ANALYSIS OF APOPTOTIC SIGNALING PATHWAYS INDUCED BY NITROPARABENS IN MELANOMA CELLS

Isabella Hildebrandt

This thesis is submitted in partial fulfillment of the requirements of the Research Honors Program in the Department of Chemistry

Marietta College

Marietta, OH

April 28, 2016
Acknowledgements:

Honors Thesis Committee:

Dr. Kimberly Suzanne George Parsons & Dr. Tej Gautam

Marietta College Chemistry Department

Dr. Steven Spilatro

Sean Merrill
Abstract

Novel nitroparabens, mononitroparaben and dinitroparaben, were investigated for potential to replace the most commonly used paraben, methylparaben, which is shown to be toxic at the 5mM level. Human melanoma M624 cells were treated with 2.5mM, 5mM, and 10mM mononitroparaben and 2.5mM and 5mM dinitroparaben in the presence and absence of 25 mJ/cm² ultraviolet B light. Apoptosis was analyzed through PARP protein analysis via western blot. Minimum inhibitory concentrations were determined for the nitroparabens and methylparaben on *Escherichia coli*, *Proteus vulgaris*, *Staphylococcus aureus*, and *Enterococcus faecalis*. Mononitroparaben was the best antimicrobial agent with the greatest MIC being at 0.8mM. Mononitroparaben induced apoptosis at 10mM, while dinitroparaben induced apoptosis at 5mM. Nitroparabens have shown to induce apoptosis when exposed to UV-B irradiation at all concentrations. Mononitroparaben would need to be tested on normal human keratinocytes and the 0.8mM level to determine whether it could be a viable substitute for methylparaben on the commercial market.

**Key words:** nitroparaben, parabens, methylparaben, melanoma, ultraviolet, apoptosis
# Table of Contents

INTRODUCTION ............................................................................................................................................. 5  
FIGURE 1: MONONITROPARABEN AND DINITROPARABEN ........................................................................... 6  
OBJECTIVE .................................................................................................................................................. 15

METHODOLOGY ............................................................................................................................................ 16

RESULTS ....................................................................................................................................................... 20  
FIGURE 2: TOXICITY AND PHOTOTOXICITY STUDIES ........................................................................... 20  
FIGURE 3: MONONITROPARABEN WESTERN BLOT QUANTIFICATION .................................................. 22  
FIGURE 4: DINITROPARABEN WESTERN BLOT QUANTIFICATION ....................................................... 23  
FIGURE 5: MINIMUM INHIBITORY CONCENTRATION ASSAYS .................................................................. 25

DISCUSSION ................................................................................................................................................. 26

CONCLUSION ................................................................................................................................................. 30

REFERENCES .................................................................................................................................................. 31

APPENDIX A: BRADFORD PROTEIN CONCENTRATION ASSAY ............................................................ 35

APPENDIX B: WESTERN BLOT NUMERICAL DATA AND ALTERNATIVE ANALYSIS FIGURE ....................... 36
**Introduction**

Parabens are a class of commonly used preservatives in a majority of personal care products, including cosmetics, due to their antimicrobial characteristics in preventing fungal and microbial growth. However, parabens in topical skin products are rapidly absorbed through the skin and a small amount is not hydrolyzed by esterases (1, 2). These intact parabens in the human body that cause a multitude of adverse side effects: endocrine-disrupting effects, sensitization with damaged skin, genotoxic activity, enzyme inhibition and unfavorable reproductive effects (1, 2). Parabens are all esters of \( p \)-hydroxybenzoic acid and include methyl, ethyl, butyl, heptyl, and benzyl parabens. Methylparaben is the most widely used paraben in topical skin care products (3). There have been two studies showing the detrimental effects of methylparaben and ultraviolet B (UV-B) radiation first on human skin HaCaT keratinocytes (4) and then in transformed human melanoma cells, M624 (5).

Handa et al. studied HaCaT keratinocytes treated with methylparaben and UV-B radiation. It was shown that there is a correlation between the methylparaben treatment concentrations and cell death. As the compound treatment increases, the cell death also increases. When the same treatment groups are exposed to UV-B radiation, the cells have an increased percentage of cell death from the no UV-B treated group. The detrimental potential of methylparaben was also shown to be dependent on dose and time (4). In our laboratory, Wood et al. studied M624 human melanoma cells treated will methylparaben (1mM and 5mM) and UV-B radiation. At 5mM, the methylparaben alone and combined with UV-B radiation were each shown to be toxic to the cells after 24 hours. The toxicity was amplified when cells were exposed to UV-B radiation at all concentrations of methylparaben (0mM, 1mM, 5mM). This effect was not as prominent after only a 4 hours exposure. Methylparaben at 5mM showed the induction of
PARP I cleavage, an apoptotic indicator, with and without UV-B radiation and was able to inhibit UV-B induced PARP II expression, which is a cellular damage indicator (5).

Due to recent studies such as the ones mentioned above and consumer concern, the number of paraben-free cosmetics is on the rise but parabens are still used in 75%-90% of cosmetic products on the market (3). Parabens continue to stay popular in the industry when compared to natural products due to a broad spectrum of antimicrobial activity, lack of odor, and stability at different pH and temperatures (4). An ideal substitute would be a paraben that has the mentioned ideal preservative properties and less cytotoxicity when compared to methylparaben. Marietta College’s Organic Chemistry Lab has synthesized novel parabens by adding nitro groups to methylparaben. There were two novel parabens created, mononitroparaben (MNP) and dinitroparaben (DNP), shown below in Figure 1. The nitroparabens have been shown in preliminary studies to cause less toxicity than methylparaben. This study will further investigate the potential for these parabens to be used in the skin care industry as a safer substitute for more volatile parabens.

