 mM EDTA, 1.0 M MOPS, 1.0 M Tris base, and 7.7 pH) diluted to 1X. The gel was run for 40 minutes at 200 V and 100 mA.

The Immobilon®-PVDF Membrane (MilliporeSigma, Burlington, MA) was soaked in 100% methanol. The blotting membrane was coated in a solution that was 10% methanol in NuPAGE™ Transfer Buffer (20X) (Thermo Fisher Scientific, Waltham,

MA). The Immobilon®-PVDF membrane was then placed on top of the blotting paper and the "sandwich" for the protein transfer was made with the blotting paper on top, gel, PVDF membrane, and blotting paper on the bottom. To complete the transfer of the protein from the gel onto the PVDF membrane the "sandwich" was electroblotted at 54 mA for 1 hour.

To make the blocking solution 0.5 g dry milk and 10 mL TBS-T were placed in a 15 mL centrifuge tube. After the protein transfer was complete the PVDF membrane was incubated for 1 hour on a shaker with the blocking solution.

Each of the primary antibody solutions was made in a 15 mL centrifuge tube and consisted of 10 mg bovine serum albumin (BSA), 10 mL tris-buffered saline with Tween 20 (TBS-T), and the appropriate amount of primary antibody. The p53 primary antibody (p53 (DO-1) Santa Cruz Biotechnology Inc., Dallas, TX) solution included 50 μL of primary antibody solution at a 1:200 dilution. The Bax primary antibody (Bax (B-9) Santa Cruz Biotechnology Inc., Dallas, TX) solution consisted of 50 μL of primary antibody solution at a 1:200 dilution. The α-tubulin primary antibody (α Tubulin (10D8) Santa Cruz Biotechnology Inc., Dallas, TX) solution consisted of 50 μL of primary antibody solution at a 1:200 dilution. The α-tubulin was used as the loading control. The JNK primary antibody (Human/Mouse/Rat Phospho-JNK (T183/Y185) Antibody, Catalog Number: AF1205, R&D Systems, Inc., Minneapolis, MN) solution included 5.7 μL of primary antibody solution. The ERK1/2 primary antibody (Human/Mouse/Rat Phospho-ERK1 (T202/Y185)/ERK2 (T185/Y187) Antibody, Catalog Number: AF1018, R&D Systems, Inc., Minneapolis, MN) solution included 1.69 μL of primary antibody solution. The p38 primary antibody (Human Phospho-p38α (T180/Y182) Antibody,

Catalog Number: MAB8691, R&D Systems, Inc., Minneapolis, MN) solution included 16 µL of primary antibody solution. For the JNK, ERK1/2, and p38 primary antibody solutions 100 µL of sodium azide was added. The primary antibodies were incubated with the PVDF membrane for at least 1 hour to overnight. The primary antibody solutions were collected after the incubation periods.

Each of the secondary antibody solutions was made in a 15 mL centrifuge tube and consisted of 10 mg BSA, 10 mL TBS-T, and 1 µL of each corresponding secondary antibody at a 1:10,000 dilution. The p53 and Bax primary antibodies originated from mice, so their secondary was IRDye® 800CW Infrared Dye goat anti-mouse (Lot# C80116-04, LI-COR Biosciences, Lincoln, NE). The p38 primary antibody originated from mice; its secondary antibodies were IRDye® 680RD Infrared Dye goat anti-mouse (Lot# C70908-04, LI-COR Biosciences, Lincoln, NE) and IRDye® 800CW Infrared Dye goat anti-mouse (Lot# C80116-04, LI-COR Biosciences, Lincoln, NE). The JNK and ERK1/2 primary antibodies originated from rabbits; their secondary antibodies were IRDye® 680RD Infrared Dye goat anti-rabbit (Lot# C71214-05, LI-COR Biosciences, Lincoln, NE) and IRDye® 800CW Infrared Dye goat anti-rabbit (Lot# C80118-01, LI-COR Biosciences, Lincoln, NE). The PVDF membranes were incubated for 1 hour with the secondary antibodies. The PVDF membranes were imaged using an Odyssey® CLx Imaging System (LI-COR Biosciences, Lincoln, NE).

