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Induced Cell Proliferation Initiated by Roxarsone (ROX) is not Sufficient to Prevent Acrylamide Impairments in Beas-2B Cells.

Brett Lavigna Afiwa Tapoyo

INDUCED CELL PROLIFERATION INITIATED BY ROXARSONE (ROX) IS NOT SUFFICIENT TO PREVENT ACRYLAMIDE IMPAIRMENTS IN BEAS-2B CELLS

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Professor Desirée Jackson, Ph.D., Advisor

Natural environmental contaminants and xenobiotic compounds consumed by humans are likely to be deleterious to human health. This study evaluates the effects of the organoarsenic compound roxarsone and the organic compound acrylamide on BEAS-2B cells. Arsenic occurs naturally in the environment in inorganic and organic forms. Inorganic arsenics are carcinogenic to humans. Organic arsenics (organoarsenics) are less toxic, yet their level of toxicity cannot be ignored. Roxarsone (4-hydroxy-3-nitrobenzenearsonate) is a compound used as a poultry feed additive and antimicrobial agent. It promotes cell proliferation and possesses angiogenesis properties. Roxarsone (ROX) can be found in chicken breast meat; along with a metabolite of ROX, thiolated roxarsone, which is more toxic than Roxarsone. Acrylamide is used in the manufacturing of paper, dye, and industrial products. It is also found in starchy food cooked at high temperatures. The body can be simultaneously exposed to ROX and acrylamide through

food consumption. Acrylamide is known to cause cell damage, impair cell viability, and it affects cell proliferation. Experimental findings showed that 3.5 mM acrylamide destroys about half the population of BEAS-2B cells. Since acrylamide has deleterious effects on cells, it may be possible for ROX which stimulates cell proliferation, to ameliorate the deleterious effects of acrylamide. Previous studies showed that 10 μM Roxarsone induces cell proliferation. The specific aim of this study to demonstrate that the stimulation of BEAS-2B cell proliferation by 10 μM Roxarsone is not sufficient to protect the cells from damage induced by acrylamide. The first aim of this study is to demonstrate that treatment of BEAS-2B cells with ROX followed by treatment with acrylamide will alter cell morphology. The working hypothesis is that treatment of BEAS-2B cells with 10 μM ROX followed by 3.5 mM acrylamide will cause crenation, causing changes in cell morphology. The second aim of this study is to demonstrate that changes in cell morphology due to crenation will alter the PI3K/AKT pathway. The working hypothesis is that change in cell morphology will alter the proper functioning of genetic material and cellular responses, which is likely to destroy the PI3K/AKT cell survival pathway. Observation of cell morphology in response to ROX show that ROX induced cell proliferation in BEAS-2B cells with no morphological changes observed. Treatment of BEAS-2B cells with 10 μM ROX followed by 3.5 mM acrylamide changed cell morphology, which suggests that acrylamide is responsible for changes in cell morphology. Acrylamide-induced cell shrinkage and increased cell death were observed in treated BEAS-2B cells. ROX induced cell proliferation and caused significant upregulation of Ak strain transforming (AKT) levels after 24 hours of exposure. Cell proliferation stopped when the cells reached their

maximum confluency at 48 hours. AKT and downstream genes in the PI3K/AKT pathway were analyzed in BEAS-2B cells treated with Roxarsone, or a combination of Roxarsone and acrylamide.

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LIST OF SYMBOLS AND ABBREVIATIONS

ACTB	Actin Beta
AKT	AK Strain Transforming
APAF-1	Apoptotic protease activating factor-1
ATCC®	American Type Culture Collection
BAD	BCL2 Associated agonist of cell Death
BAX	BCL2-Associated X Protein
BIM	BCL-2 Interacting Mediator of cell death
BCL-2	B-Cell Lymphoma 2
BCL-XL	B-Cell Lymphoma Extra Large
BEAS-2B	Bronchial Epithelium transformed with Ad12-SV40 2B
CDC	Centers for Disease Control and Prevention
CM^2	Centimeter Square
CO_2	Carbon Dioxide
DEPC	Diethyl Pyrocarbonate
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic Acid
FBS	Fetal Bovine Serum
FDA	The United States Food and Drug Administration
H	Hour
H_2O	Water

HRP	Horseradish Peroxidase
kg	Kilogram
LD	Lethal Dose
M	Molar
Min	Minutes
mM	Millimolar
mTOR	mammalian Target of Rapamycin (mTOR)
OD	Optical Density
p53	Tumor protein 53
PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate-buffer saline
PI3K	Phosphatidylinositol 3- Kinase
PIP	Phosphatidylinositol-1, 4, 5-triphosphate
PKB	Protein Kinase B
PKBα	Protein Kinase B Alpha
PKBβ	Protein Kinase B Beta
PKBγ	Protein Kinase B Gamma
RIPA	Radio Immunoprecipitation Assay
ROS	Reactive Oxygen Species
Rpm	Revolutions per minute
SDS	Sodium Dodecyl Sulfate
µg	Micrograms
µL	Microliters

µM	Micromolar
mL	Milliliters

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CHAPTER 1

INTRODUCTION

Humans are exposed to various toxicants most often without their knowledge. Acrylamide, a toxic chemical, occurs in various starchy foods prepared under certain conditions, and is also found in industrial settings. Roxarsone (ROX) an organoarsenic compound, used as a feed additive, is administered to poultry and meant to be withdrawn before the poultry is placed for sale in the consumer market. ROX is known to have angiogenic properties and increases cell proliferation. Despite efforts to remove this organoarsenic toxicant, it remains in some tissues and is inevitably consumed by the general population. Acrylamide is known to be deleterious to cells and 10 μM ROX is known to cause cell proliferation and increase cell viability (Zhu et al., 2013). Eventually, both toxicants may be present in a human organism at the same time. The consequence of repeated and long-term exposure to these toxicants has not been assessed. This study, which involves BEAS-2B cells treated with a combination of 10 μM ROX and 3.5 mM acrylamide has never been investigated until now.

Roxarsone

Organoarsenical compounds are derived from arsenic and are generally assumed to be less toxic than inorganic arsenic. The lack of data on the toxicity of organoarsenics contributes to the thought that they are nontoxic (Luvonga et al., 2020). The benzenearsonate, Roxarsone (ROX), is commonly used as a feed additive for its angiogenic and antimicrobial properties in poultry (Bayse et al., 2013). Although it has been widely

used for over 70 years, several U.S. manufacturers voluntarily halted production and marketing of ROX. The U.S. Food and Drug Administration (FDA) has not yet approved its ban (Center for Veterinary Medicine, 2021a; Center for Veterinary Medicine, 2020). Because some studies report the ability of ROX to metabolize into inorganic arsenic, the FDA conducted research to assess the presence of inorganic arsenic levels in chicken livers by comparing the level of residues in ROX-treated and untreated chickens (Center for Veterinary Medicine, 2021b). Although the results showed a higher level of inorganic arsenic in ROX-treated chickens, the FDA still needs to assess these levels in various edible tissues in chickens.

Roxarsone Properties

Organoarsenics can be extracted using a methanol and water mixture (Cubadda, 2007). ROX has been shown to be soluble in methanol in different studies (Chen et al., 2019; Zhang et al., 2015). Its chemical formula is $C_6H_6AsNO_6$ with a molecular weight of 263.04 g/mol. Studies show that at a low concentration, ROX has angiogenic properties similar to inorganic trivalent arsenic (Zhang et al., 2015), whereas at a high concentration it is cytotoxic and moderately genotoxic (Zhang et al., 2012). ROX is used as an antimicrobial agent, preventing enteric diseases in chicken. It also improves growth and feed efficiency. After ROX is administered to poultry, a considerable percentage is excreted into the soil presenting ecological and human health concerns because it can rapidly convert into inorganic arsenic.

Acrylamide

Acrylamide, also named 2-propenamide is widely used in industrial settings (e.g., as a grouting agent, as a coagulant in water treatment or papermaking processes) (Kumar et al., 2018). It is a solid substance that is white, crystalline, odorless, and soluble in water. Its chemical formula is C_3H_5NO with a molecular weight of 71.08 g/mol. It exists in monomer and polymer forms, while the latter form is nontoxic. The monomer form can affect the nervous system inducing hallucinogenic effects and drowsiness. Human exposure to the monomer form of acrylamide is observed in exposed workers via dermal contact with acrylamide while injecting grouting agents into concrete cracks and by inhaling acrylamide dust and vapor (Kim et al., 2017). Repeated and prolonged exposure results in the onset of neuropathies such as peripheral neuritis, cerebellar alterations, and ataxia (Pennisi et al., 2013). With more cases emerging, additional research is necessary to provide conclusive data on the mechanism of action for this toxin.