**Figure 1: Mononitroparaben and dinitroparaben**

To assess how destructive the novel nitroparabens are to the cell, toxicity and phototoxicity testing is performed on a transformed human melanoma cell line, M624. The cell culture technique for toxicity testing has been proven to be the simplest, quickest, most reproducible and
relatively inexpensive test to be used for testing toxic chemicals (6). The cell culture technique is the process where cells are grown under controlled aseptic conditions in a tissue culture plate. The cells from one plate are split into multi-well plates to generate wells with the same number of genetically identical cells for testing. In cytotoxicity testing, the cultured cells are treated with different concentrations of the compound of interest. The cells are exposed to the compound for a certain exposure time then the compound is removed and cell viability, or the percentage of living cells when compared to a control that is not treated, is determined (5).

The cell viability can be measured in multiple ways including quantifying DNA, quantifying protein, counting individual cells or using stained dye absorbance in live cells. In this study, stained dye absorbance is used for its efficiency and its good correlation with routine cell counting (6). The cells have to be stained in order to get an absorbance reading since the small amount of them is essentially colorless. Cells are dyed by crystal violet in methanol. The methanol fixes the cells to the bottom of the wells so they do not wash off yielding false results while the crystal violet is absorbed by viable cells giving them a purple color. This coloration allows the cell proliferation to be measured by absorption spectroscopy. The stained cells are read in a plate reader at 570nm, the maximum absorbance wavelength for crystal violet. The higher the absorbance, the more living cells in the treatment group and therefore, the less toxic the substance in that given concentration. The control cells do not receive a chemical treatment and are considered to be 100% viable. All of the treated wells’ absorbance are divided by the control’s to obtain the percentage of cell proliferation for each treatment (7). Phototoxicity follows the same procedure but includes UV-B exposure during the compound treatment.

Ultraviolet B (UV-B) radiation (290-320nm) is emitted from the sun and has been an increased environmental and health concern with connections to cellular damage. Over the past
few decades, there has been a depletion of stratospheric ozone, or O\textsubscript{3}, due to the increase in pollutants that deplete the amount of the O\textsubscript{3} layer. The stratospheric layer of the ozone is primarily responsible for the blocking of harmful UV light radiation shorter than 310 nm (8). The reductions in the ozone create holes for radiation, primarily harmful UV-B radiation, to reach the Earth’s surface and be absorbed by human skin. Human skin is at high risk since UV-B radiation only penetrates the dermis (9). UV-B radiation has been identified as a primary mutagen that increases the risk for skin cancer by being the most absorbed form of UV light by the DNA.

DNA is the main target for UV-B damage since DNA contains ring structures of conjugated systems, which are alternating single and multiple bonds. Conjugated systems in organic molecules, such as DNA monomers, absorb UV radiation from 200 nm to over 300 nm, which generates chemical reactions. The genes located in the absorbing DNA are easily damaged and harmful mutations can follow after exposure (9). UV-B induces DNA photoproducts that may or may not be repaired depending on the individual’s genetics (10). Not only does direct DNA damage occur, but the formation of free radical species also occurs that further damages the cell (9). Extensive cell damage can lead to the activation of cellular pathways resulting in apoptosis, which is programmed cell death. UV-B from the sun causes sunburns that lead to skin cancer.

Melanoma is the deadliest type of skin cancer that accounts for the majority of skin cancer deaths and has the fastest rising incidence of any other cancer with 160,000 new cases every year (11). Melanoma has multiple forms: mucosal melanoma, nodular melanoma, lentigo maligna, superficial melanoma (most common) and acral melanoma (5, 11). Along with predisposed genetic and environmental factors, all forms of melanoma are causally related to UV exposure from the sun. Normal melanocytes have a balance of physiological processes
through signal transduction pathways. These pathways are regulated by both external and internal signaling, such as a ligand binding to a receptor in the cell membrane or an amount of a substance within the cell’s cytoplasm. Cancer develops when there is a disruption within these pathways causing uncontrollable cell proliferation rather than apoptosis (9).

Apoptosis, or programmed cell death, is characterized by distinct morphological characteristics and energy-dependent biochemical mechanisms (12). Apoptosis is a vital component for many processes including development, aging, tissue homeostasis, and defense mechanisms (12). There are four stages of apoptosis: cell shrinkage, membrane blebbing, nuclear breakdown, DNA fragmentation, and budding (13). Cell shrinkage makes the cell smaller by making the cytoplasm denser and the organelles packer tighter. As the cell is shrinking, the irreversible process of pyknosis also occurs, which involves the DNA and chromatin to condense in the nucleus (12). Next, extensive membrane blebbing occurs which uses an actin-myosin system. Actin and myosin are proteins of the cytoskeleton that are responsible for all intracellular movement. In apoptosis, these proteins use their contractile force to form ‘blebs’, swelling of the membrane into spherical bubbles (14). The nucleus then breaks down allowing the DNA to be fragmented. Once all the DNA is fragmented, the ‘blebs’ containing cytoplasm, organelles and nuclear fragments bud off to create multiple apoptotic bodies. These bodies are then phagocytosed, or ‘eaten’, by macrophages where the contents are degraded (12). This process of controlled cell death is a vital component for many processes including embryonic development, aging, tissue homeostasis, and defense mechanisms (5, 12).