Results

alamarBlue® Results
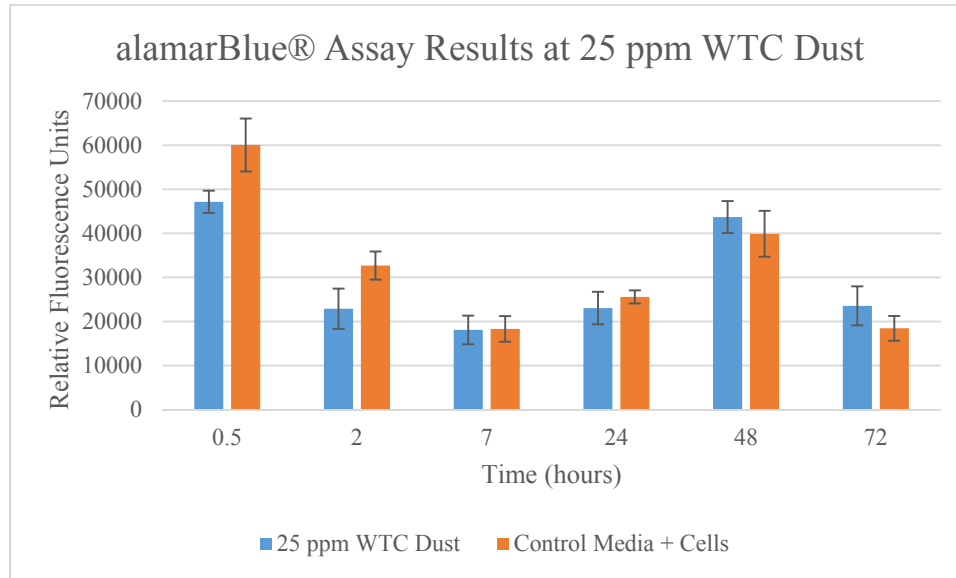
Figure 9. alamarBlue® time course study over 72 hours at 25 ppm WTC dust.
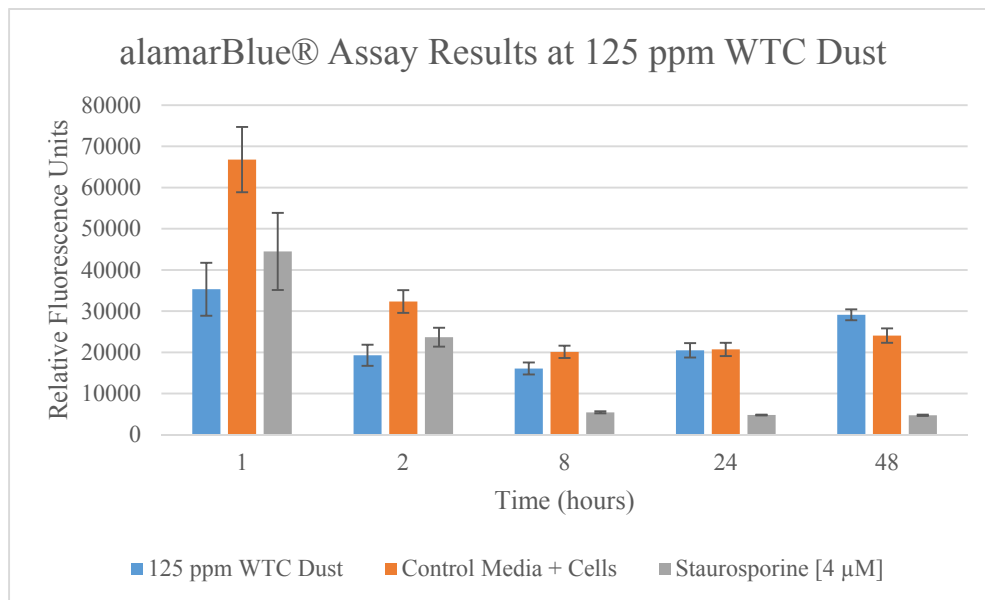
Figure 10. alamarBlue® time course study over 48 hours at 125 ppm WTC dust. Staurosporine [4 µM] was used as the positive control.