Exposure to the monomer form of acrylamide can happen through diet, which has elicited questions regarding the potential neurotoxicity of dietary acrylamide. Acrylamide is found in carbohydrate-rich foods when fried, baked, or roasted, especially when the temperature exceeds 120°C (Visvanathan, 2014). It is also present in roasted coffee, cigarettes, and certain meats. During the heating process of carbohydrate-rich foods, the Maillard reaction takes place and results in the production of acrylamide under certain conditions. The Maillard reaction is responsible for the nonenzymatic browning of food as well as changes in aroma and taste (Hellwig & Henle, 2014; Lund & Ray, 2017). For acrylamide to be formed, the amino acid asparagine must react with reducing sugars (e.g., glucose and fructose) (Virk-Baker et al., 2014) and the temperature needs to be at least

120°C for the reaction to occur. The reaction with asparagine is the major route to obtain acrylamide; however, in its absence, acrolein and ammonia are responsible for acrylamide production in lipid-rich food (Visvanathan, 2014). Because of its suspected carcinogenic properties, some solutions have been proposed to mitigate the occurrence of acrylamide in foodstuff. Controlling the presence of reducing sugars and asparagine in varieties of potatoes and cereals seems to be the most impactful solution (Visvanathan, 2014).

Studies show that acrylamide increases oxidative stress in cells by promoting the formation of reactive oxygen species (ROS) (Celik et al., 2018). It creates an oxidant and antioxidant imbalance which ultimately decreases cell viability and stimulates tumorigenesis. Acrylamide has also been shown to disrupt cell morphology (Eltayeb, 2020), however the mechanisms behind these alterations are not fully understood.

Downstream Genes of the AKT Pathway

AKT protein is a downstream gene of the PI3K/AKT pathway which regulates apoptosis and promotes cell survival and cell proliferation. AKT is frequently involved in tumor growth and human cancers when it is dysregulated. AKT has also been seen to disrupt desired responses to cancer treatments (Maddika et al., 2007). AKT promotes cell survival by indirectly stimulating Bcl-xL or by inhibiting Bcl-xL inhibitors. Bcl-xL is a gene of the Bcl-2 family that promotes cell survival by preventing the release of cytochrome c from mitochondria. It is also involved in longevity and successful aging (Borrás et al., 2020). Bcl-xL serves as an anti-apoptotic protein and can regulate apoptosis by inhibiting the action of Bax.

Bax is a gene of the Bcl-2 family that acts as a pro-apoptotic protein by promoting the release of cytochrome c from mitochondria. It is inhibited by AKT for cell survival to

be maintained (Kanehisa Laboratories, 2019; Manning & Cantley, 2007). It is an important component of intrinsic apoptosis and carries out apoptosis by permeabilizing the outer membrane of the mitochondria (Robin et al., 2018). Bax is found in the cytosol in an inactive form, and it must be translocated to the mitochondrial membrane to be activated (Robin et al., 2018).

Acrylamide is known to be deleterious to cells and reduces cell viability. ROX induces proliferation and increases cell viability. This study investigates whether the proliferative action of 10 μM ROX on BEAS-2B cells will prevent the damage caused by 3.5 mM acrylamide.

CHAPTER 2

LITERATURE REVIEW

Humans are exposed to both Roxarsone (ROX) and acrylamide simultaneously through diet and occupational exposures. Little is known about ROX, an organoarsenic compound, or about health concerns arising from exposure to ROX and acrylamide. Studying chronic and long-term exposure of cell lines to ROX and acrylamide provides an approach to identifying possible long-term effects *in vivo*.

Non-malignant immortalized human bronchial epithelial cells, BEAS-2B cells, have been used to study repeated and prolonged exposure to metals and were chosen for use in this study. BEAS-2B cells originate from normal bronchial epithelium that was infected with a SV40 virus or with an Adeno 12-SV40 hybrid virus. Viral transfection gave proliferative potential to the cell line, while providing scientists with a mean to monitor cellular differentiation and carcinogenesis (Reddel et al., 1988). Studies have shown that BEAS-2B cells are suited to studies of carcinogenesis induced by heavy metals (Park et al., 2015).

ROX has the potential to metabolize into inorganic arsenic and studies have shown that remnants of ROX were present in tissues of poultry long after the withdrawal of the drug. Studies have revealed that packaged chicken ready for purchase contained arsenic. although, the mechanism behind metabolism of ROX into arsenic is not yet understood (Konkel, 2016). Arsenite, which is a very toxic inorganic arsenic, ROX, and unidentified arsenic species were present several days after withdrawal of ROX. ROX-fed chickens had

significantly higher unmetabolized ROX and unidentified arsenic species than controls (Konkel, 2016). Evaluation of human daily consumption was found to be 0.01 μg/day/kg body weight for a 70-kg adult after consumption of 3.5 ounces of ROX-fed chicken meat. This number is much lower than the World Health Organization (WHO) estimate of safe daily intake of arsenic species. There is debate about exposure to arsenic and what safe levels may be as it does not appear to have a carcinogenic threshold. This would mean that exposure to any amount of arsenic could pose risks.

Many studies determined that 10 μM ROX induces cell proliferation and angiogenesis. One study found that 10 μM ROX stimulates proliferation of Caco-2 cell by permeation of ROX from apical side to the basolateral side of the cell (Bayse et al., 2013). Other studies determined that 10 μM ROX promotes cell viability and angiogenesis of rat endothelial cells compared to untreated cells (Zhang et al., 2015; Zhu et al., 2013). This information was used to establish the concentration of ROX that was used for this study.

Acrylamide has been classified has a human carcinogen although the consequences of exposure in humans has been centered on neurotoxic effects (Chu et al., 2017). Cases of neurotoxicity were observed in workers exposed to acrylamide by dermal injection and inhalation of acrylamide-containing vapor. Acrylamide was shown to cause neuropathies and has been linked to alteration of normal thyroid and sex hormone functions, and to increased oxidative stress (Chu et al., 2017; Kim et al., 2017). Acrylamide at 0.5mM and higher concentrations have been found to induce cellular transformation, oxidative stress, and apoptosis (Park, 2002; Kacar et al., 2019; Eltayeb, 2020) Some studies found that prenatal exposure to acrylamide reduces newborn weight in humans (Chu et al., 2017). Moreover, acrylamide was shown to reduce body weights of rodents, and chronic exposure

produced tumors (Chu et al., 2017; Park, 2002). A study involving acrylamide-treated Syrian Hamster Embryo cells found that cells treated with 5 mM acrylamide or higher had no surviving colonies, whereas cells treated with lower concentrations of acrylamide still had colonies remaining (Park, 2002). Since 5 mM acrylamide may be too damaging to cells, experimental findings determined lower concentrations would yield more cell viability. From there, 3.5 mM acrylamide was found to destroy only half the population of BEAS-2B cells and was therefore used for this study to mimic the possible effects of acrylamide in vivo.

AKT Pathway (PI3K/AKT Pathway)

The AKT pathway is a pathway that regulates cell survival, cell proliferation, cell cycle progression, and apoptosis (Manning & Cantley, 2007). AKT (also called protein kinase B) is a serine/threonine kinase that has three isoforms: PKBα/Akt1, PKBβ/Akt2 and PKBγ/Akt3 (Song et al., 2005). Initial studies of AKT isoforms found that they share upstream activators and downstream effectors, however newer studies focus on their individual signaling specificity (Toker & Marmiroli, 2014). The AKT pathway can be activated through integrins, receptor tyrosine kinases, G-protein coupled receptors and various other stimuli (PI3K/Akt Signaling, 2007).

The PI3K/AKT pathway has been targeted for therapeutic purposes because of its involvement in many cellular processes. Components of the AKT pathway are often altered in human cancer (Vara et al., 2004), and dysregulation of the AKT pathway leads to cancer and neurological diseases (PI3K/Akt Signaling, 2007). Tumor cells fail to enter programmed cell death, which causes therapeutic resistance. The PI3K/AKT pathway regulates most survival signals involved. Drugs targeting these signaling molecules are

being developed (Vara et al., 2004) with PI3K inhibitors yielding promising results (Xu et al., 2020).

AKT and Downstream Genes

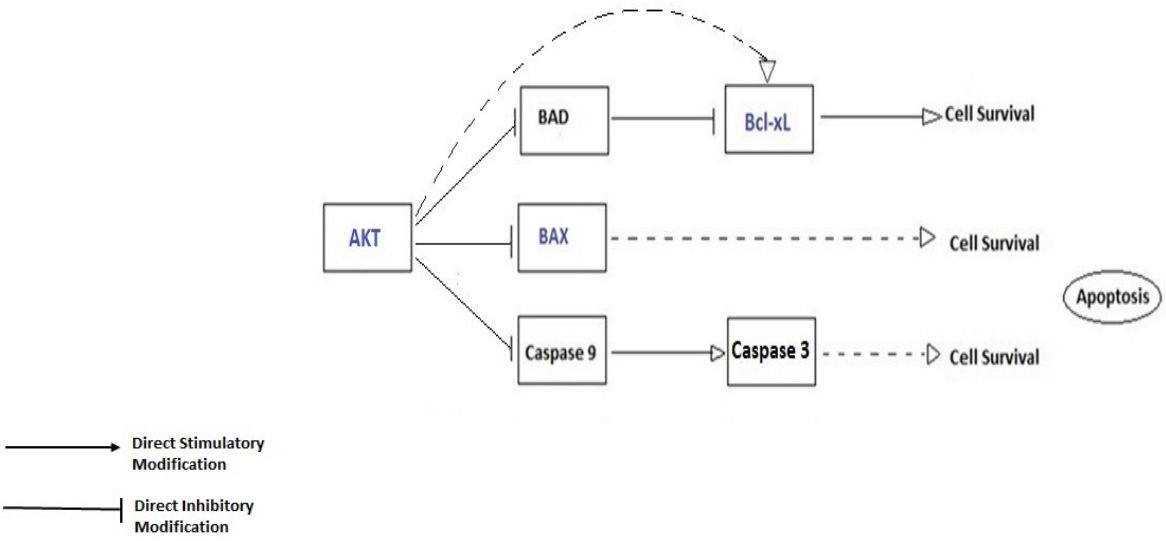

Figure 1: **Proteins Downstream of AKT in the PI3K/AKT Pathway (Kanehisa Laboratories, 2019; K. B., & Toker, A. T. 2007; Cell Signaling Technology & Yuan, 2008)**