Apoptosis can be activated by either an extrinsic or intrinsic pathway. The extrinsic pathway originates from the outside of the cell when a ligand binds to a death receptor on the cell’s membrane such as type 1 TNF receptor (TNFR1) and Fas (CD95) (15). The death
receptors extend through the membrane of the cell and contain an intracellular domain that activates an intrinsic signaling pathway when the respective external ligand binds. The intrinsic pathway is initiated within the cell; it’s also known as the mitochondrial pathway. It is triggered by internal stimuli such as irreparable genetic damage and severe oxidative stress (15). Triggering of this pathway causes cytochrome-c, a component involved in making energy in the mitochondria, to be released from the mitochondria (5). Cytochrome-c then complexes with the adaptor molecule apoptotic protease activating factor-1 (Apaf-1) and caspase 9 proteins to create an apoptosome (16). The extrinsic and intrinsic pathways converge at an irreversible point, caspase 3, a cysteine protease, which cleaves the inhibitor of the caspase-activated deoxyribonuclease, which causes nuclear apoptosis. Downstream caspases cleave components of the cell such as cytoskeleton proteins and DNA repair proteins, to further the process of apoptosis throughout the cell (15). With complicated signaling pathways, the process of apoptosis can be disrupted in multiple locations resulting in malignant cell growth.

In order for a cell to transform to become malignant, it must accumulate genetic mutations while evading apoptosis. Reduced or defective apoptosis has been proven to be a major causal factor in the development and tumorigenesis of cancer as well as resistance to anticancer drugs (17). This reduced apoptosis can occur in three ways: imbalance of pro-apoptotic proteins and anti-apoptotic proteins, reduced caspase function, and impaired death receptor signaling (15). Even though the evasion of apoptosis is a large part of carcinogenesis, it is the main focus for being the target of cancer treatments. Drug therapy and treatment are gearing their strategy towards restoring these apoptotic-signaling pathways by targeting proteins involved in the regulation of the intrinsic pathways and the components reducing cellular apoptosis.
UV-B radiation disrupts this balance by interfering with signal transduction pathways by altering the production of proteins that are involved in the pathway (9). Since UV-B radiation shows a direct mutagenic role in melanoma pathogenesis by altering the DNA sequence, the signaling pathway protein genes are disrupted by the radiation. The genes that are mutated by UV-B radiation and cause melanoma are named ‘melanoma’ genes, and include P53, P16, P14, and CDK4 (11). A majority of these genes are tumor suppressor genes, which stop the formation of tumors when intact. When mutations occur in tumor suppressor genes, there is an anti-apoptotic effect and cells lose control of their growth (11). The dysregulation of UV-B apoptosis, or programmed cell death, may result in photocarcinogenesis (5).

Poly-(ADP-ribose) polymerase, known as PARP, is a family of proteins involved in regulating DNA repair mechanisms in nuclei that includes PARP-1 and PARP-2. When a cell undergoes genotoxic stress, such as UV-B radiation, it creates DNA interruptions. PARP is then activated to turn on DNA repair systems of the DNA double helix by the addition of poly(ADP)-ribose (PAR) by poly(ADP)-ribosylation of proteins in the nucleus. The addition of PAR allows the DNA repair proteins to interact with the DNA in order to fix the mutations. The reaction is then reversed by cleaving PAR rapidly to shut off the DNA repair mechanisms by poly(ADP-ribosyl)glycohydrolase (PARG) (18).

PARP-1 is found abundantly in the nucleus of all mammalian cells but when cellular damage is too extensive to be repaired PARP-1 is cleaved and therefore an indicator of apoptosis, while PARP-2 is an indicator of cellular damage (19). The PARP proteins are activated by DNA interruptions, such as bulky mutations from UV-B radiation and DNA strand breaks (20). PARP-1 and PARP-2 are involved in cell survival/death, transcription, DNA repair, and cell division. They recognize damage in the DNA and signal transducers to activate down-
stream effectors (21, 22). If the cellular damage is too extensive for repair, the PARP proteins signal the cell to undergo apoptosis (19). PARP-1 cleaves into an 89-kDa catalytic fragment and a 24-kDa DNA binding domain fragment during apoptosis (23). The 89-kDa fragment contains the catalytic domain of the enzyme and cannot bind to the DNA for the PAR reaction to take place, therefore, it is released from the nucleus. The 24-kDa cleaved fragment stays in the nucleus, irreversibly binds to the damaged DNA where it acts as an inhibitor of active PARP-1. This binding of the 24-kDa fragment therefore inhibits DNA repair mechanisms and diminishes DNA repair during apoptosis. If PARP-1 was not inactivated by cleavage, there would be a high NAD$^+$ consumption, the substrate for the PAR reaction (24). The presence and amount of PARP-1, PARP-2 and PARP-1 fragments can be analyzed to determine whether certain cell conditions amplify cellular damage leading to apoptosis.

The western blot technique is used for protein analysis of cell lysates treated with variable concentrations of the nitroparabens and UV-B radiation in this study. The cell lysates are purified to contain only the proteins of the cell. In order to purify the proteins, the cell membranes are broken open by three different methods: change in temperature, detergent and mechanical. The cells are also centrifuged at high speeds to eliminate cellular debris then at a low speed to separate the DNA from the proteins (25).

The lysates are then separated by molecular weight using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Sodium dodecyl sulfate (SDS) is a detergent that binds to proteins in the cell lysates before samples are loaded into the well of the gel. SDS linearizes the protein and gives all proteins in the sample the same mass to charge ratio. The protein loading buffer treatment with heat and the SDS denatures, or unfolds, the proteins so only primary structure remains (25). Existence of only primary structure allows the proteins to
move through the gel solely based on the molecular weight and not the three-dimensional structure of the protein. An overall negative charge allows the proteins to move towards the positive pole in an electric field (25).