Cells Grown For Western Blots
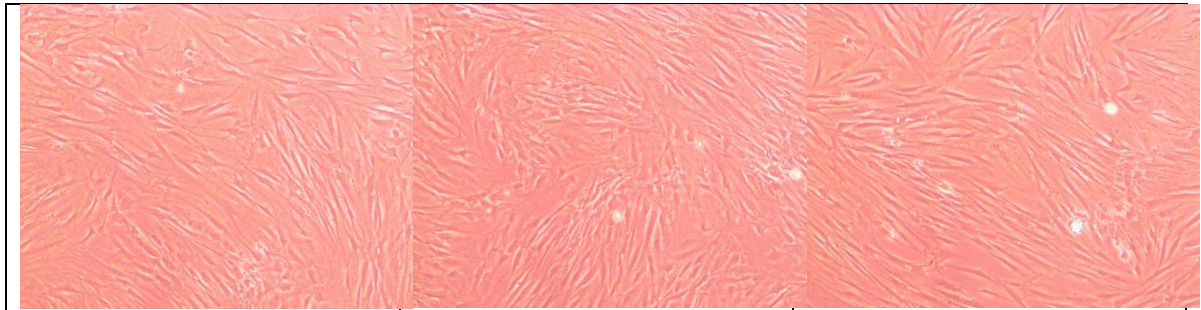
A) Before 1-hour incubation.	B) Before 2-hour incubation.	C) Before 8-hour incubation.

Figure 11. Control Media + Cells before A) 1-hour incubation, B) 2-hour incubation, and C) 8-hour incubation.
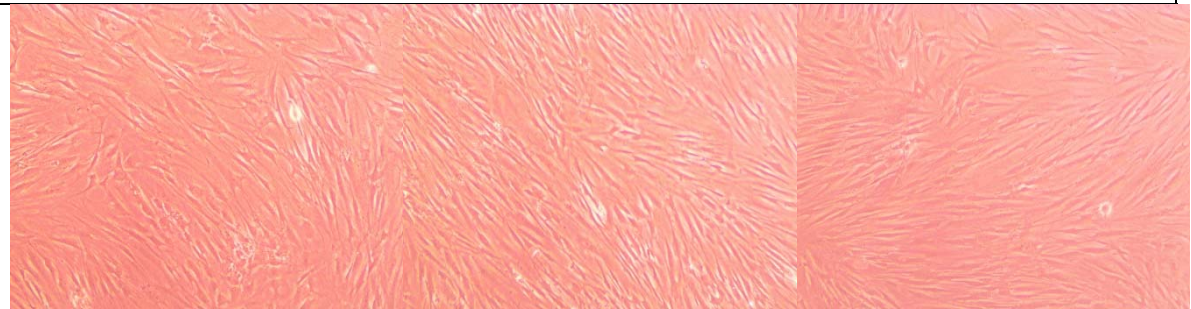
A) After 1-hour incubation.	B) After 2-hour incubation.	C) After 8-hour incubation.

Figure 12. Control Media + Cells after A) 1-hour incubation, B) 2-hour incubation, and C) 8-hour incubation.
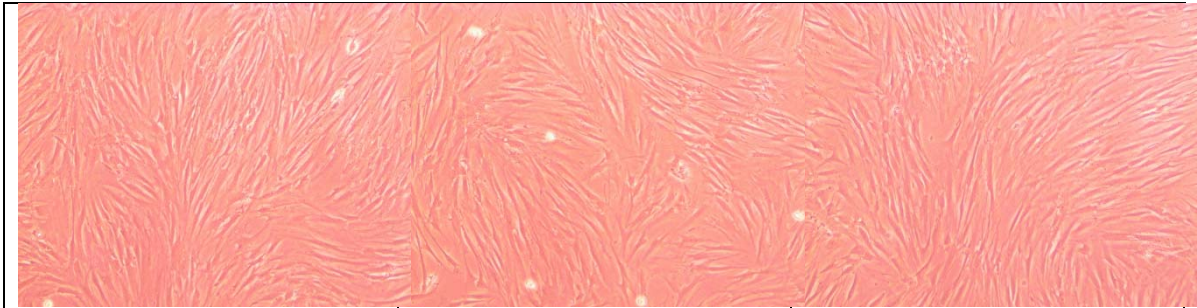
| A) Before 1-hour incubation. | B) Before 2-hour incubation. | C) Before 8-hour incubation. |