B-Cell Lymphoma-extra-large (Bcl-xL) is an anti-apoptotic protein of the Bcl2 family. It is a transmembrane molecule in mitochondria that prevents the release of cytochrome-c and therefore the activation of the apoptotic program (Hellwig & Henle, 2014). Bcl-xL can be inhibited when binding with BAD or Bim which are pro-apoptotic proteins (Maddika et al., 2007). AKT can promote cell survival by either inhibiting BAD, which allows Bcl-xL to proceed with maintaining cell survival (Figure 1), or by indirectly stimulating Bcl-xL (Figure 1). Because Bcl2 family proteins are implicated in survival of certain cancer cells, Bcl-xL is a suitable drug target to prevent survival. Studies show that Bcl-xL may promote a resistance to cancer therapy therefore, Bcl-xL inhibitors have been used in cancer therapy and seem to ameliorate results (Punnoose et al., 2016).

Bcl2-associated X protein (Bax) is a protein in the Bcl2 family that inhibits cell cycle progression and cell survival, and positively regulates apoptosis (Maddika et al., 2007) (Figure 1). The apoptosis intrinsic pathway is associated with mitochondria and is generally activated by intracellular stimuli (e.g., oxidative stress, DNA damage).The apoptosis pathway takes place when cytochrome c is released from the mitochondria into the cytoplasm, forms an apoptosome with APAF-1 and leads to the activation of caspase 9, which in turn activates cell apoptosis. Bax normally remains in the cytosol in healthy cells with minimum interaction with the outer membrane of mitochondria. However, when Bax binds and accumulates on the outer mitochondrial membrane, it forms a heterodimer with Bcl2 family proteins, permeabilizes the membrane and leads to the leaking of mitochondrial contents including cytochrome c. Some studies suggest that Bax alone can depolarize the outer mitochondrial membrane and create pores through the insertion of alpha-helical structures, that lead to the release of cytochrome c (Carpenter & Brady, 2021).

After the apoptosome complex is formed, caspase 9 is stimulated and activates effector caspases (Maddika et al., 2007). Among those effector caspases is cleaved caspase 3, which promotes DNA degradation and the inhibition of DNA repair. AKT promotes cell survival by inhibiting the action of the executioner caspase 9, preventing it from stimulating the caspase cascade, and therefore apoptosis (Figure 1).

Beta actin is a gene that is highly conserved and expressed in most eukaryotic cells. It is involved in cell motility, integrity, and in the formation of the cytoskeleton. It is considered as a housekeeping gene and is frequently used as a gel loading control (Eliseeva et al., 2019). Because of its relatively constant expression in cells, it is used to prevent

loading protein inaccurately in the SDS-PAGE phase of Western blot procedures. However, studies also infer that Beta actin may not be an efficient loading control because of its involvement in many cellular processes (Castano & Kypta, 2008; Farmer et al., 1983; Gilda & Gomes, 2013). A study found that the mTOR pathway regulates the translation of Beta actin. This study also states that the translation of Beta actin is dependent on cell type (Gilda & Gomes, 2013)

AKT, Bcl-xL, and Bax are downstream genes of the PI3K/AKT pathway. Some of the functioning of AKT protein is to promote cell survival and cell proliferation. Protein expression studies of these genes will help to detect whether ROX-induced cell proliferation of BEAS-2B cells can prevent acrylamide damage.

CHAPTER 3

DESIGN OF THE STUDY

Cell Culture

BEAS-2B cells are derived from normal human bronchial epithelial cells. For this study, cells were acquired from the American Type Culture Collection (ATCC® CRL-9609, Manassas, VA). BEAS-2B cells were first grown in Dulbecco's Modified Eagle's Medium (DMEM) media in a 75 cm^2 flask. The growth medium was supplemented with 10% Fetal Bovine Serum (FBS), 2 mM L-glutamine, 1% GlutaMAX, and 1% Penicillin/Streptomycin. After reaching 80% confluency, BEAS-2B cells were trypsinized using Gibco™ Trypsin-EDTA (0.05%) (Life Technologies, Carlsbad, CA) and split into six 60x15mm culture dishes. Each dish contained 2 million cells. BEAS-2B cells were incubated in a 5% CO_2 humidified incubator for 24h.

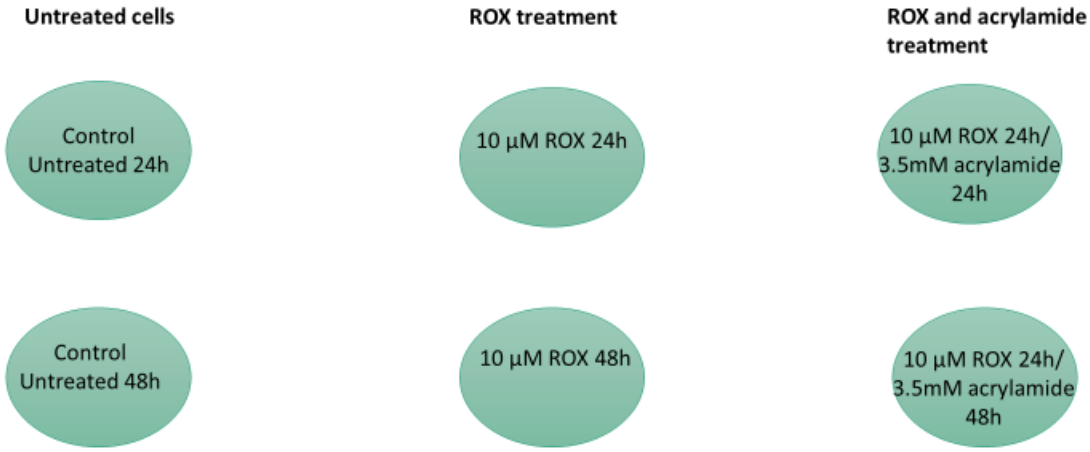

Figure 2: Treatment of BEAS-2B Cells with 10 µM Roxarsone (ROX) and 3.5 mM Acrylamide

BEAS-2B cell treatment groups were divided into 3 sets with 2 culture dishes per set (Figure 2). The first control group consisted of untreated BEAS-2B cells incubated for 24h. The second control group consisted of untreated BEAS-2B cells incubated for 48h. One set of treated cell samples contained BEAS-2B cells treated with 10 µM ROX only. The first ROX treatment consisted of BEAS-2B cells incubated with 10 µM ROX for 24h. The second ROX treatment consisted of BEAS-2B cells incubated with 10 µM ROX for 48h. Another set of treated cell samples contained a combination of 10 µM ROX and 3.5 mM acrylamide. In one sample, BEAS-2B cells were treated with 10 µM ROX for 24h followed by an additional incubation with 3.5 mM acrylamide for 24h. In another sample, BEAS-2B cells were treated with 10 µM ROX for 24h followed by an additional incubation with 3.5 mM acrylamide for 48h. Incubation for 24h allowed the cells to undergo one cell cycle in the presence of 10 µM ROX before the addition of 3.5 mM acrylamide. The choice of the concentration, 3.5 mM acrylamide, was chosen based on experimental findings in the laboratory; 3.5 mM acrylamide destroys about half a population of BEAS-2B cells.

Cell morphology of BEAS-2B cells was visualized and analyzed under a Nikon Microscopy Eclipse Ti-E Inverted Nikon Microscope, (Nikon Instruments Inc. Melville, NY). Photos of BEAS-2B cell morphology were captured at 10X magnification.

Protein Isolation

BEAS-2B cell protein was isolated using the Radio-Immunoprecipitation Assay (RIPA) reagent (Cell Signaling, Danvers, MA). Media from each cell culture dish was transferred into a 1.5 mL microcentrifuge tube and centrifuged. The supernatant was discarded while the pellet was collected. The culture dish was then placed on ice and fr lysis buffer reaction mix was added on the remaining cells on the plate. The RIPA lysis buffer (1X) was supplemented with 1 mM phenylmethanesulfonyl fluoride (PMSF), protease inhibitor (1:100), and protease I phosphate inhibitor (1:100) (Cell Signaling, Beverly, MA), according to the manufacturer's instruction. Cells were then scraped and transferred into the corresponding 1.5 mL microcentrifuge tube previously containing the pellet (cellular material). The content was gently mixed by tapping multiple times and incubated on ice for 45 min. The solution was then centrifuged for 20 min at 4°C at 14,000 revolutions per minute (rpm) (Precision, Winchester, VA). After centrifugation, the supernatant containing protein was transferred into a new 1.5 mL microcentrifuge tube. The protein samples were stored at -80°C.