The proteins are able to be separated by size due to the polyacrylamide gel, which creates a web. The small proteins are able to travel faster through the gel, therefore migrating further. The larger proteins have a harder time getting through so they appear farther up the gel. Both gels are made up of the monomer, polyacrylamide, and the crosslinker, bisacrylamide (26). The polyacrylamide gel has two sections a stacking gel and a separating, or resolving, gel. The stacking gel is the upper layer with a lower concentration of acrylamide that compresses the proteins together before the separating gel and also contains the sample wells. The separating gel is the lower level that actually separates based on size, which has a higher percentage of acrylamide. There is an electric field applied to the gel: a negative pole at the top of the gel by the sample wells and a positive pole at the bottom of the gel. The negative protein samples are repelled by the negative electrode at the top of the gel and attracted to the positive pole at the bottom, which allows them to migrate straight down the gel (26).

The proteins are then transferred to a nitrocellulose membrane through a wet transfer, which involves a horizontal electrical current to pull the proteins out of the gel and onto the surface of the membrane. The nitrocellulose membrane is blocked, and then treated with primary and secondary antibodies that bind to the protein of interest. Chemiluminescence detection allows the visualization of the PARP protein bands through immunoblotting.

For nitroparabens to replace methylparaben on the commercial market, they must maintain the same antimicrobial properties. Methylparaben has been proven to inhibit the growth of *Escherichia coli*, *Proteus vulgaris*, *Staphylococcus aureus*, and *Enterococcus faecalis* (1, 29).
These bacteria are classified by their gram reaction, morphology and oxygen requirements. If a bacterium is gram-positive, it has a thick multi-layered peptidoglycan cell wall surrounding the cell membrane and creates exotoxins in the cytoplasm that may or may not be secreted. If it is gram-negative, the bacterium has a thin single-layer of peptidoglycan between two membranes. The outer membrane has endotoxins, known as lipopolysaccharides, and also creates exotoxins. Gram-negative bacteria are considered more toxic since endotoxins cause endotoxic shock, which all blood vessels vasodilate due to infection, and the presence of both exotoxins and endotoxins (27). A gram reaction is a staining process of bacteria fixed on a microscope slide involving crystal violet, iodine, alcohol and then safranin. Crystal violet is used to stain peptidoglycan and iodine is a mordant that fixes the crystal violet to the peptidoglycan of the gram-positive bacteria appearing purple. Alcohol is then used to wash off the non-fixed crystal violet. Lastly, safranin acts as a counter stain, which stains all cell nuclei red, making gram-negative bacterium appear red (28). Morphology is the form, structure, and special features of organisms (28). Oxygen requirements can be obligate aerobes obligate anaerobes, facultative, or microaerophilic. Obligate aerobes can only grow in the presence of oxygen while obligate anaerobes can only grow in the absence in oxygen. Facultative bacteria can grow in the presence in oxygen but can switch to fermentation in the absence of oxygen. Microaerophilic bacteria need oxygen since they cannot ferment but are poisoned by high oxygen levels.

*E. coli* and *P. vulgaris* are all rod shaped, gram-negative bacteria. *E. coli* and *P. vulgaris* are facultative. *S. aureus*, and *E. faecalis* are both gram-positive cocci bacteria. *S. aureus* is specifically a cocci that grows in clusters, while *E. faecalis* grow in pairs. Both of these gram-positive bacteria are facultative. Facultative bacteria are the most common found to grow within cosmetic products since they can grow at the surface of the product (with oxygen) and within the
product (without oxygen). Most of the bacteria are commensal, meaning that they live on the human skin, making them likely to be candidates for contamination (28).

The antimicrobial activity of compounds is determined by minimum inhibitory concentration (MIC) testing. The MIC is the lowest concentration of a compound that visually inhibits growth of an organism after overnight incubation (30). The MIC test is performed by doing a serial dilution from 0 mg/ml to 0.00001 mg/ml of the chemical of interest, in this case, nitroparabens. A specific amount of a bacterial suspension is added to each of the tubes then they are incubated for 24 to 48 hours. The MIC is read in a spectrophotometer at 600nm and plotted to determine when growth starts to be inhibited (31). The centrifuged sample’s media is used as a blank to determine if growth has occurred.

**Objective**

Through toxicity and phototoxcity assays, PARP western blot protein analysis and MIC antimicrobial testing, the novel nitroparabens were characterized. The results determine the potential of novel nitroparabens to replace the current cytotoxic parabens used in skin products by investigation of the cytotoxicity, antimicrobial potential and apoptotic PARP protein expression of these novel parabens in mammalian cells.
Methodology

Cell Culture and Treatment:

Transformed melanoma cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% FBS and 1% penicillin/streptomycin. At 80% confluency, cells are treated with mononitroparaben (0mM, 2.5mM, 5mM, 10mM) and dinitroparaben (0mM, 2.5mM, 5mM) and UV-B. The cells are incubated with treated media for two hours. For UV-B treatment groups, the nitroparaben media is removed while treating the cells with 25mJ/cm² UV-B radiation and replaced after radiation, and then incubated for additional four hours.