Figure 13. Cells before being incubated with poisoned media that contained WTC dust at 125 ppm. A) Cells before 1-hour incubation. B) Cells before 2-hour incubation. C) Cells before 8-hour incubation.
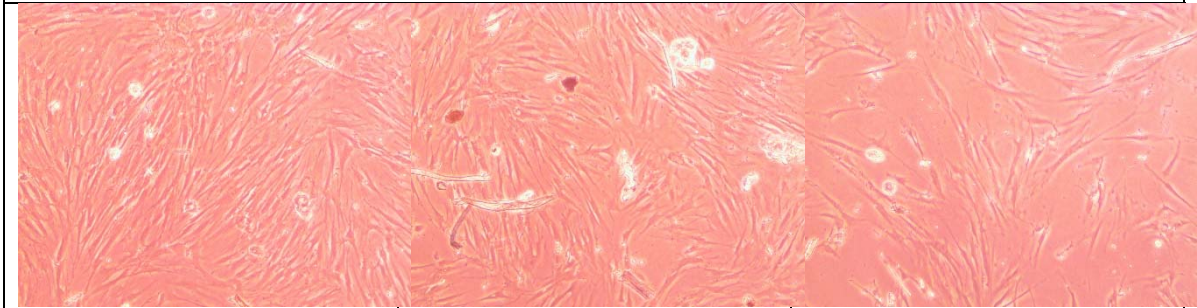
| A) After 1-hour incubation. | B) After 2-hour incubation. | C) After 8-hour incubation. |

Figure 14. Cells after being incubated with poisoned media that contained WTC dust at 125 ppm. A) Cells after 1-hour incubation. B) Cells after 2-hour incubation. C) Cells after 8-hour incubation.
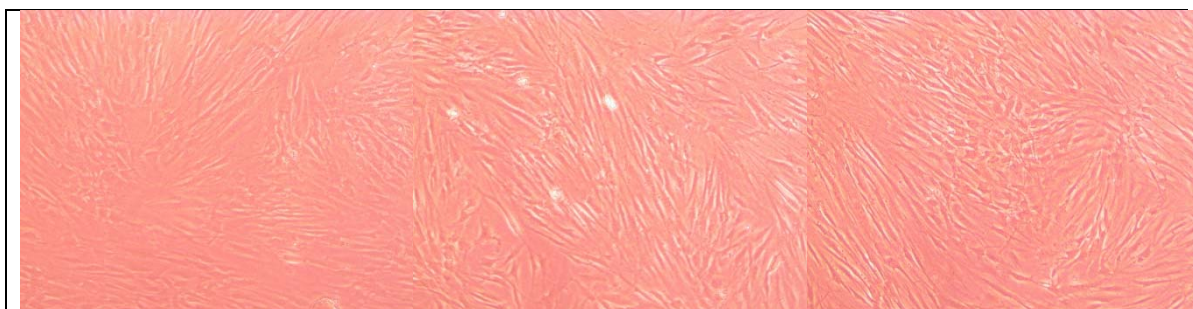
A) Before 1-hour incubation.	B) Before 2-hour incubation.	C) Before 8-hour incubation.