Protein Quantification

To determine the amount of protein extracted from the cell samples, a Spectronic BioMate 3 UV-Vis (Thermofisher™, Waltham, MA) was used to confirm the optical density of each protein sample at a wavelength of 280 nm. To perform the measurement, 997.5 µL of UltraPure™ DNase/RNase-Free Distilled Water (Thermofisher™, Waltham,

MA) and 2.5 µL of extracted protein was added to a 1.5 mL microcentrifuge tube. The mixture was gently mixed by tapping and then transferred into a reading cuvette. The OD_{280} was then measured and recorded. Each extracted protein sample was aliquoted into a 0.6 mL microcentrifuge tube and stored at -80°C to be used as a working stock.

Protein Gel Electrophoresis and Western Blot

Each isolated protein sample (100 µg) and a standard marker (Precision Plus Protein™ WesternC™ Blotting Standard (BIORAD, Hercules, CA)) were loaded on 10% Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) gels and electrophoresed using a BIORAD Mini-PROTEAN Tetra System (BIORAD, Hercules, CA). Gels were stained using 1X Coomassie Blue (BIORAD, Hercules, CA) following the manufacturer's protocol staining and Western blotting. Gels were destained in distilled water followed by visualization using a Li-COR Odyssey® Fc imager (LI-COR Biosciences, Lincoln, NE). The imaging system was used to capture photos of the gel.

For each Western blot, protein samples were electrophoresed on SDS-PAGE gels followed by transfer to nitrocellulose membranes using a Trans-Blot® Turbo™ Transfer System (BIORAD, Hercules, CA). Proteins of interest Beta Actin, Bcl-xL, AKT and Bax were detected on the Western blots. Polyclonal primary rabbit- or mouse IgG HRP-linked antibodies (Cell Signaling Technology, Danvers, MA) for Beta Actin, Bcl-xL, and Bax followed by secondary Anti-rabbit or Anti-mouse IgG HRP-linked antibodies (Cell Signaling Technology, Danvers, MA) were used to probe Western blots. Western blots were also probed with primary polyclonal rabbit AKT 1/2/3 IgG HRP-linked antibodies (Abcam, Cambridge, MA) followed by a secondary Goat Anti-Rabbit IgG H&L (HRP) (Abcam, Cambridge, MA) following the manufacturer's protocol. Proteins were detected

on the Western blots using Clarity™ and Clarity Max™ Western (ECL) Blotting Substrates (BIO-RAD, Hercules, CA) for chemiluminescent detection. Protein bands were visualized using the LICOR Odyssey® Fc imager (LI-COR Biosciences, Lincoln, NE). Protein expression was quantified using trimmed intensity signals in the Image Studio Lite Version 5.2 (LI-COR Biosciences, Lincoln, NE). Densitometric quantification of proteins bands identified by probes was followed by statistical analysis. Five percent of the highest and lowest band intensities were excluded from the trimmed intensity signal. The resulting p values were considered significant if they were lower than 0.05.

CHAPTER 4

RESULTS AND DISCUSSION

This study investigated the effects of combining Roxarsone (ROX) and acrylamide on BEAS-2B cells. Cell proliferation is induced by 10 μM ROX and previous experimental findings determined that 3.5 mM acrylamide destroys about half the population of BEAS-2B cells treated. In this study, BEAS-2B cells were treated with 10 μM ROX, and a combination of 10 μM ROX and 3.5 mM acrylamide for different incubation periods. Protein was extracted from the treated cells and analyzed using Western blotting. Western blots of protein samples from the various treatment groups were probed with antibodies for downstream genes of the AKT pathway. Overall, the treatment of BEAS-2B cells with 10 μM ROX for 24h followed by 3.5 mM acrylamide for 24h was not sufficient to prevent the damage due to 3.5 mM acrylamide. The same is true of treatment of BEAS-2B cells with 10 μM ROX for 24h followed by 3.5 mM acrylamide for 48h.

Cell Morphology

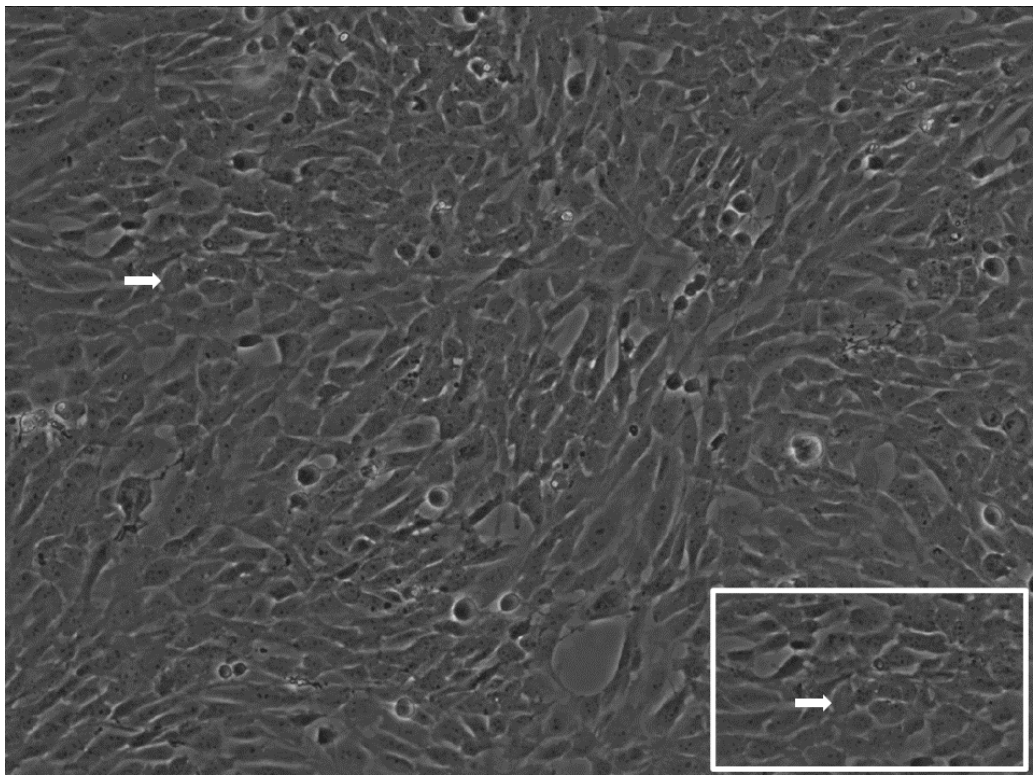

Figure 3: Untreated BEAS-2B Cells after 24h of Incubation (10X magnification) White arrow: normal cell

Figure 3 shows normal BEAS-2B cell morphology. After 24 hours the cells have completed one cell cycle and are at approximately 80% confluency.

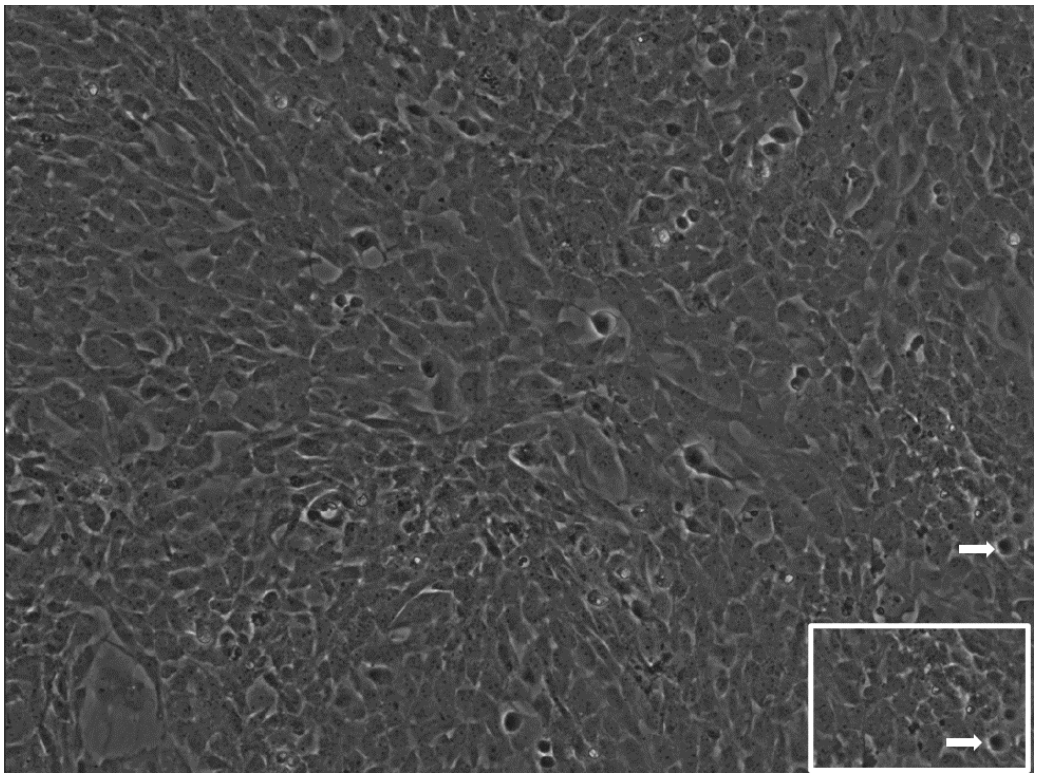

Figure 4: Untreated BEAS-2B Cells after 48h of Incubation (10X magnification) White arrow: apoptosis

In Figure 4, BEAS-2B cell morphology remains normal with the cells at approximately 90% confluency. Programmed cell death (apoptosis) is evident (white arrows).