Toxicity and Phototoxicity Assays:

Transformed M624 human melanoma cells are grow to 80% confluency in DMEM enriched with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. The cells are detached by trypsin and 100µl of the cell suspension is transferred to each well in a 6-well plate. The 6-well plates are incubated for twenty-four hours or until cells are 80% confluent. Cells are incubated with either methyl-2-nitroparaben (control, 2.5mM, 5mM, 10mM, UVB, 2.5mM+UV-B, 5mM+UV-B, 10mM+UV-B) or methyl-2,6-dinitroparaben (control, 2.5mM, 5mM, UVB, 2.5mM+UV-B, 5mM+UV-B) for twenty-four hours. The nitroparaben species media is removed and cells are washed with cold 1x Phosphate Buffered Saline (PBS). Cells stain on a shaker for 15 minutes with 0.5% crystal violet solution in 25% methanol. Staining solution is removed and cells are washed with tap water. Dry plates are then analyzed in a plate reader at 590nm. The data was standardized to the control. All assays were done in triplicate. The data was averaged and standard deviations were determined.
**Protein Sample Isolation:**

Media from the treated cells was collected and centrifuged to collect detached cells. Cells were washed with 1x cold PBS, which was discarded. Then, 0.5 mL cold PBS was added and cells were scraped from the plate into a puddle. Cell suspension was transferred to tubes on ice. Cell suspensions were centrifuged at high speed for 10 minutes. The supernatant was discarded, while the cell pellet were placed in a -20°C freezer overnight. Frozen pellets were thawed on ice for 5 minutes then resuspended in 0.5 mL PBS. The cell suspension was centrifuged at a high speed for 10 minutes and the supernatant was removed. Protease inhibitor in 0.5 mL TNET lysis buffer (25mm Tris-HCl pH7.5, 150mM NaCl, 5mM EDTA, and 0.1% Triton X-100) was added to the cell pellet. Cells were homogenized by drawing the cell suspension through a 23-gauge needle and syringes twenty times. The sheared cell suspension was then centrifuged at a low speed for 10 minutes. The protein containing supernatant was collected and transferred to a new tube stored in the freezer.

**Protein Concentration Assays:**

**Bradford Assay:** A bovine serum albumin (BSA) serial dilution was performed to create standard protein concentrations for the standard curve. The protein samples/standards and Bradford reagent (4.75% ethanol, 100 mL 8.5% phosphoric acid, 0.01% Coomassie Brilliant Blue G-250) were added to a 96 well plate in triplicate (10 µl sample/standard and 150 µl Bradford reagent). The plate was agitated and any bubbles were popped with a needle. The absorbance was read at 595 nm and the sample protein concentrations were calculated from the BSA standard curve.
**Sample Preparation:**

Protein samples were prepared by adding protein-loading buffer (0.25M Tris, pH 6.8, 10% sodium dodecyl sulfate, 0.05% bromophenol blue, 50% glycerol, 10mM β-mercaptoethanol). The samples were vortexed, boiled for five minutes, then vortexed. The prepared samples were stored in the freezer.

**SDS-PAGE and Transfer:**

SDS-PAGE was preformed using a 4% stacking and 15% separating acrylamide gel with a thickness of 1.0mm. Protein samples at varying volumes and 5 µl of protein ladder were loaded into the wells. Samples and the ladder were separated at 200 volts for 45 minutes. The protein samples and ladder were then transferred to a nitrocellulose membrane via wet transfer at 100 volts for 35 minutes. After the transfer, the nitrocellulose membrane was blocked using dried non-fat milk in 1x tris buffered saline with Tween (TBS-T) for 20 minutes.

**Western Blot:**

Western Blot was performed on the blocked nitrocellulose membrane. The nitrocellulose membrane was probed on a shaker with 2.0µl of monoclonal β-actin primary antibody (Sigma, 122M4782) in 10 mL TBS-T at room temperature for three hours or overnight at 8°C followed by three rinse wash cycles. The same membrane was then probed with 2.0µl anti-mouse secondary antibody (Sigma, SLBN5472V) in 10mL TBS-T for one hour. For PARP protein expression and cleavage detection, the nitrocellulose membrane is probed with 10mL of PARP primary antibody (ThermoScientific MA5-15031) for three hours at room temperature or overnight at 8°C and anti-rabbit secondary antibody (Sigma, SLBP3451V) for one hour at room temperature. Visualization and detection of the probed proteins was performed via chemiluminescence. Nitrocellulose membrane went through three rinse and wash cycles and then
incubated at room temperature with 6mL of chemiluminescence (SuperSignal West Dura Extended Duration Chemiluminescent Substrate) for 4 minutes. Membrane was placed between two transparent liners and exposed to film in a dark room. After exposure, the membrane is dipped in developer 20 times, rinsed in water, dipped in fixer, and then rinsed again. Blots were standardized using JImage software.

**Minimum Inhibitory Concentration Antimicrobial Assay:**

Stock solutions of methylparaben, mononitroparaben, and dinitroparaben were made at 50mg/ml concentration in methanol. Each paraben that was inhibitory at the respective maximum concentration was serial diluted from 0.2mg/ml to 0.9mg/ml and a 0mg/ml control tube was prepared. A methanol control series was performed to take into account the effects of the methanol used to dissolve the parabens. *Escherichia coli, Proteus vulgaris, Staphylococcus aureus,* and *Enterococcus faecalis* bacterial suspensions were made and 50µl were put in each series separately. Tubes are incubated at 37°C for 24 hours. Turbidity was determined spectrophotomically at 600nm. Blanks were the media from the centrifuged sample.
Results

The toxicity of novel nitroparabens and UV-B light to human melanoma M624 cells was analyzed using cell proliferation assays and western blot protein analysis. The toxic and phototoxic effects of the novel nitroparabens and UV-B light on cell viability were investigated through cell proliferation assays (Figure 2). The effects of the novel nitroparabens and UV-B light on apoptotic pathways was analyzed through cleaved PARP expression in treated lysate samples via SDS-PAGE and western blot (Figure 3).