Figure 15. Cells before being incubated with poisoned media that contained Staurosporine [4 µM]. A) Cells before 1-hour incubation. B) Cells before 2-hour incubation. C) Cells before 8-hour incubation.
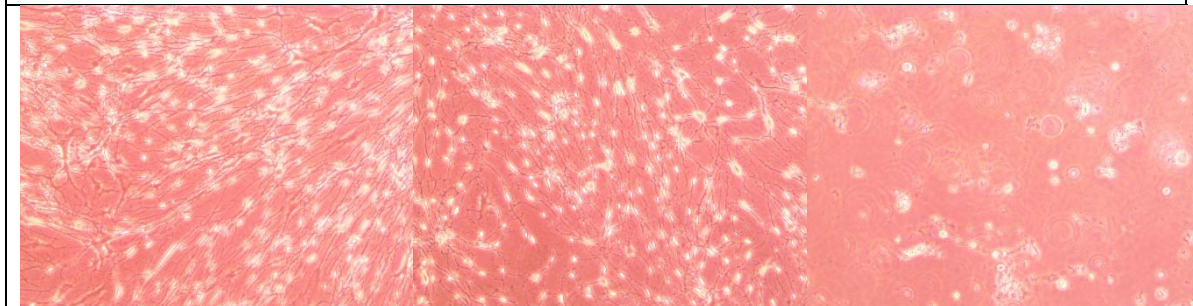
A) After 1-hour incubation.	B) After 2-hour incubation.	C) After 8-hour incubation.

Figure 16. Cells after being incubated with poisoned media that contained Staurosporine [4 µM]. A) Cells after 1-hour incubation. B) Cells after 2-hour incubation. C) Cells after 8-hour incubation.

	1	2	3	4	5	6	7	8	9
Ladder	Positive Control Staurosporine [4 µM] 1 hr 6/21/18	Negative Control (Cells + Media) 1 hr 6/21/18	WTC Dust 125 ppm 1 hr 6/21/18	Positive Control Staurosporine [4 µM] 2 hr 6/21/18	Negative Control (Cells + Media) 2 hr 6/21/18	WTC Dust 125 ppm 2 hr 6/21/18	Positive Control Staurosporine [4 µM] 8 hr 6/21/18	Negative Control (Cells + Media) 8 hr 6/21/18	WTC Dust 125 ppm 8 hr 6/21/18

Figure 17. Lane key for the Western blots that were performed with how long the cells were incubated with media that contained Staurosporine [4 µM], control media, or media with WTC dust at 125 ppm.
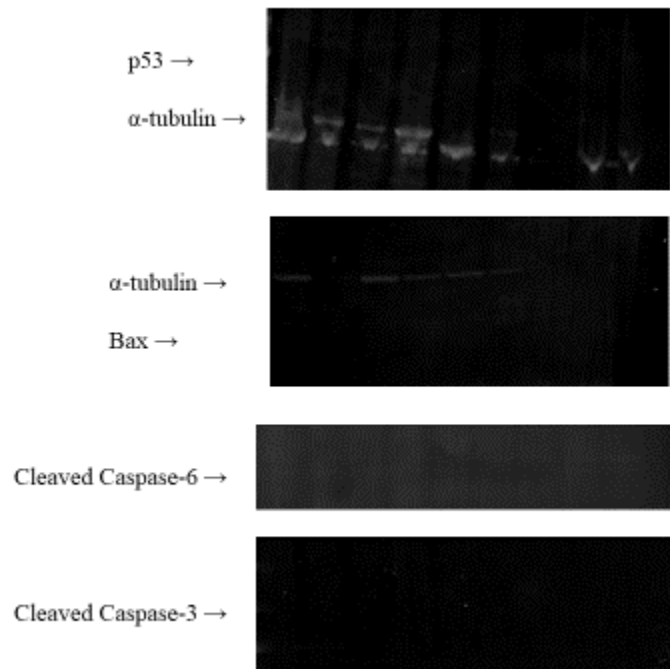
Figure 18. Western blot results for pro-apoptotic proteins.
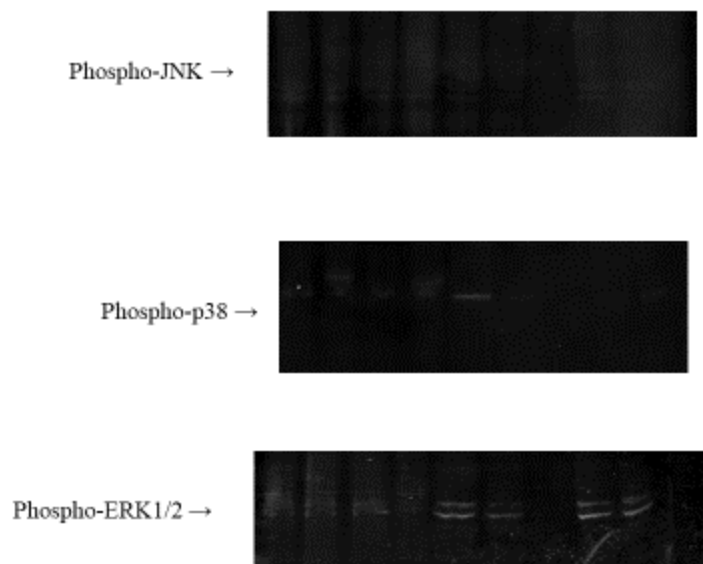
Figure 19. Western blot results for proteins associated with inflammation and cell survival.