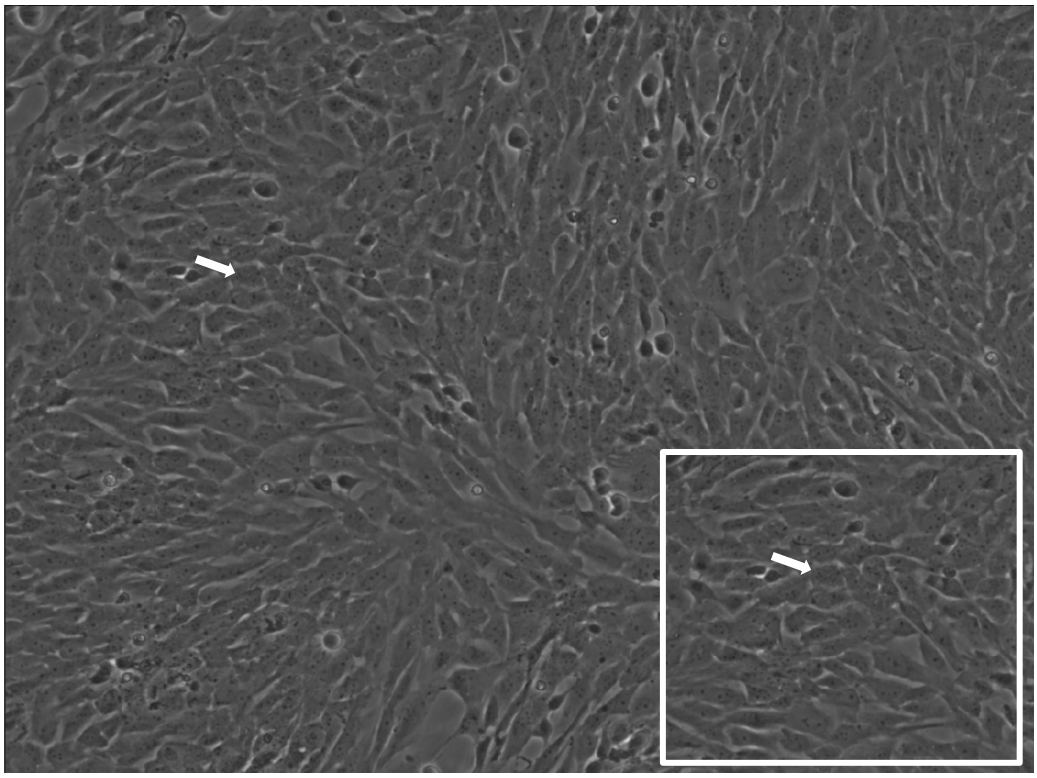

Figure 5: BEAS-2B Cells Incubated with 10 µM Roxarsone for 24h (10X magnification)
White arrow: normal cell

After incubation with 10 µM ROX (Figure 5), the cells are more confluent than untreated BEAS-2B cells at 24h. The increased proliferation is due to the addition of ROX and the cell morphology remains normal.

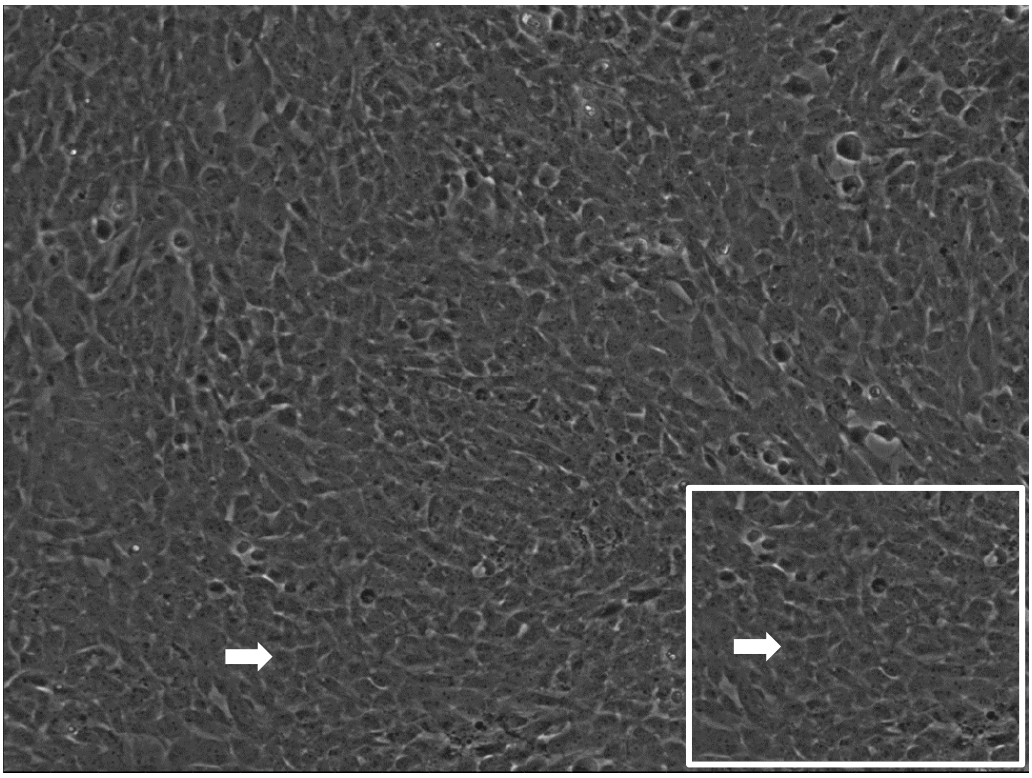

Figure 6: BEAS-2B cells incubated with 10 µM Roxarsone for 48h (10X magnification)
White arrow: normal cell

After 48h in the presence of 10 µM ROX, there is an increase in cell proliferation and the cells have reached the maximum confluency (Figure 6).

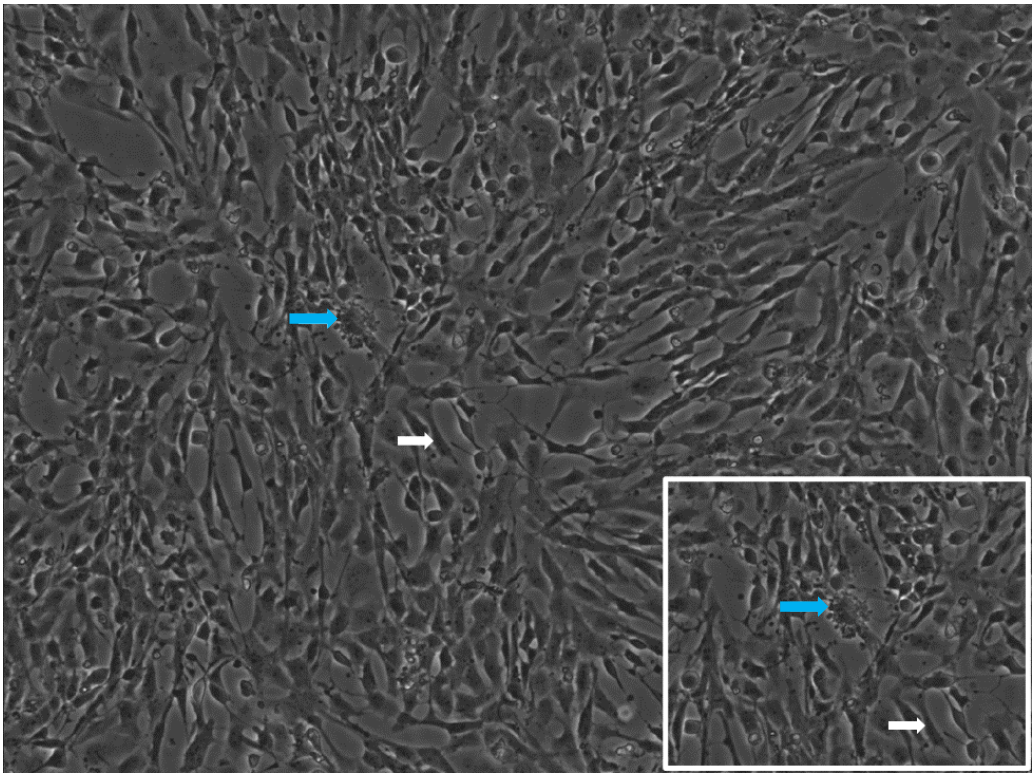

Figure 7: BEAS-2B Cells Treated with 10 µM Roxarsone for 24h Followed by an Additional Incubation with 3.5 mM Acrylamide for 24h (10X magnification)
White arrow: crenation; Blue arrow: apoptosis

After treatment of BEAS-2B cells with 10 µM ROX followed by 3.5 mM acrylamide (Figure 7), the cells appear to be under stress and began to shrink and elongate. The addition of 3.5 mM acrylamide caused the cells to have altered morphology and to lose their cytoplasmic contents despite the presence of ROX. White arrows in Figure 7 point out to cells undergoing apoptosis and crenation.