Human melanoma M624 cells were treated with mononitroparaben (2.5mM, 5mM, and 10mM) and dinitroparaben (2.5mM and 5mM) and 25mJ/cm² UV-B irradiation. As the concentrations of both nitroparabens increase, the cell viability decreases after 24 hours (Figure 2). The toxicity for dinitroparaben is greater than mononitroparaben at 5mM by a difference of 22%. With mononitroparaben and UV-B exposure, the decrease in cell viability from the control with no mononitroparaben declines at a lower rate than the non UV-B exposed cells.

Figure 2:

![Graphs showing cell viability](chart)

**Figure 2.** Toxicity and Phototoxicity studies: a) Cell proliferation assay: Human melanoma M624 cells were treated with novel nitroparabens, mononitroparaben or dinitroparaben, and 25mJ/cm² UV-B irradiation. Cells are then stained with a 0.5% crystal violet solution after 24 hours.
Western blot PARP analysis (Figure 3) shows that 2.5mM and 5mM of mononitroparaben increased PARP I expression (Figure 3a), while the 10mM concentration creates cleaved PARP (Figure 3b). PARP II expression slightly increases as concentration increases up to 5mM (Figure 3c). With the combination of UV-B and mononitroparaben treatment, the amount of PARP I expression increased with the concentration of mononitroparaben except for the 5mM concentration (Figure 3a). The amount of cleaved PARP increases from the UV-B control sample to the 2.5mM concentration of mononitroparaben plus UV-B treatment then decreases with the UV-B and 10mM treatment (Figure 3b). There was no cleaved PARP with the UV-B and 5mM treatment (Figure 3b). PARP II is constant between the UV-B control and UV-B + 2.5mM treatment then decreases in the UV-B + 5mM and UV-B +10mM treatment (Figure 3c).
Figure 3: Mononitroparaben western blot quantification. Treated whole cell lysate protein samples of PARP I (a), cleaved PARP (b), and PARP II (c) were quantified and standardized using β-actin western blot.

The western blot analysis for the dinitroparabens (Figure 4) showed an increase in PARP I expression from the control to the 2.5mM concentration, then a decrease in PARP I from 2.5mM to 5mM (Figure 4a). This trend was also seen with the UV-B treatment groups. The amount of cleaved PARP doubles from the control and 2.5mM to the 5mM treatment (Figure 4b). With UV-B, the amount of cleaved PARP doubles from the control to the 2.5mM and stays constant in the 5mM treatment compared to the 2.5mM treatment (Figure 4b). PARP II expression quadruples from the control to 2.5mM and doubles from 2.5mM to 5mM treatments.
(Figure 4c). Numerical data and alternate analytical figure for western blot of nitroparabens can be found in Appendix B.

Figure 4:

a)  

![Graph](image1)

b)  

![Graph](image2)

c)  

![Graph](image3)

Figure 4. Dinitroparaben western blot quantification. Treated whole cell lysate protein samples of PARP I (a), cleaved PARP (b), and PARP II (c) were quantified and standardized using β-actin western blot.
Minimum inhibitory concentration (MIC) assays show that methylparaben has an MIC of less than 0.4mg/ml for *S. aureus* (Figure 5a), 0.4mg.ml for *E. coli* (Figure 5b), 0.12mg/ml for *P. vulgaris* (Figure 5c), and greater than 1.2mg/ml for *E. faecalis* (Figure 5d). Mononitroparaben has an MIC of less than 0.4mg/ml for *S. aureus* (Figure 5a), less than 0.4mg.ml for *E. coli* (Figure 5b), 0.08mg/ml for *P. vulgaris* (Figure 5c), and 0.16mg/ml for *E. faecalis* (Figure 5d). Dinitroparaben has an MIC of less than 0.04mg/ml for *S. aureus* (Figure 5a), 1mg.ml for *E. coli* (Figure 5b), less than 0.04mg/ml for *P. vulgaris* (Figure 5c), and 1mg/ml for *E. faecalis* (Figure 5d)
Figure 5: Minimum inhibitory concentration assays for methylparaben (MP), mononitroparaben (MNP), and dinitroparaben (DNP) on *Staphylococcus aureus* (a), *Escherichia coli* (b), *Proteus vulgaris* (c) and *Enterococcus faecalis* (d). Sample absorbance was read at 600nm.
Discussion

Parabens are the most commonly used preservatives used in commercial products, such as cosmetics, personal care products, and food. However, these parabens are shown to create havoc in the human body when absorbed by the skin (1, 2). Methylparaben is the most commonly used and has been shown to have detrimental effects with UV-B irradiation in HaCaT cells by Handa et al. and in M624 cells by Wood et al. Novel nitroparabens have shown in primary studies to have decreased cytotoxic properties when compared to methylparaben. This is the first study to investigate the potential for these novel nitroparabens to replace methylparaben on the market as a preservative.

Toxicity and phototoxicity studies performed on M624 human melanoma cells have shown that both mononitroparaben and dinitroparaben have decreased cytotoxicity when compared to methylparaben. Methylparaben is toxic to the M624 cells at the 5mM level with cell viability below 10% and exposure to UV-B increased this toxicity (5). The mononitroparaben allowed for 82% cell viability, while dinitroparaben has above 65% cell viability at the 5mM level. When the nitroparaben treated cells were exposed to UV-B irradiation, the decrease in cell viability from the 0mM control was less than the decrease without the UV-B irradiation except for the 2.5mM concentration of mononitroparaben, which was 3% higher than the non UV-B treated cells. The decrease in cell viability decline in the UV-B treatment group shows that the nitroparabens have a protective effect on the cells from the UV-B irradiation. This pattern can be explained by the nitroparabens highly conjugated structure, meaning molecules with alternating single and double bonds.