Discussion

alamarBlue® Cell Viability Assay

      This assay examined cell viability by measuring how much resazurin, a non-fluorescent compound, was converted into resorufin, a fluorescent compound. As observed in Figure 9, until the 2-hour mark the Control Media + Cells show more cell viability than the cells which were incubated with the 25 ppm WTC dust. At the 7-hour time point it appears that the cells that were incubated with the 25 ppm WTC dust and the Control Media + Cells appear to show a similar amount of cell viability because the error bars overlap close together. The 24-hour time point shows a higher average of cell viability, however, the error bars overlap for the cells that were incubated with the 25 ppm WTC dust and the Control Media + Cells. The 48-hour time point shows higher cell viability for the cells that were incubated with the 25 ppm WTC dust than the Control Media + Cells but there is overlap in the error bars. The 72-hour time point also shows higher cell viability for the cells that were incubated with the 25 ppm WTC dust than the Control Media + Cells but there is overlap in the error bars. However, this overlap is less than the 7-hour, 24-hour, and 48-hour time points.

      As seen in Figure 10, until the 2-hour mark the Control Media + Cells show more cell viability than the cells that were incubated with the 125 ppm WTC dust. At the 8-hour time point it appears that the Control Media + Cells are slightly more viable than the cells that were incubated with the 125 ppm WTC dust. The 24-hour time point for the cells that were incubated with the 125 ppm WTC dust and the Control Media + Cells appear to show a similar amount of cell viability because the error bars overlap close together. The 48-hour time point shows higher cell viability for the cells that were

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incubated with the 125 ppm WTC dust than the Control Media + Cells. At this time point for this concentration, the error bars do not overlap.

It appears that the longer the MRC-5 cells were exposed to the WTC dust, the higher the cell viability was for those cells. This could be because the WTC dust is inhibiting p53 along with other pro-apoptotic proteins and/or activating MAPKs associated with cell viability. The results show that the cells are viable when exposed to WTC dust at both 25 ppm and 125 ppm concentrations.

Even though a different assay was performed, these results are different from previous cell viability results (Hernandez, Choi, & DiLorenzo, 2012; Lambroussis et al., 2009) because the results of the alamarBlue® presented in this research showed higher cell viability ratios at the 24-hour time point. Also, the alamarBlue® assay was a time course assay performed over 72 hours for the 25 ppm WTC dust concentration and 48 hours for the 125 ppm WTC dust concentration, while Hernandez et al. (2012) and Lambroussis et al. (2009) performed their research at one time point. Therefore, it is suggested that the cells need to lose some viability before they can become stimulated toward survival.

Western Blot

Figures 11-16 are photographs showing the physical appearance of MRC-5 human lung fibroblast cells before and after experimental conditions. Figures 11 and 12 show Control Media + Cells before and after their incubations, respectively. The cells look very similar in both sets of photos. Figure 13 shows the MRC-5 cells before being incubated with 125 ppm WTC dust. The cells appear to be healthy. Figure 14 shows the

MRC-5 cells after being incubated with 125 ppm WTC dust for different amounts of time. As more time passed, more damage was done to the cells and they became less confluent. Figure 15 shows the MRC-5 cells before being incubated with Staurosporine [4 µM]. The cells appear to be healthy. Figure 16 shows the MRC-5 cells after being incubated with Staurosporine [4 µM] for different amounts of time. As more time passed, the cells became more damaged and at by 8 hours there was much less viable cells left compared to the amount of cells that were used to begin the incubation. These cells were used harvested and their lysates were used in the western blot experiments. All western blots were set up according to the lane key that is shown in Figure 17.