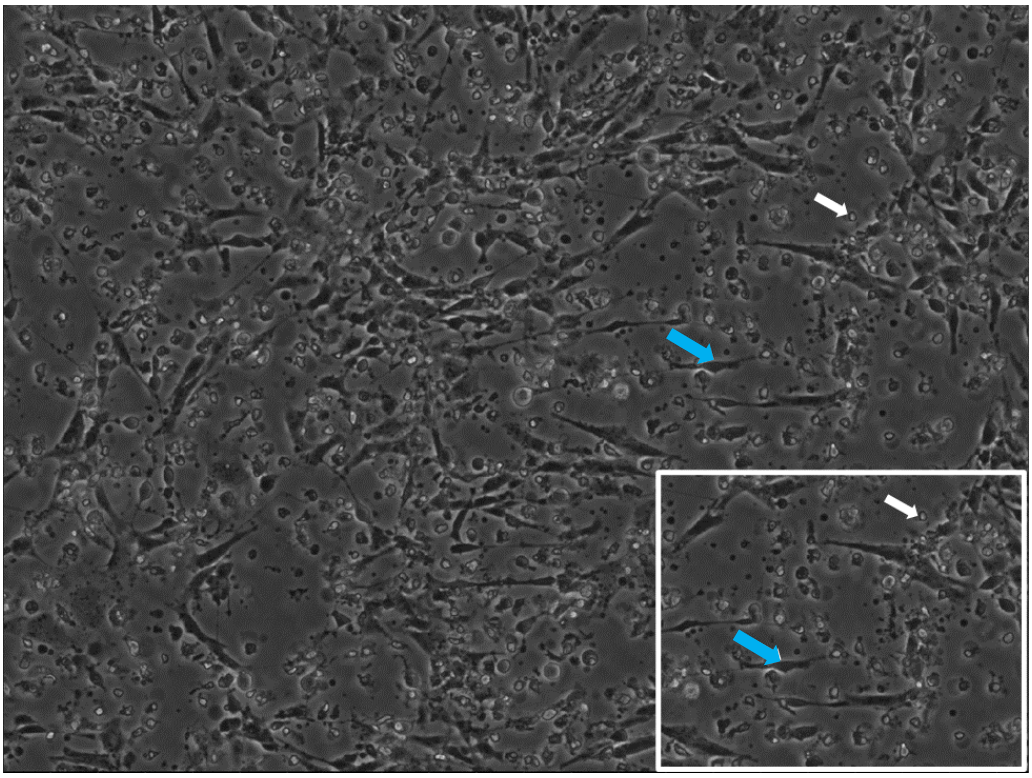

Figure 8: BEAS-2B Cells Incubated with 10 µM Roxarsone for 24h followed by an Additional Incubation with 3.5 mM Acrylamide for 48h (10X magnification)
White arrow: dead cell; Blue arrow: crenation

BEAS-2B cells incubated with 10 µM Roxarsone for 24h followed by an additional incubation with 3.5 mM acrylamide for 48h **(**Figure 8) show increased cell shrinkage and cell death. Cell shrinkage is caused by the loss of cytoplasmic contents (crenation). Arrows (Figure 8) point out crenated cells and dead cells.

Protein Expression

Figure 9 shows an SDS-PAGE gel loaded with experimental samples from this study. Protein expression appears to be reduced for proteins with molecular weights below 60 kilodaltons (kDa). Protein expression also appears to be reduced in cell samples treated with a combination of ROX followed by acrylamide (lanes 5 and 6).

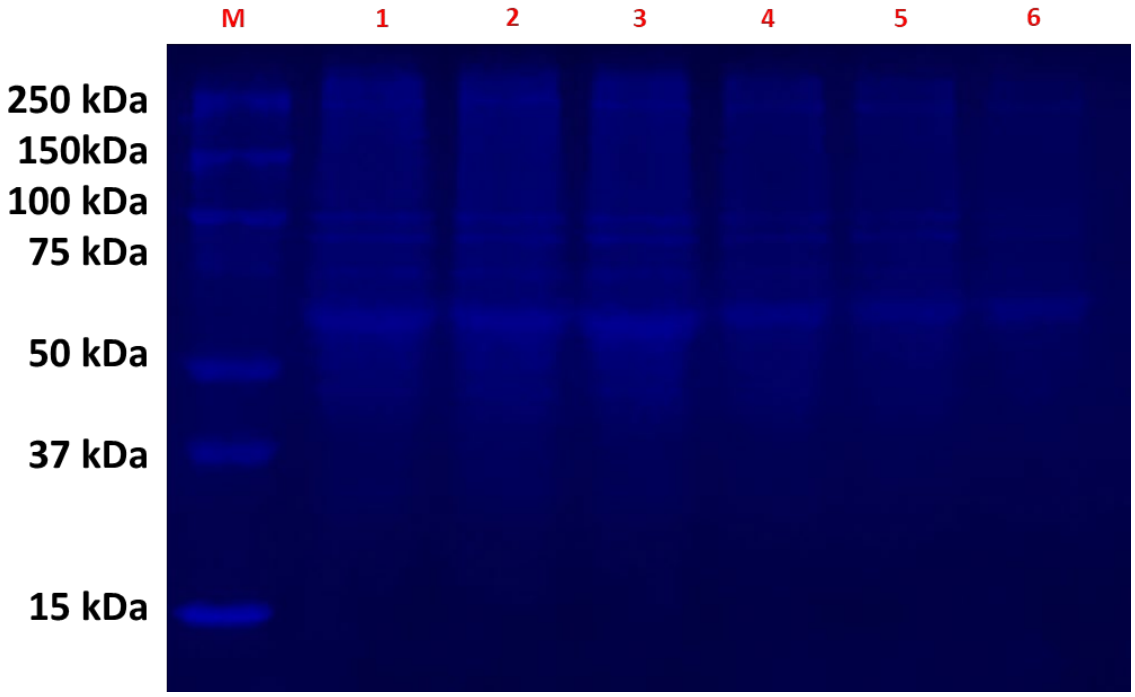

Figure 9: SDS-PAGE Analysis of Protein from BEAS-2B Cells Treated with Roxarsone and Acrylamide

Lane 1: Untreated BEAS-2B cells incubated for 24h; Lane 2: Untreated BEAS-2B cells incubated for 48h; Lane 3: BEAS-2B cells incubated with 10 µM ROX for 24h; Lane 4: BEAS-2B cells incubated with 10 µM ROX for 48h; Lane 5: BEAS-2B cells incubated with 10 µM ROX for 24h followed by additional incubation with 3.5 mM acrylamide for 24h; Lane 6: BEAS-2B cells incubated with 10 µM ROX for 24h followed by additional incubation with 3.5 mM acrylamide for 48h; M: Protein marker.

Analysis of Cell Morphology and Total Protein

Reduced protein expression is observed in BEAS-2B cells treated with 10 μM ROX followed by 3.5 mM acrylamide (Figure 9). This result indicates that acrylamide at a 3.5 mM concentration affects protein expression in BEAS-2B cells.

Increased cell proliferation was observed in BEAS-2B cells incubated with 10 μM ROX for 24h (Figure 5) when compared to untreated BEAS-2B cells (Figure 3). An increase in cell proliferation was also observed in BEAS-2B cells treated with 10 μM ROX for 48h (Figure 6) when compared to untreated BEAS-2B cells (Figure 3). This cell proliferation is due to the addition of 10 μM ROX which is known to induce cell proliferation. BEAS-2B cells treated with 10 μM ROX for 24h followed by 3.5 mM acrylamide for 24h (Figure 7) shrank and became elongated. Treatment of BEAS-2B cells with 10 μM ROX for 24h followed by 3.5 mM acrylamide for 48h (Figure 8) was more damaging to cells, causing increased cell death and cell shrinkage. These changes are most likely due to crenation which is known to cause the loss of cytoplasmic contents. Crenation occurs when the environment outside of a cell becomes hypertonic. This makes the interior of the cell hypotonic, and forces cell contents to move into the external environment. BEAS-2B cells retained normal cell morphology when treated with 10 μM ROX; however, the addition of 3.5 mM acrylamide induced alteration to their morphology. Acrylamide at 3.5 mM concentration appears to be deleterious to BEAS-2B cells, induces changes in cell morphology and reduction of protein expression.