The nitro groups added onto the aromatic ring of methylparaben increased the conjugation, meaning that additional p-orbitals where added in order to delocalize electrons in
the molecule. This feature usually decreases the energy and stabilizes the molecule (32). When a molecule absorbs light, it increases the energy that excited the ground state electrons. In a conjugated molecule, the ground and excited states are closer in energy therefore changing the color. Not only does the addition of the nitro groups make the parabens more stable, but also allows it to absorb UV-B light and provide a protective effect.

The novel nitroparabens are yellow in color with the mononitroparaben being lighter than the dinitroparaben. The observed color is the complementary color to the absorbed wavelength. The nitroparabens are highly conjugated so they absorb in the lower wavelength range of the visible spectrum, which is complementary to yellow on the color wheel (32). With high energy and shorter wavelengths being absorbed in the violet range, the molecule is able to absorb the UV-B light at a wavelength of 280-310nm. As the concentration of the parabens increases, more molecules are present to absorb the light and therefore there is a larger difference from the non UV-B to the UV-B cells as the concentration increases. However, the excitation of the nitroparabens by UV-B irradiation causes PARP I cleavage, an indicator of apoptosis, by the excitation of the molecule which create reactive species as seen in the western blot analysis.

The western blot analysis analyses the amount of PARP protein in each of the cell lysates treated with the nitroparabens. The PARP protein is an indicator of cellular damage and apoptosis. When DNA is damaged, DNA interruptions are formed, which activate PARP I and PARP II. PARP I is present in all mammalian cells and is up regulated with damage, PARP I cleavage is an indicator of apoptosis induction and PARP II expression is an indicator of cellular damage. If the DNA damage is too severe for repair, PARP I and PARP II signal that the cell is going though apoptosis (22). Results show that mononitroparaben and dinitroparaben induce apoptosis at all concentration levels with UV-B exposure excluding 5mM mononitroparaben,
which was concluded to be an outlier. This could be caused by the nitroparabens absorption of the UV-B irradiation, which excites the molecule causing it to be more metabolically reactive. The nitro group can cause oxidation/reduction reactions as well as creation of radical species. An increase in these reactions and species would cause the cell to have too much damage, therefore cleaving PARP I to induce apoptosis (33).

Apoptosis also occurred in the mononitroparaben treatments at the 10mM concentration. The amount of PARP I increased from 0mM to 5mM but decreased at the 10mM concentration of mononitroparaben. This decrease is due to the fact that PARP I cleaved to form the cleaved PARP, indicating apoptosis. Since cleaved PARP is derived from PARP I, the total amount of PARP I decreases due to the cleavage. PARP II is also increased at the 5mM level, which indicates that there is cellular damage at this concentration. In the mononitroparaben + UV-B treatment group, there is a steady increase in PARP I as the concentration increases excluding the 5mM concentration (concluded an outlier). PARP II expression was induced after the UV-B treatment, which indicates cellular damage. The UV-B induced PARP II expression was enhanced by the 2.5mM concentration, while the 5mM and 10mM concentrations inhibited PARP II expression.

The dinitroparaben cell lysates showed apoptosis occurring at the 5mM concentration. PARP I increased from the 0mM to the 2.5mM concentrations then decreased at the 5mM concentration due to PARP I cleaved at the 10mM level. In the dinitroparaben + UV-B treatment group, PARP I increased from the 0mM to the 2.5mM concentrations then decreased slightly at the 5mM concentration. The slight decrease is caused by the greater amount of cleaved PARP at the 5mM level. PARP II increased significantly from 0mM to 2.5mM, then again from 2.5mM to 5mM. The dinitroparaben potentiates UV-B induced PARP II expression as the concentration
increases. As the PARP II increases with the concentration, so does the cellular damage. Overall, nitroparabens increase in toxicity when exposed to UV-B irradiation indicated by the cleavage of PARP I.

The minimum inhibitory concentration (MIC) determines the growth of bacteria through a series of concentrations. The bacteria remain unaffected in growth until the MIC is reached because the MIC is the lowest concentration of a compound that inhibits the growth. In the graphs, this is indicated by a constant absorbance from the control during the unaffected concentrations then a consistent decrease when the MIC is reached. For *Staphylococcus aureus*, all parabens experienced a drop in absorbance at 0.04mg/ml. Mononitroparaben has the largest decrease followed by dinitroparaben and methylparaben respectively. This means that all of the parabens have an MIC less than 0.04mg/ml. However, after the 0.04mg/ml, methylparaben stays at a constant absorbance from 0.04mg/ml to 0.2mg/ml then decreases linearly from 0.2mg/ml. The nitroparabens start a linear decrease at a steeper slope when compared to methylparaben at 0.04mg/ml. Monoparaben has a lower absorbance at each concentration meaning it is more effective at inhibiting growth at all concentrations. *Escherichia coli* is most sensitive to mononitroparaben with an MIC less than 0.04mg/ml. Methylparaben is second with an MIC of 0.4mg/ml, then dinitroparaben with a MIC of 1mg/ml. *Proteus vulgaris* is more sensitive to dinitroparaben with an MIC less than 0.04mg/ml, followed by mononitroparaben at 0.08mg/ml and methylparaben at 0.12mg/ml. *Enterococcus faecalis* is the more resistant bacteria with the highest MICs and most gradual slopes. Mononitroparaben is the most effective at 0.16mg/ml. Dinitroparaben is inhibitory at 1mg/ml, while a methylparaben MIC was not observed meaning it is greater than 1.2mg/ml. Overall, mononitroparaben is more effective than methylparaben in all strains of bacteria tested.
Conclusion