Figure 18 presents the negative results of western blots for a series of known proteins in the intrinsic apoptotic pathway. Each of the proteins selected in this work, p53, Bax, Cleaved caspase-3, and Cleaved caspase-6, were selected to determine if the WTC dust would stimulate change in protein expression. There was no change observed in protein expression in this biochemical pathway. Since Bax, a pro-apoptotic protein, was not detected while cell viability was detected, further studies should investigate the expression of Bcl-2 which is an anti-apoptotic protein. These negative results are in agreement with the alamarBlue® cell viability assay. This pointed to the need for further investigation of proteins involved in inflammation and cell survival pathways.

Figure 19 showed no expression of JNK but did show expression of p38 and ERK1/2. This correlates well with the biochemical flowchart shown in Figure 6. The expression of p38 is associated with cell growth, differentiation, apoptosis, and inflammation. The expression of ERK1/2 is associated with cell growth, adhesion, survival, differentiation, apoptosis, and cell cycle progression.

According to the alamarBlue® cell viability assay and the western blots for pro-apoptotic proteins, it does not appear that p38 and ERK1/2 are affected pro-apoptotic proteins. These data point toward the need for further investigation of proteins associated with cell survival and inflammation pathways. Some of the stimuli that MAPKs, including p38 and ERK1/2, respond to include proinflammatory cytokines, heat shock, osmotic stress, and mitogens.

Further studies should include performing western blots with MRC-5 cells incubated with media that contained 125 ppm WTC dust at the 48 hour time point. The alamarBlue® cell viability assay in Figure 10 showed that the cells were more viable at the 48-hour time point for the 125 ppm WTC dust concentration than at the 1-hour, 2-hour, and 8-hour time points. Using that time point may provide more proteins of interest to use as starting material.

It can be concluded that the WTC dust is not inducing apoptosis in the MRC-5 cells. However, this does not mean that the WTC dust is harmless. Original plans for this experimental research led to western blots that looked for expression of pro-apoptotic proteins associated with the intrinsic apoptotic pathway. This did not lead to a definitive answer as seen in Figure 18. However, when the alamarBlue® cell viability assay were used in conjunction with proteins associated in the cell survival and inflammation pathways as seen in Figure 19, positive indication of protein expression was observed, especially in ERK1/2.

Future Research

The lab has also looked at the effect of reactive oxygen species (ROS) on the cells. Previous work done in the lab determined that the higher the concentration of WTC dust added to the cells, the more ROS was detected (Seder, 2018). It is possible that the ROS that the cells are producing after exposure to the WTC dust are activating the MAPKs. Previous studies have shown that ROS activate MAPK pathways (McCubrey, LaHair, & Franklin, 2006). Further studies should be done to determine if the MAPKs are being activated by the ROS produced from WTC dust exposure. Further studies should also be conducted to determine if the dust is causing lung cancer.

These experiments suggest more research should follow the expression of genes associated with pro-apoptotic proteins. If those genes are being expressed as mRNAs, it should be investigated why those mRNAs are not being translated into proteins.

The original goal for this project was to look at how WTC dust affected the expression of the pro-apoptotic proteins p53, Bax, cleaved caspase-3, and cleaved caspase-6. These proteins were chosen because they are well studied. P53 is known as "the guardian of the genome" (Strachan, T., & Read, A. P., 2001). This is because it is inactivated in the majority of human cancers (Surget, Khory, & Bourdon, 2013). When p53 is active it can suppress most cancers. The original thinking was that the WTC dust may be causing the MRC-5 cells to undergo apoptosis. If this was the case we would have seen an upregulation of p53 and Bax expression. However, this was not what was observed.

The MRC-5 cells were not observed to be undergoing apoptosis. When this was realized different kinase proteins were tested because they are involved in inflammation and inflammation had been observed with human epithelial lung cells (Wang et al., 2010). The inflammation related proteins were p38, JNK, and ERK1/2. Further studies should be performed to observe what kind of immunological responses the MRC-5 cells produce after exposure to the WTC dust.

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