Beta Actin Protein Expression

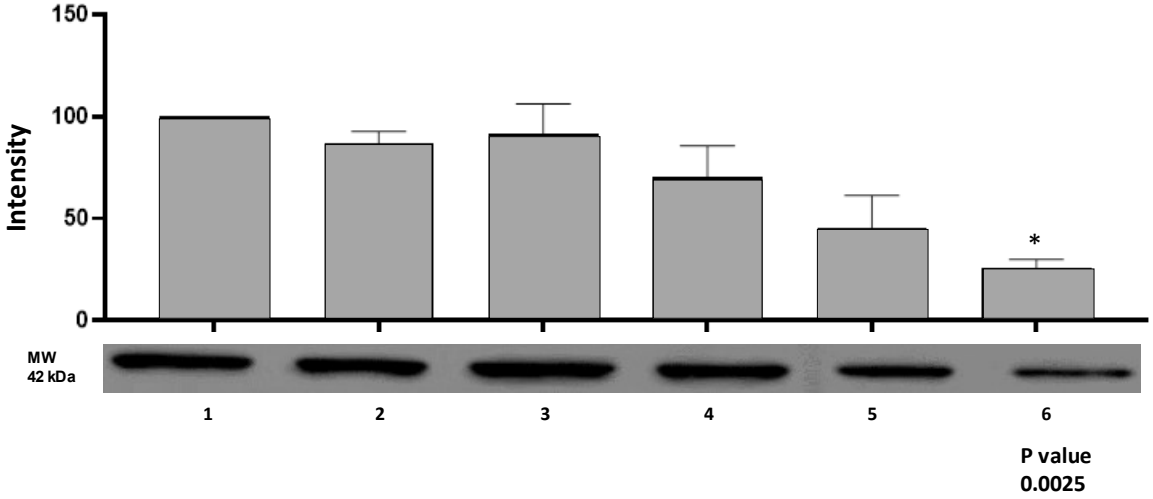

Figure 10: Western Blot of BEAS-2B Cell Proteins Probed with Antibodies for Beta Actin

Lane 1: Untreated BEAS-2B cell proteins after 24h incubation; Lane 2: Untreated BEAS-2B cell proteins after 48h incubation. Lane 3: BEAS-2B cell proteins after incubation with 10 µM ROX for 24h; Lane 4: BEAS-2B cell proteins after incubation with 10 µM ROX for 48h; Lane 5: BEAS-2B cell proteins after incubation with 10 µM ROX for 24h followed by additional incubation with 3.5mM acrylamide for 24h; Lane 6: BEAS-2B cell proteins after incubation with 10 µM ROX for 24h followed by additional incubation with 3.5 mM acrylamide for 48h.

Beta actin is a gene that is highly conserved and expressed across all eukaryotic cells. It is involved in the formation of the cytoskeleton as well as cell integrity, structure, and motility. The results of Western blot analysis (Figure 10) show a significant downregulation of Beta actin levels in BEAS-2B cells treated with 10 µM ROX for 24h followed by 3.5 mM acrylamide for 48h (Lane 6; P value = 0.0025) when compared to untreated cells (Lane 1). This downregulation is due to the addition of 3.5 mM acrylamide causing a deleterious effect on the cells, altering cell morphology. Treatments with 10 µM ROX did not alter cell morphology. However, when BEAS-2B cells were incubated with

10 μM ROX for 24h followed by 3.5 mM acrylamide for 24h (Lane 5), the cells became elongated. The effects appear to be more severe when cells were incubated with 10 μM ROX for 24h followed by 3.5 mM acrylamide for 48h (Lane 6), resulting in increased crenation and cell death. The addition of 3.5 mM acrylamide altered cell morphology and likely disrupted the proper functioning of the genetic material and cellular responses, altering protein expression. Because Beta actin is involved in the formation of the cytoskeleton, the downregulation of Beta actin levels resulting from incubation with 10 μM ROX followed by 3.5 mM acrylamide appears to impact the integrity of BEAS-2B cell morphology. Acrylamide at 3.5 mM concentration appears to have a deleterious effect on the expression of Beta actin.

AKT Protein Expression

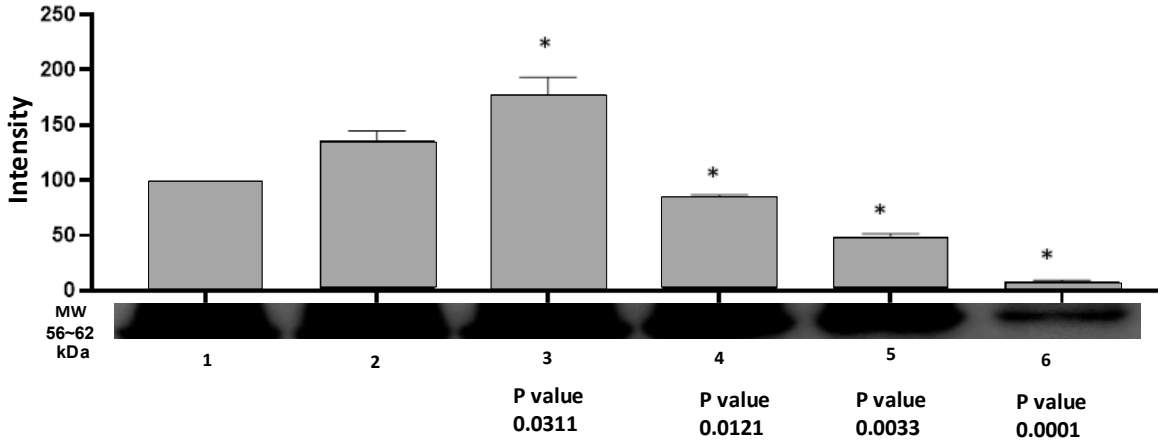

Figure 11: Western Blot of BEAS-2B Cell Proteins Probed with Antibodies for AKT

Lane 1: Untreated BEAS-2B cell proteins after 24h incubation; Lane 2: Untreated BEAS-2B cell proteins after 48h incubation. Lane 3: BEAS-2B cell proteins after incubation with 10 μM ROX for 24h; Lane 4: BEAS-2B cell proteins after incubation with 10 μM ROX for 48h; Lane 5: BEAS-2B cell proteins after incubation with 10 μM ROX for 24h followed by additional incubation with 3.5mM acrylamide for 24h; Lane 6: BEAS-2B cell proteins after incubation with 10 μM ROX for 24h followed by additional incubation with 3.5 mM acrylamide for 48h.

AKT regulates cell survival and cell growth. The probe used in this experiment detected the three isoforms of AKT protein. A significant upregulation of AKT protein levels was observed in BEAS-2B cells treated with 10 μM ROX for 24h (Lane 3; P value = 0.0311), when compared to untreated BEAS-2B cells (Lane 1). Upregulation of AKT protein expression resulted from the addition of 10 μM ROX which stimulates cell proliferation and ultimately cell growth.

There was significant downregulation of AKT protein levels in cells treated with 10 μM ROX for 48h (Lane 4; P value = 0.0121) when compared to untreated cells (Lane 1). Cells reached maximum confluency after 48 hours of incubation. Cell division ceased

and cells began to die. This change in cell division accounts for the reduced levels of AKT proteins.

There was significant downregulation of AKT protein levels in BEAS-2B cells treated with 10 µM ROX for 24h followed by 3.5 mM acrylamide for 24h (Lane 5); P value = 0.0033), when compared to untreated cells (Lane 1). The addition of 3.5 mM acrylamide altered cell morphology which likely disrupted the proper functioning of genetic material and cellular responses where protein expression was affected. There was also significant downregulation of AKT levels in BEAS-2B cells treated with 10 µM ROX for 24h followed by 3.5 mM acrylamide for 48h (Lane 6; P value = 0.0001,) when compared to untreated cells. The effects of 3.5 mM acrylamide on cells appears to be more severe after 48h of incubation, resulting in increased cell shrinkage and cell death.

AKT levels in BEAS-2B cells treated with 10 µM ROX for 24h followed by 3.5 mM acrylamide for 24h are approximately 4-fold less (Lane 5) than AKT levels in BEAS-2B cells treated with 10 µM ROX for 24h (Lane 3). Moreover, AKT levels in BEAS-2B cells treated with 10 µM ROX for 24h followed by 3.5 mM acrylamide for 48h are approximately 20-fold less (Lane 6) than BEAS-2B cells treated with 10 µM ROX for 24h (Lane 3). This result suggests that despite cell proliferation induced by 10 µM ROX, 3.5 mM acrylamide has deleterious effects on AKT expression.