Mononitroparaben is proven to be a more effective preservative over dinitroparaben in all categories. Mononitroparaben is less cytotoxic than dinitroparaben with having higher cell viability at the 5mM level and can go up to the 10mM level without being toxic to mammalian cells. Both nitroparabens are less cytotoxic than methylparaben, which had less than 10% cell viability at 5mM. Mononitroparaben induced apoptosis indicated by cleaved PARP at 10mM, while dinitroparaben and methylparaben induced apoptosis at 5mM. Both mononitroparaben and dinitroparaben induced apoptosis at all concentrations with UV-B irradiation. Methylparaben induced apoptosis only at the 5mM level with UV-B exposure. The apoptotic induction at all concentration levels with UV-B exposure makes the nitroparabens more toxic to the M624 cells. However, nitroaromatic groups have been used in chemotherapy to specifically target cancer cells while leaving normal cells unharmed (33). To determine true applicability to commercial use, the nitroparabens have to be tested on normal human keratinocytes, such as HaCaTs. Mononitroparaben is the most effective against all the bacterial types. The maximum MIC of 0.16mg/ml (0.8mM) is below the minimum tested 2.5mM in cell viability assays. To determine the ability to substitute for methylparaben on the commercial market, mononitroparaben would need to be tested at the 0.8mM range to determine if UV-B apoptotic induction occurs.
References


5. Wood, RS; Greenstein, RS; Hildebrandt, IM; Parsons, KSG. The apoptotic effect of methylparaben and ultraviolet B light on M624 human melanoma cells. (submitted for publication).


14. Coleman, ML; Sahai, EA; Yeo, M; Bosch, M; Dewar, A; Olson, MF. Membrane blebbing during apoptosis results from caspase-mediated activation of ROCK I. *Nature Cell Biology* **2001**, *4*, 339-345.


22. Sousa, F; Matuo, R; Soares, D; Escargueil, E; Henriques, J; Larsen, A; Saffi, J. PARPs and the DNA damage response. *Carcinogenesis*. **2011**, *33*, 1433-1440.


31. Rodriguez-Tudela, JL; Barchiesi, F; Bille, J; Chryssanthou, E; Cuenca-Estrella, M; Denning, D; Donnelly, JP; Dupont, B; Fegeler, W; Moore, C; Richardson, M; Verweij, PE. Method for the determination of minimum inhibitory concentration (MIC) by broth dilution of fermentative yeasts. *Clinical Microbiology and Infection*. **2003**, *9*, i-viii.


Appendix A: Bradford Protein Concentration Assay

Bradford protein concentration assay: The novel nitroparaben treated cytosolic cell lysates protein concentration was determined by the Bradford method at 595nm with bovine serum as a standard.
### Appendix B: Western Blot Numerical Data and Alternative Analysis Figure

<table>
<thead>
<tr>
<th></th>
<th>PARP I</th>
<th>Cleaved PARP</th>
<th>PARP II</th>
</tr>
</thead>
<tbody>
<tr>
<td>M0</td>
<td>1</td>
<td>1.000002101</td>
<td>0.999996603</td>
</tr>
<tr>
<td>M2.5</td>
<td>1.060113572</td>
<td>1.000002101</td>
<td>1.028225529</td>
</tr>
<tr>
<td>M5</td>
<td>1.685504185</td>
<td>1.000002101</td>
<td>1.47780814</td>
</tr>
<tr>
<td>M10</td>
<td>0.9899791144</td>
<td>5.613242724</td>
<td>1.271025688</td>
</tr>
<tr>
<td>UVM0</td>
<td>1.01831796</td>
<td>1.000002101</td>
<td>1.203680335</td>
</tr>
<tr>
<td>UVM2.5</td>
<td>1.233638342</td>
<td>11.03678156</td>
<td>1.352857871</td>
</tr>
<tr>
<td>UVM5</td>
<td>0.796179776</td>
<td>0.00272057</td>
<td>0.495286811</td>
</tr>
<tr>
<td>UVM10</td>
<td>1.324022383</td>
<td>7.419238859</td>
<td>0.581292359</td>
</tr>
<tr>
<td>D0</td>
<td>1</td>
<td>0.999999169</td>
<td>1.000002959</td>
</tr>
<tr>
<td>D2.5</td>
<td>1.734678468</td>
<td>0.952024448</td>
<td>1.000002959</td>
</tr>
<tr>
<td>D5</td>
<td>1.526976697</td>
<td>2.152978789</td>
<td>1.000002959</td>
</tr>
<tr>
<td>UVD0</td>
<td>1.008708203</td>
<td>1.079102951</td>
<td>1.000002959</td>
</tr>
<tr>
<td>UVD2.5</td>
<td>1.350175383</td>
<td>2.233555651</td>
<td>4.041012365</td>
</tr>
<tr>
<td>UVD5</td>
<td>1.030431982</td>
<td>2.148737639</td>
<td>7.283983022</td>
</tr>
</tbody>
</table>

Numerical data from western blot analysis for nitroparabens. All samples were standardized with the \( \beta \)-actin western blot. Each individual treatment group was standardized against the control.
Alternate analysis figure. The bar graph shows the PARP I quantification for each sample standardized against β-actin western blot. Below, western blot of whole cell lysate protein samples of cleaved PARP and PARP II.