Bcl-xL Protein Expression

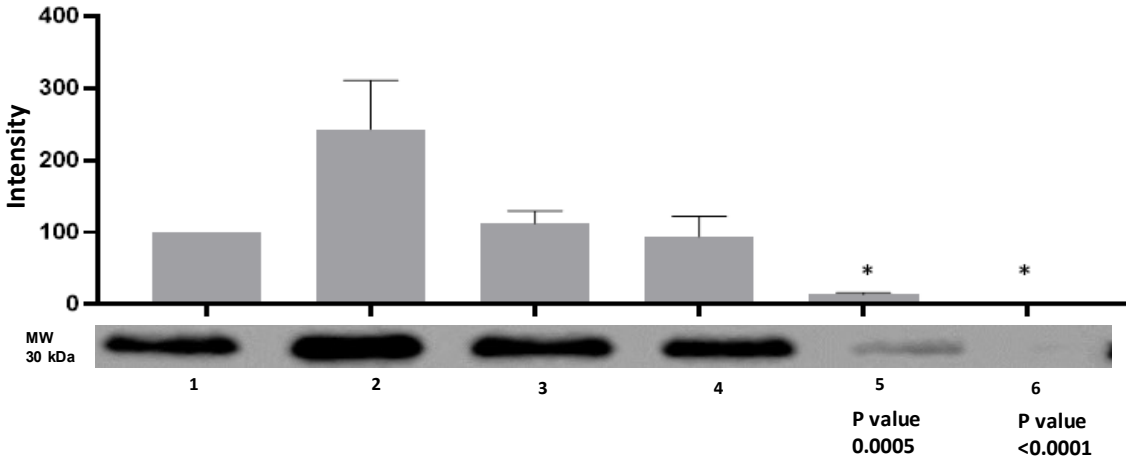

Figure 12: Western Blot of BEAS-2B Cell Proteins Probed with Antibodies for Bcl-xL

Lane 1: Untreated BEAS-2B cell proteins after 24h incubation; Lane 2: Untreated BEAS-2B cell proteins after 48h incubation. Lane 3: BEAS-2B cell proteins after incubation with 10 µM ROX for 24h; Lane 4: BEAS-2B cell proteins after incubation with 10 µM ROX for 48h; Lane 5: BEAS-2B cell proteins after incubation with 10 µM ROX for 24h followed by additional incubation with 3.5mM acrylamide for 24h; Lane 6: BEAS-2B cell proteins after incubation with 10 µM ROX for 24h followed by additional incubation with 3.5 mM acrylamide for 48h

Bcl-xL is a mitochondrial transmembrane molecule that acts as an anti-apoptotic protein. For Bcl-xL to proceed with cell survival functions, it can either be indirectly stimulated by AKT or AKT may inhibit the gene BAD (BAD inhibits Bcl-xL) which allows Bcl-xL to carry on. These results show that Bcl-xL levels were significantly downregulated in BEAS-2B cells treated with 10 µM ROX for 24h followed by 3.5 mM acrylamide for 24h (Lane 5; P value= 0.0005) when compared to untreated cells (Lane 1). The addition of 3.5 mM acrylamide altered cell morphology and likely impacted the proper functioning of the genetic material and cellular responses which affected protein expression. There was also a significant downregulation of Bcl-xL levels in cells treated with 10 µM ROX for

24h followed by 3.5 mM acrylamide for 48h (Lane 6; P value= <0.0001) when compared to untreated cells (Lane 1). The effects of this treatment were similar to those observed when BEAS 2B cells were incubated with 10 μM ROX for 24h followed by 3.5 mM acrylamide for 24h (Lane 5); however, there was more cell damage, and the level of protein expression was lower. It appears that 3.5 mM acrylamide has deleterious effects on the expression of Bcl-xL.

Bax Protein Expression

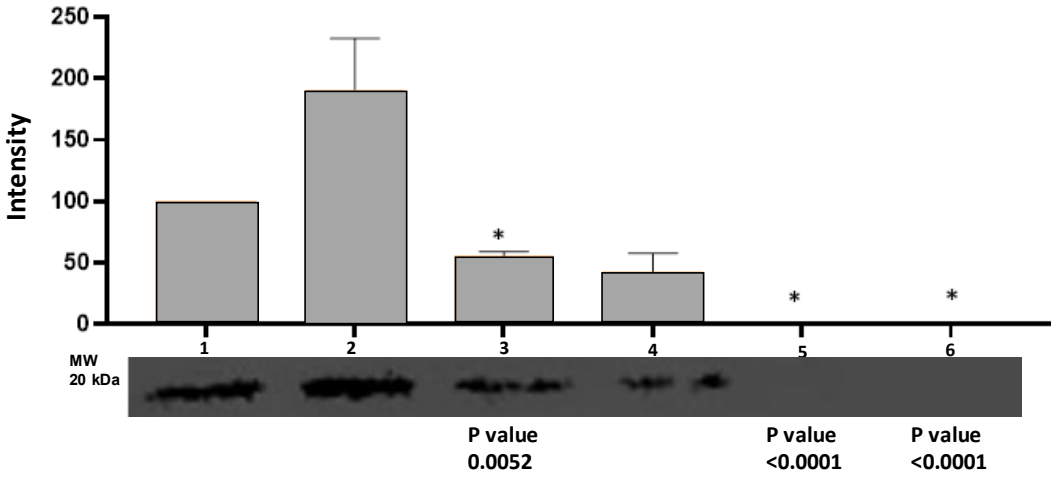

Figure 13: Western Blot of BEAS-2B Cell Proteins Probed with Antibodies for Bax

Lane 1: Untreated BEAS-2B cell proteins after 24h incubation; Lane 2: Untreated BEAS-2B cell proteins after 48h incubation. Lane 3: BEAS-2B cell proteins after incubation with 10 µM ROX for 24h; Lane 4: BEAS-2B cell proteins after incubation with 10 µM ROX for 48h; Lane 5: BEAS-2B cell proteins after incubation with 10 µM ROX for 24h followed by additional incubation with 3.5mM acrylamide for 24h; Lane 6: BEAS-2B cell proteins after incubation with 10 µM ROX for 24h followed by additional incubation with 3.5 mM acrylamide for 48h.

Bax is a pro-apoptotic protein but when inhibited by AKT, cell survival proceeds. Western blot analysis (Figure 13) shows significant downregulation of Bax levels in BEAS-2B cells treated with 10 µM ROX for 24h (Lane 3, P value = 0.0052). After 24h of incubation, cell proliferation is induced by 10 µM ROX and AKT levels were high (Figure 11, lane 3) when compared to untreated cells (Figure 11, Lane 1). AKT can promote cell survival and cell proliferation by inhibiting Bax (Figure 1). Because cell proliferation and cell survival occurred when BEAS-2B cells were incubated with 10 µM ROX (Lane 3), Bax levels were low.

There was significant downregulation of Bax levels in BEAS-2B cells treated with 10 μM ROX for 24h followed by 3.5 mM acrylamide for 24h (Lane 5; P value = <0.0001) when compared to untreated cells (Lane 1). The addition of 3.5 mM acrylamide caused changes in BEAS-2B cell morphology and affected the normal functioning of the genetic material and cellular responses, ultimately affecting Bax protein expression. A similar scenario is seen in cells treated with 10 μM ROX for 24h followed by 3.5 mM acrylamide for 48h (Lane 6; P value= <0.0001) with more cell damage observed. The addition of 3.5 mM acrylamide significantly downregulated Bax levels and ultimately appears to have deleterious effects on Bax.

Figures 10 through 13 show the expression of AKT 1,2,3, Bcl-xL, Bax, and Beta actin proteins in BEAS-2B cells treated with 10 μM ROX, or 10 μM ROX and 3.5 mM acrylamide. Downregulation of protein expression was observed across all genes when BEAS-2B cells were treated with 10 μM ROX followed by 3.5 mM acrylamide. In all cases, 3.5 mM acrylamide appears to have deleterious effects on protein expression of the genes tested.

CHAPTER 5
SUMMARY, CONCLUSIONS, AND RECOMMENDATIONS

The goal of this study is to demonstrate that the stimulation of BEAS-2B cells with 10 μM Roxarsone (ROX) is not sufficient to protect BEAS-2B cells from damage due to incubation with 3.5 mM acrylamide. Findings of this study show that 10 μM ROX induced cell proliferation in BEAS-2B cells; normal cell morphology was maintained. However, incubation of BEAS-2B with 10 μM ROX followed by 3.5 mM acrylamide abrogated cell proliferation and changes in cell morphology were observed. These results suggest that incubation with 3.5 mM acrylamide caused alterations to BEAS-2B cell morphology.

Analysis of AKT protein expression showed levels of AKT protein were upregulated in BEAS-2B cells treated with 10 μM ROX. Once the BEAS-2B cells reached maximum confluency, the cells began to die and the level of AKT was downregulated. ROX at 10 μM concentration appears to have an indirect effect on the level of Bax protein, lowering Bax protein levels probably through its action on AKT protein levels.

Overall, 3.5 mM acrylamide appears to reduce protein expression for Beta actin, AKT, Bcl-xL and Bax genes evidenced by downregulation of protein expression. This study suggests that the reduced expression of proteins in the AKT pathway in BEAS-2B cells is most likely due to crenation, known to cause the loss of cytoplasmic contents, which

caused cell shrinkage and cell death. Incubation of BEAS-2B cells with 10 μM ROX followed by 3.5 mM acrylamide caused increase in cell death and appears to cause breakdown of the PI3K/AKT cell survival pathway. Moreover, alteration of cell morphology in BEAS-2B cells is likely the cause of DNA inactivation. Beta actin which was used as a loading control was not equally expressed throughout BEAS-2B cell treated with 10 μM ROX or with 10 μM ROX and 3.5 mM acrylamide. This confirms studies indicating that its efficient use as a loading control differs with cell treatments and toxicants involved.

These findings suggest that incubation of BEAS-2B cells with 3.5 mM acrylamide induced crenation, which lead to cell morphology alteration, cell death, and the breakdown of the PI3K/AKT pathway. Roxarsone at 10 μM concentration induces cell proliferation but is not sufficient to prevent cell damage caused by 3.5 mM acrylamide.

Future studies include investigating the presence of DNA damage in unstable (microsatellite) DNA region and DNA recombinant region. Also, investigating more genes involved in the PI3K/AKT pathway that may have been altered by the treatments can provide more insight on whether DNA damage occurred.

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